N-alkylphenazines (phenazine metho- and ethosulphates) as electron acceptors[17]. However, if prepared as described by KING[7] (Procedure 1 of the present paper), this enzyme can recombine with respiratory particles deprived of succinate dehydrogenase, but containing the other components of the respiratory chain, and can reconstitute the complete succinate oxidase system, transporting electrons from succinate to molecular oxygen[7,11,18]. The reconstitutively active dehydrogenase can be obtained only if the enzyme is isolated in the presence of succinate[11]. We have observed that if the enzyme is made free of succinate after the isolation, *e.g.* by subsequent Sephadex filtration, it also loses its reconstitutive activity.

In order to examine the effect of oxaloacetate on the reconstitution ability, a series of experiments was carried out in which the enzyme was incubated 2 min with oxaloacetate, oxaloacetate was then removed by transamination with glutamate, and finally succinate dehydrogenase-deficient particles were added and oxygen uptake was measured polarographically. The nonreconstitutive activity was determined by adding 2 mM KCN followed by PMS and by measuring the O_2 uptake. As shown in Table IV and Fig. 5, the ability of succinate dehydrogenase to reconstitute the suc-

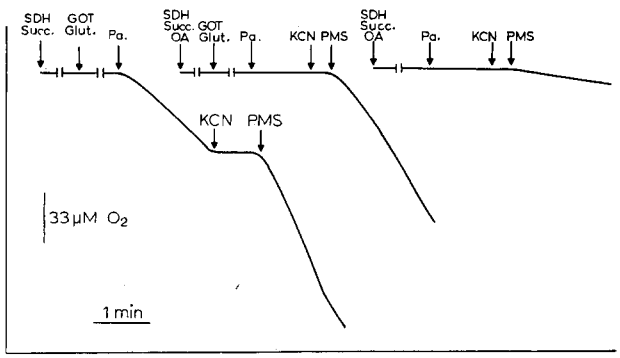

Fig. 5. Reconstitution of the succinate oxidase system. Polarographic traces of oxygen uptake. The assay medium contained 50 mM phosphate and 1 mM EDTA (pH 7.4); temp. 30°; total vol. 3.0 ml. Additions (and abbreviations): succinate dehydrogenase, crude preparation (Procedure 1) (SDH), containing succinate (Succ.) at final concentration in the assay medium 9 mM; oxaloacetate (OA), 0.6 mM; glutamate (Glut.), 6.7 mM; glutamate–oxaloacetate transaminase (GOT); succinate dehydrogenase-deficient particles (Pa.), 3 mg protein; KCN, 1 mM; PMS, 2.2 mM. Time lapse between the addition of succinate dehydrogenase + succinate (or succinate dehydrogenase + succinate + oxaloacetate) and that of glutamate + glutamate–oxaloacetate transaminase was 2 min; between glutamate + glutamate–oxaloacetate transaminase and succinate dehydrogenase-deficient particles, 3 min; and between succinate dehydrogenase + succinate + oxaloacetate and succinate dehydrogenase-deficient particles (last trace), 5 min.

cinate oxidase system was irreversibly lost after a short contact with oxaloacetate, in spite of the subsequent removal of oxaloacetate by transamination. Contrary to this, the activity with the artificial electron acceptor PMS was partly restored. The enzyme preparation used usually contained enough glutamate–oxaloacetate transaminase, so that the omission of externally added transaminase had only a little effect on the recovery of succinate dehydrogenase activity (nonreconstitutive). However, the addition of glutamate was obligatory (Table IV). The irreversible inactivation of the reconstitution was caused by oxaloacetate and not by the products of transamination, aspartate or α-oxoglutarate. As shown in Table IV, Expt. 2, aspartate and α-oxo-

TABLE IV

EFFECT OF OXALOACETATE ON THE RECONSTITUTION OF THE SUCCINATE OXIDASE SYSTEM

All conditions as in Fig. 5; concentrations of aspartate and oxoglutarate, 0.7 mM each.

Expt. No.	*Pretreatment*		*Activity (relative values)*	
	2 min	*3 min*	*Reconstituted**	*With PMS***
1	—	Glutamate + glutamate–oxaloacetate transaminase	100	100
	Oxaloacetate	—	0	0
	Oxaloacetate	Glutamate + glutamate–oxaloacetate transaminase	0	65
	Oxaloacetate	Glutamate	0	46
2	—	Glutamate + glutamate–oxaloacetate transaminase	100	100
	Oxaloacetate	—	0	0
	Oxaloacetate	Glutamate + glutamate–oxaloacetate transaminase	0	35
	Oxaloacetate	Glutamate	0	33
	Oxaloacetate	Glutamate–oxaloacetate transaminase	0	0
	—	Aspartate + glutamate	95	
	—	α-Oxoglutarate + glutamate	68	68
	—	α-Oxoglutarate	0	11

* Succinate → O_2.

** Succinate → PMS → O_2.

glutarate had no or little effect on the reconstitution, provided that glutamate was present, probably in order to prevent the formation of minute amounts of oxaloacetate by a reversed transamination (with endogenous aspartate or α-oxoglutarate).

DISCUSSION

The peculiar pattern of the inhibition of succinate dehydrogenase by oxaloacetate, as characterized by the increasing degree of inhibition in time, is a unique feature not observed, to the authors' knowledge, with other enzymes and their competitive inhibitors nor with other competitive inhibitors of succinate dehydrogenase. It has been repeatedly observed[19–21] that the inhibitory effect of oxaloacetate on succinate oxidation in mitochondria is not instantaneous but is preceded by a lag. Factors such as permeability of mitochondrial membranes to oxaloacetate and processes which remove oxaloacetate from mitochondria may be partly responsible for this phenomenon (*cf.* ref. 21). However, it seems probable that the unique property of succinate dehydrogenase to be inhibited only slowly by low concentrations of oxaloacetate may also be involved.

First reported by us[5], the inhibitory effect of oxaloacetate, increasing with time, has been confirmed by ZEYLEMAKER AND SLATER[6]. However, in disagreement with us, these authors report an immediate, although relatively small, inhibition, while in our experiments there was almost no immediate effect. The other point in which our results are in disagreement with those of ZEYLEMAKER AND SLATER[6] is that we have

observed a constant level of the inhibition only after a lag of 30 sec to a few minutes, while ZEYLEMAKER AND SLATER claim to be able to distinguish a linear, although short, portion of the trace immediately following the addition of oxaloacetate. They also report that spectral changes of succinate dehydrogenase[4] as produced by oxaloacetate are complete within 4 sec after the addition of oxaloacetate. These discrepancies require a further critical examination. It may be interesting to note here that, according to observations of DERVARTANIAN AND VEEGER[4], spectral changes of purified succinate dehydrogenase as produced by oxaloacetate markedly differ from changes caused by malonate and other competitive inhibitors. This may also indicate that the nature of the inhibition by oxaloacetate is different.

As shown in the present study, the increasing inhibition of succinate oxidation after the addition of oxaloacetate is due to the effect of oxaloacetate itself and not to the reaction products or the electron acceptors. The peculiar kinetics of the inhibition by oxaloacetate can only be observed with low (micromolar) concentrations of oxaloacetate or, rather, within certain limits of the ratio between oxaloacetate and succinate (and probably the enzyme protein). At high oxaloacetate concentrations, the inhibition is practically instantaneous. This is probably the reason why this lag has been overlooked by many investigators.

The inhibition constant for oxaloacetate determined in the present investigation is several times lower than that found by PARDEE AND POTTER[3] and DERVARTANIAN AND VEEGER[4]. ZEYLEMAKER AND SLATER[6] found that K_i for the immediate inhibition varied between 1.7 and 6.3 μM and for the secondary (final) one between 0.6 and 0.7 μM. The latter values are in a fairly good agreement with those reported by us for the final (constant) inhibition phase, namely between 0.12 and 0.35 μM. K_m and K_i values for malonate determined in the present study are similar to those values found by others[4,16–18,22].

The reactivation of succinate dehydrogenase inhibited by oxaloacetate, as described in the present paper, is similar to the activation of soluble succinate dehydrogenase observed by KEARNEY[16]. It may be speculated that both processes have a common background and that, in fact, the enzyme isolated by KEARNEY was already inhibited due to a previous contact with oxaloacetate.

It seems likely that the effect of phospholipids as described here is of a different nature than that studied by CERLETTI *et al.*[9]. Both processes differ in two respects: (1) Various phospholipids were equally active in the system described here, while only acidic phospholipids were active under experimental conditions of CERLETTI *et al.*[23]; (2) the activating effect was produced in our system not only by phospholipids but also, and even better, by succinate or malonate, whereas the activation observed by CERLETTI was specific to phospholipids and differed from the activating effect of succinate[23].

The peculiar behaviour of succinate dehydrogenase towards oxaloacetate may be explained by two hypotheses: (1) that oxaloacetate is a *sensu stricto* competitive inhibitor of succinate dehydrogenase, forming reversibly a complex with the enzyme, but the formation and the splitting of this complex occur only slowly; (2) that the enzyme exists in two forms, active and inactive, and that the transition from the active form into an inactive one is promoted by oxaloacetate, while the reverse process is induced by succinate or certain other substances. The existence of two forms of succinate dehydrogenase exhibiting high and low activities, respectively, has been already postulated by the group of SINGER[24] and supported by recent kinetic studies

of ZEYLEMAKER AND JANSEN[25]. According to the second hypothesis, as proposed above, oxaloacetate would not be a true competitive inhibitor, although the kinetics of the inhibition may imitate a competitive inhibition, the ratio of the active to the inactive forms of the enzyme being controlled by the ratio of succinate to oxaloacetate.

The first hypothesis postulates a relatively stable complex between oxaloacetate and the dehydrogenase. The binding site of succinate dehydrogenase for succinate presumably contains two positively charged groups, able to attract the two carboxylic groups of succinate. Malonate, a three-carbon-atom analogue of succinate, probably fits even better to this binding site, as indicated by the K_i value which is 20–30 times lower than the K_m value. On the contrary, unsaturated and substituted dicarboxylic four-carbon-atom acids, such as fumarate, methylene succinate, D-chlorosuccinate and malate, are weak inhibitors[4,16,26]. Therefore it seems puzzling why oxaloacetate which is the carbonyl derivative of succinate is such a potent inhibitor. It should be supposed that in this case not only the two carboxyls, but also the carbonyl group or the enol structure which may be formed, is involved in the binding with the enzyme. This additional binding would also be responsible for the slowness of the formation and of the splitting of the enzyme–inhibitor complex. It may be speculated that the acid-labile SH group or the non-heme iron is involved in this binding. However, this might also be any other moiety of the enzyme protein, even not necessarily essential for the enzymatic reaction. Succinate, malonate and, to a lesser degree, phosphate and phospholipids compete for the positive binding sites, thus enabling the other binding to be slowly split. The stability of the dehydrogenase–oxaloacetate complex should be, however, not high enough to sustain repeated Sephadex filtration or prolonged dialysis against Tris buffer.

The other hypothesis assumes that conformation changes in the enzyme protein are induced by oxaloacetate. These changes may be reversed by succinate, by its analogues, *e.g.* malonate or fumarate, or by certain other substances probably showing an affinity to the substrate binding site of the enzyme, *e.g.* phospholipids and phosphate (inhibition of succinate dehydrogenase by phosphate has been observed by SLATER AND BONNER[27]). The transition from the inactive to the active states of the enzyme must, however, occur also spontaneously, as the reactivation also proceeds, at least partly, on simply removing oxaloacetate. In consequence, experiments described here do not allow us to decide unequivocally between these two hypotheses. We failed more directly to demonstrate the existence of a stable complex of succinate dehydrogenase with oxaloacetate or to isolate an inactive form of the enzyme which would be stable enough in the complete absence of oxaloacetate. Further work and perhaps a new experimental approach to this problem are needed.

At present there is one more direct experimental evidence for changes in the molecule of succinate dehydrogenase caused by a transient contact with oxaloacetate, namely the loss of the ability of the enzyme to reconstitute the complete succinate oxidase system. This change is, however, irreversible and is thus not similar to the reversible inactivation of the dehydrogenase activity. The hypothesis of conformation changes produced by oxaloacetate in succinate dehydrogenase is also compatible with suggestions expressed elsewhere and based on the behavior of succinate dehydrogenase in aged mitochondria[28].

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KINETIC STUDIES OF SUCCINATE DEHYDROGENASE BY ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY

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SUMMARY

The electron paramagnetic resonance signals of succinate dehydrogenase (succinate: (acceptor) oxidoreductase, EC 1.3.99.1) were studied during reductive titrations, and the kinetics of their appearance and disappearance were followed on rapid reduction of the enzyme with succinate and after treatment of the reduced enzyme with ferricyanide, fumarate or O_2. At most 1 equiv of unpaired electrons per mole of enzyme-bound flavin is accounted for in the EPR signal at $g = 1.94$. Thus, according to present information on the structure showing this signal[14] 2 iron atoms out of the total 6–8 present per enzyme molecule are involved in the oxidoreductive process observed by EPR. Only a fraction (about 16%) of this iron, however, responds rapidly to reduction by succinate, whereas after complete reduction with succinate, reoxidation of a larger portion of the iron with ferricyanide and fumarate is rapid. After reduction with NADH and phenazine methosulfate, reoxidation by fumarate is slow as is reoxidation by O_2 under any conditions. During reduction the amount of iron which rapidly reacts does not depend on substrate concentration, whereas the extent of free radical formation does, rising with increasing substrate concentration. The maximal radical concentration observed accounted for 20–30% of the bound flavin present.

INTRODUCTION

SINGER AND KEARNEY[1,2] and independently WANG *et al.*[3] reported in 1955 that preparations of succinate dehydrogenase (succinate: (acceptor) oxidoreductase, EC 1.3.99.1) contain iron which is not bound to porphyrin. Subsequently, different types of succinate dehydrogenase preparations were shown by chemical analysis to contain amounts of iron varying from 2 to 8 iron atoms per mole of flavin[3–7]. In 1960 BEINERT AND SANDS[8] found a new type of electron paramagnetic resonance (EPR) signal in reduced succinate dehydrogenase preparations, which they tentatively attributed to

Abbreviation: PMS, phenazine methosulfate.

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iron. Since then this signal has been seen by other workers[9–11], and it has been inferred from data on simpler iron–sulfur proteins that it is due to an iron complex of unknown structure, presumably containing sulfur[12–17].

Kinetic studies by ZEYLEMAKER *et al.*[19] indicated that all enzyme molecules in a preparation as obtained[20] have the same turnover number (50–65 sec^{-1} at 25°). Incubation of such a preparation with succinate will then convert all enzyme molecules into a more active form (110–130 sec^{-1} at 25°)[19]. Thus far, except for a preliminary statement[18], no kinetic studies on succinate dehydrogenase preparations have been published which cover reaction rates of the EPR-detectable components in the time range of the turnover of either the activated or not activated form of the enzyme.

The main reason for the fact that thus far no such attempts have been made to obtain quantitative information on the reaction rates of the components of this otherwise thoroughly studied enzyme must be found in the difficulties introduced by this "activation" phenomenon. These difficulties have been discussed and summarized by SINGER[21]. Briefly stated, efficient activators are either substrates or inhibitors of the enzyme, and their effect is said to be at least partly reversed after their removal[22]. Because of the requirements of the EPR technique, it is not feasible to dilute these substances out, as is usually done in routine activity assays. Also for the studies intended here, the enzyme should obviously not be fully or even partly reduced, which again is irrelevant for routine assays. It also appears at this time that certain aspects of the activation phenomenon are not completely understood and remain controversial.

We were not able to overcome these difficulties in a satisfactory manner in the present work. Fortunately, however, only the results on the rates of reduction of the various enzyme components can be affected. The enzyme used for reoxidation experiments must certainly have been activated by exposure to succinate, and the results on the titrations should also be free of interference by the activation phenomenon. We, nevertheless, present our results on rates of reduction, although their interpretation may be ambiguous because of the unknown activation state of the enzyme. However, much work has been done in the past few years with this and related enzyme preparations at similarly poorly defined states of activation, and we feel therefore that the data obtained contain useful information on the behavior of succinate dehydrogenase under such conditions.

With these reservations in mind, in this paper we report quantitative data on rates and extent of participation of the EPR-detectable components, *viz.* the iron complex and a free radical, presumably flavin semiquinone, in the turnover of succinate dehydrogenase.

MATERIALS AND METHODS

The enzyme used in this work was the preparation described by WANG *et al.*[3] as modified by DERVARTANIAN AND VEEGER[20]. This type of enzyme preparation at the purity used contains 4–5 μmoles of flavin and 35–40 μgatoms of iron per g of protein[7]. The protein was stored in small lots under liquid N_2 and only thawed once, immediately before use. All solutions were 0.1 M with respect to potassium phosphate buffer (pH 7.4) and were equilibrated with "high purity" N_2 unless otherwise specified. Enzyme solutions contained 1 mM EDTA. All manipulations involving the purified enzyme were carried out with the exclusion of O_2 as far as was practical without resorting to

elaborate techniques. In the kinetic experiments, however, previously described anaerobic techniques[23] were used. The N_2 gas used in these experiments, as well as in the titrations with NADH and phenazine methosulfate (PMS)[24], was passed through alkaline pyrogallol before use. In the titration the level of PMS used was quite critical. In a total reaction mixture of 0.275 ml, 5 μl of a 0.0002% solution of PMS were optimal.

The titration procedure using dithionite has been briefly described elsewhere[14,25] and will be published in the near future[62]. Helium ($\leq$ 1 ppm of O_2) was used in this case. Iron was determined by the method of VAN DE BOGART AND BEINERT[26].

The apparatus and techniques used in EPR spectroscopy and rapid freezing have been described in various recent papers[23,25,27-31]. The cavity used for the measurements was a standard rectangular V-4531 cavity of Varian Associates with a loaded Q of 4500–5000 under the conditions of our experiments. The concentrations of reactants given are final concentrations, *i.e.*, after mixing with various additions, by hand or in the rapid mixing apparatus. As in previous work using the rapid freezing technique the reaction times given refer to the times calculated from the dimensions of the apparatus and the speed of the syringe drive but neglect the unknown freezing time[31], which appears to be less than 10 msec.

The spectra obtained represent the first derivative of the EPR absorption curve (as for instance shown in Fig. 8). In the evaluation of the data obtained in the various experiments, we have normalized all signal heights that were observed on different days, on the basis of a standard of solid copper sulfate and to an enzyme concentration, after mixing, of 12.5 mg/ml, which was representative of the majority of experiments. The presence of isopentane in the frozen mixtures was also taken into account in this normalization. In agreement with previous work[30] 43% of the volume was found to be occupied by the frozen aqueous phase. The conditions of EPR spectroscopy chosen for normalization were: 23 mW microwave power; 12 gauss modulation amplitude; scanning rate of 100 gauss/min; time constant 0.5 sec; and temperature 98°K. The data from the titration experiment of Fig. 3 were treated separately as the observation temperature was 82°K in this case.

Since we did not observe any significant changes in signal shape, the observed "heights" of the derivative signals can be taken to be proportional to signal intensities and therewith to concentrations of the components represented. The proportionality factors are given by double integrations of derivative signals of the dehydrogenase and comparison with suitable standards. Thus in the experiment of Fig. 4 the iron signal obtained with dithionite corresponds to 30 μM in the sample after mixing, and the radical signal obtained at 6 min with the higher succinate concentration corresponds to 5.6 μM flavin, 11% of the enzyme-bound flavin present in this experiment. For the iron signals at $g = 1.94$ and $g = 4.3$, a copper–EDTA standard was used; for the radical signals, the flavoprotein from Azotobacter[32,33], which is quantitatively converted to the semiquinone by dithionite, was used. The semiquinone concentration for this protein can thus be based on a flavin determination. For integration, the radical signals were measured at 10 μW, where saturation appears to be $<$ 20% for the enzyme samples as well as for the standard. Because of the relatively low intensities of the signals and the signal-to-noise ratio of the spectrometer, operation at lower power did not result in an advantage in overall accuracy. With the iron signals, corrections were applied for transition probabilities[34] and population of states as far as presently known[35]. With several substances we have examined (iron–conalbumin,

iron–EDTA), the signal at $g = 4.3$ only accounted for 30–50% of the iron present even after the mentioned corrections were applied (*cf.* refs. 35–37). We have therefore assumed that this same situation holds for the signal of succinate dehydrogenase and have made the additional correction of doubling the values in order to approximately account for this. The values given in this paper for the signal at $g = 4.3$ are therefore subject to an appreciable uncertainty. The temperature at which reactions were allowed to proceed was 22°. A relative measure of the spin relaxation rates was obtained by comparing signal heights over several orders of magnitude of incident microwave power. The logarithm of the signal height in arbitrary units divided by the square root of the power, $\log(S/\sqrt{P})$ (abscissa), was plotted *versus* the logarithm of the power. Details are found in recent publications[25,38]. Light absorption of the liquid samples contained in the round quartz tubes, as used for EPR spectroscopy, was measured with the aid of a specially constructed attachment, which will be described in the near future[63].

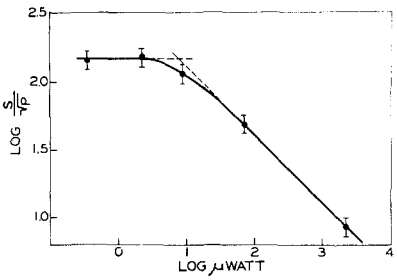
RESULTS

EPR signals of succinate dehydrogenase

The EPR signals of succinate dehydrogenase have been previously described[8–10,13,39–41]. The only EPR signal in the enzyme, as isolated, was that at $g = 4.3$, which is typical of Fe^{3+} in the high spin state[42]. This signal represents an amount of iron of the order of the flavin content of the enzyme. This iron is partially and slowly reduced by succinate and, when previously reduced, is rapidly reoxidized by ferricyanide or fumarate. The significance of this signal is not clear (see DISCUSSION).

On reduction by substrate or by a chemical reducing agent, a signal at $g = 2.00$, typical of a free radical, can be observed at room[39] and at low temperature[8–11], and concomitantly a signal appears at $g = 1.94$ (*cf.* Fig. 8), which is only seen at low temperature. It is reasonable to attribute the radical signal to a semiquinoid form of the bound flavin of the enzyme. According to the results obtained on simpler iron proteins, exhibiting a signal at $g = 1.94$ on reduction, the corresponding signal of succinate dehydrogenase is due to an iron complex[12,14]. For the sake of simplicity, it will be called the iron signal, and appearance of this signal will be referred to as indicating "iron reduction", although details of the electron distribution in the iron complex are not known. The radical signal is rather readily saturated with microwave power at the temperature necessary for observation of the iron signal. Saturation is observed at powers of 3–10 μW (110°K), the lowest at which the signals obtained in this work could still be measured with acceptable precision. Saturation varies to some extent with the conditions under which the radical is generated, as will be discussed below. The iron signal is not saturated at 77°K up to powers of 250 mW but is saturated at 4°K at powers as low as 3 μW (Fig. 1). The power of half saturation (*cf.* ref. 38) is approx. 10 μW at 4°K.

On quantitative determination, maximally 30% of the bound flavin and, after reduction with dithionite, maximally 16% of the total iron were accounted for in the respective signals. Low recoveries in the signal at $g = 1.94$ with respect to the total iron present have rather generally been observed, particularly with the more complex nonheme iron proteins[12,25,44–46]. Possible interpretations of this finding will be offered below. In the case of succinate dehydrogenase the low recovery is not due to the



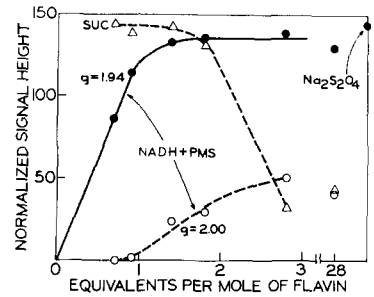


Fig. 1. Saturation of $g = 1.94$ signal ($g_\perp$) of succinate dehydrogenase at 4°K. The enzyme was prepared according to ref. 43. Enzyme prepared according to ref. 6 showed the same behavior within the limits of error of the method. The logarithm of the power incident on the cavity (abscissa) is plotted against the logarithm of the ratio of signal height, in arbitrary units, to the square root of the power (ordinate) (*cf.* refs. 25 and 38). The conditions of EPR spectroscopy were microwave power as indicated; modulation amplitude, 3.5 gauss; scanning rate approx. 200 gauss/min; time constant 0.3 sec; and temperature 4° K.

Fig. 2. Anaerobic titration of succinate dehydrogenase with NADH as reductant and phenazine methosulfate as mediator (*cf.* ref. 24). After manual mixing of the reactants in the dark, the samples were incubated in the dark for 1 h at room temperature, then frozen and their EPR spectrum was recorded. The sample tubes were subsequently evacuated, filled with N_2 and an excess of solid sodium succinate was added under N_2 to each tube, the samples were refrozen and their EPR spectrum was recorded. Signal (first derivative of absorption) heights measured in mm were normalized to routine settings of the EPR apparatus. Unless otherwise specified, these values refer to an enzyme concentration of 12.5 mg protein per ml after mixing, at a microwave power of 23 mW, modulation amplitude of 12 gauss, scanning rate of 200 gauss/min and time constant of 0.5 sec, at 98°K. In this experiment as well as all others, 0.1 M potassium phosphate buffer (pH 7.4), containing 1 mM EDTA, was used for the enzyme preparations and for the reactants the same buffer without EDTA. All reactant concentrations reported are final, and the temperature at mixing was approx. 22°. ○—○, radical signal; ●—●, iron signal; △ - - △, radical signal after addition of solid succinate (SUC).

TABLE I

CONCENTRATION OF UNPAIRED ELECTRONS IN SUCCINATE DEHYDROGENASE AFTER REDUCTION WITH DITHIONITE

Concentrations of iron and flavin are derived from protein determination by the biuret method on the assumption of a molecular weight of 240 000 and 1 mole of flavin per mole of enzyme (*cf.* ref. 7).

Iron (mM)	*Flavin (mM)*	*Temp. (°K)*	*Unpaired electrons in EPR signal at $g \approx 1.94$ and $g \approx 2.01$* (mM)*
Not determined	0.123	32	0.099
Not determined	0.123	81	0.10
Not determined	0.123	103	0.09
0.394	0.061	81	0.065

* On extensive reduction with dithionite radical signals at $g = 2$ are negligible.

temperature dependence of the signal, as shown in Table I. At least at temperatures higher than 30°K the signal merely broadens, probably through increased spin relaxation, but the integrated intensity remains unchanged up to temperatures (about 100°K) at which broadening sets a practical limit (*cf.* ref. 40).

Anaerobic reduction

Titration with NADH and phenazine methosulfate. It was shown by VAN GELDER[24] and VAN GELDER AND SLATER[47] that cytochrome *c* or cytochrome *c* oxidase can be titrated with NADH, when PMS is used as a mediator. Attempts to apply[18] this technique to succinate dehydrogenase showed that the EPR-detectable iron and flavin components can indeed be reduced and titrated with these reagents, when sufficient time is allowed for equilibration. Since in approx. 30 min maximal EPR signals were obtained, we routinely let the samples stand in the dark for 1 h at 22°. A titration curve is shown in Fig. 2. It can be seen that with 1 electron equiv of titrant the maximal iron signal was produced and that radical signals were only seen when more reductant was added. The amount of radical at its maximal development corresponded to 2% of the bound flavin. This must mean that out of the approximately eight iron atoms that are present per molecule, the equivalent of at most one is reduced, although according to findings with simpler iron proteins[14,48] at least two iron atoms may be involved in the one electron process giving rise to the $g = 1.94$ signal. Flavin is apparently only reduced after the iron complex has taken up an electron*. Since appearance of radical signals does not *per se* mean reduction, but could equally well indicate oxidation, we applied an independent test to the state of the flavin in the enzyme at the various stages of the titration. After addition of NADH *plus* PMS, incubation, freezing and measurement, the sample tubes were kept frozen with solid CO_2 and were again evacuated, filled with N_2 and thawed. The tops were removed and, while N_2 was blown down into the tubes, a small amount of solid sodium succinate was added, and the contents were frozen immediately after mixing (approx. 1 min). The radical signals observed after this treatment are shown by the open triangles in Fig. 2. Radical signals, amounting to 8% of the bound flavin, appeared in the samples with up to 2 reducing equiv of NADH, whereas with 3 and more reducing equiv of NADH present, succinate had less influence on radical concentration. Obviously succinate elicits strong radical signals as long as no radical has been formed by NADH *plus* PMS, whereas succinate in fact diminished the radical concentration, after NADH *plus* PMS has produced maximal radical signals. We take this to indicate that flavin was indeed left in its oxidized form with 1 electron equiv of NADH per mole of flavin but was reduced with larger quantities of NADH. Under these conditions, succinate served only to reduce flavin even further, thus diminishing the signal.

Titration with sodium dithionite. Fig. 3 shows the results of an anaerobic titration of succinate dehydrogenase with solid sodium dithionite, diluted to weighable quantities by KCl. The left-hand side of Fig. 3 refers to the EPR measurements on the frozen samples, while the right-hand side refers to the light absorption measurements on the

* Changes in the signal at $g = 4.3$ were not measured during this titration. Since it was observed in the titration with dithionite (see below) that flavin radical is not formed unless the iron represented in the signal at $g = 4.3$ is reduced, it is possible that the delay of flavin radical formation, presumably indicating flavin reduction, is related to the behavior of the signal at $g = 4.3$ rather than to that at $g = 1.94$.

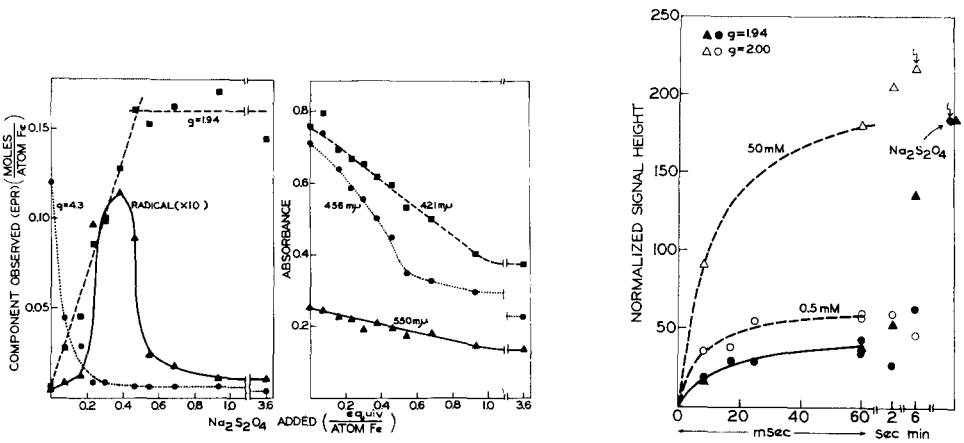


Fig. 3. EPR signal heights observed during titration of succinate dehydrogenase with dithionite. Enzyme, 0.4 mM with respect to iron, was titrated with solid dithionite, which was diluted to weighable quantities by solid KCl (refs. 14 and 25). Before freezing and observation of the EPR spectra, light absorption was measured in the EPR sample tubes. This was done by an accessory to the Cary spectrophotometer, Model 14, to be described elsewhere (ref. 49). The effective light path under these conditions was 2.8 mm. The left side of the figure refers to the EPR measurements and the right side to light absorption. The ordinate referring to the optical measurements gives the actual absorbance values measured under the conditions just described. The ordinate referring to the EPR measurements gives the concentrations of the three EPR-detectable components, as calculated from double integrations of the observed signals divided by the total iron concentration of the preparation, as determined chemically. Note that the values on the ordinate should be divided by 10, when applied to the concentration of flavin semiquinone. The conditions of EPR spectroscopy were: microwave power, 27 mW; modulation amplitude, 6 gauss; scanning rate, 200 gauss/min; time constant, 0.5 sec; and temperature 82°K for the signal at $g = 1.94$; the same conditions, except 12-gauss modulation for the signal at $g = 4.3$; and 0.2 mW, 6-gauss modulation, the same scanning rate, and a temperature of 101°K for the radical. The symbols used in the right half of the figure refer to absorbance at the wavelengths indicated and those in the left half to EPR signals as follows: ●·····●, $g = 4.3$; ■— — —■, $g = 1.94$; ▲—▲, radical at $g = 2.0$.

Fig. 4. Signal heights during anaerobic reduction of succinate dehydrogenase at two levels of succinate. Samples were frozen at the times indicated. The conditions of EPR spectroscopy were those of Fig. 2. ○ — —○, 0.5 mM succinate, radical signal; ●—●, 0.5 mM succinate, iron signal; △— — —△, 50 mM succinate, radical signal; ▲—▲, 50 mM succinate, iron signal.

same samples, immediately before freezing. The EPR results show that a portion of the first equivalents to enter the enzyme is used for reduction of the high spin Fe^{3+} represented in the signal at $g = 4.3$ and another portion is accomodated in the iron complex, which after electron uptake, exhibits the signal at $g = 1.94$.

According to double integrations of the signals obtained, the maximal signal at $g = 1.94$ (at 0.92 equiv of reductant) accounted for 1.1 equiv of unpaired electrons per mole of flavin or 16.5% of the total iron found by chemical analysis, *i.e.*, 1/6 of the iron. The signal at $g = 4.3$ accounted for 0.3–1 gatom of iron per mole of flavin and the maximal radical signal (at 0.375 equiv of reductant) for 7% of the flavin in this particular experiment, whereas in other experiments as much as 20–30% of the flavin was accounted for in the radical signal after dithionite reduction. Since the two iron species are present in roughly equal amounts (within the limits of error of the determination of the high spin ferric species) the question may be raised whether, when ferric, the iron represented in the $g = 4.3$ species may be the same that on reduction produces the signal at $g = 1.94$. However, neither the titration curves shown in

Fig. 3 nor the kinetic course of the reduction and reoxidation of these two species are compatible with the idea that the $g = 4.3$ species gives rise to the $g = 1.94$ species on reduction. They must be separate entities. It does, however, appear that the high spin ferric species ($g = 4.3$) may be able to accept electrons *via* flavin, by intra- or intermolecular oxidoreduction since flavin reduction only begins when the high spin species is reduced. Similar observations were previously made on NADH–cytochrome *c* reductase[50]. Flavin semiquinone formation is maximal while the $g = 1.94$ species is partly developed. Further reduction of flavin sets in as soon as the iron complex ($g = 1.94$) has been saturated with reducing equivalents. With 0.55 equiv added per iron all EPR-detectable components are reduced. If we assume that the enzyme contained eight atoms of iron per flavin, 0.55 equiv per iron would mean 4.4 equiv consumed per flavin. It appears then that of these 4–5 equiv per flavin, 2 are consumed in complete reduction of the flavin and approx. 1 each for production of the $g = 1.94$ species and the reduction of the high spin Fe^{3+} ($g = 4.3$). The light absorption data show, however, that additional electron acceptors are present which are most likely additional nonheme iron components. Similar observations have been reported for the titration of aldehyde oxidase from rabbit liver[25].

These observations indicate that those additional nonheme iron components, which are not detected by EPR in our experiments, have an absorptivity of the same order of magnitude as the EPR-detectable ones and can therefore not readily be distinguished from the latter. It appears from the plots of the light absorption data at the different wavelengths that the curve describing primarily flavin absorption (456 mμ) shows a break at the position where flavin reduction is complete, according to EPR, in contrast to the curves more related to iron reduction.

Kinetics of reduction with succinate. Fig. 4 shows the time-course of anaerobic reduction of iron and flavin in succinate dehydrogenase at final succinate concentrations of 50 and 0.5 mM. It can be seen that both signals appear at similar rates but that only a fraction of the maximal iron signal, which can be obtained with dithionite, is readily produced initially with succinate. A slow reduction of iron then follows. The half-time for the rapid phase of the reduction is of the order of 10 msec, in agreement with the results of measurements by the stopped-flow technique[19]. The number of observations points is insufficient to make a closer distinction. It is apparent that the intensity of the iron signal at early reaction times does not significantly depend on the succinate concentration, whereas the radical concentration is 3 times higher at the higher succinate level. In the time range of minutes, the higher succinate concentration also leads to stronger iron signals. The points indicated by the arrows in the figure correspond to 30 μM iron and 5.6 μM flavin.

The concentration of enzyme-bound flavin was 52 μM. Enzyme preparations of the type used here have a turnover number of approx. 65 moles of succinate per mole of flavin and per sec at 25° with ferricyanide or PMS as acceptor[51]. Depending on the electron accepting and transfer arrangement in the enzyme (*cf.* footnote 2 of refs. 52 and 46), one might then expect a cycling of oxidation–reduction catalysts in the enzyme every 8–16 msec. The rate of reduction of the components observed in the experiment of Fig. 4 at 22° is compatible with these numbers derived from overall activity assays.

An experiment similar to that illustrated in Fig. 4 was carried out with succinate–coenzyme *Q* reductase*. With 20 mM succinate and 50 μM enzyme the rapid phase

* These experiments were done in collaboration with Dr. D. M. ZIEGLER.

of iron reduction was 60% completed in 6 msec and complete in 60 msec. Semiquinone formation followed a similar course and was maximal at 60 msec. The intensity of the rapidly produced $g = 1.94$ signal was only 10% of that elicited by dithionite.

During the anaerobic reduction of succinate dehydrogenase with D-malate, which has been shown to be a substrate of the enzyme[51], only small signals were observed. The iron and flavin signals developed simultaneously as with succinate. At 16 msec less than 1% of the maximally observed (with succinate) radical or $g = 1.94$ signal is seen and at 2 sec 14% of the radical and 7% of the iron signal. The turnover number of D-malate with succinate dehydrogenase is approx. 1.5 mmoles of D-malate per mole of flavin and per sec at 25° (*cf.* ref. 20). It is only possible to deduce approximate rates from the available EPR data, but the EPR observations are in general agreement with the conclusions drawn from overall rate studies and support the relationship to enzyme activity of the components observed by EPR spectroscopy.

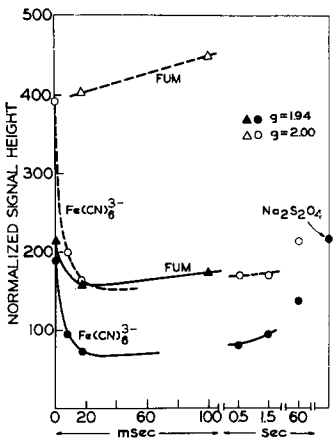
Reoxidation of reduced enzyme

Kinetics with ferricyanide. The enzyme was reduced anaerobically, either with a small quantity of succinate or with NADH and PMS and reoxidation of the enzyme was attempted by ferricyanide, fumarate or O_2.

Fig. 5 shows an experiment with ferricyanide or fumarate as oxidant. Since the enzyme is reoxidized very rapidly and is inactivated with large amounts of ferricyanide, we chose a ferricyanide concentration of 0.5 mM, one tenth of the original succinate concentration. At the concentration used (52 μM), the enzyme will turn over approx. 5 times. From the turnover number at an infinite ferricyanide concentration, it can be concluded that the activated enzyme turns over every 8–10 msec. From the dependency of the reaction rate on both donor and acceptor concentrations for this enzyme[19], it can be calculated that under the conditions of the experiment it takes approx. 250–300 msec for one turnover. Fig. 5 shows that oxidation of the reduced enzyme by ferricyanide to a steady-state level is approx. 85% complete in 8 msec, the first point of measurement. In the steady state observed after 16 msec, the iron is about 35% reduced as compared to the initial reduced enzyme. As expected, after 500 msec very little change in steady-state level is observed; however after 1.5 sec the reduction level starts to increase. In the reduced enzyme 90% of the $g = 1.94$ signal that can be produced by dithionite was observable. This experiment indicates that the reduction of the enzyme is a slower reaction than reoxidation by ferricyanide. Similar observations were made with NADH dehydrogenase[45].

Kinetics with fumarate. Reoxidation by 5 mM fumarate of the iron represented in the signal at $g = 1.94$ is a similarly rapid reaction. The radical signal is slightly increased with fumarate. In view of the ratio of the affinities of succinate and fumarate (K_D succinate/K_i fumarate = 27)[51] for the dehydrogenase it is not surprising that at an equimolar ratio of these substrates reoxidation of the iron does not exceed 25%.

In order to eliminate the competition between succinate and fumarate and to measure what might be the true reoxidation rate by fumarate, in another experiment, the enzyme was anaerobically reduced with 1.5 electron of equiv NADH (in the presence of PMS) per bound flavin and was reoxidized with 5 mM fumarate. This is shown in Fig. 6. It can be seen that reoxidation by fumarate is not a rapid reaction under these conditions. The half-time lies at approx. 1 sec. Unless the low velocity of reoxidation is due to the presence of PMS or NADH, this is an interesting observation



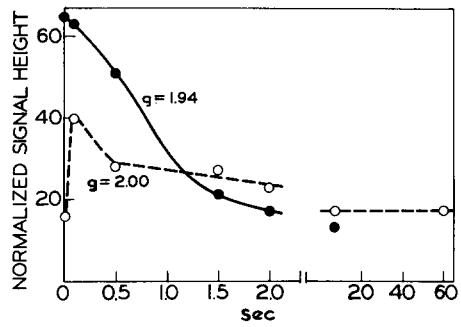


Fig. 5. Time-course of reoxidation by potassium ferricyanide or fumarate (FUM), of enzyme reduced by 10 mM succinate. The conditions of EPR spectroscopy were those of Fig. 2. Symbols of the curves referring to reoxidation by fumarate (5 mM) are: △— — —△, flavin radical; ▲—▲, iron signal. Symbols of the curves describing reoxidation by potassium ferricyanide (0.5 mM) are :○— — —○, flavin radical; ●—●, iron signal.

Fig. 6. Reoxidation by 5 mM fumarate of enzyme reduced by 0.5 equiv of NADH per mole of flavin as in the experiment of Fig. 2. ○— — —○, radical signal; ●—● iron signal. The conditions of EPR spectroscopy were those of Fig. 2.

insofar as it would imply that succinate conditions the enzyme for reoxidation.

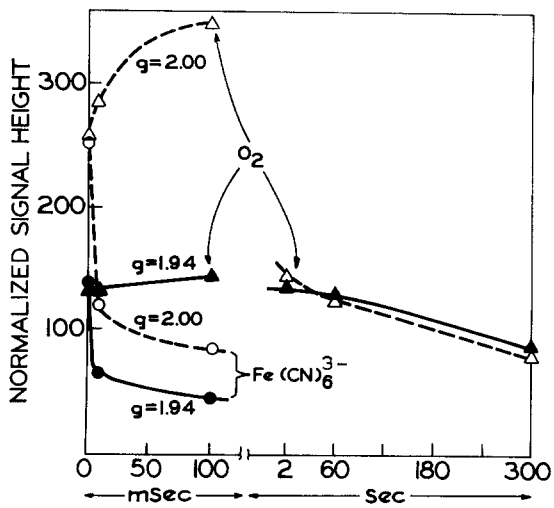
Kinetics with O_2. In a third type of experiment, the enzyme was first reduced anaerobically with an excess of succinate and was then mixed with O_2-saturated buffer. As shown in Fig. 7, O_2 causes first a rise and then a fall of the radical concentration and a decline of the iron signal. This behavior is most easily interpretated as a reoxidation, during which the radical passes through a maximum at the state of partial reduction and then vanishes as oxidation progresses. The decline of these EPR signals in the presence of O_2 has also been observed by KING *et al.*[9].

In the same series of experiments reoxidation by ferricyanide was repeated (Fig. 7) with results analogous to those obtained in Fig. 5.

Changes in signal shape and spin relaxation rate

It was observed that at the earliest times during reoxidation of succinate dehydrogenase, previously reduced with succinate, a change in the shape of the signal occurs in the $g = 2$ region. The effect is more pronounced with ferricyanide than with fumarate as oxidant. An example is shown in Fig. 8. The $g = 2.01$ peak of the iron signal is shifted 5–6 gauss downfield while the flavin signal is shifted by approximately half this value. Samples collected at 100 msec after initiation of reoxidation or later do not show this effect. No unique explanation can be given for this effect. It obviously belongs to the class of subtle changes, previously reported on[25,38,46,53] which include minor, but reproducible, changes in signal shape and electron spin relaxation rates, depending on the type of reactants present and on the time allowed for interaction.

Along these lines differences in electron spin relaxation of the radical signal were also observed with succinate dehydrogenase as they were with other flavo-



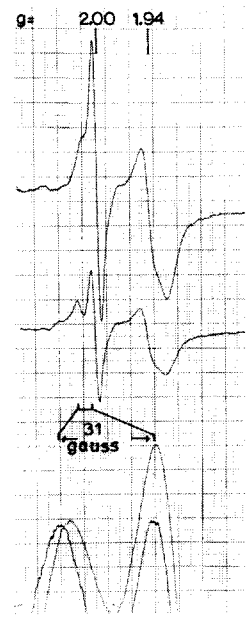


Fig. 7. Effect of O_2 on the EPR signal heights of enzyme reduced by 2.5 mM succinate. An equal volume of buffer saturated with O_2 was mixed with the anaerobically reduced enzyme. The conditions of EPR spectroscopy were those of Fig. 2. △— — —△, flavin radical; ▲—▲, iron signal. In the same experiment reoxidation of the same enzyme by 0.5 mM ferricyanide was repeated. The corresponding curves are: ○— — —○, radical signal; ●—●, iron signal.

Fig. 8. EPR spectra (first derivative) of reduced succinate dehydrogenase before and after partial reoxidation by ferricyanide. The conditions of EPR spectroscopy were: microwave power, 27 mW; modulation amplitude, 12 gauss; scanning rate, 200 gauss per min; and temperature 101° K for the two upper curves and scanning rate, 100 gauss per min; and temperature 82° K for the lowermost curves. The final concentrations of reactants were: enzyme, 8.65 mg/ml (approx. 0.036 mM); succinate, 6 mM; ferricyanide, 1 mM. Uppermost curve: reduced blank; center curve: 8 msec after mixing with ferricyanide; bottom curve: expanded scan (as indicated) of the samples used for the two upper curves at lower temperature, showing details of the shifts of the peaks (see text). The shifts as shown in the figure are subject to a number of inaccuracies of recording, whereas the gauss values given in the text and on the figure were derived from measurements with a proton probe. The latter values should therefore be used for quantitative evaluations.

proteins[25,38,46,54], depending on conditions. The differences were, however, not as pronounced as with aldehyde oxidase[25,52] or dihydroorotate dehydrogenase[46,53]. Particularly, the presence of fumarate or ferricyanide in addition to succinate had little effect, while effects of the time allowed for interaction with succinate were more pronounced.

DISCUSSION

The present work has shown that two of the EPR-detectable components of succinate dehydrogenase, a substance that can occur in the form of a stable free radical, and iron ($g = 1.94$) react at rates compatible with their participation in the catalytic turnover of the enzyme. These two components are thought to be the flavin prosthetic group and an iron–sulfur complex of the kind found in several other nonheme iron proteins[12,14,25,30,45,46,48]. A third EPR-detectable component, namely the high spin

Fe^{3+} represented by the signal at $g = 4.3$, is not considered a catalytically significant component, as will be discussed below.

It is at first sight disturbing that only a small fraction of the iron found in the enzyme by chemical analysis reacts rapidly. The following quantitative balance, derived from Fig. 4, illustrates this. In this experiment the concentration of bound flavin was approx. 0.05 mM so that 8×0.05 mM $=$ 0.4 mM iron should have been present. On reduction with dithionite, 30 μM iron was accounted for in the signal at $g = 1.94$ and 15% of this, or 1% of the total iron present, had reacted at 17 msec. Although this overall balance may at first glance suggest that we are observing a negligible and possibly insignificant fraction of a component of the enzyme, the following considerations are pertinent: (1) The titration experiments of Figs. 2 and 3 show clearly that the total observable signal at $g = 1.94$ is produced by 1 equiv only per mole of enzyme. This information is independent of absolute quantitative evaluation of EPR signals which may involve some unknown errors but depends solely on the amount of reductant added and on the observation of maximal signal size by EPR, which cannot be significantly in error. When the $g = 1.94$ signal obtained with 1 reducing equiv per mole was quantitatively evaluated, between 0.4 and 1 equiv* were accounted for in different experiments. On the basis of this information from the titration experiment, at most the equivalent of one iron atom per flavin is represented in the $g = 1.94$ signal, which brings the fraction of rapidly reacting iron to 8% of that which we can actually follow by EPR spectroscopy. (2) Studies on a variety of proteins containing nonheme iron indicate that the minimum unit required for the appearance of the $g = 1.94$ iron signal on reduction consists of two iron atoms, although only one electron is taken up by this unit. A similar two-iron unit is very likely to be operative in succinate dehydrogenase. The fraction of iron involved in the oxidation–reduction process is therefore in fact twice that apparent from quantitative determination of the signal. (3) An additional point to be raised here concerns the behavior of the iron signal in the reduction experiment of Fig. 4 as compared to the reoxidation experiments of Figs. 5 and 7. It is evident from these experiments (Figs. 5 and 7) that on prolonged exposure (10 min) of the enzyme to succinate over 90% of the $g = 1.94$ signal, which can be produced by dithionite, does appear. On reoxidation with ferricyanide this signal disappears within a few msec (Figs. 5 and 7) and with fumarate a smaller fraction (25%) also disappears rapidly. While it could be argued that ferricyanide is a chemical oxidant, this argument does not apply to fumarate. An explanation for this unusual behavior of the iron signal may be found in the well-known requirement of succinate dehydrogenase for "activation" (*cf.* refs. 22 and 55). It is very difficult if not impossible to have preactivated enzyme under the conditions of concentration needed for EPR spectroscopy, without having either substrate present (in which case the enzyme would be reduced to start with), or an inhibitor (fumarate, malonate, *etc.*). It is easier to circumvent these problems in the usual catalytic assays for enzymes, where substrates or inhibitors can be effectively diluted out and the oxidation state of the enzyme at the outset of the experiment is not relevant, as only turnover is observed, *e.g.*, by product formation or reduction of an acceptor. It might thus be suggested that a fully activated enzyme should have a

* The higher recoveries were obtained in more recent experiments, when an improved temperature and humidity control system allowed us to record spectra at approx. 80° K during all seasons.

larger fraction of rapidly reacting iron complex of the type producing the $g = 1.94$ signal, although we were not able to design experiments to verify this directly.

The maximal free radical in the experiment of Fig. 4 amounted to 11% of the total bound flavin and to approx. 2% at 17 msec at 0.5 mM succinate and 6% at 50 mM succinate. Since it is a feature, to our knowledge only observed with one flavoprotein[33,33], that all the flavin should be present in the semiquinone form, these recoveries appear entirely reasonable.

Two interesting aspects of the reduction experiment are as follows: (1) the rate of formation of the radical and $g = 1.94$ signals is independent of the succinate concentration, in agreement with the catalytic mechanism of ZEYLEMAKER *et al.*[19] and (2) the quantity of radical produced is 3 times higher at the higher succinate concentration. The half-time of appearance of the radical is not affected by the succinate concentration and has within experimental error the same value as that of the appearance of the signal at $g = 1.94$. These observations have a number of implications and are not readily explained in a unique fashion. In work of the kind described here one must always be aware of the possibility that different molecular species may be present and that different signals, which are observed (*e.g.* the radical and $g = 1.94$ signals), do not necessarily originate from the same molecules. Such heterogeneity of species could explain the kinetic behavior observed on reduction (Fig. 4). On the other hand, such behavior can also be explained on the assumption of a homogeneous population of molecules, if a sufficient number of intermediates with a particularly chosen set of rate constants for their interconversion is postulated.

The observation that the quantity of radical formed increases with succinate concentration, whereas the intensity of the $g = 1.94$ signal is independent of succinate concentration, suggests the presence of at least one more intermediate in addition to the ones proposed from kinetic experiments by ZEYLEMAKER *et al.*[19] in the reaction of succinate with the oxidized enzyme. It also militates against the suggestion that the flavin and nonheme iron react in a concerted fashion, simply each one accomodating 1 reducing equiv from an electron pair (hydride ion) transferred from the substrate. An argument against such a suggestion may also be seen in the titration experiment of Fig. 2, which indicated that the iron complex, but not the flavin, retains the first electrons which enter the enzyme. It should be kept in mind, however, that the titration experiments are concerned with an equilibrium situation and not with initial events and that the mechanism of reduction by NADH and PMS does not have to be identical with that by succinate.

The significance of the third EPR-detectable component, namely the high spin Fe^{3+}, with an EPR signal at $g = 4.3$ remains doubtful, although it is present in an amount approximately equivalent to the flavin and the nonheme iron complex that shows the signal at $g = 1.94$ in its reduced state. The reasons for questioning the significance of the high spin ferric component are these: (a) Signals of this kind are rather generally found in inorganic and organic matter and have been seen in many proteins, even in proteins which are not known as iron proteins. (b) The presence of these signals depends on the treatment of proteins during purification and is particularly observed when iron chelators such as EDTA are added or have been added at some stage during purification. Succinate dehydrogenase as used here was prepared in the presence of EDTA and EDTA was also added to all reaction mixtures. We have not attempted in this series of experiments to prepare the enzyme in the absence of

EDTA, but it has been found in previous work that in succinate and NADH dehydrogenase prepared without EDTA by other methods the signals at $g = 4.3$ were very weak (*cf.* ref. 56). (c) The signal at $g = 4.3$ does not respond to reduction by succinate to an extent or at a rate which would clearly indicate that it is a catalytically active component.

It is not certain whether the iron represented in this signal is adventitious iron picked up by the enzyme during manipulations or whether it is a constituent of the enzyme. In either case it is likely that we are dealing here with a ternary protein iron–EDTA complex, as has been suggested previously[56] and has also been postulated by other workers[57].

The experiments of Figs. 5 and 6 show that fumarate does effectively reoxidize the carriers in the enzyme and, at least in the presence of succinate, rapidly reoxidizes them. Fig. 7 relates to the question of whether the electron carriers in succinate dehydrogenase are oxidized by O_2. To our knowledge all flavoproteins are oxidized by O_2 although at low rates in some instances. MASSEY AND SINGER[58] established that soluble succinate dehydrogenase reacted with O_2 very slowly, *viz.*, at 0.02% of the rate shown with PMS. This has been confirmed by HÜFNER *et al.*[59]. On the other hand, a number of apparently contradictory effects have been observed with succinate dehydrogenase in concentrated solutions as they are required in EPR spectroscopy. HOLLOCHER AND COMMONER[39] reported that in the presence of O_2 the maximal radical concentration depended on the ratio of succinate to fumarate, while DERVARTANIAN *et al.*[10] found that under anaerobic conditions, the addition of fumarate of any concentration to a succinate-reduced enzyme caused a decline in both the radical and iron signals, which they ascribed to the formation of a complex between oxidized enzyme and fumarate. VAN VOORST *et al.*[60] observed that when the succinate-reduced enzyme was exposed to O_2, the radical signal declined and the iron signal increased, an effect which was not reversed when the system was again made anaerobic. However, when an enzyme reduced with D-malate was exposed to O_2, there was a complete loss of radical signal with no effect on the iron signal. On the other hand, in the presence of equimolar amounts of succinate and fumarate, exposure to O_2 caused an increase in both the radical and iron signals. GRIFFIN AND HOLLOCHER[61] have confirmed the observations that the EPR signals in the absence of O_2 are more intense and that O_2 causes a decline in the radical signal when succinate is present.

The results of the experiment of Fig. 7, namely an initial increase of the radical signal followed by a decline of both iron and radical signals, are most readily explained by assuming that a slow oxidation takes place, in disagreement with GRIFFIN AND HOLLOCHER[61] who concluded that oxidation was not involved in the signal changes of the kind described above.

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L-AMINO-ACID OXIDASE

I. EFFECT OF pH

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SUMMARY

The effect of pH on K_m and upon the rate constants associated with the reduction of oxidized enzyme by substrate and with the oxidation of reduced enzyme has been examined for the case of purified L-amino-acid oxidase (L-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.2) of Eastern Diamondback Rattlesnake *(Crotalus Adamanteus)*, using L-leucine as a substrate at 25°, 30° and 35°.

The effect of pH on K_m yields two pK's: one in the range 5.7–5.9 corresponding to the enzyme–substrate (ES) intermediate and one in the range 8.2–8.4 corresponding to the free enzyme. The variation of the two sets of pK's with temperature both yield an apparent enthalpy of ionization of 7.5 kcal/mole. These data lead to the conclusion that histidyl is most likely involved in the catalysis at the active center.

The variation of the reduction rate constant k_{obs} with pH parallels the variation of K_m with pH, indicating that the variation of k_{obs} with pH depends on the variation in the availability of ES intermediate with pH.

The oxidation rate constant, k_{ox}, does not vary appreciably with pH, suggesting that the oxidation process proceeds *via* a direct combination of oxygen and reduced form or forms of the enzyme without the mediation of catalytic groups which may be titrated in the range of pH studied.

INTRODUCTION

The effect of pH on the kinetic parameters of an enzyme–substrate system is an important area of study, since it may enable one to gain some insight into the nature of the protein groups participating in the catalytic interactions at the enzyme active center.

L-Amino-acid oxidase (L-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.2) is an important flavin enzyme suitable for the investigation of the effect of pH on appropriate kinetic parameters and is readily available in purified form.

The reaction whose pH dependence is being considered is shown in Eqn. 1, where $[E]_{ox}$ and $[E]_{red}$ are oxidized and reduced forms of the flavoprotein enzyme, respectively.

$$[E]_{ox} + \text{L-leucine} \rightleftarrows \text{intermediate state(s)} \rightleftarrows [E]_{red} + \text{product} \quad (1)$$

Because the enzyme exhibits substrate inhibition, it is necessary to perform such studies of pH dependence at substrate concentrations where inhibition is not encountered[1]. Eqn. 1 represents the sequence in the action mechanism which is rate limiting under the conditions of low substrate concentration where substrate inhibition does not occur. The steps in the reaction are written as equilibria since the overall reaction has been shown to be reversible[2]. The classical *ES* complex is termed an intermediate state in order not to imply any specific number of kinetically distinct bound forms of enzyme and substrate.

MATERIALS AND METHODS

L-Amino-acid oxidase was obtained in purified form from Worthington Biochemical Corp. Purified L-leucine was obtained from Schwartz Bioresearch Corp. The buffers used were 0.05 M Tris–maleate for pH 5.3–8.8 and 0.05 M glycine–NaOH for pH 9.0–9.6. All solutions were 0.2 M in KCl. The pH of all buffers was determined as a function of temperature using a Sargent Model DR pH meter.

The concentration of active enzyme, $[E]_0$, was initially determined using a Cary 14 spectrophotometer by measuring the absorbance difference at 450 nm between the completely oxidized and completely reduced forms of the enzyme arising from the addition of an amount of 0.1 M leucine in great excess of the stoichiometric amount under anaerobic conditions. An approximate (maximal) molar absorbance coefficient for the absorbance difference between oxidized and reduced forms of the enzyme was taken to be $2.2 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ at 450 nm, assuming two FAD groups per active enzyme molecule[3]. The total active enzyme concentration determined in this manner was related to the slope of the straight line obtained at each temperature studied when $\log [O_2]_t$ was plotted *vs.* t for the reaction of enzyme and 0.100 M leucine in Tris–maleate buffer (pH 7.4) initially saturated with oxygen. Under these conditions the overall kinetics are very nearly first-order in oxygen and constitute a convenient way to determine the active enzyme concentration before each experiment with reference to that of the spectrophotometrically standardized enzyme. This procedure was necessary because the concentration of active enzyme decreased slowly from day to day during storage at 4°.

The rate of oxygen uptake was measured using a biological oxygen monitor (Yellow Springs Instrument Co.). The total volume of reaction mixture was 3 ml. Leucine solutions in buffer were saturated with oxygen at 25°, 30° or 35°. At pH values less than 6.5, the reaction was sufficiently slow that it was necessary to correct the primary data for oxygen leakage from the cell assembly of the oxygen monitor and for oxygen consumption by the polargraphic oxygen sensor.

The enzyme preparations used did not contain any detectable amount of catalase. Experiments were performed at several pH values using $8.0 \cdot 10^{-4}$ M leucine dissolved in buffer solutions initially saturated with oxygen at 25°, 30° and 35°. At all three temperatures, the initial concentration of oxygen was greater than the initial substrate concentration. It was found that, within experimental error, a stoichiometric amount of oxygen was consumed during the course of the reaction. If catalase were present in significant amount, then the amount of oxygen consumed would have been less than $8.0 \cdot 10^{-4}$ M.

All velocity measurements were made at an oxygen percent saturation corresponding to $8.6 \cdot 10^{-4}$ M at each temperature. K_m values were determined from Lineweaver–Burk plots. The substrate concentrations were chosen to give the best intervals of $1/[S]$ in the range where substrate inhibition did not occur. This range varied from 0.1 M or less (pH 5.3) to $1.5 \cdot 10^{-3}$ M or less (pH 9.6).

The apparent enthalpy of ionization corresponding to each set of pK values was calculated from a simple Van 't Hoff plot of log K *vs.* $1/T$ by multiplying the slope by the factor $-2.3\,R$.

The second-order forward rate constant, k_{obs}, associated with the reduction of oxidized enzyme by leucine was determined from experiments in which the initial substrate concentration was nearly equal to the initial oxygen concentration. Assuming the active enzyme concentration, $[E]_0$, is approximately equal to the steady-state concentration of the oxidized form of the enzyme, a plot of ln $[S]_t$ *vs.* t gives a straight line with slope $-k_{obs}\,[E]_0$, where $[S]_t = [S]_{t=0} - ([O_2]_{t=0} - [O_2]_t)$.

The second-order forward rate constant, k_{ox}, associated with the overall reaction at substrate concentrations of approx. 0.05 M or greater where the reoxidation of the reduced form or forms of the enzyme determines the overall reaction rate, was determined at 38° using 0.1 M leucine in buffers initially saturated with oxygen. Recent work by MASSEY AND CURTI[4] suggests that substrate inhibition in this system can be explained by invoking an oxidation step of a reduced enzyme–amino acid complex which is slower than the reoxidation of other reduced enzyme species. If such is the case, then k_{ox} would correspond to the specific rate of the reoxidation of this reduced enzyme–amino acid species. At pH values greater than 6.5, a plot of ln $[O_2]_t$ *vs.* t was initially linear. At pH values less than 6.5, the first-order plots exhibited a downward curvature, and the tangent to the curve at time zero was taken as an estimate of the slope for the purpose of comparison with values at pH values greater than 6.5. The slope of the first-order plot at each pH was taken to be equal to $-k_{ox}\,[E]_0$.

The rate constants, k_{obs} and k_{ox}, are second-order rate constants in that they correspond to the reaction of enzyme *plus* substrate, either leucine or oxygen, respectively. Since the total enzyme concentration is a constant during the course of a given experiment, the overall kinetics appear to be pseudo-first-order in substrate.

RESULTS

Effect of pH on K_m

Figs. 1, 2 and 3 show the effect of pH on pK_m for leucine at 25°, 30° and 35°, respectively. Each solid curve was calculated using Eqn. 2 (ref. 5) and pK values which gave the best agreement with the experimental observations.

$$pK_m = \text{constant} + \log\left[1 + \frac{H^+}{K_{ES}}\right] - \log\left[1 + \frac{H^+}{K_E}\right] \qquad (2)$$

Table I tabulates these pK values. Useful data beyond pH 9.6 were not obtainable under these experimental conditions, since the enzyme rapidly loses activity at higher pH. At pH values lower than 5.3 the reaction rate becomes too slow to be measured accurately with the apparatus used.

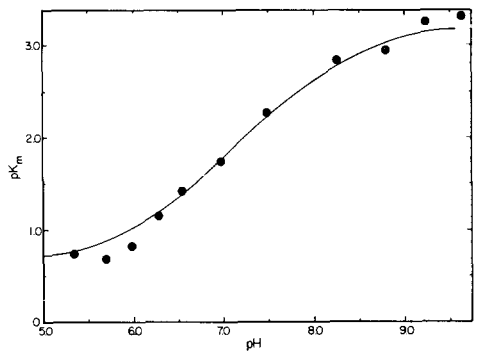

Fig. 1. pH dependence of $-\log K_m$ or pK_m of the L-amino-acid oxidase catalyzed reaction of L-leucine at 25°. Each point was obtained from a Lineweaver–Burk plot.

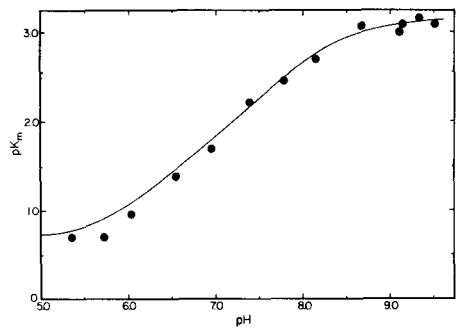

Fig. 2. pH dependence of pK_m of the L-amino-acid oxidase catalyzed reaction of L-leucine at 30°. Each point was obtained from a Lineweaver–Burk plot.

Effect of pH on k_{obs}

The effect of pH on the forward rate constant of the reaction studied is shown in Fig. 4 for the three temperatures used. Within the precision of k_{obs}, the curves are parallel and have the same general features of the pK_m *vs.* pH curves.

Effect of pH on k_{ox}

Fig. 5 shows the variation of k_{ox} with pH. Within the pH range studied there seems to be little change in the second-order rate constant associated with the oxidation of reduced enzyme.

TABLE I

pK VALUES DETERMINED KINETICALLY WITH PURIFIED L-AMINO-ACID OXIDASE AND L-LEUCINE AT 25°, 30° AND 35°

The given pK values are considered accurate to ± 0.10 pK unit. Values beyond this latitude did not permit a satisfactory theoretical representation of the experimental data. Moreover, the pK_m *vs.* pH curves indicate a consistent displacement of 0.1 pK unit for each 5° temperature interval, thus giving added weight to the accuracy of the calculated enthalpy of ionization.

	pK values at		
	25°	*30°*	*35°*
ES intermediate	5.90	5.80	5.70
Free enzyme	8.40	8.30	8.20

DISCUSSION

A theoretical treatment of the effect of pH on enzyme–substrate affinity has been discussed in detail by DIXON[6] and by DIXON AND WEBB[7]. According to theory, the observed pH effect on K_m and the maximal velocity V can be interpreted in terms of the pK values of groups situated in the free enzyme, in the free substrate and in the enzyme–substrate intermediate state *ES*. The theory of DIXON states that a plot of

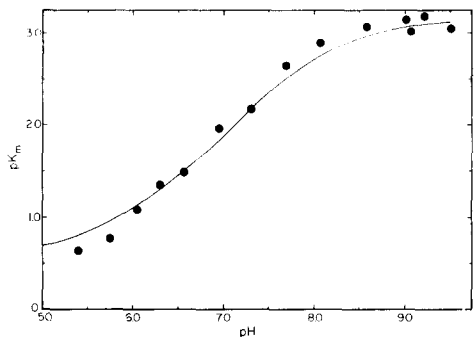

Fig. 3. pH dependence of pK_m of the L-amino-acid oxidase catalyzed reaction of L-leucine at 35°. Each point was obtained from a Lineweaver–Burk plot.

pK_m *vs.* pH will be composed of straight line segments having integral slopes connected by short curved segments which indicate the pK of an ionizing group in one of the components of the components of the system. An upward bend in the curve is produced by each pK of a group situated in the *ES* intermediate. Each pK of a group in the free enzyme or free substrate produces a downward bend.

The graphs of pK_m *vs.* pH exhibit changes generally as predicted by theory; the calculated curves (solid lines in Figs. 1–3) describe the behavior of the experimental data quite well. The upward bend in acid pH for each of the curves corresponds to the pK of a group situated in the *ES* intermediate. The experimental curve appears to break more sharply at acidic pH values than is predicted theoretically for a simple

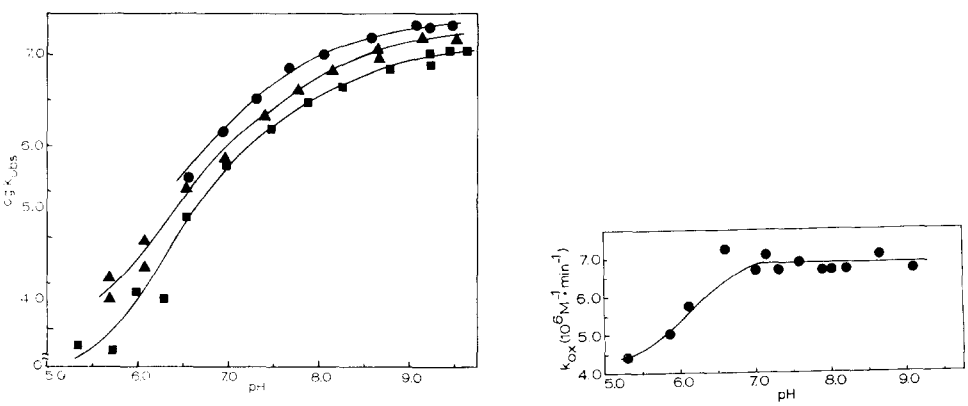

Fig. 4. pH dependence of log k_{obs}, the second-order forward rate constant associated with the reduction of oxidized L-amino-acid oxidase by L-leucine at 25° (■—■), 30° (▲—▲) and 35° (●—●). Each point at 25° was determined from the slope of a plot of ln $[S]_t$ *vs.* t determined from oxygen uptake data using $1.2 \cdot 10^{-3}$ M leucine in buffers initially saturated with oxygen at 25° ($[O_2]_0 = 1.2 \cdot 10^{-3}$ M). Data at 30° and 35° were determined in a similar manner (see MATERIALS AND METHODS) using $1.0 \cdot 10^{-3}$ M leucine in buffers initially saturated with oxygen at the temperature studied.

Fig. 5. pH dependence of k_{ox}, the pseudo-second-order rate constant associated with the oxidation of reduced L-amino-acid oxidase at 38°. Each point was obtained from the slope of a plot of ln $[O_2]_t$ *vs.* t as determined from oxygen uptake data using 0.10 M L-leucine in buffers initially saturated with oxygen at 38°.

ionization of a single group. This might be due to the influence of other charged groups near the group responsible for the measured pK.

The variation of these pK values with temperature yield an enthalpy of ionization of 7.5 kcal/mole for the group situated in the ES intermediate. The unit slope of the rising straight segments of the curves indicates a $+1$ change of charge when the ES intermediate dissociates into free enzyme and free substrate. This process would correspond to: $(ES)^n \rightleftarrows E^{n+1} + S$. Since leucine would be in the zwitterionic form throughout the pH range under consideration and since the free enzyme appears to have a pK at 8.2–8.4, the example above is a reasonable representation of the actual process of dissociation.

The downward bend in basic pH corresponds to the pK of a group in the free enzyme. These pK values yield an enthalpy of ionization of 7.4 kcal/mole. It is unlikely that these pK values are associated with an ionizable group of free leucine since they are more than one pK unit too low for the pK of the amino acid ammonium group (which is 9.6). Furthermore, the enthalpy of ionization of such an ammonium group is on the order of 11 kcal/mole, which is significantly greater than that which is observed in the present case[8]. Curves of the nature of Figs. 1–3 generally correspond to an ionizing group not directly involved in the bonding between enzyme and substrate in the ES intermediate, whose pK value in the free state is perturbed to a different pK value in the different environment of the ES intermediate. In the present case, the ionizing group in the ES intermediate has a pK of 5.7–5.9. As the intermediate dissociates to give free enzyme and free substrate in the pH range 6–8, the pK_m *vs.* pH curves indicate that a group of the free enzyme gains a positive charge. The group of the free enzyme corresponding to the pK values of 8.2–8.4 is most likely this group. After being freed from the influence of substrate, the pK values of 8.2–8.4 would render this group capable of gaining a positive charge in the pH range of 6–8. Therefore, the two sets of pK values observed in the present case most likely correspond to the same group in different environments.

Although the observed ΔpK of 2.5 units between pK_{ES} and pK_E is large, it is not unreasonable to assign the same group in different charge environments as being responsible for both. Since the enzyme exhibits reversible inactivation in the absence of mono-negative anions, it was necessary to perform all experiments in the presence of 0.2 M Cl^-. It is conceivable that chloride binding at the active site could account for the rather high pK assigned to histidyl in the free enzyme active center, the effect of the negatively charged species being to increase the pK of the imidazole group of histidyl. The proximity of the dipolar amino acid zwitterion to the imidazole side chain of a histidyl residue in the ES intermediate could have a profound effect on the pK of the imidazole group in comparison to the same group in the free enzyme. It is well known that the adjacency of a positively charged body to an ionizable group can lower its pK_a drastically. For example, the pK_a of free imidazole has been determined[9] to be 6.95. Substitution of an 2-ethylammonium side chain in the 4(5) position results in a depression[10] of the pK_a to 5.78*.

The pK values of 5.7–5.9 for a group in the ES intermediate and of 8.2–8.4 for a group in the free enzyme, together with the fact that both groups exhibit apparent enthalpies of ionization of 7.5 kcal/mole, all lead to the conclusion that histidyl is a

* A differentiation between electrostatic and inductive effects is not possible with these data but it is worth noting that the pK_a of 4(5)-methylimidazole is perturbed upwards[9] to 7.52.

group which is probably involved with the catalytic interactions at the active center. From a consideration of the reversible inactivation process exhibited by L-amino-acid oxidase, KEARNEY AND SINGER[11] have postulated earlier that histidyl might be present at the active center. The present study serves to strengthen this hypothesis.

DE KOK AND VEEGER[12] recently found a group with an apparent pK of 7.8 in the free form of L-amino-acid oxidase which participates in the binding of the inhibitor *o*-aminobenzoate. They suggested the group might be a histidyl group. In view of the close similarity between the pK value which they observed and those reported for the free enzyme in the present work, it is probable that the same group, possibly a histidyl, is involved in the binding of the inhibitor and of the substrate.

The variation of log k_{obs} with pH, as shown in Fig. 4, indicates that the specific rate of the overall reaction parallels the availability of the intermediate form of enzyme and bound substrate as a function of pH. This suggests that effects on binding rather than base catalysis may explain the overall pH dependence of the forward reaction (Eqn. 1), but this point deserves further investigation.

The variation of k_{ox} with pH, as shown in Fig. 5, shows that the oxidation process in the action mechanism seems to be insensitive to changes in pH. The slight downward trend in the curve at low pH is probably a manifestation of the fact that the absolute rate of the reduction step becomes less than that of the oxidation process due to the great decrease in k_{obs} at the low end of the pH range.

The application of the theory of DIXON appears to successfully explain the pH behavior of the L-amino-acid oxidase–L-leucine kinetic system. In view of the variable pH dependence of the D-amino-acid oxidase system with different substrates[13], it should be noted that there is a possibility that the present results may not necessarily represent the pH behavior of L-amino-acid oxidase with amino acids other than leucine. In particular, one might not expect the value of pK_{ES} to be the same with substrates other than leucine. However, the probable involvement of a histidyl residue in the binding of at least one substrate leads to additional interesting experiments on solvent isotope effects, on histidine photooxidation experiments and with possible specific irreversible inhibitors. Further studies are in progress on this system to investigate the nature of the elementary processes involved in the reduction of oxidized enzyme by substrate.

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OCTOPINE DÉSHYDROGÉNASE

PURIFICATION ET PROPRIÉTÉS CATALYTIQUES

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SUMMARY

Octopine dehydrogenase: Purification and catalytic properties

1. Octopine dehydrogenase, an NAD^+ enzyme, which catalyzes the dehydrogenation of octopine into arginine *plus* pyruvate, has been purified from muscles of *Pecten maximus*. It is homogeneous on analytical ultracentrifugation and disc electrophoresis.

2. The enzyme contains no such metal cofactor as Fe^{2+}, Mn^{2+}, Zn^{2+}.

3. The Michaelis constants are: $1.5 \cdot 10^{-3}$ M for octopine, arginine and pyruvate, $1.5 \cdot 10^{-4}$ M for NAD^+ and $4 \cdot 10^{-5}$ M for NADH.

4. Substrate analogs with a guanidyl (guanidinobutane) or a carboxyl group (valeric acid) or both of them (δ-guanidinovaleric acid) are competitive inhibitors of the octopine forming reaction. In the dehydrogenation reaction, compounds with only one of these groups are competitive inhibitors whereas those possessing both guanidyl and carboxyl groups are not.

INTRODUCTION

L'octopine déshydrogénase, extraite du muscle de certains invertébrés marins, est un enzyme à NAD^+, catalysant la réduction réversible du produit de condensation chimique de l'arginine et du pyruvate[1-4].

Chez les invertébrés renfermant de l'octopine déshydrogénase dans leurs muscles, la lactate déshydrogénase musculaire est très peu active ou totalement absente[4,5]. Il serait donc possible que, chez ces invertébrés, le pyruvate provenant de la glycolyse assure, principalement avec l'arginine libérée de la phosphoarginine et grâce à l'octopine déshydrogénase, la formation de l'octopine, la synthèse de l'acide lactique devenant tout à fait secondaire. L'octopine déshydrogénase jouerait le rôle de la lactate déshydrogénase dans le métabolisme de l'acide pyruvique.

D'autres travaux ont mis en évidence, dans les tissus végétaux cancéreux, l'existence de l'octopine déshydrogénase[6,7] et d'un enzyme catalysant le même type de réaction avec la lysine ou l'ornithine et le pyruvate, produisant respectivement la

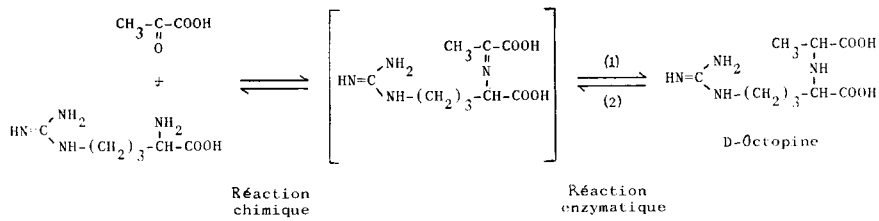


Fig. 1. Réaction catalysée par l'octopine déshydrogénase.

lysopine et l'acide octopinique[6-8]. On pourrait en rapprocher aussi la formation enzymatique de saccharopine, formée par condensation de la lysine (par son groupement ε-NH_2) avec l'α-cétoglutarate dans les tissus animaux[9,10] ou chez les micro-organismes[11]. Cette réaction pourrait, d'après les auteurs, jouer un certain rôle dans le métabolisme de la lysine.

L'octopine déshydrogénase semble donc faire partie d'un nouveau groupe de déshydrogénases, toutes caractérisées par le même type de substrat, une base de Schiff, et conduisant toutes au blocage d'un produit essentiel de métabolisme, le pyruvate ou l'α-cétoglutarate.

Il nous a semblé intéressant d'approfondir le mécanisme d'action de l'octopine déshydrogénase et, éventuellement, de la comparer avec les autres déshydrogénases du même type. Nous avons donc entrepris sa purification à partir des muscles de *Pecten maximus* L. et abordé l'étude de la réaction enzymatique.

MATÉRIEL ET MÉTHODES

Produits utilisés

Les acides gras ou aminés ou α-cétoniques et les chélateurs sont des produits commerciaux, recristallisés au besoin.

Le guanidinobutane et l'acide δ-guanidinovalérique sont préparés par amidination de l'amine ou de l'acide δ-aminé correspondant au moyen de la *S*-méthylisothiourée[12].

L'octopine a été synthétisée par la méthode de IZUMIYA *et al.*[13].

NAD^+ et NADH (Calbiochem, grade A) sont utilisés tels quels. Leur concentration est déterminée d'après leur coefficient d'extinction molaire respectif: $18 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ à 260 mμ pour NAD^+ et $6.22 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ à 340 mμ pour NADH.

Les gels de Sephadex G-100 et de DEAE-Sephadex A-50 sont préparés et régénérés d'après les indications du fabricant (Pharmacia, Uppsala). Le gel de phosphate de calcium est préparé d'après TISELIUS *et al.*[14].

Mesure de l'activité enzymatique

Celle-ci est déterminée spectrophotométriquement d'après l'apparition ou la disparition de NADH, suivant le sens de la réaction.

Les mesures sont effectuées à 33° et à 340 mμ, au spectrophotomètre Unicam SP 800, avec un enregistreur Electro-synthèse P-62 permettant d'apprécier les variations d'absorbance à 0.001 unité près. Les variations sont linéaires pendant au moins 1 min d'enregistrement.

L'enzyme est ajouté au milieu réactionnel, préincubé pendant 5 min à 33° et dont la composition est la suivante: octopine $6 \cdot 10^{-3}$ M, NAD^+ $1 \cdot 10^{-3}$ M, tampon glycine–soude 0.1 M et pH 9.8 pour la Réaction 2; L-arginine $4 \cdot 10^{-3}$ M, pyruvate de sodium $4 \cdot 10^{-3}$ M, NADH $2.5 \cdot 10^{-4}$ M, tampon phosphate 0.1 M et pH 6.6 pour la Réaction 1.

Dans les expériences d'inhibition par les analogues de substrat ou par les chélateurs, le produit est ajouté au milieu réactionnel avant la préincubation.

L'unité enzymatique correspond à la quantité d'enzyme qui, dans les conditions de mesure adoptées, provoque l'apparition ou la disparition d'1 μmole de NADH en 1 min.

Mesure des protéines

Celles-ci sont évaluées spectrophotométriquement d'après les absorbances mesurées à 280 et 260 mμ, suivant la formule de Warburg et Christian[15].

Ultracentrifugation

Elle a été réalisée au laboratoire de Mme Filitti-Wurmser.

Electrophorèse préparative sur gel de polyacrylamide

Nous avons utilisé l'appareil "Poly-Prep" de Buchler et la technique décrite par ORNSTEIN ET DAVIS[16,17] et JOVIN *et al.*[18].

La migration des protéines vers l'anode s'effectue dans un système discontinu de tampons alcalins, le tampon supérieur étant Tris–glycine 0.052 M (pH 8.9), le tampon inférieur et celui d'élution Tris–HCl 0.1 M (pH 8.1).

À titre d'exemple: pour 57 mg de protéines, nous avons utilisé 60 ml de gel de résolution (7.5% d'acrylamide) polymérisé chimiquement et 12 ml de gel de concentration (2.5% d'acrylamide) photopolymérisé. La protéine est dissoute dans 9 ml de tampon Tris–phosphate 0.059 M (pH 7.2) contenant dithiothréitol 0.1 mM, sucrose 3% et 0.1 ml de bleu de bromophénol 0.01%. Un courant de 50 mA sous tension de 250 V a été appliqué. L'appareil d'électrophorèse a été relié à un collecteur de fractions permettant de recueillir des fractions de 3 ml. L'élution s'effectuait à la vitesse de 0.6 ml/min. L'enzyme est apparu 60 min après la sortie du bleu de bromophénol.

Electrophorèse analytique sur gel de polyacrylamide

Nous avons utilisé l'appareil Acrylophor (Pleuger, Wijnegem, Belgique) et la méthode, légèrement modifiée, d'ORNSTEIN ET DAVIS[16,17], adaptée par REISFELD *et al.*[19].

L'électrophorèse s'effectue sur des colonnes de gel de polyacrylamide de 0.6 cm de diamètre et 3.5 cm de longueur. La colonne de gel est composée de deux couches superposées: l'inférieure consiste en 0.85 ml de gel de résolution et la supérieure en 0.15 ml de gel de concentration dont la composition est celle utilisée pour l'électrophorèse préparative.

L'échantillon de protéine de 5–50 μg contenant du sucrose 5–10% est déposé au sommet du tube ainsi qu'une goutte de solution de bleu de bromophénol 0.001%. Le système de tampons discontinu est également celui de l'électrophorèse préparative. Un courant d'environ 3 mA par tube, sous une tension de 80 V, est appliqué pendant 1 h.

Après la migration, les colonnes de gel sont placées pendant 30 min dans le bain de coloration composé de 6 g d'Amidoschwarz par l d'acide acétique 7%. La décoloration s'opère également par électrophorèse dans l'acide acétique 7% avec un courant de 7 mA par tube et sous tension de 80 V.

Purification de l'octopine déshydrogénase

A. *Extrait brut.* Les muscles adducteurs de Pecten sont disséqués, lavés à l'eau distillée et congelés à −20°. Ils peuvent être gardés ainsi plusieurs mois. Ils sont ensuite coupés en morceaux et homogénéisés avec de l'eau distillée glacée, par portions de 700 g de muscles dans 3 fois leur poids d'eau. Après une agitation de 30 min à 4°, la suspension est centrifugée 30 min à 27 700 × *g*. Le surnageant est gardé et le résidu musculaire réextrait avec 1 fois 1/2 son poids d'eau distillée à 4° pendant 30 min. Après une centrifugation de 30 min à 27 700 × *g*, le surnageant réuni au premier extrait constitue l'extrait brut. Toutes les opérations ultérieures sont conduites à 4°.

B. *Fractionnement au $(NH_4)_2SO_4$.* L'extrait brut est additionné à froid de 288 g de $(NH_4)_2SO_4$ cristallisé par l de solution et ajusté à pH 7.5. Après 30 min d'agitation, le précipité formé est éliminé par centrifugation de 30 min à 16 300 × *g*. Le surnageant est ensuite additionné de la même façon de 237 g de $(NH_4)_2SO_4$ cristallisé par l de solution. Après une nuit à 4°, le précipité est recueilli par centrifugation à 16 300 × *g*, puis dissous dans du tampon phosphate $5 \cdot 10^{-3}$ M, EDTA $1 \cdot 10^{-4}$ M (pH 6), à raison de 150 ml pour 700 g de muscles traités. Ceci constitue la Fraction B.

C. La Solution B est dialysée 20 h contre 16 l du même tampon. L'abondant précipité blanchâtre est éliminé par centrifugation pendant 5 h à 75 000 × *g*. Le surnageant constitue la Fraction C.

D. *Traitement par le gel de phosphate de calcium.* La Fraction C est ajustée à pH 7.0 avec NH_4OH diluée et traitée par une quantité de gel de TISELIUS *et al.*[14] correspondant à 5 fois le poids de protéines. L'enzyme n'est pas adsorbé. Après centrifugation à 16 300 × *g*, le surnageant est réservé et le gel lavé une fois avec le tampon phosphate $5 \cdot 10^{-3}$ M (pH 7). Les surnageants réunis constituent la Fraction D, qui est ensuite précipitée par 600 g de $(NH_4)_2SO_4$ par l de solution et additionnée de dithiothréitol à une concentration finale de $1 \cdot 10^{-5}$ M.

À ce stade et sous forme de suspension en solution concentrée de $(NH_4)_2SO_4$, l'enzyme est suffisamment stable pour être conservé à 4° et accumulé en attendant d'être soumis aux opérations ultérieures de purification.

E. *Chromatographie sur DEAE-Sephadex.* La Fraction D résultant de 4 traitements successifs de 700 g de muscles congelés est centrifugée. Le précipité est dissous dans 160 ml de tampon Tris–HCl $2 \cdot 10^{-2}$ M, arginine $1 \cdot 10^{-3}$ M, EDTA $1 \cdot 10^{-4}$ M et dithiothréitol $1 \cdot 10^{-5}$ M (pH 7.5), contenant NaCl $5 \cdot 10^{-2}$ M. L'enzyme est ensuite dialysé une nuit contre le même tampon. Après la dialyse, la solution contenant 6230 mg de protéines est amenée à environ 18 mg de protéines par ml. Elle est partagée en 2 portions de 174 ml chacune, qui sont filtrées simultanément sur deux colonnes (62 cm × 3.2 cm) de DEAE-Sephadex, préalablement équilibrées avec le même tampon. Les colonnes sont développées par 260 ml du même tampon et lavées avec 3 fois leur volume de ce tampon additionné de NaCl $1 \cdot 10^{-1}$ M. Une grande quantité de protéines inactives est ainsi éliminée. L'enzyme est ensuite élué à la

vitesse de 60 ml/h par le même tampon, additionné de NaCl $1.8 \cdot 10^{-1}$ M. Les fractions ayant une activité spécifique supérieure à 300 sont réunies et concentrées par dialyse contre une solution saturée de $(NH_4)_2SO_4$ renfermant EDTA $1 \cdot 10^{-4}$ M, dithiothréitol $1 \cdot 10^{-5}$ M et arginine $1 \cdot 10^{-3}$ M (pH 7).

F. *Chromatographie sur Sephadex G-100.* La Fraction E est centrifugée et le précipité dissous dans 7.5 ml de tampon Tris–HCl 50 mM, KCl 0.1 M, arginine $1 \cdot 10^{-3}$ M, EDTA $1 \cdot 10^{-4}$ M, dithiothréitol $1 \cdot 10^{-5}$ M (pH 7.5). La solution est filtrée sur une colonne (86 cm × 2.8 cm) de Sephadex G-100, préalablement équilibrée avec le même tampon. Le filtrat est recueilli par portions de 5 ml à raison de 30 ml/h. Les fractions ayant une activité spécifique d'environ 900 sont réunies. On ajoute à la solution NAD^+ $5 \cdot 10^{-3}$ M et on précipite par 600 mg/ml de $(NH_4)_2SO_4$.

RÉSULTATS

Purification de l'enzyme

Le Tableau I rend compte des différentes étapes de la purification et de son rendement. À partir de 2800 g de muscles de Pecten on peut obtenir environ 50 mg d'enzyme pratiquement pur.

TABLEAU I

PURIFICATION DE L'OCTOPINE DÉSHYDROGÉNASE DE *P. maximus*

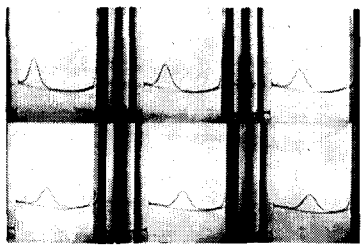
Fractions	*Protéines totales (mg)*	*Activité enzymatique*		*Rendement (%)*
		Unités enzymatiques totales	*Unités enzymatiques par mg*	
A. Extrait brut		386 100		100
B. Fractionnement au $(NH_4)_2SO_4$	37 050	364 000	10.2	94.5
C. Dialyse et centrifugation prolongée	11 160	318 000	28.5	82.5
D. Gel de phosphate de calcium	6 250	268 000	43	70
E. DEAE-Sephadex	490	183 500	375	47.5
F. Sephadex G-100	50	45 500	910	12

L'analyse à l'aide de l'ultracentrifugation, effectuée sur la Fraction F utilisée à la concentration de 10 mg/ml, permet de constater l'homogénéité de la protéine (Fig. 2).

Au contraire, l'électrophorèse analytique de la même fraction (Fig. 3B) montre la présence d'une trace d'impureté à côté d'une bande protéique principale. L'impureté est éliminée après électrophorèse préparative dans l'appareil Poly-Prep de Buchler (voir MATÉRIEL ET MÉTHODES) et on obtient une fraction enzymatique d'activité spécifique = 1000 (Fig. 3C).

Recherche d'un cofacteur métallique éventuel

Un certain nombre de déshydrogénases contiennent du zinc qui joue un rôle à la fois catalytique et structural (alcool déshydrogénases de levure et de foie de cheval,



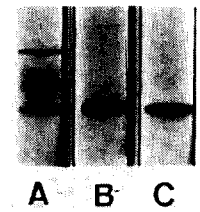


Fig. 2. Ultracentrifugation analytique de l'octopine déshydrogénase purifiée. Tampon phosphate 0.1 M (pH 7.4); protéines: 10 mg/ml; vitesse de régime: 59 780 tours/min; température: 4°; prises de vue après 96, 128, 160, 176, 192 et 200 min.

Fig. 3. Electrophorèse sur gel de polyacrylamide de l'octopine déshydrogénase (0.020 mg de protéines) (A) après DEAE-Sephadex; (B) après Sephadex G-100; (C) après électrophorèse préparative sur gel de polyacrylamide.

glutamodéshydrogénase). Nous avons donc entrepris la recherche d'un cofacteur métallique éventuel dans l'octopine déshydrogénase.

Nous avons d'abord essayé l'action inhibitrice de différents chélateurs de métaux. Comme l'indique le Tableau II, l'action de ces corps, même à des concentrations relativement élevées, est assez faible. Les inhibitions les plus nettes ont été obtenues avec le zincon, le diéthyldithiocarbamate de sodium et le NaN_3.

Les essais d'inactivation par dialyse contre l'*o*-phénanthroline $2 \cdot 10^{-3}$ M ou le diéthyldithiocarbamate de sodium $1 \cdot 10^{-3}$ M n'ont pas donné de résultats.

TABLEAU II

INHIBITION INSTANTANÉE DE L'OCTOPINE DÉSHYDROGÉNASE PAR DES CHÉLATEURS

Inhibiteurs	*Concn. (M)*	*Inhibition (%)*
α,α'-Dipyridyl	$2.5 \cdot 10^{-2}$	10
EDTA	$8 \cdot 10^{-2}$	7.5
	$1.6 \cdot 10^{-1}$	19
o-Phénanthroline	$5 \cdot 10^{-3}$	8
	$9 \cdot 10^{-3}$	19
Zincon	$5 \cdot 10^{-4}$	30
Diéthyldithiocarbamate	$1 \cdot 10^{-2}$	10
	$2 \cdot 10^{-2}$	22
	$3 \cdot 10^{-2}$	47
NaN_3	$2 \cdot 10^{-1}$	33
	$4 \cdot 10^{-1}$	58
	$8 \cdot 10^{-1}$	86

Une analyse par spectrophotométrie d'absorption atomique effectuée sur l'enzyme pur n'a pas permis de trouver des quantités appréciables de Zn^{2+}, de Cu^{2+} ou de Fe^{2+}. Des traces seulement de ces métaux ont pu être décelées.

Détermination des constantes de Michaelis

Dans la réaction de déshydrogénation (Réaction 2), les courbes de saturation de l'enzyme par l'octopine (Fig. 4A) ou par NAD^+ (Fig. 4B) ont fourni pour valeurs respectives de K_m $1.5 \cdot 10^{-3}$ M dans le premier cas et $1.5 \cdot 10^{-4}$ M dans le second.

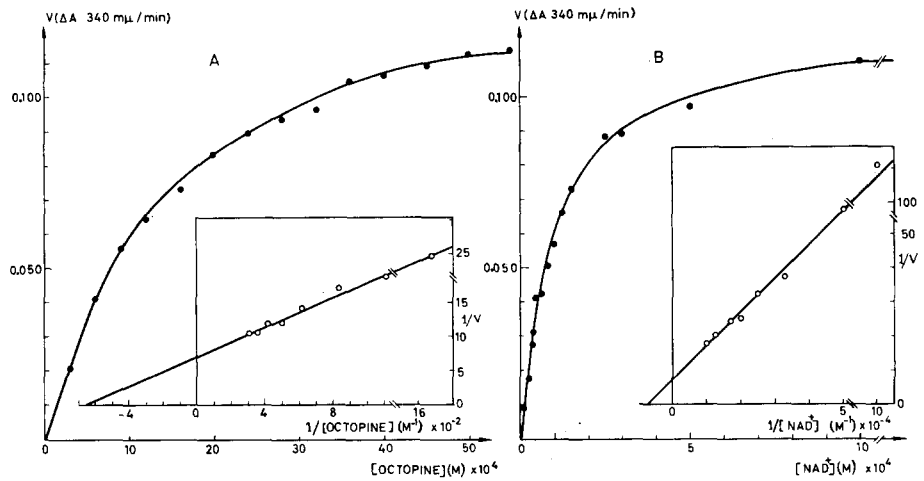


Fig. 4. Déterminations de K_m pour la Réaction 2. (A) Concentrations croissantes d'octopine. (B) Concentrations croissantes de NAD^+.

Dans la réaction d'hydrogénation (Réaction 1), le véritable substrat étant le produit de condensation de L-arginine et de pyruvate[3], la représentation graphique de la saturation de l'enzyme par leurs mélanges équimoléculaires donne des courbes sigmoïdes (Fig. 5), expression d'une réaction bimoléculaire.

Les courbes n'affectent le type michaelien que lorsqu'elles sont tracées en fonction des quantités croissantes de l'un des constituants du mélange arginine *plus* pyruvate en présence d'une concentration saturante de l'autre ($4 \cdot 10^{-3}$ M). Les

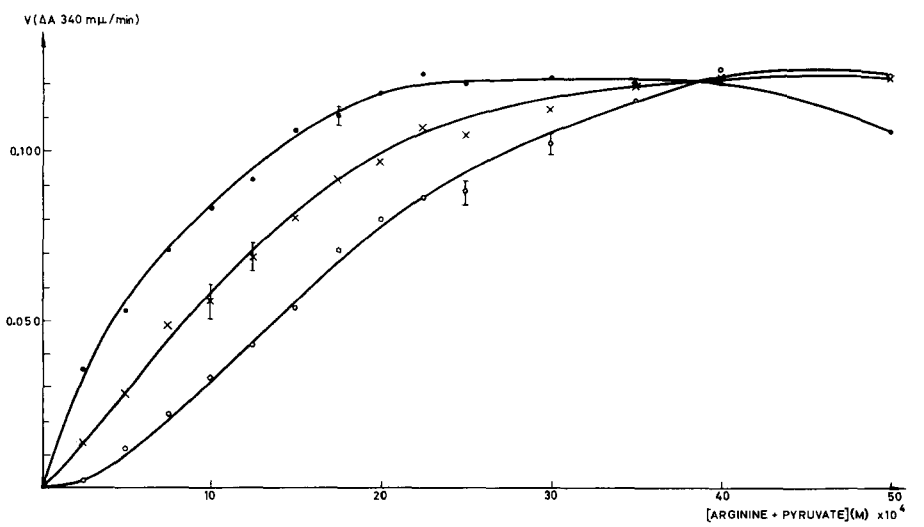


Fig. 5. Courbe de saturation de l'octopine déshydrogénase par des mélanges équimoléculaires arginine *plus* pyruvate (○ — ○); les mêmes mélanges en présence de l'arginine $2 \cdot 10^{-3}$ M (× — ×) et en présence de l'arginine $6 \cdot 10^{-3}$ M (● — ●).

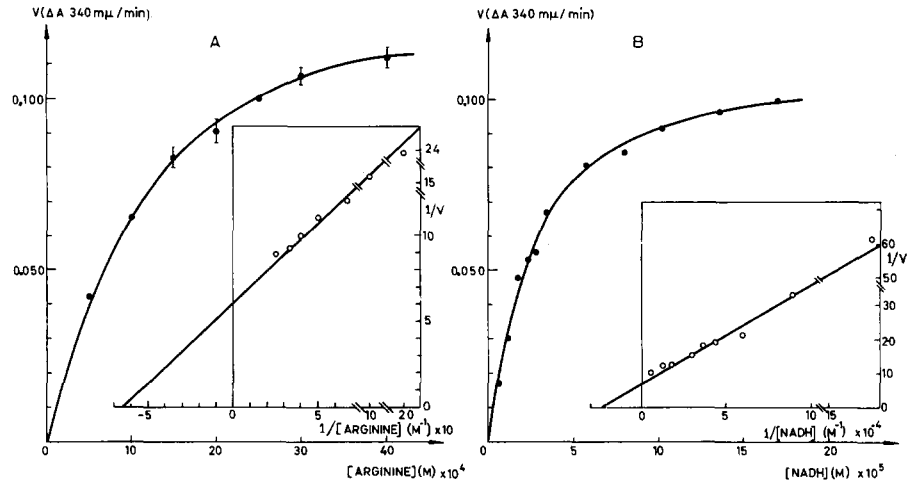


Fig. 6. Détermination de K_m pour la Réaction 1. (A) Concentrations croissantes en substrat. (B) Concentrations croissantes en NADH.

valeurs du K_m calculées sont respectivement de $1.5 \cdot 10^{-3}$ M pour le substrat (Fig. 6A) et de $4 \cdot 10^{-5}$ M pour NADH (Fig. 6B).

Effets des analogues de substrat

Pour préciser la nature des interactions enzyme–substrat, nous avons étudié l'action sur l'enzyme d'un certain nombre d'analogues de substrat, possédant une ou plusieurs fonctions caractéristiques de ce dernier: groupements guanidique, carboxylique et aminé, seuls ou associés. Le Tableau III montre l'effet inhibiteur des composés utilisés.

Nous avons voulu ensuite préciser le type de ces inhibitions. La Fig. 7 montre

TABLEAU III

INHIBITION (%) DE L'OCTOPINE DÉSHYDROGÉNASE PAR DIFFÉRENTS ANALOGUES DE SUBSTRAT

Inhibiteurs	*Concentration (M)*					
	$1 \cdot 10^{-4}$	$1 \cdot 10^{-3}$	$5 \cdot 10^{-3}$	$1 \cdot 10^{-2}$	$4 \cdot 10^{-2}$	$1 \cdot 10^{-1}$
A. Réaction de déshydrogénation						
Acide δ-guanidinovalérique		71	92			
Acide γ-guanidinobutyrique		33	71	85		
L-Arginine		28	68	81		
D-Arginine			16		56	66
Homoarginine			15		48	61
Agmatine			14		37	45
Guanidinobutane			20		62	85
Acide aminovalérique					29	47
B. Réaction de formation d'octopine						
Acide δ-guanidinovalérique	59		100			
Octopine		27	48*			
Guanidinobutane					55	70
Acide aminovalérique					35	65

* Pour $2 \cdot 10^{-3}$ M.

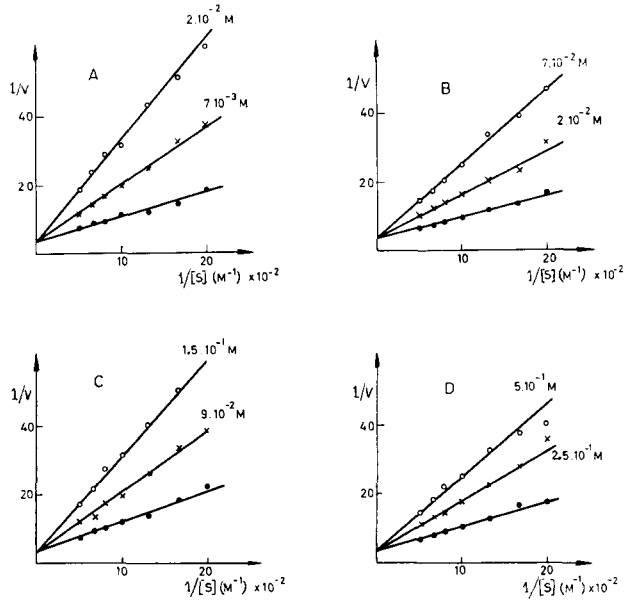


Fig. 7. Inhibitions compétitives par les analogues de substrat, dans la Réaction 2. (A) Guanidinobutane $7 \cdot 10^{-3}$ et $2 \cdot 10^{-2}$ M. (B) Agmatine $2 \cdot 10^{-2}$ et $7 \cdot 10^{-2}$ M. (C) Acide valérique $9 \cdot 10^{-2}$ et $1.5 \cdot 10^{-1}$ M. (D) Acide γ-aminobutyrique $2.5 \cdot 10^{-1}$ et $5 \cdot 10^{-1}$ M. Substrat: octopine.

que dans la Réaction 2 l'inhibition exercée par le guanidinobutane, l'agmatine, l'acide valérique et l'acide γ-aminobutyrique est compétitive. Celle produite par l'acide δ-aminovalérique et par l'arginine est non-compétitive (Fig. 8). L'action de l'acide δ-guanidinovalérique est plus complexe: la Fig. 8 montre qu'avec l'augmentation de la concentration en inhibiteur, la v_{max} diminue, mais la K_m diminue également.

Dans la Réaction 1 (Fig. 9), les inhibitions sont compétitives, qu'il s'agisse du guanidinobutane, des acides valérique et δ-guanidinovalérique, ou encore de l'octopine qui peut être considérée, dans ce sens-ci, comme l'analogue hydrogéné de substrat. Les résultats sont les mêmes, que l'on fasse varier l'un ou l'autre des composants du mélange arginine *plus* pyruvate, l'autre étant maintenu à concentration saturante.

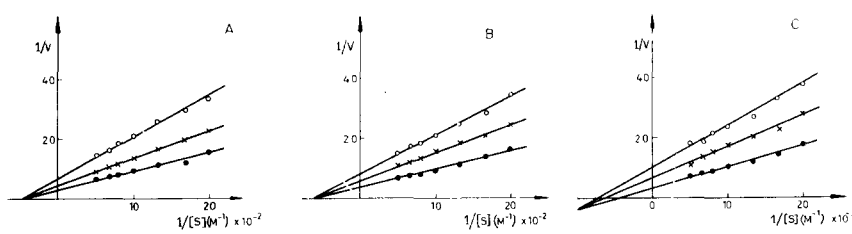


Fig. 8. Inhibitions non compétitives par les analogues de substrat, dans la Réaction 2. (A) Acide δ-aminovalérique $3 \cdot 10^{-2}$ et $1 \cdot 10^{-1}$ M. (B) L-Arginine $9 \cdot 10^{-4}$ et $2 \cdot 10^{-3}$ M. (C) Acide δ-guanidinovalérique $1.5 \cdot 10^{-4}$ et $4 \cdot 10^{-4}$ M. Substrat: octopine.

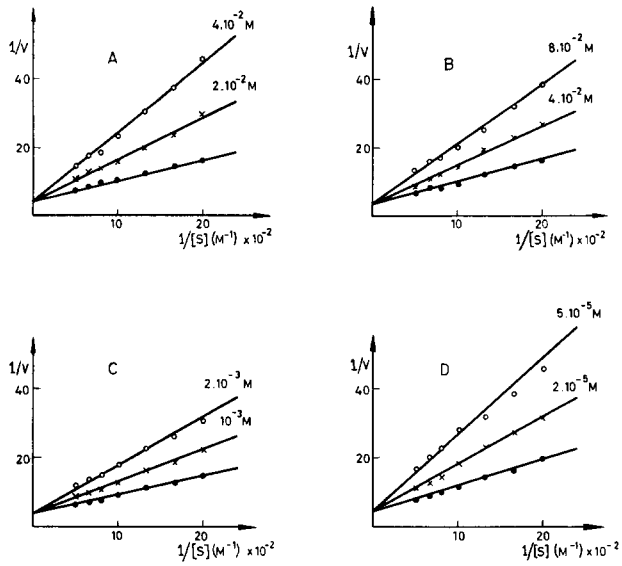


Fig. 9. Inhibitions compétitives par les analogues de substrat, dans la Réaction 1. (A) Guanidinobutane $2 \cdot 10^{-2}$ et $4 \cdot 10^{-2}$ M. (B) Acide valérique $4 \cdot 10^{-2}$ et $8 \cdot 10^{-2}$ M. (C) Octopine $1 \cdot 10^{-3}$ et $2 \cdot 10^{-3}$ M. (D) Acide δ-guanidinovalérique $2 \cdot 10^{-5}$ et $5 \cdot 10^{-5}$ M. Substrat: arginine *plus* pyruvate.

DISCUSSION

La méthode de purification décrite plus haut permet d'obtenir des préparations enzymatiques homogènes à l'ultracentrifugation analytique et à l'électrophorèse sur gel de polyacrylamide. L'activité spécifique du produit final est très élevée.

L'octopine déshydrogénase ne semble pas contenir de métal. Elle ressemble en cela à la plupart des déshydrogénases lactiques et diffère des autres déshydrogénases telles que les alcool déshydrogénases de levure et de foie de cheval, et la glutamodéshydrogénase, qui contiennent du Zn^{2+}, ce métal jouant un rôle à la fois structural et catalytique.

Les effets inhibiteurs, d'ailleurs peu importants, qu'exercent certains chélateurs sur l'octopine déshydrogénase sont comparables à ceux signalés par VESTLING *et al.*[20] sur la lactate déshydrogénase de foie de rat. Ils pourraient être dus aux interactions d'une autre nature que la formation des complexes métalliques.

Dans l'étude cinétique de la réaction de formation de l'octopine (Réaction 1), la forme sigmoïde de la courbe de vitesse en fonction des quantités équimoléculaires croissantes d'arginine et de pyruvate reflète la superposition de deux réactions[3], l'une chimique: la condensation spontanée de l'acide α-aminé et de l'oxoacide, l'autre enzymatique: la réduction de la base de Schiff à l'aide de NADH. En favorisant la formation du produit de condensation par l'emploi à concentration saturante de l'un des constituants de la réaction, on obtient à nouveau les courbes michaeliennes classiques.

La possibilité que l'octopine déshydrogénase soit un enzyme allostérique a été écartée du fait que les différents analogues des substrats et du coenzyme, comme le

changement du pH de la réaction, ne modifiaient guère la cinétique de celle-ci. Cet enzyme de petit poids moléculaire (environ 50 000) n'est d'ailleurs pas dissociable en sous-unités (travaux non publiés).

L'étude de l'inhibition par les analogues de substrats semble montrer qu'il existe sur l'enzyme au moins deux sites impliqués dans la fixation du substrat: l'un réagissant avec le groupement guanidique du substrat, l'autre avec l'un des carboxyles de ce dernier. En effet, dans la Réaction 1, l'inhibition exercée par les analogues de substrat est compétitive, qu'ils possèdent seulement un groupement guanidique (guanidinobutane) ou un groupement carboxylique (acide valérique) ou les deux associés (acide δ-guanidinovalérique, octopine).

L'inhibition est du même type, que l'on fasse varier l'un ou l'autre des composants du mélange arginine *plus* pyruvate, l'autre étant maintenu à concentration saturante.

Dans la Réaction 2, le guanidinobutane et l'acide valérique sont des inhibiteurs compétitifs. Par contre, les composés: arginine, acides δ-amino- et δ-guanidinovalérique, bien que possédant à la fois un groupe basique et un groupe acide séparés par une longueur de chaîne adéquate et, par conséquent, capables de bloquer sur l'enzyme les deux sites de fixation correspondants, ne sont pas des inhibiteurs compétitifs.

Il est possible que les complexes binaires octopine déshydrogénase–NAD^+ et octopine déshydrogénase–NADH, de conformation moléculaire sans doute différente, réagissent de façons diverses aux mêmes analogues de substrat. L'étude actuellement en cours, de spectrophotométrie différentielle des complexes binaires et ternaires, doit apporter d'autres précisions à ce sujet.

REMERCIEMENTS

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RÉSUMÉ

1. L'octopine déshydrogénase, enzyme à NAD^+, qui catalyse la déshydrogénation de l'octopine en arginine *plus* pyruvate, a été purifiée à partir des muscles de *Pecten maximus*. L'enzyme est homogène à l'ultracentrifugation analytique et à l'électrophorèse sur gel de polyacrylamide.

2. L'enzyme ne renferme pas de cofacteur métallique du type Fe^{2+}, Mn^{2+}, Zn^{2+}.

3. Les constantes de Michaelis sont de $1.5 \cdot 10^{-3}$ M pour l'octopine, l'arginine et le pyruvate, $1.5 \cdot 10^{-4}$ M pour NAD^+ et $4 \cdot 10^{-5}$ M pour NADH.

4. Les composés chimiques possédant un groupement guanidique (guanidinobutane) ou carboxylique (acide valérique) ou les deux fonctions associées (acide δ-guanidinovalérique) sont inhibiteurs compétitifs dans la réaction de formation de l'octopine. Dans la déshydrogénation de celle-ci, les analogues de substrat possédant une seule des fonctions précédentes sont inhibiteurs compétitifs; au contraire, ceux des produits renfermant à la fois les deux groupements guanidique et carboxylique ne sont pas inhibiteurs compétitifs.

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MECHANISM OF THE SALICYLATE HYDROXYLASE REACTION*

II. THE ENZYME–SUBSTRATE COMPLEX

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SUMMARY

1. Salicylate hydroxylase (salicylate, NADH:oxygen oxidoreductase (1-hydroxylating, 1-decarboxylating)) from *Pseudomonas putida* forms an enzyme–substrate complex with salicylate.

2. The complex could be detected by a new absorption maximum around 480 nm. By spectrophotometric titration, it was found that a molar ratio of apoenzyme, FAD and salicylate in the complex was 1:1:1.

3. The complex was more stable than the holoenzyme under any tested conditions, *i.e.*, heat, acid and proteinase treatments.

4. The FAD moiety of the complex was reduced with NADH under anaerobic conditions, and the reoxidation of the reduced complex with air resulted in product formation. The stoichiometric relation in each reaction was demonstrated by using substrate level amounts of the enzyme. A mechanism for salicylate hydroxylation reaction is proposed.

INTRODUCTION

It has already been established that salicylate hydroxylase (salicylate, NADH: oxygen oxidoreductase (1-hydroxylating, 1-decarboxylating)) from a pseudomonad†, which catalyzes the conversion of salicylate to catechol with the stoichiometric consumption of O_2 and NADH, is a flavoprotein with FAD as the prosthetic group[4–6]. In 1957, HAYAISHI AND SUTTON[7] demonstrated that lactate oxidative decarboxylase from *Mycobacterium phlei* catalyzes the incorporation of O_2 into substrate and requires

* The preceding paper in this series is ref. 1. A part of the results was presented at the meeting of the Japan–U.S.A. Symposium on Oxygenases in Kyoto, 1966 (ref. 2).

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† Kindly identified by Dr. G. Hegeman of Department of Bacteriology, University of California, Berkely as a new pseudomonad which is quite similar to *Pseudomonas putida*[3].

FMN as a coenzyme. Since then, a number of flavin(FAD or FMN)-requiring monooxygenases have been studied. However, there has been little experimental evidence, except for several speculations, clarifying the function of the flavin in the monooxygenase reaction. Salicylate hydroxylase with molecular weight of 57 000 provides an especially favorable system for investigating the function of flavin in a monooxygenase reaction since it shows unusual alteration of absorption spectrum in the presence of the substrate[1,2]. Upon addition of salicylate, the absorption peaks are shifted to longer wavelengths with a marked shoulder around 480 nm. These spectral changes suggest the formation of an enzyme–substrate complex. Since the new shoulder around 480 nm is characteristic of the complex, we can directly demonstrate the ratio of apoenzyme, FAD and salicylate in the complex by spectrophotometric titration.

This paper describes spectrophotometric experiments which indicate that salicylate hydroxylase combines specifically with the substrate to form a complex in which the ratio of apoenzyme, FAD and salicylate is 1:1:1. Furthermore, we report preliminary experiments designed to show that this ternary complex is an actual intermediate involved in the overall reaction of salicylate hydroxylase.

MATERIALS

Reagents

FAD (approx. 90% purity) was supplied by Dr. E. Ohmura, Takeda Research Laboratories, Osaka. NADH was obtained from Calbiochem. Co.; DEAE-cellulose from Brown Co.; Sephadex G-25, G-100 and DEAE-Sephadex A-50 from Pharmacia; salicylic acid from Nakarai Chemical, Kyoto; benzoic acid and catechol from Merck Co.; 2,3-, 2,4-, 2,5- and 3,4-dihydroxybenzoic acids, *p*-aminosalicylic acid, 1-hydroxy-2-naphthoic acid, 3-methylsalicylic acid, *m*- and *p*-hydroxybenzoic acids from Tokyo Kasei, Tokyo. Catechol, *m*- and *p*-hydroxybenzoic acids were recrystallized from toluene. 1-Hydroxy-2-naphthoic acid and 2,3-, 2,4- and 3,4-dihydroxybenzoic acids were recrystallized from water. All of the acids used were neutralized with NaOH. Bacterial proteinase, "Nagarse", was donated by Dr. K. Kusai of Nagase and Co., Amagasaki.

Preparation of salicylate hydroxylase

A purified preparation of salicylate hydroxylase was prepared according to a modification of the method reported previously[5]. Cells were cultivated essentially under the same conditions as described previously[4], except that 2 mg of $FeSO_4$ were further supplied to 1 l of the medium.

All subsequent procedures were carried out at below 5°. The buffer used was K_2HPO_4–KH_2PO_4 (pH 7.0), except when otherwise stated. Centrifugations were carried out at 13 000 × *g* for 20 min. A typical protocol is presented in Table I.

Step 1: Crude extract. The cells (100 g, wet wt.) were ground for 45 min in a prechilled mechanical mortar (20.5 cm diameter) with 2 times their weight of aluminum oxide (Wako W, 800) and were mixed with 500 ml of 33 mM buffer. The resultant slurry was centrifuged off. The precipitate together with alumina was resuspended in 300 ml of the same buffer and was centrifuged. The supernatant fluids from the two centrifugation steps were combined to yield a crude extract.

Step 2: $(NH_4)_2SO_4$ precipitation. To 500 ml of the crude extract, 113 g of solid

TABLE I

SUMMARY OF PURIFICATION OF SALICYLATE HYDROXYLASE

Step	*Total vol. (ml)*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*	*Yield (%)*
Crude extract	715	9290	7090	0.76	100
$(NH_4)_2SO_4$ fractionation	172	4610	5020	1.09	71
DEAE-cellulose	193	3330	4940	1.49	70
Sephadex G-100	87	714	4160	5.83	59
DEAE-Sephadex A-50	30	384	3840	10.0	54

$(NH_4)_2SO_4$ were added with mechanical stirring. After standing for more than 15 min, the mixture was centrifuged, and 75 g of $(NH_4)_2SO_4$ were further added per 500 ml of supernatant fluid. The precipitate was collected by centrifugation, was dissolved in about 150 ml of 10 mM buffer and was then dialyzed for 3 h against 3 l of the same buffer with two changes.

Step 3: DEAE-cellulose treatment. The dialysate was passed through a column (4.0 cm × 20 cm) of DEAE-cellulose equilibrated with 10 mM buffer, under which the enzyme was not adsorbed on the column. The enzyme fraction was rapidly eluted with the same buffer and was immediately applied to a gel-filtration column.

Step 4: Sephadex G-100 gel filtration. The eluted enzyme solution (193 ml) was applied to a column (9.0 cm × 50 cm) of Sephadex G-100 equilibrated with 10 mM buffer, and the column was developed with the same buffer at the flow rate of 60 ml per h. To the eluate containing the enzymatic activity (450 ml), 225 g of $(NH_4)_2SO_4$ were added. The precipitate was removed by centrifugation, was dissolved in 10 mM K_2HPO_4 solution freshly prepared to give about 50 ml of the enzyme solution and was dialyzed for 3 h against 10 mM K_2HPO_4.

Step 5: DEAE-Sephadex chromatography. The dialyzed material was applied to a DEAE-Sephadex A-50 column (2.5 cm × 30 cm) equilibrated with a fresh solution of 10 mM K_2HPO_4. The enzyme was eluted with a linear gradient established between 300 ml of 50 mM K_2HPO_4 (mixing vessel) and 300 ml of 0.2 M K_2HPO_4 (reservoir). The enzyme fractions (250 ml) were combined, 125 g of $(NH_4)_2SO_4$ were added and the resulting precipitate was removed by centrifugation. The preparation, dissolved in a minimum amount of 33 mM buffer, was dialyzed for 3 h against the same buffer.

Step 6: Reconstitution of holoenzyme. Since the flavin was partially leached from the protein moiety of the enzyme during the purification procedure, 2 mg of FAD per 100 mg protein were added to the dialyzed enzyme solution at the final step. The mixture was then applied to a Sephadex G-25 column (2.5 cm × 25 cm) equilibrated with 33 mM buffer. The column was subsequently developed with the same buffer. The holoenzyme thus obtained was completely free of unbound FAD and was used as the source of enzyme, except when stated otherwise. The enzyme was quite stable for a few weeks when stored at −20°. Disc electrophoresis of this preparation by the method of DAVIS[8] showed a single band, and the enzyme had a specific activity of 10.

Crystallization of salicylate hydroxylase

The $(NH_4)_2SO_4$ precipitate obtained at Step 5 was used as the starting material for crystallization. The precipitate was redissolved in a minimum amount of 50 mM

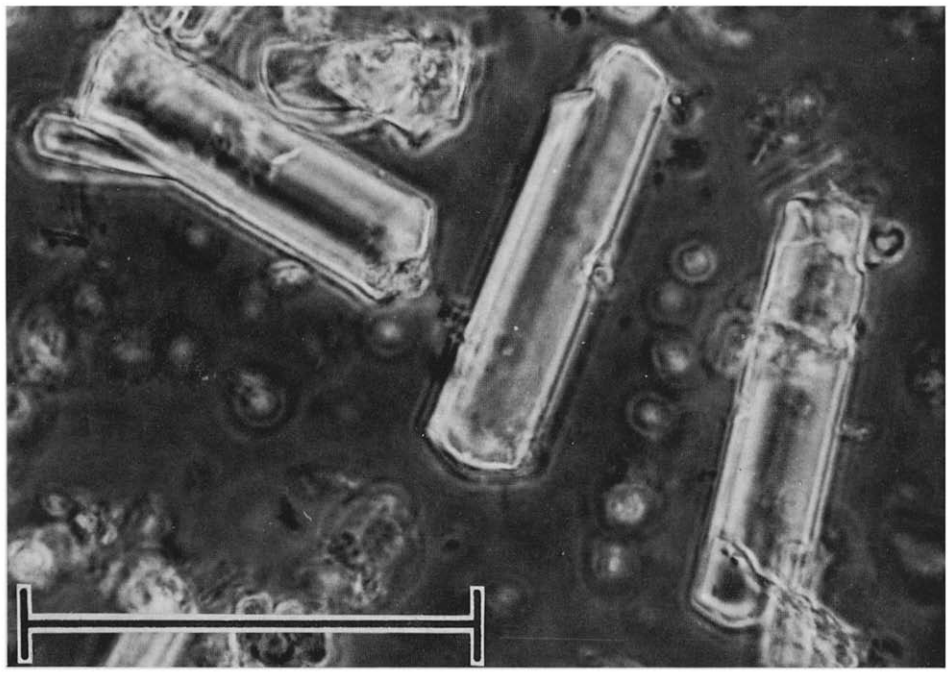


Fig. 1. A phase-contrast photomicrograph of crystalline salicylate hydroxylase. The indicated scale represents 0.1 mm.

Tris–HCl buffer (pH 8.0) containing a few mg of FAD, and solid $(NH_4)_2SO_4$ was added slowly to the solution until a slight turbidity appeared. The pH of the solution was adjusted to 8.0 with aqueous ammonia. The solution was left to stand at 5° for a few hours, and the precipitate was then removed by centrifugation. This procedure was repeated until the first traces of yellow precipitate started to settle. This solution was left to crystallize at 5°. Yellow crystals were slowly formed, and over a period of several days the crystal size increased (Fig. 1). The specific activity was not increased by this procedure. Therefore, crystallization was not used for purification of the enzyme.

Preparation of apo-salicylate hydroxylase

The apoenzyme was prepared by the acid–$(NH_4)_2SO_4$ method in the same way described previously[4]. This preparation of apoenzyme was stable for several days when stored at −20°.

Metapyrocatechase

This was prepared from *Pseudomonas putida*, T-2, essentially as described by NOZAKI *et al.*[9]. The enzyme was stored as crystals suspended in 50 mM potassium phosphate buffer (pH 7.5) containing 10% acetone.

METHODS

Estimation of enzymatic activities

The units of salicylate hydroxylase activity and the assay procedures have been described elsewhere[5]. The amount of enzyme protein was estimated by the method of

LOWRY *et al.*[10], using bovine serum albumin (Sigma Chemical Co.) as a protein standard. Concentrations of the holoenzyme were determined spectrophotometrically with the use of the molar extinction coefficient of 11.2 $mM^{-1} \cdot cm^{-1}$ at 450 nm*.

Measurement of absorption spectrum

A Hitachi model EPR 3 recording spectrophotometer and a Perkin–Elmer Model 202 recording spectrophotometer were used with a 1-cm light path cuvette.

Determination of catechol or 3-methylcatechol

The amounts of catechol or 3-methylcatechol were estimated enzymatically with the use of a crystalline metapyrocatechase. To a sample containing catechol or 3-methylcatechol in 3 ml of 40 mM potassium phosphate buffer (pH 7.2), 70 ng of metapyrocatechase in 50 μl were added by means of a micropipette. After the mixture was kept at room temperature for more than 3 min, the amounts of catechol or 3-methylcatechol were determined from the changes of the absorbance at 375 nm (ε (375 nm) = $3.6 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$) or at 389 nm (ε (389 nm) = $9.6 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$), respectively. Under the assay conditions, the absorbances at these wavelengths were found to be proportional to the concentrations of catechol or 3-methylcatechol, respectively.

Titration of enzyme-bound FAD with NADH

The experiments were performed in a Thunberg-type cuvette (1-cm light path) equipped with a side arm and a vaccine cup. The cuvette was evacuated at room temperature for 15 min and was then flushed with N_2 gas, alternately twice. O_2-free N_2 was prepared with the treatment of alkaline pyrogallol. After placing it in an ice bath for 1 h, the same procedure was repeated once more. Finally the cuvette was flushed with N_2. The solution of NADH was anaerobically prepared in a Thunberg tube in the same way as above. Titration of the enzyme with NADH was performed by stepwise additions of small portions (10–20 μl) of the latter into the former solution by means of a 50-μl microsyringe.

RESULTS

Absorption spectrum of the enzyme–substrate complex

As illustrated in Fig. 2, the holoenzyme showed a slight difference in spectrum compared with free FAD. When FAD was mixed with an equimolar amount of the apoenzyme, the absorption intensity of FAD was slightly lowered, while the peaks at 450 and 375 nm were not shifted. When salicylate was added to the holoenzyme, the peaks at 450 and 375 nm were shifted to 455 and 385 nm, respectively, and a marked shoulder appeared around 480 nm (Fig. 3). Similar spectral shifts were also produced by a number of other substrates for the hydroxylase reaction, namely 2,3-, 2,4- and 2,5-dihydroxybenzoates, *p*-aminosalicylate, 1-hydroxy-2-naphthoate and 3-methylsalicylate. Some representative spectra in the absence and presence of these compounds are shown in Fig. 4. However, compounds such as benzoate, *m*- and *p*-hydroxybenzoates and 3,4-dihydroxybenzoate which were not substrates of the enzyme had little or no effect on spectral shift at 375 and 450 nm or in forming a shoulder at 480 nm. Free FAD did not replace the holoenzyme. *o*-Phenolsulfonate, a compe-

* Calculated on the basis of direct analysis of FAD released from the protein moiety by heat and acid treatments.

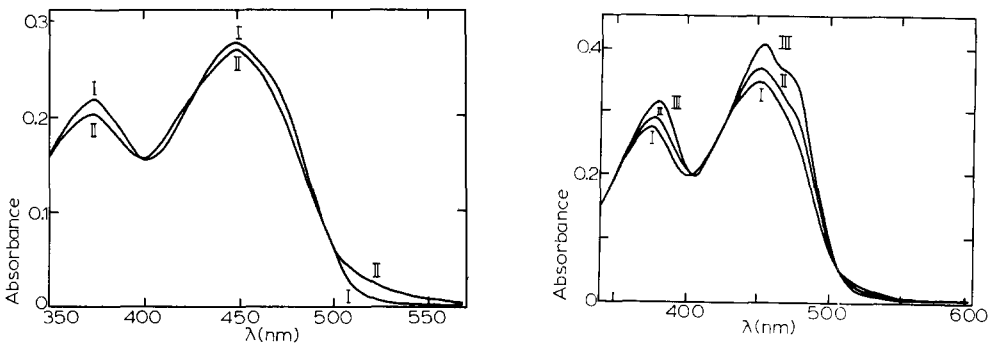


Fig. 2. Comparison of absorption spectra of FAD and salicylate hydroxylase in 33 mM potassium phosphate buffer (pH 7.0) (3.0 ml). Curve I, FAD (74 nmoles); Curve II, FAD + apoenzyme (74 nmoles).

Fig. 3. Effect of salicylate on the spectrum of salicylate hydroxylase in 33 mM potassium phosphate buffer (pH 7.0) (3.0 ml). Curve I, holoenzyme (92 nmoles); Curve II, holoenzyme + salicylate (50 nmoles); Curve III, holoenzyme + salicylate (1 μmole).

titive inhibitor for the enzyme[6], gave another type of spectral change (Fig. 4D). This compound differed in effect from salicylate, producing only a slight shift in the maximum at 375 nm to shorter wavelengths. The peaks around 450 nm were not essentially affected, and no shoulder at 480 nm was observed.

Stoichiometric relation of apoenzyme, FAD and salicylate in the enzyme–substrate complex

Since the complex formation was observed by the absorption increase at the shoulder at 480 nm, stoichiometry was determined by titration of the holoenzyme with salicylate. The enzyme-bound FAD (153 nmoles) was titrated precisely with varying amounts of salicylate (Fig. 5, Curve I). No significant difference was observed when

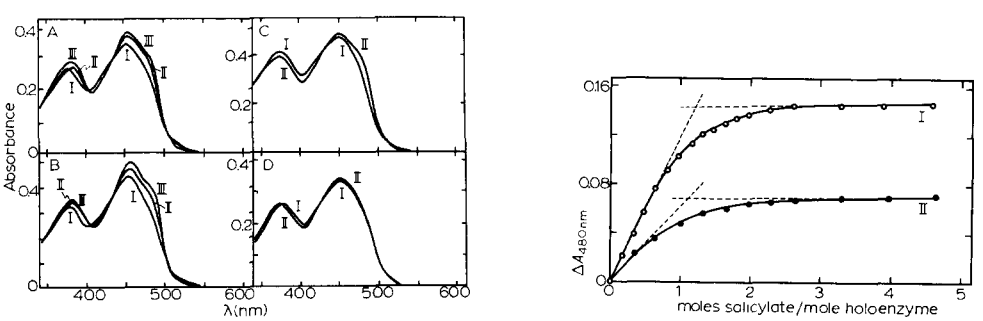


Fig. 4. Effect of other substrates and an inhibitor on the spectrum of salicylate hydroxylase in 33 mM potassium phosphate buffer (pH 7.0) (3.0 ml). A. Curve I, holoenzyme (92 nmoles); Curve II, holoenzyme + 2,5-dihydroxybenzoate (5 μmoles); Curve III, holoenzyme + *p*-aminosalicylate (5 μmoles). B. Curve I, holoenzyme (110 nmoles); Curve II, holoenzyme + 2,3-dihydroxybenzoate (5 μmoles); Curve III, holoenzyme + 2,4-dihydroxybenzoate (5 μmoles). C. Curve I, holoenzyme (129 nmoles); Curve II, holoenzyme + 3-methylsalicylate (5 μmoles). D. Curve I, holoenzyme (92 nmoles); Curve II, holoenzyme + *o*-phenolsulfonate (5 μmoles).

Fig. 5. A spectrophotometric titration of salicylate hydroxylase with salicylate. The reaction mixture contained 153 nmoles (Curve I) or 76 nmoles (Curve II) of the holoenzyme and indicated amounts of salicylate in 33 mM potassium phosphate buffer (pH 7.0) in 3.0 ml system.

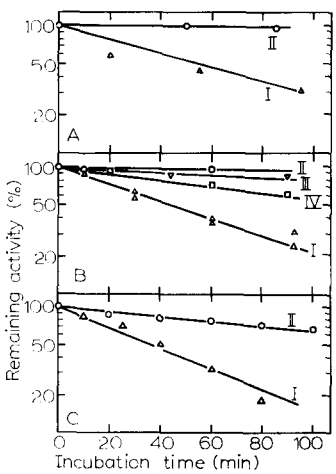
the titration was carried out in the presence of excess free FAD. A similar titration was performed with salicylate in the presence of 76 nmoles holoenzyme (Fig. 5, Curve II). In both cases, the initial slope of the titration curve intercepted the maximum value observed at 1 mole salicylate per mole enzyme-bound flavin.

The fact that the molar ratio of FAD to the apoenzyme in salicylate hydroxylase is 1:1 has been confirmed by equilibrium dialysis[5] and by fluorometric analysis[11]. From combination of these results, it is now possible to conclude that a molar stoichiometry of apoenzyme, FAD and salicylate in the complex is 1:1:1.

Since the increases in absorption at 480 nm upon addition of salicylate were assumed to be proportional to the amounts of the complex, it was possible to estimate the dissociation constant from data of the titration curve shown in Fig. 5. A mean value of the dissociation constant of salicylate in the complex was calculated to be 3.5 μM.

Stability of the enzyme–substrate complex

Experiments demonstrated in Figs. 6A and 6B indicate that the holoenzyme is rapidly inactivated at 41° or at pH 4.2. However, the complex which was formed in the presence of salicylate was found to be completely stable under these conditions. The protective effect of salicylate in a proteinase treatment was also similar to that



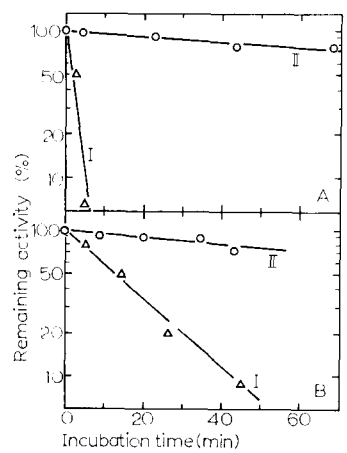


Fig. 6. Effect of substrate on acid (A), heat (B) and Nagarse (C) inactivations of salicylate hydroxylase. A. The reaction mixture (2.0 ml) containing 5.7 nmoles of the holoenzyme, 60 μmoles of acetate buffer (pH 4.2) and 800 nmoles of salicylate was incubated at 20°. Samples were withdrawn at time intervals, and assayed for activity. B. The reaction mixture (2.0 ml) containing 5.7 nmoles of the holoenzyme and 60 μmoles of potassium phosphate buffer (pH 7.0) was supplemented with 800 nmoles of the substrates and incubated at 41°. C. The reaction mixture (3.0 ml) containing 5.7 nmoles of the holoenzyme, 48 μmoles of Tris–HCl buffer (pH 8.0), 10 μg of Nagarse and 800 nmoles of salicylate was incubated at 29°. Curve I, without substrate; Curve II, with salicylate; Curve III, with 2,5-dihydroxybenzoate; Curve IV, with *p*-aminosalicylate.

Fig. 7. Effect of substrate on the inactivation of the apoenzyme by heat (A) and Nagarse (B). A. The reaction mixture (2.0 ml) containing 1.5 nmoles of the apoenzyme, 60 μmoles of potassium phosphate buffer (pH 7.0) and 800 nmoles of salicylate, was incubated at 41°. B. The reaction mixture was as described in A, except that 10 μg of Nagarse were further added. The reaction mixture was incubated at 20°. Curve I, without salicylate; Curve II, with salicylate.

observed in the heat and acid inactivation experiments (Fig. 6C). Under the conditions in which the holoenzyme lost 80% of its initial activity, the complex lost only 30%.

To determine whether the addition of other substrates related to salicylate would also affect the stability of the enzyme, the holoenzyme was incubated with such compounds at 41°. The data collected in Fig. 6B show that the stability is significantly increased by incubation with such compounds, while other substances such as catechol or benzoate afforded scarcely any significant protection.

These results provide an additional evidence that the holoenzyme combines with salicylate to form a complex having a more rigid structure than the holoenzyme.

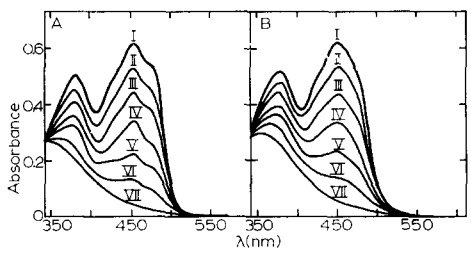
As shown in Fig. 7A, the apoenzyme was also spontaneously destroyed at 41°. Enzymatic activity was completely lost in 10 min. However, an appreciable protective effect was observed in the presence of salicylate. When the apoenzyme was incubated with Nagarse, its activity decreased considerably. This inactivation was depressed by the addition of salicylate, as shown in Fig. 7B. Similar protective effects were observed with substances related to salicylate but not with substances which were not substrates for the enzyme.

Spectrophotometric and stoichiometric demonstration that the enzyme–substrate complex is involved in the catalysis

Anaerobic titration of the enzyme–substrate complex with NADH. To elucidate the catalytic mechanism of FAD in the complex, the effect of NADH on the spectrum of the complex was investigated. Fig. 8A shows the results of anaerobic titration of the complex with NADH. Curve I represents oxidized form. Upon addition of appropriate amounts of NADH to a solution of the complex, the absorption over the whole spectral range diminished instantaneously (Curves II–VI), and no increase in absorption at a wavelength greater than 530 nm, which could be attributed to a stable one-electron reduced form of the enzyme–flavin, was observed. The resulting spectra were very similar to those of the fully reduced form of flavin, and the amount of the change in absorbance at 450 nm was proportional to the amount of added NADH. After addition of 1 mole of NADH per mole of the complex, the spectrum of the reaction mixture showed full reduction (Curve VII).

Anaerobic titration of the holoenzyme with NADH. In the absence of salicylate, the holoenzyme was also reduced with NADH, and the situation was quite similar to the case in its substrate complex. As shown in Fig. 8B, a fully reduced form appeared throughout the titration, and the full reduction was observed on the addition of 1 mole of NADH per mole of enzyme-bound FAD.

Reaction of the reduced enzyme–substrate complex with O_2. To show that NADH-reduced complex is an actual intermediate in the overall hydroxylation reaction, attempts were made to demonstrate that the reduced complex is capable of aerobic hydroxylation of the substrate. Upon introducing air into the reduced form of the complex, the reduced flavin moiety was rapidly reoxidized, and the absorption in the visible region increased to the same level as that of the untreated preparation. Under these conditions, the reaction product, catechol, was stoichiometrically produced in the reaction mixture. As shown in Fig. 9, the stoichiometric relation was apparently maintained until 1 mole of NADH per mole of the enzyme-bound FAD had been added. When the reduced complex was heated before admitting air, no product was observed. This indicated also that anaerobiosis had been completely achieved. When



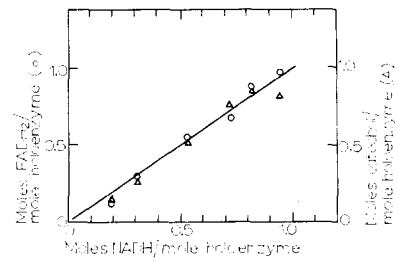


Fig. 8. Anaerobic titration of salicylate hydroxylase with NADH in the presence (A) and absence (B) of 5 μmoles salicylate. Curve I, oxidized enzyme (142 nmoles) in 33 mM potassium phosphate buffer (pH 7.0) (3.0 ml); Curves II–VII, after addition of 0.19, 0.37, 0.55, 0.73, 0.92 and 1.10 moles of NADH per mole of enzyme-bound flavin, respectively.

Fig. 9. A graphic representation of stoichiometry of salicylate hydroxylase reaction. The reaction mixture contained, in the main vessel of a Thunberg-type cuvette with 1-cm light path, 100 μmoles of potassium phosphate buffer (pH 7.0), 5 μmoles of salicylate and 142–165 nmoles of the enzyme in a total volume of 2.9 ml, and indicated amounts of NADH (in 0.1 ml) in the side arm at 20°. The cuvette was evacuated according to the same procedure as described in METHODS. After the addition of NADH into the main vessel, the amount of enzyme-bound $FADH_2$ was calculated by the decrease of the absorbance at 450 nm, taking an extinction coefficient of 10.3 $mM^{-1} \cdot cm^{-1}$. Then air was introduced into the cuvette and the amount of catechol produced in the reaction mixture was estimated with the use of metapyrocatechase.

the holoenzyme was reduced with NADH in the absence of salicylate and then was allowed to react with air after addition of salicylate, a stoichiometric amount of catechol was also detected as observed in the complex.

3-Methylsalicylate–holoenzyme was found to show spectral and enzymatic properties similar to those of salicylate–holoenzyme (Table II). The amount of the reduced complex formed was identical with that of NADH added, and upon admitting air a stoichiometric amount of 3-methylcatechol was produced from the reduced complex. When the reduced holoenzyme was allowed to react with air after addition of 3-methylsalicylate, the product was also detected stoichiometrically.

These results support the conclusion that the holoenzyme–substrate complex interacts with NADH to form an actual reduced intermediate (enzyme–$FADH_2$–substrate), which reacts with O_2 to form the product.

TABLE II

STOICHIOMETRY OF SALICYLATE HYDROXYLASE REACTION WITH 3-METHYLSALICYLATE AS SUBSTRATE

Expts. I and II, the assay conditions were as described in Fig. 9, except that 5 μmoles 3-methylsalicylate were used as a substrate. Expt. III, after the enzyme had been reduced, 5 μmoles substrate were added from the side arm, and the reaction mixture was then exposed to air.

Expt. No.	*Enzyme (nmoles)*	*3-Methylsalicylate (μmoles)*	*NADH (nmoles)*	*Enzyme reduced (nmoles)*	*3-Methylcatechol formed (nmoles)*
I	111	5	58	47	67
II	107	5	107	98	107
III	109	—	110	116	113

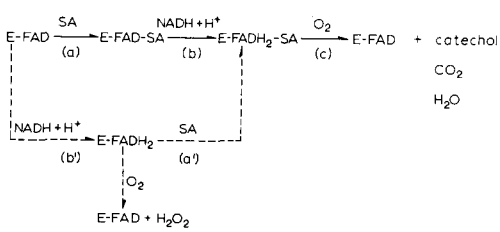
DISCUSSION

The interaction of substrate with salicylate hydroxylase is characterized by an absorption spectrum in which a new shoulder is formed at about 480 nm. The appearance of the shoulder is considered to be due to the formation of an enzyme–substrate complex. The dissociation constant of salicylate in the complex is found to be very small, so that we can now directly demonstrate the stoichiometry between apoenzyme, FAD and salicylate to be 1:1:1 by spectrophotometric titration with salicylate. Recently we have carried out a fluorometric analysis of the enzyme–substrate complex of salicylate hydroxylase and have found that the enzyme combines specifically with the substrate to form a fluorescent complex; the molar ratio of the components in the complex is 1:1:1 (refs. 11 and 12). This is in good accordance with the results of spectrophotometric investigations described here.

Spectral shifts, similar to those observed with salicylate hydroxylase in the presence of salicylate, have been reported in a study of a benzoate complex of D-amino-acid oxidase[13]. Attempts have been made to explain the spectral shifts seen in the spectrum of D-amino-acid oxidase after the addition of benzoate[13,14]. As demonstrated in this paper, the substrate complex of salicylate hydroxylase is the really "active" form of the enzyme, so that the fine mechanism of the spectral shift in the flavin, induced by the binding of the enzyme and the substrate, may be a key to the solution of the real mechanism involved in the monooxygenase reaction.

The protective effect of substrate against inactivation of salicylate hydroxylase is an additional evidence that the holoenzyme combines with salicylate to firm the substrate complex which has a more rigid structure than the holoenzyme. It is of some interest that the stability characteristics of the apoenzyme in the presence of substrate are very similar to those of the substrate complex of the holoenzyme. The effect of salicylate on the stability of the apoenzyme can be interpreted in terms of stabilization of the protein structure. The fluorometric experiments reported in the subsequent paper[11] show that the apoenzyme forms a binary complex with salicylate at a molar ratio of one to one.

Although more kinetic experimental information is required to establish the mechanism, the data collected in this study form the basis of a tentative hypothesis shown in Scheme I. We suppose that the primary reaction of salicylate hydroxylase involves the interaction of substrate with the holoenzyme as indicated in Reaction a of Scheme I. The enzyme–salicylate complex can now receive electron from NADH (Reaction b) and in the presence of O_2, produces the hydroxylated product, catechol (Reaction c). Another possibility involves the interaction of NADH rather than



Scheme I. Proposed mechanism of salicylate hydroxylase reaction. E and SA denote the apoenzyme and salicylate, respectively.

salicylate, the holoenzyme was found to be reduced with NADH. When the reduced holoenzyme was mixed with salicylate under anaerobic conditions and then O_2 was introduced, the stoichiometric formation of catechol was observed. These results suggest the possible process of the alternative reaction sequence b', a' and c for salicylate hydroxylation. However, Reaction b' is not an efficient one for salicylate hydroxylation, since the K_m for NADH determined by the decrease of absorbance at 340 nm is about 400 times larger in the absence than in the presence of salicylate[6], while the affinity of the holoenzyme for salicylate is not influenced by the presence of NADH as reported here. Kinetic studies by a stopped flow technique, which will be published later, have supported this hypothesis. Thus it is now possible to say that the reaction sequence a, b and c should be predominant in the overall catalytic process of salicylate hydroxylation.

ACKNOWLEDGMENTS

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MECHANISM OF THE SALICYLATE HYDROXYLASE REACTION*

III. CHARACTERIZATION AND REACTIVITY OF CHEMICALLY OR PHOTOCHEMICALLY REDUCED ENZYME–FLAVIN

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SUMMARY

1. The spectral natures of chemically- and photochemically-reduced salicylate hydroxylase (salicylate, NADH:oxygen oxidoreductase (1-hydroxylating, 1-decarboxylating)) from *Pseudomonas putida* have been investigated.

2. When salicylate hydroxylase in the presence of substrate was titrated with a limited amount of dithionite or was illuminated in the presence of EDTA, a new species with a weak absorption band at a long wavelength appeared, whereas an entirely different species which was typical for the "red flavoprotein radical" was detected in the absence of substrate. The latter was converted to the former upon anaerobic addition of substrate.

3. Reoxidation of the reduced enzyme–substrate complex with air formed the product in an amount stoichiometric with the reduced flavin.

INTRODUCTION

Salicylate hydroxylase (salicylate, NADH:oxygen oxidoreductase (1-hydroxylating, 1-decarboxylating)) combines specifically with the substrate to form a new enzyme–substrate complex in which the ratio of apoenzyme, FAD and substrate is 1:1:1 (refs. 3 and 4). The enzyme–substrate complex is converted by the anaerobic addition of an external electron donor, NADH, to the stable two-electron reduction intermediate in which the stoichiometric relation between added NADH and formed $FADH_2$ has been demonstrated. This reduced species of the enzyme has been shown to be an active intermediate capable of supporting the aerobic hydroxylation of the substrate bound to the enzyme[3,4]. These results suggest that the enzyme-bound

* A part of the results was presented at the meeting of the 7th International Congress of Biochemistry in Tokyo, 1967 (ref. 1), and a preliminary report has been presented[2].

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$FADH_2$ formed by NADH is an essential electron donor in the hydroxylation reaction. The claim could be greatly strengthened if independent methods (especially redox systems most frequently used in studies with hydrogenating flavoproteins) other than reduction with NADH would lead to the same conclusions.

This paper presents studies indicating that the complex reduced either with sodium dithionite or by illumination in the presence of EDTA does react with O_2 to convert enzyme-bound salicylate into catechol.

MATERIALS AND METHODS

Sodium dithionite, FMN and EDTA were obtained from Wako Pure Chemical Co., Osaka. Other reagents used in this work have been described in a preceding paper[4]. Purified salicylate hydroxylase was prepared from *Pseudomonas putida*, S-1, grown in salicylate according to the method described in a preceding paper[4]. Crystalline metapyrocatechase was prepared from *Ps. putida*, T-2, essentially as described by NOZAKI *et al.*[5].

Optical assays under anaerobic conditions were performed in a Thunberg-type cuvette according to the procedure described in a preceding paper[4]. Spectra were recorded with a Hitachi model EPR-3 recording spectrophotometer and with a Perkin–Elmer Model 202 recording spectrophotometer.

Sodium dithionite solution of a known concentration was prepared in a Thunberg tube with a side arm and an open mouth fitted tightly with a vaccine cup. After placing 33 mM potassium phosphate buffer adjusted to pH 7.0 and the appropriate amount of solid sodium dithionite in the main vessel and in the side arm, respectively, the container was immediately closed and freed from O_2 by at least three cycles of alternate evacuation and flushing with O_2-free N_2. After the tube had been made anaerobic, the solid powder of dithionite in the side arm was introduced into the buffer solution. The concentration of dithionite was determined by titrating FMN under anaerobic conditions. Anaerobic titrations were carried out with the use of a microsyringe through the vaccine cup. The dithionite solution was stable during the experiment under these conditions if it was kept anaerobically in an ice bath.

The EDTA-photochemical reduction was carried out by the method of MASSEY AND PALMER[6]. The reaction mixture for illumination contained EDTA at the concentrations described in the legends for the appropriate figures and tables. The enzyme in the anaerobic cuvette was irradiated using a conventional slide projector through a glass water bath with 10-cm light path containing 100 g $CuSO_4$ per l as a filter.

Experiments for electron spin resonance (ESR) were carried out with a Varian V-4500 spectrometer using 100-kcycles magnetic field modulation, and spectra were measured by the use of an anaerobic tube as described by BEINERT AND SANDS[7].

RESULTS

Reduction of salicylate hydroxylase with sodium dithionite

Upon anaerobic titration of salicylate hydroxylase with a limited amount of dithionite in the presence of substrate, the absorption over the whole spectral range diminished instantaneously, and a new absorption band extending beyond 500 nm was formed (Fig. 1A). The intensity at long wavelength was maximal with 0.5 mole

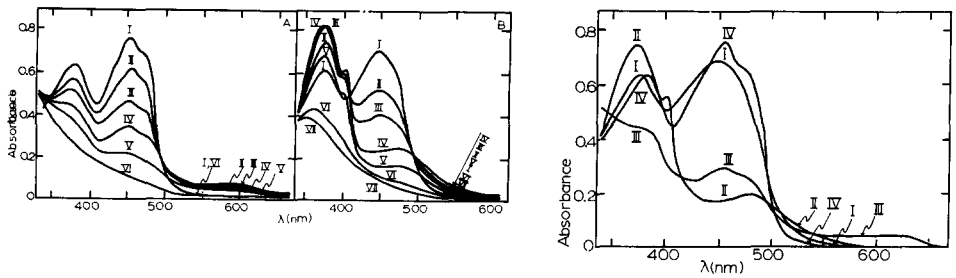


Fig. 1. Sodium dithionite titration of salicylate hydroxylase in the presence (A) and absence (B) of 5 μmoles salicylate. Assay conditions: 147 nmoles of enzyme, 100 μmoles of potassium phosphate buffer (pH 7.0) and $Na_2S_2O_4$ as indicated in a total volume of 3 ml. A. Curve I, oxidized enzyme; Curves II–VI, after addition of 0.29, 0.52, 0.73, 0.95 and 1.39 moles of $Na_2S_2O_4$ per mole of FAD, respectively. B. Curve I, oxidized enzyme; Curves II–VII, after addition of 0.23, 0.47, 0.70, 0.94, 1.07 and 1.40 moles of $Na_2S_2O_4$ per mole of FAD, respectively.

Fig. 2. Conversion of the red intermediate to the species with a long wavelength absorption band by the addition of salicylate. Curve I, oxidized enzyme (147 nmoles); Curve II, after the addition of 0.7 mole of $Na_2S_2O_4$ per mole of FAD; Curve III, after the subsequent addition of 5 μmoles salicylate; Curve IV, after admitting air into the reaction mixture. Other conditions were the same as in Fig. 1.

dithionite per mole flavin. The resulting spectra were not similar to those which had been found on titration of the enzyme with an external electron donor, NADH[3,4]. The flat long wavelength band disappeared completely after full reduction was obtained with 1 mole of dithionite per mole of the enzyme-bound flavin. Similar absorption spectra were also produced in the presence of other substrates for the hydroxylation reaction, namely, 2,5-dihydroxybenzoate and 3-methylsalicylate.

When the titration was carried out in the absence of substrate, the reddish color appeared indicating the possible formation of a semiquinoid intermediate (Fig. 1B). Its spectrum exhibited maxima at 375, 400 and 480 nm. The intensity at 520 nm was maximal with 0.5 mole dithionite per mole flavin. The complete reduction of the enzyme was obtained with 1 mole of dithionite per mole flavin. The conversion between two types of intermediates in the presence and absence of substrate, respectively, was obtained from the experiment depicted in Fig. 2. The enzyme was first reduced anaerobically with dithionite to produce a red intermediate (Curve II). Salicylate in the side arm was then mixed anaerobically with the red intermediate in the main tube. As expected, the color changed from red to yellow. The resulting intermediate was found to have the same spectral properties as an intermediate reduced in the presence of salicylate (Curve III). The addition of 2,5-dihydroxybenzoate or 3-methylsalicylate to the red intermediate resulted in similar changes in the spectrum.

Photoreduction of salicylate hydroxylase

Spectral changes very similar to those observed upon dithionite titrations were also observed when salicylate hydroxylase was photoreduced. Fig. 3A shows the changes in spectrum produced on light irradiation in the presence of EDTA. A rapid development of a flat long wavelength band very similar to that which appeared during the anaerobic dithionite titration was observed. Prolonged light irradiation resulted in a gradual disappearance of the long wavelength band and a concomitant appearance of the spectrum of the fully reduced enzyme. Upon admitting air into the

cuvette, the original oxidized spectrum was completely restored. A similar species could be obtained when the enzyme was irradiated with light in the presence of 2,5-dihydroxybenzoate or 3-methylsalicylate.

In the absence of substrate, as shown in Fig. 3B, illumination resulted in the formation of a red intermediate which exhibited the same characteristic features as those found in dithionite reduction, the peaks being situated at 375, 400 and 480 nm. Photoreduction of the enzyme at an alkaline pH produced more typical semiquinoid spectrum than at neutral pH.

The addition of substrate to a red intermediate resulted in the conversion of it to a species with a long wavelength band (Fig. 4). Reoxidation of the reduced enzyme with air was rapid, and the original oxidized spectrum was regained.

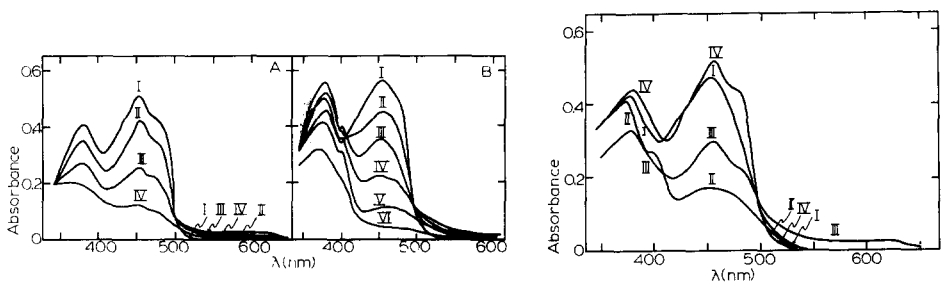
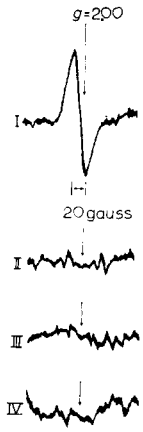


Fig. 3. Comparison of the spectra of salicylate hydroxylase during photoreduction in the presence (A) and absence (B) of 5 μmoles salicylate. Assay conditions: 100 μmoles of potassium phosphate buffer (pH 7.0) and 150 μmoles of EDTA in a total volume of 3 ml. Illumination was carried out with 100 W (A) or 1 kW (B) tungsten lamp for the times shown at 20°. A. Curve I, oxidized enzyme (110 nmoles); Curves II–IV, after illumination for 70, 180 and 570 min, respectively. B. Curve I, oxidized enzyme (121 nmoles); Curves II–VI, after illumination for 20, 40, 60, 100 and 400 sec, respectively.

Fig. 4. Conversion of the red intermediate to the species with a long wavelength absorption band by the addition of salicylate. Curve I, oxidized enzyme (111 nmoles); Curve II, after illumination for 138 min with 100-W tungsten lamp; Curve III, after the subsequent addition of 5 μmoles salicylate; Curve IV, after admitting air into the reaction mixture. Other conditions were the same as in Fig. 3.

Spectral changes of the red intermediate described above were correlated with the appearance of a free radical signal as determined by ESR spectrometer. As shown in Curve I of Fig. 5, a typical signal was observed with the red intermediate. The signal converged toward 20 gauss peak to peak width, the g value being 2.00. However, this signal gradually disappeared as the illumination was continued for prolonged times. The absorption spectrum and ESR signal of the red intermediate of salicylate hydroxylase represent a semiquinoid form of the enzyme-bound flavin. No ESR signal was demonstrable when the enzyme was illuminated anaerobically in the presence of the substrate such as salicylate, 3-methylsalicylate or 2,5-dihydroxybenzoate (Curves II–IV in Fig. 5).

The substrate has a marked effect on the rate of photoreduction of salicylate hydroxylase. As shown in Fig. 6, the holoenzyme was completely reduced in 20 min, while in the presence of the substrate, the rate became slow. The maximum photoreduction required 180 min with salicylate or 120 min with 3-methylsalicylate, respectively. A similar effect was observed in the presence of *o*-phenolsulfonate, a



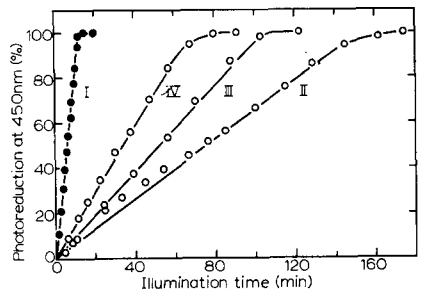


Fig. 5. Comparison of ESR spectra of salicylate hydroxylase as photoreduced in the presence and absence of substrate. The assay system (0.3 ml) contained: 27 nmoles of enzyme, 4.7 μmoles of EDTA, 10 μmoles of potassium phosphate buffer (pH 7.0) and 1.3 μmoles of substrate indicated. The assay mixture was illuminated for the time required to half reduce the flavin with 750-W tungsten lamp. Curve I, without substrate; Curve II, with salicylate; Curve III, with 2,5-dihydroxybenzoate; Curve IV, with 3-methylsalicylate.

Fig. 6. Comparison of rates of photoreduction of salicylate hydroxylase with EDTA in the presence and absence of substrate. The assay system (2.6 ml) contained: 33 nmoles of enzyme, 86 μmoles of potassium phosphate buffer (pH 7.0), 15 μmoles of EDTA and 5 μmoles of substrate indicated. Illumination was carried out at 20° with 1-kW tungsten lamp for the times shown. Curve I, without substrate; Curve II, with salicylate; Curve III, with 3-methylsalicylate; Curve IV, with *o*-phenolsulfonate.

competitive inhibitor for the enzyme; it required 80 min for the maximum photoreduction. However, no appreciable effect was observed with other nonsubstrate substances such as benzoate.

Stoichiometric demonstration that artificially reduced flavin is "active" as an electron donor

Attempts were made to demonstrate that the reduced enzyme thus obtained is capable of aerobic hydroxylation of the substrate. In the first approach to this problem, sodium dithionite was mixed with the enzyme anaerobically in the presence of salicylate. Air was introduced into the cuvette after the amount of the reduced flavin was estimated. Reoxidation of the reduced flavin with air was rapid, and the original oxidized spectrum was regained. When a limited amount of dithionite was added, it was found that the amount of added dithionite was equivalent to that of the enzyme-bound flavin reduced and the catechol formed, respectively (Expts. IA and IB in Table I). That a stoichiometric relation is apparently maintained between dithionite, flavin and product is strongly supported by these results. The enzyme reduced with dithionite in the absence of salicylate was reacted with air. The addition of salicylate before the introduction of air resulted in the stoichiometric formation of catechol (Expt. IC). However, when the reduced enzyme was mixed with air before addition of salicylate, no product formation was detected (Expt. ID). The results indicate that the reduced enzyme is also capable of hydroxylating the substrate once salicylate is added to the system.

TABLE I

REACTIVITY OF THE ENZYME–FLAVIN REDUCED WITH DITHIONITE AND LIGHT IRRADIATION

Each reaction was carried out in a Thunberg-type cuvette with 3 ml assay system containing 100 μmoles of potassium phosphate buffer (pH 7.0) and the components indicated. Illumination (Expt. II) was performed with the use of 100-W tungsten lamp as in MATERIALS AND METHODS in the presence of 150 μmoles of EDTA. The amount of reduced enzyme-bound FAD was estimated by taking the molecular extinction coefficient as 10.3 $mM^{-1} \cdot cm^{-1}$ at 450 nm. Catechol was determined after air was admitted into the cuvette as described in preceding paper[4].

Expt. No.	*Enzyme (nmoles)*	*Salicylate (μmoles)*	*Reductants*		*Enzyme reduced (nmoles)*	*Catechol formed (nmoles)*
			$Na_2S_2O_4$ (nmoles)	*Light (min)*		
IA	147	5	205		147	174
IB	147	5	76		79	64
IC*	147	—	103		115	107
ID**	147	—	206		147	0
IIA	77	5		181	58	41
IIB	110	5		568	101	98
IIC*	110	—		138	60	48
IID**	110	—		551	105	0

* After the enzyme had been reduced, 5 μmoles of substrate were added from the side arm and the reaction mixture was then exposed to air.

** The reduced enzyme was exposed to air before the addition of substrate.

A stoichiometric relationship quite similar to that observed on dithionite reduction was obtained on light irradiation of salicylate hydroxylase (Expt. II in Table I).

As shown in Table II, the results with 3-methylsalicylate–holoenzyme indicated that a stoichiometric amount of 3-methylcatechol was produced from the reduced complex, prepared either with dithionite or by light irradiation.

TABLE II

STOICHIOMETRY IN THE HYDROXYLATION REACTION WHEN 3-METHYLSALICYLATE WAS USED AS A SUBSTRATE

The assay conditions were the same as those described in the legend of Table I, except that 3-methylsalicylate was used as the substrate, and 1-kW tungsten lamp was used for photoreduction. 3-Methylcatechol produced was estimated as described in preceding paper[4].

Expt. No.	*Enzyme (nmoles)*	*3-Methylsalicylate (μmoles)*	*Reductants*		*Enzyme reduced (nmoles)*	*3-Methylcatechol formed (nmoles)*
			$Na_2S_2O_4$ (nmoles)	*Light (sec)*		
IA	65	5	72		61	53
IB*	64	—	70		72	74
IC**	117	—	150		118	0
IIA	131	5		300	133	98
IIB*	131	—		60	124	100
IIC**	122	—		170	121	0

* After the enzyme had been reduced, 5 μmoles of substrate were added from the side arm and the reaction mixture was then exposed to air.

** The reduced enzyme was exposed to air before the addition of substrate.

DISCUSSION

In an attempt to gain some insight into the mechanism of the salicylate hydroxylase reaction, efforts have been made to establish a reaction sequence in which NADH, salicylate and O_2 interact with the enzyme. Recent communications from this laboratory[3,4,8,9] have firmly established that salicylate hydroxylase combines specifically with substrate to form a substrate–enzyme complex in which the ratio of apoenzyme, FAD and salicylate is 1:1:1 and its complex is the actual intermediate involved in the overall reaction of salicylate hydroxylation. With the role of flavin, it was suggested that the flavin moiety of the enzyme can participate in the hydroxylation reaction as the sole electron donor. These conclusions were based on the demonstration that enzyme-bound $FADH_2$ produced by NADH is enzymatically reactive as an electron donor and that the hydroxylation reaction was coupled with the electron transfer from the reduced flavin to O_2. The results of the present investigation have confirmed and extended the conclusion that the reduced flavin, which is produced whether the reducing agent is dithionite, EDTA–light or NADH, is enzymatically "active" as an electron donor in the hydroxylation reaction.

The results indicated that the anaerobic reduction of the enzyme proceeds by way of two spectroscopically distinguishable species depending on the substrate. In the absence of substrate, spectral changes considered to be associated with the formation of semiquinoid form of flavoprotein were observed upon dithionite or EDTA–light reduction of the enzyme. The spectrum of the red intermediate seems to be very similar to those shown in photoreduction of D- and L-amino-acid oxidases, oxynitrilase and glucose oxidase[6]. Recently MASSEY AND PALMER[6] demonstrated two different spectral types of flavoprotein semiquinone which they called "red" and "blue". Furthermore they postulated that the red species was the anion radical, the blue species the neutral semiquinone. This was confirmed by EHRENBERG *et al.*[10]. According to this classification, the red intermediate of salicylate hydroxylase belongs to the red flavin radical species (E. G. ERIKSSON, personal communication). The red intermediate formed the species having a long wavelength absorption band, when mixed anaerobically with salicylate. From the ESR results this appeared not to be due to the blue neutral radical, although it had a similar absorption spectrum. Recently, YAGI *et al.*[11] observed an interesting phenomenon in the case of D-amino-acid oxidase. The red radical of D-amino-acid oxidase was easily converted into a blue-colored substance without any change in its paramagnetic nature only by mixing with benzoate under anaerobic conditions. In this case, the blue-colored substance appeared to be identical with the blue radical described by MASSEY AND PALMER[6]. The flat long wavelength band arising when salicylate hydroxylase is reduced in the presence of substrate is somewhat more difficult to interpret. A specific interaction between reduced flavin and substrate, possibly of the charge transfer type is not eliminated. It should be noted that no long wavelength absorption band or semiquinoid form other than steady transition stage from oxidized to the fully reduced form was observed when the enzyme was reduced anaerobically with NADH in the presence or absence of the substrate[3,4]. The rapid reaction techniques will be required to provide further information in this connection. Absorption spectra of the enzyme in the dynamic steady states of the hydroxylation reaction are currently under investigation in this laboratory.

The substrate–holoenzyme complex was more resistant to the photoreduction

than the holoenzyme itself. These results are consistent with the evidences that the enzyme is more resistant to acid and heat inactivations and proteolytic digestion in the presence of substrate[4]. The effect of substrate on photoreduction, therefore, should be attributed to a difference of a protein conformation between holoenzyme and substrate–holoenzyme. MCCORMICK *et al.*[12], who observed that benzoate decreased the rate of photoreduction of D-amino-acid oxidase, have recently reported a quite similar phenomenon with D-amino-acid oxidase.

ACKNOWLEDGMENTS

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MECHANISM OF THE SALICYLATE HYDROXYLASE REACTION*

IV. FLUOROMETRIC ANALYSIS OF THE COMPLEX FORMATION

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SUMMARY

1. Binary and ternary complexes between apoenzyme of salicylate hydroxylase (salicylate, NADH:oxygen oxidoreductase (1-hydroxylating, 1-decarboxylating)) and components such as FAD, substrate and NADH were fluorometrically studied to determine dissociation constant and stoichiometric ratio of each component. This was based on the changes in fluorescence intensity when the apoenzyme was mixed with these components.

2. A protein-denaturing agent or an alkali treatment was shown to depress the fluorescence emission of the apoenzyme with the red shift of the maximum.

3. The addition of FAD to the apoenzyme resulted in a marked decrease in both FAD and protein fluorescences.

4. Either substrate or NADH formed 1:1 complex with the apoenzyme. This was indicated by both quenching of protein fluorescence and enhancement of substrate or NADH fluorescence.

5. The holoenzyme combined specifically with substrate to form a fluorescent ternary complex in which the ratio of apoenzyme, FAD and substrate was 1:1:1.

6. Upon formation of these complexes, the blue shift occurred in the fluorescence spectrum of protein, salicylate, NADH or FAD.

INTRODUCTION

To elucidate the mechanism of enzymatic catalysis, it is essential to study the intermediate complexes of an enzyme and a substrate. Salicylate hydroxylase (salicylate, NADH:oxygen oxidoreductase (1-hydroxylating, 1-decarboxylating)) is a particularly appropriate enzyme for such a study. The formation of the complex between salicylate hydroxylase and substrate is characterized by the appearance of a new shoulder at 480 nm in the absorption spectrum[2,3]. This change in spectrum has been used to determine the stoichiometry and dissociation constant of the complex as described in the preceding paper[3].

* A preliminary report of this work has been presented[1].

This report presents the results of fluorometric experiments which indicate that binary and ternary complexes are formed between salicylate hydroxylase apoenzyme and components such as FAD, substrate and NADH. These measurements have also made it possible to determine the stoichiometries and dissociation constants of the various complexes.

MATERIALS AND METHODS

Holo- and apoenzymes of salicylate hydroxylase were prepared by the methods described in the preceding paper[3]. Guanidine·HCl, urea, 2-chloroethanol, dioxane and riboflavin were obtained from Wako Pure Chemical Co., Osaka. NAD^+, NADH, $NADP^+$ and NADPH were purchased from Calbiochem. Co. Other chemicals were from the same sources as described in the preceding papers[3,4]. FAD, FMN and riboflavin were further purified by gel filtration on a column (1.5 cm × 60 cm) of Sephadex G-15 (Pharmacia) employing 10 mM Tris–HCl buffer (pH 8.0).

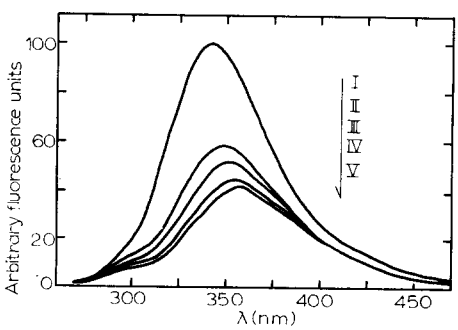
The fluorometric measurements were performed in a Farrand spectrofluorometer, in which a xenon arc lamp (150 W) was used as the exciting source and the slit width was 5–20 nm. All wavelengths reported were uncorrected. All measurements were performed in 10 mM Tris–HCl buffer (pH 8.0) at 20° with the use of quartz cuvettes of 10- and 2-mm light paths.

The enzyme activity of salicylate hydroxylase was determined according to the method described previously[5].

RESULTS

Fluorescence characteristics of the apoenzyme

As shown in Fig. 1, the fluorescent emission spectrum of the apoenzyme was formed with maximum at 342 nm when activated by light of 292 nm. Upon the addition of guanidine to a solution of the apoenzyme, the fluorescence was quenched with the red shift of the maximum to 360 nm (Curves II–V in Fig. 1). In the presence of 7.5 M



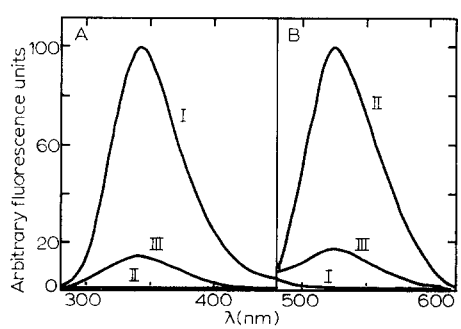


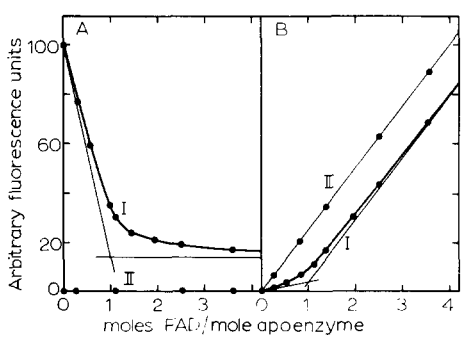
Fig. 1. The effect of guanidine on the emission spectrum of the apoenzyme activated at 292 nm. 4 μM apoenzyme and guanidine were mixed in 10 mM Tris–HCl buffer (pH 8.0) at 20°. The concentrations of guanidine used were as follows: I, 0; II, 1.5 M; III, 2.0 M; IV, 4.0 M; V, 7.5 M.

Fig. 2. Emission spectra of the apoenzyme, FAD and their mixture activated at 292 nm (A) and at 450 nm (B). I, 7.0 μM apoenzyme; II, 7.4 μM FAD; III, apoenzyme + FAD.

guanidine, the fluorescence of the apoenzyme decreased to about 40% of the original intensity. These spectral changes proceeded instantly at room temperature, and further incubation showed no appreciable effect on the fluorescence intensity. Similar effects were observed with protein-denaturing agents such as urea, 2-chloroethanol and dioxane. However, these spectral changes were not induced by increasing an ionic strength in the solvent; no appreciable effects were observed even with 4 M KCl. The fluorescence intensity at 342 nm of the apoenzyme was quenched at alkaline pH values above 9. At pH 13.8, it decreased to about 20% of the original value with the red shift of the maximum to 350 nm.

Holoenzyme formation

Fig. 2 illustrates the fluorescence behavior of the complex between the apoenzyme and FAD. When FAD was added to the apoenzyme solution, the protein fluorescence at 342 nm markedly decreased to 14% of the original intensity (Fig. 2A). The FAD solution emitted fluorescence light at 525 nm when activated at a wavelength of 450 nm. This fluorescence was also quenched by the apoenzyme to 17% of the original intensity (Fig. 2B). The blue shift of 2–3 nm occurred in the fluorescence maximum of either the apoenzyme or FAD. These results indicate that the apoenzyme combines with FAD to form the holoenzyme. Since the formation of the holoenzyme was indicated by quenching of both protein and FAD fluorescences, the stoichiometry and dissociation constant of the holoenzyme could be determined by fluorometric titration. Upon the addition of appropriate amounts of FAD to a solution of the apoenzyme (10–0.1 μM), the fluorescence at 342 nm decreased as indicated by the titration curve in Fig. 3A, and an inflection occurred at the equivalent point. When a similar titration was performed at 525-nm emission band, a clearly defined inflection occurred that indicated 1 mole of FAD was bound to 1 mole of the apoenzyme (Fig. 3B). This



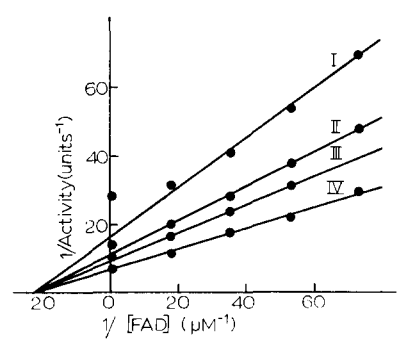


Fig. 3. Fluorometric titration of the apoenzyme with FAD. Fluorescence activated at 292 nm was measured at 342 nm (A) and at 525 nm (B), respectively. Titrations were carried out with 0.5 μM apoenzyme (closed circles in Curve I). The thick line in Curve I was calculated theoretically by using the equation: [apoenzyme] [FAD]/[holoenzyme] = 45 nM. The equivalent point is indicated by the asymptotic lines. Curve II shows the changes of fluorescence caused by FAD alone.

Fig. 4. Determination of Michaelis constant for FAD by a Lineweaver–Burk plot. The apoenzyme was incubated with varying amounts of FAD in the standard assay mixture without NADH. After 5 min preincubation at 20°, the enzymatic assay was performed by the addition of NADH. The concentrations of the apoenzyme used were as follows: I, 48 nM; II, 100 nM; III, 135 nM; IV, 241 nM.

TABLE I

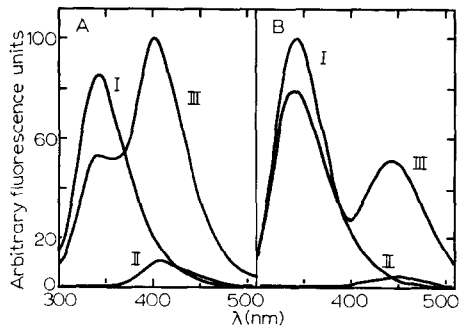
DISSOCIATION CONSTANTS AS MEASURED BY THE STERN–VOLMER EQUATION

Compound	K_d (μM)
Salicylate	1.8
3-Methylsalicylate	6.6
2,3-Dihydroxybenzoate	19
2,4-Dihydroxybenzoate	19
2,5-Dihydroxybenzoate	5.7
2,6-Dihydroxybenzoate	3.6
p-Aminosalicylate	6.8
p-Hydroxybenzoate	660
m-Hydroxybenzoate	840
o-Phenolsulfonate	23
Catechol	440
Benzoate	1160
NADH	11
NADPH	15
NAD^+	180
$NADP^+$	200
FMN	24

value is in accordance with results of equilibrium dialysis[5] or of the direct analysis of the holoenzyme[2]. The dissociation constant of FAD in the holoenzyme could be obtained from these curves. It was calculated to be 45 nM. In order to confirm this value, the Michaelis constant for FAD was determined from changes of hydroxylase activity with variation of FAD and enzyme concentration in the assay system. Fig. 4 illustrates a typical Lineweaver–Burk plot for FAD at different concentrations of the apoenzyme. The K_m value for FAD was found to be 45 nM, which agreed with the dissociation constant obtained from the fluorometric titrations. When FMN at 10 μM level, which was ineffective as a coenzyme of salicylate hydroxylase, under the standard assay conditions was added to the apoenzyme (0.5 μM), the protein fluorescence was also quenched. The dissociation constant of FMN was found to be 24 μM as shown in Table I. These results indicate that the apoenzyme may bind FMN although much less tightly than FAD.

Formation of the apoenzyme–substrate complex

Fig. 5A illustrates the emission spectra of the apoenzyme, of salicylate and of their mixture activated at 292 nm. Salicylate quenched the protein fluorescence at 342 nm, while the apoenzyme enhanced the salicylate fluorescence at 405 nm (Curve III in Fig. 5A). The blue shift of 2–3 nm occurred in the fluorescence maximum of either the apoenzyme or salicylate. The maximum of the excitation spectrum of salicylate was at 305 nm, so that these phenomena seem to be due to the energy transfer. Quite similar spectral changes were also observed when salicylate was replaced by any one of the other substrates for the hydroxylase reaction, namely 2,5-dihydroxybenzoate, 3-methylsalicylate and *p*-aminosalicylate or by a competitive inhibitor, *o*-phenolsulfonate. The emission spectra obtained with 2,5-dihydroxybenzoate are shown in Fig. 5B. However, other substances such as benzoate, *m*-hydroxybenzoate and catechol did not affect the original spectrum of the apoenzyme at 10 μM concentration levels. These results indicate that the apoenzyme combines specifically with the substrate or



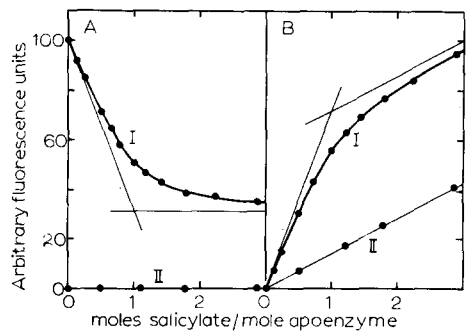


Fig. 5. Emission spectra of the apoenzyme, substrate and their complex activated at 292 nm. A. I, 0.53 μM apoenzyme; II, 3.1 μM salicylate; III, apoenzyme + salicylate. B. I, 0.53 μM apoenzyme; II, 3.1 μM 2,5-dihydroxybenzoate; III, apoenzyme + 2,5-dihydroxybenzoate.

Fig. 6. Fluorometric titration of the apoenzyme with salicylate. Fluorescence activated at 292 nm was measured at 342 nm (A) and at 405 nm (B), respectively. Titrations were carried out with 15 μM apoenzyme (closed circles in Curve I). The thick line in Curve I was calculated theoretically by using the equation: [apoenzyme] [salicylate]/[binary complex] = 1.8 μM. The equivalent point is indicated by the asymptotic lines. Curve II shows the change of fluorescence caused by salicylate alone.

with the inhibitor to form a fluorescent binary complex. This conclusion is consistent with the evidence that the apoenzyme is more resistant to acid and heat inactivations and to proteolytic digestion in the presence of substrate[3].

Since the formation of the complex was indicated by both quenching of the protein fluorescence at 342 nm and enhancement of the salicylate fluorescence at 405 nm, the stoichiometry of the apoenzyme and salicylate in the complex could be determined by titration of the apoenzyme with salicylate. It was graphically derived from the titration curves of Fig. 6 that 1 molecule of salicylate is bound to 1 molecule of the apoenzyme in the fluorescent binary complex. Furthermore, the equilibrium dialysis (K. SUZUKI AND M. KATAGIRI, unpublished work) was adopted to confirm this ratio. The number of molecules of salicylate bound to 1 molecule of the apoenzyme was calculated to be approx. 1 molecule. The dissociation constant of salicylate in the binary complex was calculated to be 1.8 μM when the level of the apoenzyme concentration was 1.0–15 μM. The dissociation constant was also analyzed by using the Stern–Volmer equation[6]:

$$K_d = \frac{Q}{F^\circ/F - 1}$$

where F° and F mean protein fluorescence in the absence or presence of substrate, and Q represents a concentration of substrate. A typical plot in the case of salicylate is illustrated in Fig. 7A. The dissociation constant of salicylate, derived by this equation, was 1.8 μM, which was in accordance with the value obtained by the fluorometric titration. The calculated values of dissociation constant for various substrates are given in Table I.

Formation of the apoenzyme–pyridine nucleotide complex

The emission spectra of the apoenzyme, NADH and their binary complex

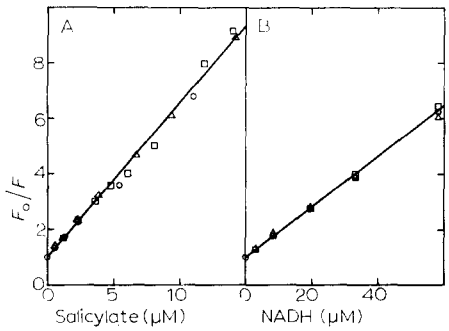
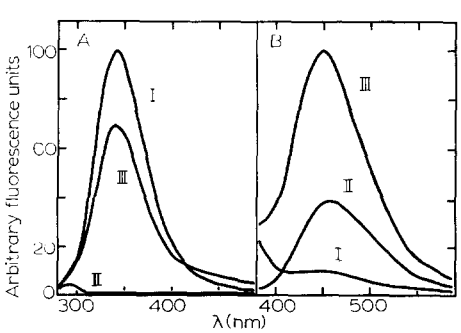


Fig. 7. Determination of dissociation constant of the complex by the Stern–Volmer equation. A. 0.1 μM (○), 0.5 μM (△), and 1 μM (□) apoenzyme were mixed with varying amounts of salicylate as indicated. B. 0.5 μM (○), 1 μM (△) and 4 μM (□) apoenzyme were mixed with varying amounts of NADH as indicated. F° and F are the fluorescence intensities at 342 nm in the absence and presence of salicylate when activated at 292 nm. In the case of A, the fluorescence intensity remaining at the large excess of salicylate was deducted from F° and F.

activated at both the protein exciting light (292 nm) and the NADH exciting light (365 nm) are illustrated in Fig. 8. The protein fluorescence was quenched by NADH (Fig. 8A), while the NADH fluorescence having the maximum at 460 nm was considerably enhanced by the apoenzyme (Fig. 8B). There was the blue shift of 2–3 nm at the emission maximum in both cases.

NADPH, which was less effective than NADH as an electron donor of salicylate hydroxylase[5], gave the similar fluorescence behavior. However, oxidized pyridine nucleotides such as NAD^+ and $NADP^+$ gave no effect on the emission spectrum of the apoenzyme at 10 μM concentration levels.

As shown in Fig. 9, the results from the titration at the concentration level of 1–40 μM apoenzyme indicate that 1 molecule of NADH is bound to 1 molecule of the apoenzyme in the fluorescent binary complex. Because of the high dissociation



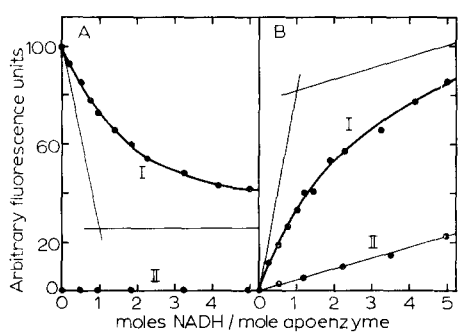


Fig. 8. Emission spectra of the apoenzyme, NADH and their complex activated at 292 nm (A) and at 365 nm (B). I, 4 μM apoenzyme; II, 4 μM NADH; III, apoenzyme + NADH.

Fig. 9. Fluorometric titration of the apoenzyme with NADH. Activation was at 292 nm (A) or at 365 nm (B). Fluorescence intensities were measured at 342 nm (A) or at 452 nm (B), respectively. Titrations were carried out with 10 μM apoenzyme (closed circles in Curve I). The thick line in Curve I was calculated theoretically by using the equation: ([apoenzyme] [NADH])/[binary complex] = 11 μM. The equivalent point is indicated by the asymptotic lines. Curve II shows the change of fluorescence caused by NADH alone.

constant of this complex, the definite inflection could not be observed even at 40 μM apoenzyme, but the titration curves at several concentrations of the apoenzyme agreed with the theoretical curve calculated on the assumption of a 1:1 stoichiometry for the binary complex. The dissociation constant of NADH in the complex was calculated to be 11 μM. This value was further confirmed by using the Stern–Volmer equation (*cf.* Fig. 7B). It was found to be 11 μM at the concentration level of 1–40 μM apoenzyme. However, the affinity of oxidized pyridine nucleotides to the apoenzyme was less than that of NADH and NADPH. The calculated values of dissociation constant are summarized in Table I.

The formation of the ternary complex of apoenzyme, FAD and substrate

When the holoenzyme was excited at 292 mμ, the emission spectrum had two maxima, one (at 340 nm) due to the protein fluorescence and the other (at 523 nm) due to the FAD fluorescence. When salicylate was added to the holoenzyme, the spectral changes produced were quite different from those of the binary complex mentioned above. The protein and salicylate fluorescences were quenched to 80 and 50% of the original intensity, respectively (Fig. 10A and 10B), but FAD fluorescence excited at 450 nm was enhanced to 165% of the original intensity (Fig. 10C). The emission maxima at salicylate and FAD fluorescences were blue-shifted to 15 and 2–3 nm, respectively. These changes in fluorescence spectra were measured under the condition that once added, the third component was incorporated into a ternary complex, leaving hardly any excess of the free component.

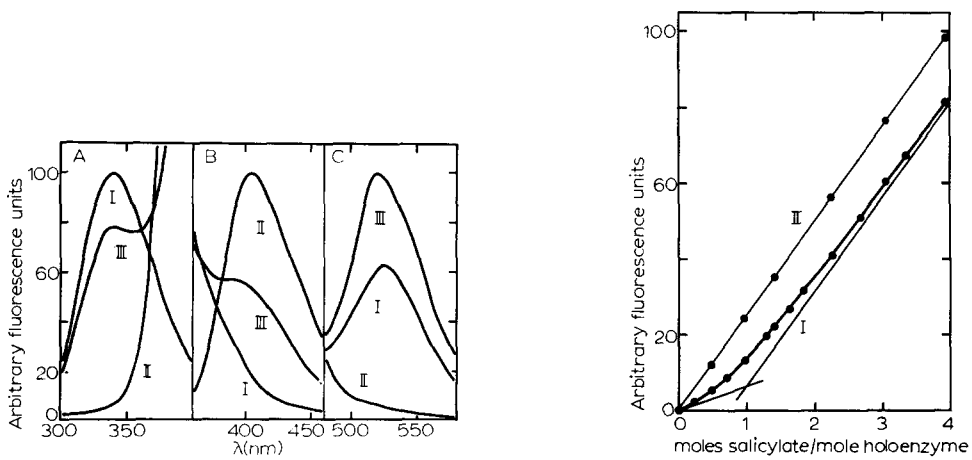


Fig. 10. Emission spectra of the holoenzyme, salicylate and their complex. Activation wavelengths were 292 nm (A and B) and 450 nm (C), respectively. A. I, holoenzyme (7.0 μM apoenzyme + 7.4 μM FAD); II, 20 μM salicylate; III, holoenzyme + salicylate. B. I, holoenzyme (5.6 μM apoenzyme + 7.4 μM FAD); II, 3.9 μM salicylate; III, holoenzyme + salicylate. C. I, holoenzyme (6.1 μM apoenzyme + 5.0 μM FAD); II, 20 μM salicylate; III, holoenzyme + salicylate.

Fig. 11. Fluorometric titration of holoenzyme with salicylate. Fluorescence activated at 292 nm was measured at 405 nm. Titrations were carried out with the mixture of 10 μM apoenzyme and 58 μM FAD (closed circles in Curve I). The thick line in Curve I was calculated theoretically by using the equation: ([holoenzyme] [salicylate])/[ternary complex] = 3.2 μM. The equivalent point is indicated by the asymptotic lines. Curve II shows the change of fluorescence caused by salicylate alone.

The addition to the holoenzyme of 2,5-dihydroxybenzoate, *p*-aminosalicylate and *o*-phenolsulfonate caused similar spectral changes. However, no effect was observed when substances which were not substrates for the hydroxylation reaction were added. These results indicate that the holoenzyme combines specifically with the substrate to form a fluorescent ternary complex.

As shown in Fig. 11, the initial slope of the titration curve was depressed by salicylate and a definite inflection occurred at the equivalent point. Thus 1 molecule of salicylate is bound to 1 molecule of the holoenzyme, and so the ratio of apoenzyme, FAD and salicylate in the ternary complex is 1:1:1. The dissociation constant of salicylate in the ternary complex was calculated to be 3.2 μM. This value coincides well with that determined by spectrophotometric titration[3].

DISCUSSION

The apoenzyme of salicylate hydroxylase showed the fluorescent emission spectrum with a maximum around 342 nm when activated by light at 292 nm. Since, under these conditions, the emission maxima of tryptophan and tyrosine were 348 and 304 nm, respectively, this observation strongly indicates that the fluorescence behavior of the apoenzyme depends on tryptophan residues of the protein moiety. A red shift of the emission peak with decrease of fluorescence intensity was induced by the addition of denaturing agents for proteins. These effects have been observed in the denaturation of other proteins such as human and bovine serum albumins[7]. On the contrary, the denaturing agents enhanced the protein fluorescence of trypsin and chymotrypsin[7]. These results were interpreted by STEINER AND EDELHOCH[8] as meaning that the intensity of fluorescence of the tryptophan residues is a sensitive function of their environment. Therefore, the fluorescence changes caused by denaturing agents here may be regarded as a reflection of a conformational change of the protein near to the tryptophan residues.

The fluorescence of the apoenzyme at 342 nm was quenched by either FAD, substrate or pyridine nucleotide. There was a detectable blue shift of the emission maximum in the case of FAD or pyridine nucleotide, although the red shift was observed in the presence of the denaturing agents. The fluorescence maximum of tryptophan has been reported to be at 355 nm in an aqueous solution but to be blue-shifted to 335 nm upon increasing the polarity of the solution[7]. Therefore, the above phenomena of the apoenzyme may be interpreted as indicating that the complex formation of the apoenzyme makes the surrounding of tryptophan residues of the protein more nonpolar, while the denaturing agent or high pH makes it more polar. The blue-shift was also observed in the case of salicylate, NADH and FAD fluorescences when the apoenzyme was added to these compounds. These results may indicate the increment of the nonpolarity in the regions of binding sites of these compounds.

Although the rate of the reaction between apoenzyme and salicylate or NADH was rapid, the reaction between apoenzyme and FAD was considerably slower and the reaction required more than a few minutes at room temperature to go to completion at the concentration level of 0.1 μM apoenzyme. In the enzymatic assay of the apoenzyme with excess FAD, it was also observed that maximum regeneration of enzyme activity required prolonged incubation. These phenomena suggest that conformational

changes in the apoenzyme probably occur during the incubation period which generate a structure capable of binding FAD.

Now considering the fluorescence behavior of the apoenzyme, it may be suggested that three different types of sites exist in the apoenzyme. There are (a) the substrate-binding site, (b) the pyridine nucleotide-binding site and (c) a site which appears to be specific for FAD. Titration experiments have shown that the apoenzyme combines with salicylate at a molar ratio of 1 and that the dissociation constant for salicylate is 1.8 μM, somewhat lower than the value of 3.2 μM calculated in the holoenzyme complex. As shown in Table I, the dissociation constants for other substrates were approx. 10–1.0 μM and were somewhat higher than the value obtained with salicylate. On the other hand, the dissociation constants for the substrate analogues, such as benzoate or *m*- and *p*-hydroxybenzoates were in the range of 1.0–0.1 mM. It should be noted that a reaction product, catechol, exhibits a low affinity similar to those described above. These results indicate that there appears to be the relationship between the dissociation constant and the substrate specificity of the hydroxylation reaction.

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IDENTITY OF β-GLUCOSIDASE AND β-XYLOSIDASE ACTIVITIES IN RAT LIVER LYSOSOMES

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SUMMARY

1. Subcellular distribution studies of rat liver β-glucosidase (β-D-glucosidase glucohydrolase, EC 3.2.1.21) and β-xylosidase (β-D-xyloside xylohydrolase, EC 3.2.1.37), using *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside as a substrate, respectively, show these enzyme activities to be localized in the lysosomes. The β-glucosidase to β-xylosidase ratio in all subcellular fractions is constant.

2. Both of the activities are more than 80% bound to the lysosomal membrane.

3. Maximum hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside occurs at pH 5.2. The K_m with *p*-nitrophenyl-β-D-glucopyranoside is 1.45 mM and with *p*-nitrophenyl-β-D-xylopyranoside it is 4.05 mM.

4. Glucono-(1→4)-lactone, a specific competitive inhibitor of β-glucosidase, inhibited the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside with a K_i of 1.7 mM. *p*-Chloromercuribenzoate also inhibits both the activities with a K_i of 12.5 μM.

5. The pH stability of the two activities at 37, 50, and 60° is similar.

6. Both the activities are eluted from DEAE-cellulose by 0.122–0.155 M NaCl.

7. The results of these studies strongly support the conclusion that a single enzyme is responsible for both of the activities.

INTRODUCTION

Previous studies[1] indicated that β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) and β-xylosidase (β-D-xyloside xylohydrolase, EC 3.2.1.37) activities of rat liver lysosomes may be of a single enzyme. ROBINSON AND ABRAHAMS[2] have shown these two activities to reside in a single enzyme in the supernatant portion of pig kidney. This paper presents properties of β-glucosidase and β-xylosidase activity of rat liver lysosomes which show that a single enzyme is responsible for hydrolysis of both *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

MATERIALS AND METHODS

p-Nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, 4-methylumbelliferyl-β-D-glucopyranoside, and 4-methylumbelliferyl-β-D-xylopyranoside were obtained from Pierce Chemical Co. *p*-Chloromercuribenzoate (PCMB) was obtained from California Foundation for Biochemicals. Glucono-(1→4)-lactone, Triton X-100, β-glycerophosphate and sodium deoxycholate were obtained from Sigma Chemical Co.; Triton WR 1339, from Ruger Chemical Co.; and DEAE-cellulose from Bio-Rad Laboratories. All other chemicals were of reagent grade.

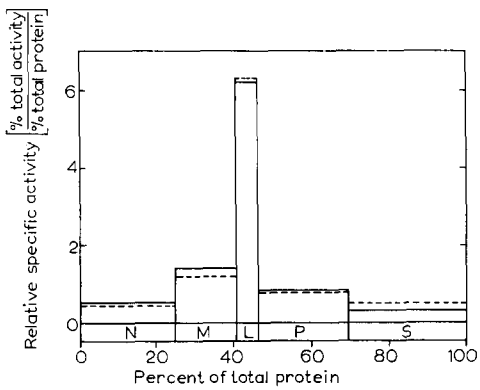


Fig. 1. Subcellular distribution of β-glucosidase (———) and β-xylosidase (- - - -) in rat liver. N, nuclear; M, mitochondrial; L, light mitochondrial; P, microsomal; and S, supernatant fractions. The specific activities of β-glucosidase and β-xylosidase in the light-mitochondrial fraction were 95.8 and 26.0 nmoles of substrate hydrolyzed per mg of protein per h, respectively.

Liver fractionation

Fractionation of rat livers into principal subcellular fractions was done according to the procedure of RAGAB *et al.*[3]. In some experiments (Fig. 1) the livers were homogenized and fractionated into nuclear, cytoplasmic extract, mitochondrial, light mitochondrial, microsomal and supernatant fractions according to the procedure of DE DUVE *et al.*[4]. Triton WR 1339-filled lysosomes were prepared as described by WATTIAUX *et al.*[5].

Separation and solubilization of membrane bound enzyme

Isolated liver lysosomes suspended in 0.25 M sucrose were frozen and thawed 10 times and were then centrifuged at 100 000 × *g* for 60 min to separate the soluble fraction from the membrane fraction. The membrane fraction was suspended in 0.25 M sucrose and homogenized in a Potter–Elvehjem homogenizer.

Since treatment by freezing and thawing solubilized only 17% of β-glucosidase and β-xylosidase activities, it was necessary to solubilize the activities by a different treatment. The membrane fraction suspended in 0.25 M sucrose was treated with 1.0 mg of sodium deoxycholate per mg of membrane protein. The mixture was stirred at 3–4° for 1.5–2 h, unless otherwise stated, and was then centrifuged at 100 000 × *g* for 60 min to separate the soluble fraction from the residue. The soluble portion is

called deoxycholate-solubilized fraction. This treatment also solubilizes other lysosomal membrane bound enzymes, *e.g.* *N*-acetyl-β-D-glucosaminidase, *N*-acetyl-β-D-galactosaminidase and sialidase.

Chromatography of deoxycholate-solubilized fraction of rat liver lysosomes on DEAE-cellulose

DEAE-cellulose was treated as described by PETERSON AND SOBER[6]. The DEAE-cellulose was equilibrated with 5 mM potassium phosphate buffer (pH 7.6) and poured into a 2.0 cm × 30 cm column. The deoxycholate-solubilized fraction was dialyzed against 5 mM potassium phosphate buffer (pH 7.6) for 8 h. A sample of the dialyzed material that contained 25 mg of protein was applied to the column. Fractions were eluted with a linear NaCl gradient and collected in 4.0 ml fractions. Protein in the eluate was estimated by absorbance at 280 nm, using bovine serum albumin as a standard. All operations were done at 4°.

Enzyme determinations

Unless otherwise stated, the reaction mixtures for the assay of β-glucosidase and β-xylosidase contained 4 mM *p*-nitrophenyl-β-D-glucopyranoside or *p*-nitrophenyl-β-D-xylopyranoside, 200–400 μg of lysosomal protein and 60 mM citrate–phosphate buffer (pH 5.2) in a total volume of 1.0 ml. The reaction mixture for measurement of *N*-acetyl-β-D-glucosaminidase contained 2.5 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, 10–15 μg lysosomal protein and 50 mM citrate–phosphate buffer (pH 4.2). Incubations were done at 37° for 30–60 min. The reaction was stopped by addition of 1.5 ml of 2.0 M NH_4OH–HCl buffer (pH 10.7). In assays which contained homogenate, the reaction was stopped by addition of 0.5 ml of 25% trichloroacetic acid. After cooling and centrifuging 1.0 ml of the supernatant portion was used for color development with 1.5 ml of 2.0 M NH_4OH–HCl buffer (pH 10.7). The absorbance of the liberated *p*-nitrophenol was measured at 420 nm. Appropriate substrate, enzyme and reagent blanks were included. Acid phosphatase activity was determined according to GIANETTO AND DE DUVE[7]. The protein was determined according to the method of MILLER[8].

RESULTS

Subcellular distribution of β-glucosidase and β-xylosidase

Two methods were used to study the intracellular distribution of these enzymes. Fig. 1 summarizes the result of fractionation according to the procedure of DE DUVE *et al.*[4]. More than 70% of β-glucosidase and β-xylosidase activities in the cytoplasmic extract was sedimentable with the mitochondrial, light-mitochondrial and microsomal fractions. The highest relative specific activities of β-glucosidase, β-xylosidase and acid phosphatase were found in the light-mitochrondrial fraction. The pattern of distribution for β-glucosidase and β-xylosidase is similar to other lysosomal enzymes studied by DE DUVE *et al.*[4]. The similarity of the distributions of these two activities is apparent. The recoveries of the total activities of acid phosphatase, β-glucosidase and β-xylosidase ranged from 93 to 105%.

Table I presents the specific activities of β-glucosidase and β-xylosidase in purified liver fractions and in Triton-filled liver lysosomes; acid phosphatase is included

TABLE I

β-GLUCOSIDASE AND β-XYLOSIDASE ACTIVITY IN PURIFIED RAT LIVER SUBCELLULAR FRACTIONS

Specific activity is expressed as nmoles of substrate hydrolyzed per mg of protein per h. Determinations of enzyme activities were done as described in MATERIALS AND METHODS.

Liver fraction	*Specific activity*			*Ratio**
	Acid phosphatase	*β-Glucosidase*	*β-Xylosidase*	*β-Glucosidase / β-Xylosidase*
Homogenate	1 290	15.5	4.2	3.7
Mitochondrial	2 320	33.4	9.6	3.5
Microsomal	1 100	30.0	7.9	3.9
Supernatant	983	4.5	1.4	3.2
Lysosomal	33 280	380	99.5	3.8
Lysosomal membrane	17 420	1150	310	3.7
Lysosomal soluble	51 600	105	27.8	3.8
Deoxycholate-solubilized membrane	33 200	1530	424	3.6
Triton homogenate	1 020	12.3	3.3	3.7
Triton lysosomes	35 500	413	103	4.0

* The values for the ratios can be multiplied by a factor of 0.68 to correct for the v_{max} and K_m for the two activities.

for comparison. The increases (24-fold) in the specific activity for both of the activities over the homogenate was almost identical. Table I also shows that the β-glucosidase to β-xylosidase ratio in the fractions remained almost constant from homogenate to deoxycholate-solubilized fraction. The constancy of the ratio in the fractions emphasizes the identity of these two activities.

Distribution of β-glucosidase and β-xylosidase between soluble and membrane fractions of liver lysosomes

Usually, repeated freezing and thawing procedures make lysosomal enzymes available to their substrates, but not all of the enzymes are released from the particulate

TABLE II

CONCURRENCE OF β-GLUCOSIDASE AND β-XYLOSIDASE ACTIVITY IN MEMBRANE AND SOLUBILIZATION BY DEOXYCHOLATE

Soluble fraction refers to the nonsedimentable portion of lysosomes obtained after freezing and thawing 10 times and centrifuging at 100 000 × *g* for 1.0 h. Membrane fraction refers to the sedimentable portion. Deoxycholate treatment is described in MATERIALS AND METHODS.

Fraction	*Percent of total activity*	
	β-Glucosidase	*β-Xylosidase*
Soluble	17	17
Membrane	83	83
Solubilized by deoxycholate treatment		
0.5 h	54	55
1.0 h	75	75
2.0 h	83	83

material into soluble form to the same extent. This has been demonstrated with liver lysosomes[3] and kidney lysosomes[9,10]. The results in Table II show that the freezing and thawing of rat liver lysosomes 10–15 times solubilized only 17% of the total β-glucosidase and β-xylosidase activities. Table II also shows the extent of solubilization by deoxycholate treatment. β-Glucosidase and β-xylosidase are solubilized to the same extent, having 83% of the activities solubilized by 2 h of treatment.

Effect of pH

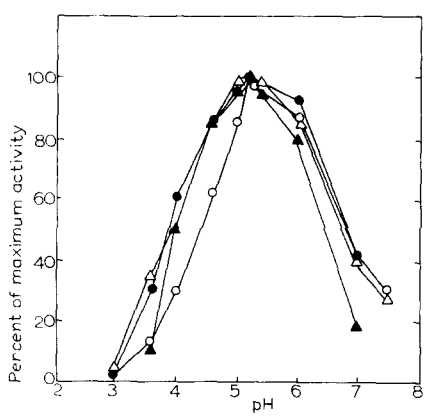
Maximum hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside or *p*-nitrophenyl-β-D-xylopyranoside by rat liver homogenate, lysosomes, lysosomal soluble fraction, lysosomal membrane fraction and deoxycholate-solubilized fraction occurred at pH 5.2. The pH–activity curves for β-glucosidase and β-xylosidase in homogenate and deoxycholate-solubilized fraction are shown in Fig. 2.

Effect of time and protein concentration

β-Glucosidase and β-xylosidase activities increased linearly with time up to 60 min of incubation when assayed in a total volume of 1.0 ml with 300 μg of lysosomal membrane protein and both of the activities were proportional to the amount of enzyme added up to 400 μg lysosomal membrane protein.

Effect of substrate concentration

From LINEWEAVER–BURK[11] plots for β-glucosidase and β-xylosidase activity, the K_m values were determined. The K_m was 1.45 mM with *p*-nitrophenyl-β-D-glucopyranoside as substrate while it was 4.05 mM with *p*-nitrophenyl-β-D-xylopyranoside



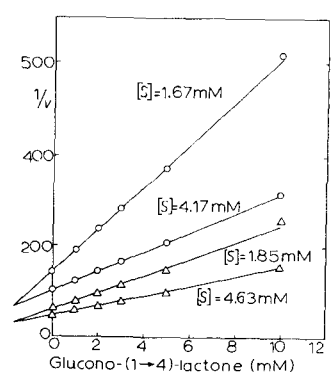


Fig. 2. β-Glucosidase and β-xylosidase activity as a function of pH. Substrate used was 4 mM *p*-nitrophenyl-β-D-glucopyranoside or *p*-nitrophenyl-β-D-xylopyranoside in 60 mM citrate–phosphate buffer. β-Glucosidase activity in homogenate (○—○) and in deoxycholate-solubilized fraction (●—●); β-xylosidase activity in homogenate (△—△) and in deoxycholate-solubilized fraction (▲—▲).

Fig. 3. Effect of glucono-(1 → 4)-lactone on β-glucosidase (○—○) and β-xylosidase (△—△) activity. The reaction mixture contained 260 μg of protein from the dialyzed deoxycholate-solubilized fraction, 60 mM citrate–phosphate buffer (pH 5.2) and the indicated amounts of substrate and glucono-(1 → 4)-lactone in a total volume of 1.0 ml. Incubations were conducted for 30 min for β-glucosidase and 60 min for β-xylosidase. $1/v$ is multiplied by 10^5 for β-glucosidase and 10^4 for β-xylosidase activity, respectively.

as substrate. The difference between the K_m values of these two activities reflects the affinities of this enzyme for these substrates.

Effect of glucono-(1→4)-lactone

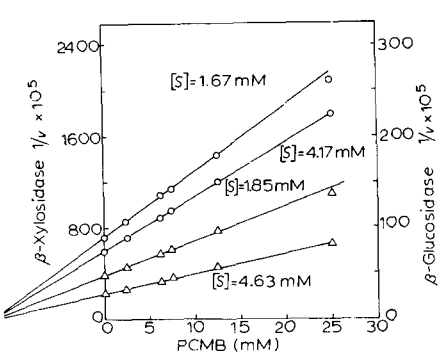
Glucono-(1→4)-lactone has been shown by CONCHIE AND LEVVY[12] to be a specific competitive inhibitor of β-glucosidase activity. The effect of glucone-(1→4)-lactone on β-glucosidase and β-xylosidase activity is shown in Fig. 3. The data are plotted by a DIXON[13] plot. The K_i, 1.7 mM, is the same for both the activities. The K_i is similar to the K_m for β-glucosidase, which indicates equal affinity for glucono-(1→4)-lactone and *p*-nitrophenyl-β-D-glucopyranoside. Since the K_i with *p*-nitrophenyl-β-D-xylopyranoside or *p*-nitrophenyl-β-D-glucopyranoside as substrate is the same, this indicates the probability of a single catalytic site for hydrolysis of the substrates.

Inhibition by PCMB

β-Glucosidase from *Saccharomyces cerevisiae* has been shown to be inhibited by PCMB[14]. The inhibition of β-glucosidase and β-xylosidase activity by PCMB is shown in Fig. 4. Both β-glucosidase and β-xylosidase activities are strongly and noncompetitively inhibited. The K_i for both the activities is the same, being 12.5 μM PCMB. The strong inhibition of both the activities by PCMB indicates the involvement of an -SH group at the active site. The fact that the K_i is the same for both of the activities strongly indicates that a single catalytic site is involved in the hydrolysis of both *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside.

Inhibition of β-glucosidase by 4-methylumbelliferyl-β-D-xylopyranoside and β-xylosidase by 4-methylumbelliferyl-β-D-glucopyranoside

The inhibitions of β-glucosidase by 4-methylumbelliferyl-β-D-xylopyranoside,



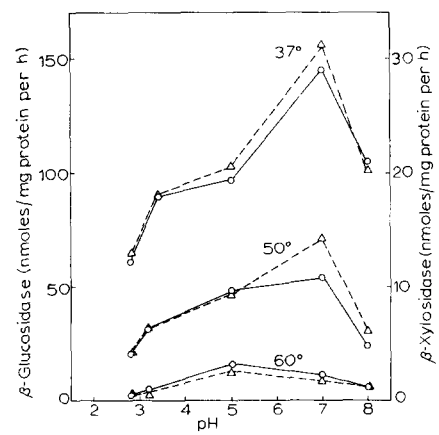


Fig. 4. Effect of PCMB on β-glucosidase (○—○) and β-xylosidase (△—△) activity. The reaction conditions are as described in Fig. 3.

Fig. 5. pH and heat stability of β-glucosidase (○—○) and β-xylosidase (△- - -△). The deoxycholate-solubilized fraction was brought to different pH levels in 1 mM citrate–phosphate buffer. Enzyme assays were done with 80 mM citrate–phosphate buffer, 4 mM substrate and 260 μg of the deoxycholate-solubilized protein. The temperature treatment is described in RESULTS.

a substrate for β-xylosidase, and of β-xylosidase by 4-methylumbelliferyl-β-D-glucopyranoside, a substrate for β-glucosidase, were found to be competitive. The data plotted by a DIXON[13] plot gave a K_i of 6.4 mM for β-glucosidase and 2.3 mM for β-xylosidase. The competitive inhibition of both the activities by these substrates very strongly suggests that the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside occurs at the same catalytic site.

pH and heat stability

Aliquots of the deoxycholate-solubilized fraction were brought to different levels of pH in 1 mM citrate–phosphate buffer, with final concentration of 1 mg of protein per ml. The mixtures were brought to 37, 50 and 60° and held for 10 min. The fractions were then cooled in a dry ice–acetone mixture (approx. −60°). The enzyme assays were done as described in MATERIALS AND METHODS except that the final buffer concentration was 80 mM. Incubations for measuring activity after the 50 and 60° treatment were 1.5–3 h for β-glucosidase and 3–6 h for β-xylosidase. The effects of pH and temperature are shown in Fig. 5. At 37 and 50° both β-glucosidase and β-xylosidase activities were most stable at pH 7.0. However, at 60° they were most stable at pH 5.0. The pH–stability curves at 37, 50 and 60° are similar for both the activities.

DEAE-cellulose chromatography

The deoxycholate-solubilized fraction was used for the fractionation. The experimental conditions are described in MATERIALS AND METHODS. The results in Fig. 6 show that both the activities are eluted between 0.122 and 0.155 M NaCl. Fig. 6 also shows the elution patterns for β-*N*-acetylglucosaminidase and acid phosphatase, which are separated from β-glucosidase and β-xylosidase activities. The similarity in elution pattern and constancy of β-glucosidase to β-xylosidase ratio throughout the column fractions emphasize their identity.

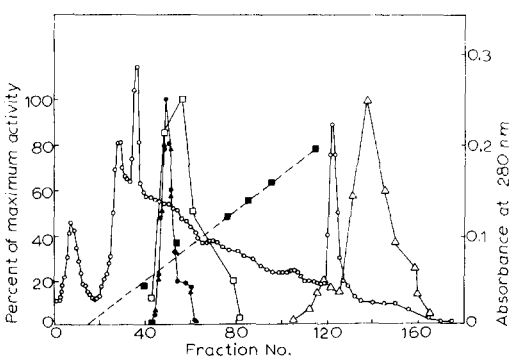


Fig. 6. Chromatography of deoxycholate-solubilized fraction of lysosomal membrane on DEAE-cellulose at pH 7.6 in 5 mM potassium phosphate buffer. Protein (○—○) was eluted with a linear gradient (■- - - -■) of 0–0.4 M NaCl at Fraction 120 and then by 0.8 M NaCl from Fractions 120–180. Enzyme activities were as follows: β-glucosidase, ●—●; β-xylosidase, ▲—▲; β-*N*-acetylglucosaminidase, □—□; and acid phosphatase, △—△. Maximum specific activity for β-glucosidase and β-xylosidase was obtained in Fraction 59. Fraction 59 gave 253-fold purification of β-glucosidase and 257-fold for β-xylosidase over homogenate. The recovery of each activity applied to the column was 15%.

DISCUSSION

Lysosomes are known to be involved in the degradation of proteins, glycoproteins and other complex substances[15–18] and are known to contain a large number of glycosidases[19]. In addition to β-glucosidase and β-xylosidase there are: β-glucuronidase (EC 3.2.1.31), β-galactosidase (EC 3.2.1.23), α-glucosidase (EC 3.2.1.20), α-mannosidase (EC 3.2.1.24), β-*N*-acetylglucosaminidase, β-*N*-acetylgalactosaminidase, lysozyme, and hyaluronidase. β-Glucose occurs in gangliosides and β-xylose occurs in proteoglycans and glycoproteins. β-Glucosidase and β-xylosidase appear to be involved in the detachment of these sugar molecules. β-Xylose has been shown to form a linkage between carbohydrate and protein moieties of proteoglycans[20–22] and glycoproteins[23]. Although the possibility that β-xylosidase activity is involved in cleaving the xylosyl–serine linkage is attractive and in fact has been suggested by ROBINSON AND ABRAHAMS[2], we have found that the lysosomal preparations from rat liver and kidney fail to hydrolyze xylosyl–serine. ARONSON AND DE DUVE[16] also found no hydrolysis of xylosyl–serine by rat liver lysosomes. These latter findings may account, in part, for excretion of significant amounts of xylosyl–serine in human urine, which may arise from degradation of proteoglycans or glycoproteins as has been demonstrated by TOMINGA *et al.*[24]. Even though hydrolysis of the dipeptide xylosyl–serine by a lysosomal enzyme has not been found, it is not known whether or not the bond is hydrolyzed if it is a part of a longer peptide. Structural similarity between β-D-glucose and β-D-xylose makes it possible for a single enzyme to exhibit activities towards *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside. The enzyme apparently does not differentiate between the $-CH_2OH$ group attached to the C-5 in the pyranose form of β-D-glucose and the –H atom on C-5 of β-D-xylose. This possibility has been suggested by BAUMANN AND PIGMAN[25] and GOTTSCHALK[26]. Competitive inhibition of both the activities by glucono-(1→4)-lactone, and analogous substrates supports this suggestion.

Other work also emphasizes the identity of β-glucosidase and β-xylosidase. We found that all subcellular fractions from dog and rabbit liver show the same ratio of β-glucosidase to β-xylosidase activity. Hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside by lysosomes from the livers of these animals occurred maximally at pH 5.2. β-Glucosidase and β-xylosidase have been shown to be identical in human liver also[27].

Experimental evidence presented in this paper strongly suggests that a single enzyme is responsible for β-glucosidase and β-xylosidase activities. It is tempting to postulate that β-xylosidase activity is always thusly associated with β-glucosidase activity in mammalian tissue. These two activities are separate in plants[28,29], which would be expected since compounds which contain β-xylose are abundant in plants. It is noteworthy that we have observed hydrolysis of xylosyl–serine by homogenates of the snail *(Helix pomatia)*. This is not surprising since snails feed on plants. FISHER *et al.*[30] reported hydrolysis of xylosyl–serine by β-xylosidase obtained from commercial almond emulsion. A preliminary report by MANNERS AND MITCHELL[31] indicates that almond β-xylosidase and β-glucosidase activities may be due to a single enzyme.

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THE INFLUENCE OF MONOVALENT CATIONS AND HYDROSTATIC PRESSURE ON β-GALACTOSIDASE ACTIVITY*

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SUMMARY

Monovalent cation activation of β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) was investigated utilizing *o*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-galactopyranoside and lactose as substrates. With these substrates the affinity of β-galactosidase for Na^+ was found to be higher than for K^+. Activation of *p*-nitrophenyl-β-D-galactopyranoside hydrolysis by K^+ was inhibited by Na^+, while the activation of *o*-nitrophenyl-β-D-galactopyranoside hydrolysis by Na^+ was stimulated by K^+.

Hydrostatic pressure stimulated the rate of *o*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-galactopyranoside and lactose hydrolysis in the presence of saturating levels of Na^+ and substrate. In contrast, activation by K^+ with these substrates was consistently depressed by the application of hydrostatic pressure. These results are interpreted on the basis of volume change of an activated enzyme–substrate complex. It was concluded that a decrease in the volume of the complex occurs in the presence of Na^+, while the converse occurs in the presence of K^+, and that the mechanism of hydrolysis is different in the case of Na^+-activated as opposed to K^+-activated substrate hydrolysis.

INTRODUCTION

The activation of β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) from *E. coli* by monovalent cations previously has been demonstrated[1-9]. When lactose, *p*-nitrophenyl-β-D-galactopyranoside and *p*-nitrophenyl-β-D-arabinopyranoside were used as the substrates, maximal activation was obtained in the presence of K^+. When *o*-nitrophenyl-β-D-galactopyranoside or *o*-nitrophenyl-β-D-arabinopyranoside was used as the substrate, the greatest activation was observed from the addition of Na^+. It appears that the position of the nitro group on the aglycone is critical in determining whether Na^+ or K^+ activates to the greatest extent. The affinity of β-galactosidase for any one of the above substrates was altered by the addition of the monovalent cations and was greatest in the presence of Na^+ (ref. 9).

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Several hypotheses have been suggested to explain monovalent cation activation of β-galactosidase. According to one proposal, monovalent cation activation resembles the modification of enzyme activity caused by changes in H^+ concentration[4]. NEVILLE AND LING[8], utilizing graphical methods of DIXON[10], have determined (a) the dissociation constants for the substrate, *o*-nitrophenyl-β-D-galactopyranoside, in the presence of several Na^+ concentrations and (b) the dissociation constant for Na^+ in the presence of several substrate concentrations. They found that a change in the dissociation constant for *o*-nitrophenyl-β-D-galactopyranoside produced by the addition of Na^+ was proportional to the change in the dissociation constant for Na^+ resulting from the addition of *o*-nitrophenyl-β-D-galactopyranoside. They also suggested that the monovalent cations were not reacting with the uncharged substrate and that a reaction of cations with product would not be important because initial rates were measured in the assays. Another postulated mode of activation is that monovalent cations surround a negatively charged species near an imidazolium group on the enzyme and that protection of this group from the negatively charged species facilitates an increased dissociation resulting in a shift of the pK toward a more acidic position[9].

This paper presents the results of an investigation concerning the mechanism of Na^+ and K^+ activation of β-galactosidase. The effect of hydrostatic pressure on this cation activation was also investigated with the intent of providing some insight into the mechanisms involved.

MATERIALS AND METHODS

Preparation of β-galactosidase

Cultures of *Escherichia coli*, ML 308, constitutive for β-galactosidase, were a generous gift from Mr. Richard Newton (Department of Chemistry, University of Oregon, Eugene, Oreg.). The cells were grown overnight at 37° in a 100-l fermentor. Each liter of the culture medium contained 13.2 g $Na_2HPO_4 \cdot 7H_2O$, 3.0 g KH_2PO_4, 1.0 g NH_4Cl, 0.4 g $MgSO_4$ and 10 ml of glycerol. After harvesting with a Sharples centrifuge, the cells (700 g wet weight) were suspended in 2 l of Buffer A which contained 0.05 M acetic acid, 0.028 M thioglycollic acid, 0.01 M $MgCl_2$, and solid Tris (pH 7.0) (Trizma, Sigma Chemical Company, St. Louis). The cellular suspension was stored at −90° until utilized.

All purification experiments were performed at 0–5° unless otherwise designated. Part of the cellular suspension (850 ml) was broken by means of a French press with a pressure of approx. 8 tons/inch^2. A crude extract was obtained by centrifugation of the macerated cells for 1 h at 34 000 × g. The initial steps in the purification scheme were performed according to the procedure of HU *et al.*[11] which involved a streptomycin sulfate treatment, $(NH_4)_2SO_4$ precipitation, and DEAE-cellulose column chromatography. The fractions of high specific activity from the DEAE-cellulose column were precipitated by adding 2 vol., in relation to the volume of eluate, of a saturated $(NH_4)_2SO_4$ solution. The precipitate was dissolved in 55 ml of Buffer B (0.01 M thioglycollic acid and 0.01 M $MgCl_2$ adjusted to pH 7.7 with solid Tris) and dialyzed overnight against Buffer B. The final solution was added to a Sephadex G-200 column (2.5 cm × 35 cm) and eluted with Buffer B by the reverse-flow method. The active fractions eluting directly from the column were then applied to a DEAE-Sephadex

G-50 column (2.5 cm × 35 cm) and eluted with 500 ml of a linear NaCl gradient (0–5%) prepared in Buffer B. The fractions containing β-galactosidase of the highest specific activity were combined, and 2 vol. of satd. $(NH_4)_2SO_4$ solution were added. The resultant precipitate was dissolved in 0.033 M sodium phosphate buffer (pH 6.0) containing 5% NaCl. The protein concentration of this solution was 20 mg/ml. Upon the slow addition of saturated $(NH_4)_2SO_4$ the fraction precipitating between 27 and 31% of saturation was collected by centrifugation and stored at −20° in 50% saturated $(NH_4)_2SO_4$ in Buffer B.

Prior to use, the enzyme preparation was dialyzed overnight against 250 ml of 0.1 M histidine (free base, L form, Sigma), with two changes of the dialysis solution. The histidine was recrystallized from a saturated aqueous solution by the addition of 2 vol. of redistilled absolute ethanol. A histidine buffer was chosen because a solution of the free base has a pH of 7.6 without the addition of an acid or base, and it did not appear to affect the activity of the enzyme. An appropriately diluted preparation of the enzyme and a 0.1 M histidine buffer system were used in all experiments unless indicated otherwise. In all experiments the glassware was rinsed and the reagents were prepared in glass-distilled deionized water. In those experiments in which low levels of Na^+ were added, experimental glassware was washed with 3 M HCl and rinsed with glass-distilled deionized water.

Assays for β-galactosidase

Routine assays during the enzyme purification scheme were performed at 22° utilizing Buffer A, an appropriately diluted enzyme, 2.3 mM *o*-nitrophenyl-β-D-galactopyranoside (Sigma), and 0.1 M NaCl. The enzymatic activity in this reaction mixture (3.0 ml) was determined spectrophotometrically by the change in absorbance at 410 mμ. Protein concentration was determined on the basis of absorbance at 260 and 280 mμ (ref. 12).

Assays for monovalent cation activation of the hydrolysis of either *o*-nitrophenyl- or *p*-nitrophenyl-β-D-galactopyranoside (Sigma) by β-galactosidase were conducted in a reaction containing the histidine buffer, the substrate at the desired concentration, the appropriate amount of monovalent cation chloride, and the enzyme in a total volume of 3.0 ml. The activity was measured by the change in absorbance per min at 410 mμ at 22° using a Cary Model 11 spectrophotometer.

When lactose was used as the substrate, each reaction mixture was incubated at 22° utilizing a substrate concentration at 45 mM unless indicated otherwise, the appropriate amount of cation chloride, and enzyme. The mixture (0.3 ml) was incubated for 10 min and the reaction was stopped by placing it in boiling water for 5 min. Enzymatic activity was followed by the measurement of the production of free glucose present with the Glucostat reagent (Worthington Biochemical Corporation, Reinhold, N.J.).

Hydrostatic pressure experiments

The experimental apparatus for hydrostatic pressure experiments and its operation were essentially as previously described[13]. The pressure bombs were equilibrated to 5° before use, and all pressure experiments unless otherwise indicated were performed at this temperature. The concentrations of substrate and cation chlorides were as given in Figs. 2, 3 and 4 *plus* Tables III and IV. The incubation mixture and

the diluted enzyme at 0–1° were mixed immediately before incubation and transferred to a 10 mm × 75 mm Pyrex culture tube. The reaction mixture of 4 ml completely filled each tube. A nichrome wire extending into each reaction mixture and hooked over the edge of each of the tubes facilitated the escape of a portion of the reaction solution that occurred upon the insertion of a Neoprene stopper. Tubes were transferred immediately to bombs, the bombs were closed, and the desired pressure applied. This entire operation was completed within 2–3 min. The bombs and the tubes containing the incubation mixtures were then transferred to a water bath at 5°. After incubation for 1 h, the pressure was checked and released. In those experiments in which *o*-nitrophenyl- and *p*-nitrophenyl-β-D-galactopyranoside were used as the substrate, the absorbance at 410 mμ was determined immediately. When lactose was used as the substrate the reactions were stopped by placing the tubes in boiling water for 5 min. The free glucose in a 0.3-ml aliquot was assayed as described previously.

RESULTS

Preparation of β-galactosidase

The specific activity of the purified enzyme assayed in the histidine buffer system at 22° in the presence of 100 mM NaCl and 2.3 mM *o*-nitrophenyl-β-D-galactopyranoside was 105 μmoles *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per min per mg protein. The specific activity as measured in the phosphate buffer system at 22° utilized by HU *et al.*[11] was not appreciably increased. Specific activities (μmoles *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per min per mg protein) which have been reported previously for a homogenous preparation include those of HU *et al.*[11] of 146 when assayed at 25°, and COLBY AND HU[14] of 160 when assayed at 30°.

Acrylamide gel disc electrophoresis[15] of the enzyme preparation in 0.05 M Tris and 0.38 M glycine (pH 8.3) revealed two main bands and four minor bands after fixing with acetic acid and staining with amido black[16]. Exposure of some unfixed and unstained gels to a buffered *o*-nitrophenyl-β-D-galactopyranoside solution containing 0.1 M NaCl revealed a yellow color in the areas of a centrally located main band and in another band that remained near the origin. It is estimated that these two bands compose from 80 to 90% of the protein in the gel. It seems possible that the slower moving active band represents a polymeric form of the enzyme since multiple species of this enzyme have been detected[17] by use of the ultracentrifuge.

Monovalent cation activation

The Michaelis–Menten constants for the substrates *o*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-galactopyranoside and lactose are given in Table I. In all instances the affinity in the presence of Na^+ was considerably greater than that in the presence of K^+. The apparent dissociation constants for Na^+ and K^+, and for the same substrates are given in Table II. With all substrates tested the values obtained for Na^+ are lower than corresponding values observed for K^+.

For all the substrates utilized, appreciable hydrolysis occurred without the addition of exogenous monovalent cations. This hydrolysis in the absence of added cations could be due to either small amounts of contaminating cations or the lack of an absolute cation requirement, although on the basis of Na^+ and K^+ measurements made with a flame photometer the former appears to be more probable. The apparent

TABLE I

THE APPARENT MICHAELIS–MENTEN CONSTANTS OF β-GALACTOSIDASE FOR THE SUBSTRATES *o*-NITROPHENYL-β-D-GALACTOPYRANOSIDE, *p*-NITROPHENYL-β-D-GALACTOPYRANOSIDE, AND LACTOSE IN THE PRESENCE OF Na^+ AND K^+

The enzymatic assays were performed as described in MATERIALS AND METHODS. The respective constants were determined from Lineweaver–Burk double reciprocal plots.

Substrate	*Cation*	*Cation concn. (mM)*	K_m *(mM)*
o-Nitrophenyl-β-D-galactopyranoside	K^+	5	0.54
	Na^+	2.5	0.27
p-Nitrophenyl-β-D-galactopyranoside	K^+	22.5	0.77
	Na^+	1	0.13
Lactose	K^+	20	5.3
	Na^+	5	0.58

K_A values, mentioned above and calculated on the basis of added cations, are low and this high affinity could account for the observed substrate hydrolysis in the absence of cation additions.

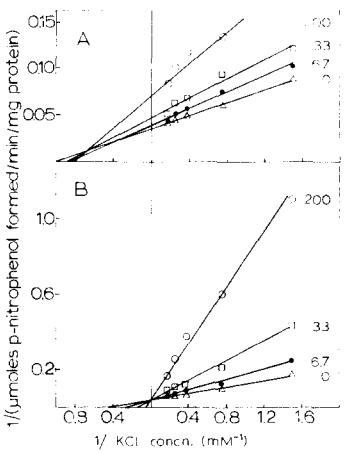
A study was conducted to determine the effect of Na^+ at different concentrations on K^+ activation of the hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside by β-galactosidase. A double reciprocal plot of these results (Fig. 1) yields either a mixed type or competitive inhibition depending upon the method of presenting these results If the results are plotted without substracting the endogenous rate (Fig. 1A) the mixed inhibition is observed. If the endogenous rate is substracted from each value the plotted data illustrate a competitive type of inhibition (Fig. 1B). When *o*-nitrophenyl-β-D-galactopyranoside was utilized as the substrate in presence of Na^+ as an activator cation, the addition of K^+ increased rather than decreased the rate of hydrolysis. The increase in rate obtained by adding the two cations together was not equivalent to the increase obtained in adding the cations separately.

TABLE II

THE APPARENT DISSOCIATION CONSTANTS OF β-GALACTOSIDASE FOR THE CATIONS K^+ AND Na^+ UTILIZING *o*-NITROPHENYL-β-D-GALACTOPYRANOSIDE, *p*-NITROPHENYL-β-D-GALACTOPYRANOSIDE, AND LACTOSE AS SUBSTRATES

The enzymatic assays were performed as described in MATERIALS AND METHODS. The respective dissociation constants were determined from Lineweaver–Burk double reciprocal plots.

Substrate	*Substrate concn. (mM)*	*Cation*	K_A *(mM)*
o-Nitrophenyl-β-D-galactopyranoside	10	K^+	0.07
	10	Na^+	0.01
p-Nitrophenyl-β-D-galactopyranoside	10	K^+	1.1
	10	Na^+	0.01
Lactose	45	K^+	4.8
	45	Na^+	0.23



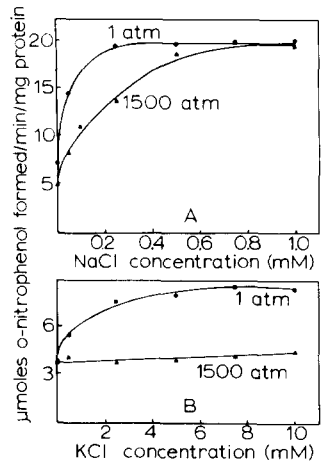


Fig. 1. The inhibition by Na^+ of the K^+ activation of β-galactosidase hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside. Assay and incubation conditions were as described in MATERIALS AND METHODS with the substrate concentration at 10 mM. A. The inverse plot of the results as they were obtained. B. The inverse plot of results where the rate in the absence of cation additions was subtracted from each value. The numerical values to the right of each line are molar Na^+ concentrations added to the respective incubation mixtures.

Fig. 2. The effect of hydrostatic pressure on the Na^+ (A) and K^+ (B) activated hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside by β-galactosidase. The incubation mixture (4 ml) consisted of 0.1 M histidine, 2.3 mM *o*-nitrophenyl-β-D-galactopyranoside, 5 mM dithiothreitol, the enzyme preparation (0.3 μg protein), and the appropriate cation chloride addition. The assay mixtures were incubated at 5° for 1 h either at 1 or 1500 atm pressure. Activity was determined on the basis of the change in absorbance at 410 mμ.

TABLE III

THE EFFECTS OF HYDROSTATIC PRESSURE ON THE RATE OF β-GALACTOSIDASE HYDROLYSIS OF *o*-NITROPHENYL-β-D-GALACTOPYRANOSIDE AND *p*-NITROPHENYL-β-D-GALACTOPYRANOSIDE

The incubation mixture (4 ml) for the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside and *p*-nitrophenyl-β-D-galactopyranoside consisted of the substrate, 5 mM dithiothreitol, the appropriate cation chloride addition and the enzyme. The assay mixtures were incubated at 5° for 1 h at the designated pressure. Activity was determined on the basis of the change in absorbance at 410 mμ. The volume change (ΔV^*) was calculated on the basis of the rate at atmospheric pressure and the rate at the applied pressure.

Substrate	*Substrate concn. (mM)*	*Cation*	*Cation concn. (mM)*	*Pressure (atm)*	ΔV^* *(cm³/mole)*
o-Nitrophenyl-β-D-galactopyranoside	2.3	K^+	10	1500	+10.1
	10	K^+	30	1500	+16.9
	2.3	Na^+	1	1500	0
	10	Na^+	3.3	1500	− 4.2
p-Nitrophenyl-β-D-galactopyranoside	2.3	K^+	20	1500	+20.4
	10	K^+	30	500	+ 4.2
	10	K^+	30	1000	+ 8.6
	10	K^+	30	1500	+17.9
	2.3	Na^+	1	1500	− 6.8
	10	Na^+	3.3	1500	− 5.0

Hydrostatic pressure experiments

A series of experiments were conducted to determine the effects of hydrostatic pressure on the monovalent cation activation of the hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside and *o*-nitrophenyl-β-D-galactopyranoside. At the beginning of these experiments it was established that the rate of hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside in presence of Na^+ at 1500 atm was linear for a period of over 1 h. Also in preliminary experiments it was demonstrated that the inhibition of the K^+ activation of the hydrolysis of *p*-nitrophenyl-β-D-galactosidase by hydrostatic pressure was reversible because hydrolysis in a reaction inhibited by pressure continued at a normal rate when the pressure was released.

The effects of hydrostatic pressure on the enzymatic hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-galactopyranoside and lactose in the presence of Na^+ and K^+ are shown in Figs. 2, 3 and 4 and Table III. Each value represents an average of three replications. In the case of *o*-nitrophenyl-β-D-galactopyranoside hydrolysis pressure did not affect the rate in the presence of saturating levels of Na^+ and 2.3 mM substrate (Fig. 2) but pressure increased the rate at 10 mM substrate (Table II). The effect of pressure on the K^+ activation of lactose hydrolysis (Fig. 4) was not as pronounced as the effect on the hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside but inhibition by pressure was still evident.

Since all of the preceeding pressure experiments were conducted at 5°, experiments were also performed at 25° using *p*-nitrophenyl-β-D-galactopyranoside as the substrate. Pressure effects at 25° were similar to those observed at 5° except that the turnover rate of the enzyme was increased.

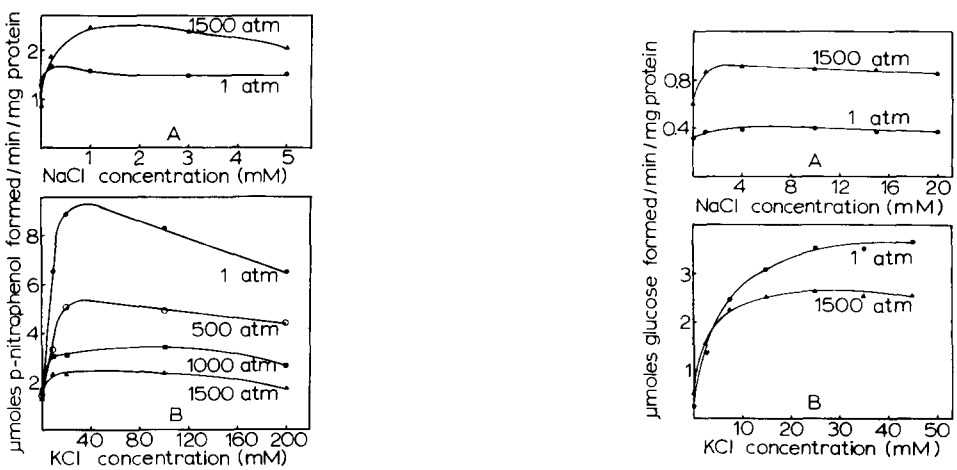


Fig. 3. The effects of hydrostatic pressure on the Na^+ (A) and K^+ (B) activated hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside by β-galactosidase. Conditions were similar to those described in Fig. 2 except that *p*-nitrophenyl-β-D-galactopyranoside (2.3 mM) was used as the substrate, the enzyme preparation consisted of 0.72 μg protein, and the pressure was varied as indicated.

Fig. 4. The effect of hydrostatic pressure on the Na^+ (A) and K^+ (B) activated hydrolysis of lactose by β-galactosidase. The conditions were similar to those described in Table II with the exception that lactose (45 mM) was substituted for *o*-nitrophenyl-β-D-galactopyranoside and the enzyme preparation consisted of 72 μg protein (A) or 3 μg protein (B). At the end of the incubation period and the release of the pressure the reaction was stopped by boiling for 5 min. A 0.3-ml aliquot was removed and assayed for glucose as indicated in MATERIALS AND METHODS.

Enzyme–substrate volume change

LAIDLER[18,19] has postulated that a volume change accompanies the transformation of the enzyme–substrate complex into an activated complex. Under conditions in which the enzyme is saturated with substrate it is this transformation which is postulated to occur just prior to hydrolysis, that is affected by pressure. The computation of the volume change was made by utilizing Eqn. 5 as given in the DISCUSSION. By the use of Eqn. 5 the volume changes of the activated enzyme–substrate complexes were calculated and the results are presented in Tables III and IV.

TABLE IV

THE EFFECTS OF HYDROSTATIC PRESSURE ON THE ENZYMATIC HYDROLYSIS OF LACTOSE BY β-GALACTOSIDASE

The assay conditions were as described in Fig. 4.

Cation addition	*Cation concn. (mM)*	ΔV^* *(cm³/mole)*
K^+	45	+ 4.5
Na^+	20	−12.0

DISCUSSION

It has been reported[8,9,20,21] that the rate limiting step in the β-galactosidase reaction is the dissociation of the enzyme–substrate complex into enzyme and products. This conclusion is based on the demonstration of the independence of the K_m and the v_{max} by cation activation studies[7] and by comparing the v_{max} with the K_m at several temperatures[20]. Results of stop-flow experiments[9,21] with several substrates further substantiate this conclusion. Since the formation of products is rate limiting and since stimulation was attained at saturating substrate concentrations, these results indicate that Na^+ and K^+ are involved in the dissociation of the enzyme–substrate complex into enzyme and products. The K_m of the enzyme for substrate also is influenced by Na^+ and K^+ (Table I and see ref. 9) and this indicates involvement of cations in the formation of the enzyme–substrate complex.

The K_A values (Table II) calculated for Na^+ and K^+ may only be relative values since, as reported by NEVILLE AND LING[8] for the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside, Na^+ can react with either the enzyme or the enzyme–substrate complex. If this were valid for all the activation studies conducted here the apparent K_A values would be less than the true K_A values due to the alteration of the equilibrium between the enzyme and metal and the enzyme–metal complex[22].

It would appear from the higher affinity of the enzyme for Na^+ than for K^+ (indicated by the kinetic determinations and the inhibition experiments) that Na^+ is bound to the enzyme in preference to K^+ and at probably the same site(s). This would indicate, as pointed out by USSING[23] that the association between the enzyme and the particular cation is occurring on the basis of the crystal or ionic radius rather than the hydrated radius. From this point of view a smaller atom, *i.e.* Na^+, would be more tightly bound or more closely associated with a negative component of the protein than a larger particle, *i.e.* K^+, of the same charge.

In the presence of high substrate and Na^+ concentrations the application of hydrostatic pressure increased in the rate of hydrolysis of all substrates tested. The rate of hydrolysis of all substrates in the presence of optimum levels of K^+ was decreased by pressure. These results can be interpreted according to the proposals of LAIDLER[18,19] which include the following rational.

Many enzymatic reactions proceed *via* an activated enzyme–substrate complex which occurs immediately prior to product formation. Furthermore, in some of these enzymatic reactions the dissociation of the enzyme–substrate complex into enzyme and products is the rate limiting step. At substrate concentrations which are considerably greater than the saturation levels for the enzyme formation of the enzyme–substrate complex would not be influenced by pressure. Under these conditions and at atmospheric pressure the rate would be related to total enzyme concentration ($[E_t]$) by the proportionally constant k_0 which can be expressed as follows:

$$v_0 = k_0[E_t] \tag{1}$$

The equation for the specific rate constant[24] of any elementary process influenced by pressure always can be written as follows:

$$k_p = k_0 e^{-P\Delta V^*/RT} \tag{2}$$

where k_0 is the rate at zero pressure, k_p is the rate at pressure P, R is the gas constant, T is the absolute temperature, and ΔV^* is the volume change accompanying the transformation. Under the specific conditions mentioned above the velocity at pressure P is given as the following:

$$v_p = k_p[E_t] \tag{3}$$

where k_p is the rate constant for the formation of products at the designated pressure. By combining Eqns. 2 and 3 an expression of the enzymatic reaction rate at pressure P can be obtained.

$$v_p = k_0[E_t]e^{-P\Delta V^*/RT} \tag{4}$$

consequently from Eqns. 1 and 4:

$$v_p = v_0 e^{-P\Delta V^*/RT} \tag{5}$$

This equation then represents a means by which the volume change of the activated enzyme–substrate complex can be calculated based on the rates in the presence of specific pressures. From this equation the volume change accompanying the formation of the activated complex can be calculated when both the substrate and the cation are at saturating levels. It is this volume change that is measured in the β-galactosidase reaction during saturating Na^+ and K^+ concentrations and that is correspondingly affected as the rates are either increased or decreased by hydrostatic pressure.

It is reported[18,19,24] that increasing pressure decreases the volume of the enzyme–substrate complex. In the experiments reported above, Na^+ activation was increased by pressure. In this case the volume of the activated enzyme–substrate–cation complex would be expected to be less in the presence of Na^+ than in its absence (Tables III and IV). The converse must be true for K^+ activation of the hydrolysis of all the substrates tested, since the application of pressure decreased the activation by K^+. Consequently, K^+ activation must occur with an increase in volume of the activated enzyme–sub-

strate–cation complex (Tables III and IV). Because a decrease in volume occurs upon ionization and an increase in volume occurs upon unfolding or association of ionized species[24], it is conceivable that Na^+ activation proceeds with an increase in ionization, while K^+ activation occurs with an increase in unfolding or an increased association of charged species.

Although the results of this investigation do not allow the prediction of a specific model for monovalent cation activation of β-galactosidase, they aid in the interpretation of results of other investigations. It is evident that both Na^+ and K^+ influence the formation of the enzyme–substrate complex as well as the hydrolysis of the substrate. During the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside, Na^+ can react with either the enzyme or the enzyme–substrate complex[8]. Multiple sites may be involved since a lower concentration of Na^+ is required for saturation than K^+ although these results could be explained on the basis of the higher affinity of the enzyme for Na^+. During the formation of the activated enzyme–substrate complex as measured by the influence of hydrostatic pressure, the mechanism of activation by Na^+ and K^+ appears to be different. A decrease in volume accompanies the formation of the complex in the presence of Na^+ while an increase in volume occurs in the presence of K^+.

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ESTERASE ACTIVITIES OF β-*N*-ACETYL-D-GLUCOSAMINIDASE PREPARATIONS

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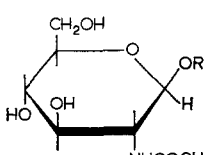
SUMMARY

Evidence indicating that the enzyme β-*N*-acetyl-D-glucosaminidase (EC 3.2.1.30) can catalyse the hydrolysis of esters has been presented. This is probably the first demonstration of catalytic action of a glycosidase upon structures other than glycosides. The specificity of the esteratic activities has been examined, and the possible significance of the results has been discussed.

INTRODUCTION

Pig epididymis is a particularly rich source of β-*N*-acetyl-D-glucosaminidase (EC 3.2.1.30) activity[1]. Although the significance of this high activity is unknown, extracts of the tissue constitute a convenient source of the enzyme for purification[2]. Certain properties of the purified enzyme have been reported[2,3], and evidence has recently been presented[4] suggesting that the enzyme catalyses the hydrolysis of 2-acetamido-2-deoxy-β-D-glucopyranose derivatives (I) by a mechanism similar to that advanced for egg-white lysozyme[5,6].



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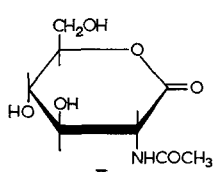

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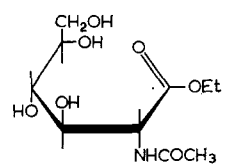
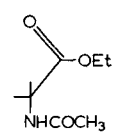


III



IV

In the course of work[4] undertaken to rationalise the high affinity of the epididymal enzyme for 2-acetamido-2-deoxy-D-glucono-(1,5)-lactone (II), binding studies using a series of simple structural analogues of the lactone (*viz.*, acetic acid, acetamide, *N*-methyl acetamide, 2-acetamidoacetic acid, ethyl 2-aminoacetate, ethyl 2-acetamidoacetate, and ethyl 2-propionamidoacetate) indicated that these materials compete with 2-acetamido-2-deoxy-β-D-glucopyranoside substrates for the highly specific 'acetamido' receptor site of the enzyme[3]. Since the latter site must lie close to that at which the catalysed reaction takes place and since there are strong similarities

of structure and of polarity between 2-acetamido-2-deoxy-β-D-glucopyranosides and competitive inhibitors such as the lactone (II) and ethyl 2-acetamidoacetate (IV), the question arises as to whether detectable catalytic action of the enzyme might be demonstrated against compounds other than the known pyranoside substrates.

The present communication describes work carried out to investigate this possibility.

MATERIALS AND METHODS

The β-*N*-acetyl-D-glucosaminidase preparations were, Stages 2, 6b and 7b of the procedure of FINDLAY AND LEVVY[2], an almond emulsin sample from British Drug Houses, and an extract of limpet visceral hump[7].

Ethyl, phenyl and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosides were prepared according to LEABACK AND WALKER[8]. Crystalline 2-acetamido-2-deoxy-D-glucono-(1,5)-lactone, which had been prepared according to British Patent 1138 367, was a gift from the late Mr. N. M. Cross. Ethyl 2-acetamido-2-deoxy-D-gluconate (m.p. 151–153°, $[\alpha]_D^{21}$ + 22.5°; *c* 1 in water) was prepared by a method analogous to the corresponding 2-benzamido compound[9].

Ethyl 2-acetamidoacetate was prepared by the addition of acetic anhydride (67 ml) over 15 min to a cooled solution of glycine ethyl ester hydrochloride (88 g) and $NaHCO_3$ (120 g) in water (II) followed by evaporation to dryness under reduced pressure after 16 h, and four extractions of the residue with ethyl acetate (200 ml). The solution was dried ($CaCl_2$) and evaporated to a syrup which crystallised. The residue was re-distilled twice at about 150° and 20 mm Mg; and recrystallized from ethyl acetate–light petroleum (b.p. 60–80°) to give 26 g ethyl 2-acetamidoacetate, m.p. 47–48°, with absorption maxima at 1750 and 1660 cm^{-1} (ref. 10, m.p. 48°). Ethyl 2-propionamidoacetate (m.p. 50–52°) was prepared similarly and showed absorption maxima at 1750 and 1660 cm^{-1}.

Unless otherwise stated, enzyme assays were carried out in 0.05 M sodium citrate buffer (pH 4.3) in the presence of 0.01% bovine serum albumin (Armour Products). After 30 min at 30° with the substrates specified the reactions were terminated and ethanol of *p*-nitrophenol were estimated by the methods described earlier[11].

RESULTS AND DISCUSSION

Moderate activities of a partially purified pig epididymal β-*N*-acetyl-D-glucosaminidase preparation (Stage 6b) were incubated with either 5 mM ethyl 2-acetamido-2-deoxy-β-D-glucopyreuoside (I, C_2H_5), with 5 mM ethyl 2-acetamido-2-doexy-D-gluconate (III) or with 200 mM ethyl 2-acetamidoacetate (IV). The results in Table I (Expt. 1) show that under the conditions specified, comparable quantities of ethanol were liberated enzymically from the ethyl esters and glycoside, whereas there was no detectable enzyme activity towards 200 mM ethyl 2-propionamidoacetate, 200 mM ethyl 2-aminoacetate, or 200 mM ethyl acetate using similar conditions. Thus, the acetamido specificity of the esterase activity parallels that of the β-*N*-acetyl-D-glucosaminidase activity of the preparation and that of the inhibition of the glycosidase by acylamido derivatives of ethyl acetate[3,4].

Results in Table I (Expts. 1, 2 and 3) show that the relative amounts of esterase

TABLE I

ESTERASE ACTIVITIES OF β-*N*-ACETYL-D-GLUCOSAMINIDASE PREPARATIONS

Enzyme preparations (sufficient to catalyse the hydrolysis of about 2 μmoles of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside in 30 min at 30° under standard conditions) were incubated with the compounds indicated; after 30 min at 30°, a pyrophosphate–glycine–semicarbazide solution[11] (3 ml) was added to the reaction mixture (0.4 ml) and the liberated ethanol assayed by the yeast alcohol dehydrogenase–NAD^+ system as described by BOROOAH *et al.*[11].

Expt. No.	*Enzyme preparation*	*Substrate*	*Substrate concn. (mM)*	*Inhibitor added*	*Ethanol liberated enzymically (μmoles)*	*Ratio of activities* b/a	c/a
1	Epididymal enzyme (Stage 6b)	(a) I, R = C_2H_5	5	—	0.09	—	—
		(b) IV	200	—	0.05	0.56	—
		(c) III	5	—	0.035	—	0.39
		(d) Ethyl 2-propionamidoacetate	200	—	<0.011	—	—
		(e) Ethyl glycinate	200	—	<0.011	—	—
		(f) Ethyl acetate	200	—	<0.011	—	—
2	Epididymal enzyme (Stage 2)	(a) I, R = C_2H_5	5	—	0.08	0.56	—
		(b) IV	200	—	0.045		
3	Epididymal enzyme (Stage 7b)	(a) I, R = C_2H_5	5	—	0.08	0.50	—
		(b) IV	200	—	0.04		
4	Epididymal enzyme (Stage 6b) after heat inactivation (66%)	(a) I, R = C_2H_5	5	—	0.07	0.43	—
		(b) IV	200	—	0.03		—
5	Epididymal enzyme (Stage 6b) but 88% heat inactivated	(a) I, R = C_2H_5	5	—	0.06	0.50	—
		(b) IV	200	—	0.03		
6	Epididymal enzyme (Stage 6b)	IV	200	a. None	0.08	—	—
				b. I, R = C_6H_5 (2.5 mM)	0.03	0.38	—
				c. I, R = C_6H_5 (5 mM)	<0.01	—	0.12
7	Epididymal enzyme (Stage 6b)	IV	200	a. None	0.08	—	—
				b. Lactone II (1.1 μM)	0.03	0.38	—
				c. Lactone II (11 μM)	<0.01	—	0.12
8	Epididymal enzyme (Stage 6b) but eluted from Sephadex G-200	(a) I, R = C_2H_5	5	—	0.075	0.53	—
		(b) IV	200	—	0.04		
9	Crude limpet extract	(a) I, R = C_2H_5	5	—	0.095	0.42	—
		(b) IV	200	—	0.04		
10	Almond emulsin	(a) I, R = C_2H_5	5	—	0.10	—	—
		(b) IV	200	—	0.04	0.40	—

and glycosidase activities remain (within the limits of the analytical technique) unchanged at various stages in the purification of the glycosidase; this was also true (Expts. 4 and 5) of residual activities after heat treatment of the enzyme preparation and of the enzyme after elution from Sephadex G-200 using a procedure similar to that described by CAYGILL *et al.*[12] (Expt. 8).

The esteratic activities were strongly inhibited by the products of the reaction and the precision of the assays was further limited by high blank readings (blanks due to acidic and basic hydrolysis of the esters under standard assay conditions, were usually equivalent to about 1 μmole of ethanol), by inhibitory products of nonenzymic substrate hydrolysis and by interference of the ester IV with the estimation of ethanol by yeast alcohol dehydrogenase. These limitations made determinations of the kinetic parameters of the esterase activities impracticable: for very small amounts of ethanol liberated (up to about 0.1 μmole), esterase activities towards ethyl 2-acetamidoacetate (IV) were maximally active at about pH 3.5, were approximately linear with enzyme concentration and with time up to about 30 min.

The esterase activity was inhibited progressively by increasing concentrations of the phenyl glycoside (I, R = C_6H_5) and by very low concentrations of the lactone II (Table I, Expts. 6 and 7, respectively). The lactone II is a powerful, competitive inhibitor with high specificity towards β-*N*-acetyl-D-hexosaminidase activities. Thus, the identity of the enzyme responsible for the esterase and glycosidase activities is indicated by studies on the specificity of the enzyme for the acetamido group, by the relative abundance of the activities in various enzyme preparations, and by the inhibition of the esterase activity by structures known to combine specifically at the active site of the glycosidase.

Preliminary experiments with crude β-*N*-acetyl-D-glucosaminidase preparations from almond emulsin and from a mollusc show similar proportions of esterase activity (Table II, Expts. 9 and 10, respectively) and suggest that the phenomenon is general. It is noteworthy that in contrast to certain 'un-natural' substrates for other enzymes (*e.g.* *p*-nitrohenyl acetate, and *p*-nitrophenyl chitobiose for chymotrypsin and lyso-

TABLE II

STABILITY OF THE 1,5-LACTONE AND β-*N*-ACETYL-D-GLUCOSAMINIDASE ACTIVITIES

(*a*) Stability of β-*N*-acetyl-D-glucosaminidase: Enzyme (Prep. 6b) was incubated at 30° for the time stated in the presence of 0.05 M sodium citrate buffer pH 4.3 and 0.01% albumin and then assayed for enzyme activity under standard conditions. (*b*) Stability of lactone II: An absolutely fresh solution (4 μM) of the crystalline lactone II was incubated at 30° for the time stated in the presence of 0.05 M sodium citrate buffer pH 4.3 and 0.01% albumin before the addition of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (5 mM) and enzyme (Prep. 6b) and assayed under standard conditions. (*c*) Inhibition by 2-acetamido-2-deoxy-D-gluconate: 200 μM lactone II was left for 16 h at 5° with 2.02 M, pH 7.0 sodium phosphate buffer, diluted to about 4 μM and incubated at 30° for the time stated with 0.05 M sodium citrate buffer pH 4.3 and assayed under standard conditions. (*d*) Stability of lactone II in presence of enzyme: 40 μM lactone II was incubated for the time stated with β-*N*-acetyl-D-glucosaminidase (Prep. 6b), 0.05 M sodium citrate buffer pH 4.3 and 0.01% albumin, then diluted 20-fold and assayed under standard conditions.

Time (min)	*(a)*		*(b)*		*(c)*		*(d)*	
	p-Nitrophenol liberated (μmoles)	*% Activity*	*p-Nitrophenol liberated (μmoles)*	*% Uninhibited activity*	*p-Nitrophenol liberated (μmoles)*	*% Uninhibited activity*	*p-Nitrophenol liberated (μmoles)*	*% Uninhibited activity*
0	0.178	100	0.132	20	0.180	100	0.135	21
15	0.178	100	0.154	23	0.177	98	0.155	23
30	0.178	100	0.166	25	0.176	98	0.173	26
45	0.176	99	0.220	33	0.175	97	0.215	35
60	0.178	100	0.250	39	0.176	98	0.250	39

zyme, respectively), the initial rates for the enzymic hydrolysis of the esters III and IV were of the same order as that for the ethyl glycoside (I).

Since the lactone (II) is the most powerful competitive inhibitor of β-*N*-acetyl-D-glucosaminidase known[2,3], decreased inhibitory power should accompany any conversion of the 1,5-lactone to the corresponding aldonic acid or to the 1,4-lactone. Any catalytic activity that β-*N*-acetyl-D-glucosaminidase might show towards the 1,5-lactone should, therefore, manifest itself as decreasing inhibitory power of the solution. Results of experiments designed to demonstrate such an effect are represented in Table II. It can be seen that at pH 4.3 and 30°, the enzyme is stable for at least 60 min, that the de-lactonised lactone recovers its inhibitory power very slowly, that fresh solutions of the lactone show a rapid fall of inhibitory power over the first 30 min but that the time-course of the process up to 60 min is not affected by the presence of the enzyme.

It was concluded that no enzyme-catalysed transformation of the lactone could be detected under conditions similar to those where hydrolytic activities towards the esters III and IV have already been demonstrated.

While there is no evidence available concerning the mechanism of the enzyme-catalysed hydrolysis of the ethyl compounds I, III and IV, it is tempting to suggest that these compounds might be hydrolysed by a mechanism (enzymic protonation of the ethoxy oxygen, *etc.*) analogous to that advanced[4] for the enzymic hydrolysis of the aryl glycosides (I), and that the lactone is not similarly hydrolysed owing to what must be a very different orientation of the lactone (ester) oxygen at the active site of the enzyme.

The present work includes what are probably the first demonstrations of catalytic activities of a glycosidase upon ester bonds. This work was greatly aided by the presence of a highly specific site for the absorption of acetamido derivatives adjacent to the catalytic centre of the enzyme. Not all enzymes would have sites with such high specificity close to the catalytic centres, and it seems possible that some of the many esterases of low substrate specificity and of unknown biological function may have unsuspected activity towards structures apparently very dissimilar (and biologically more significant) than the ester substrates commonly employed in the assay procedures.

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BBA 65962

THE FERROUS ION AS THE COFACTOR OF ARGINASE *IN VIVO*

I. PROPERTIES OF YEAST ARGINASE METALLO-COMPLEXES OF KNOWN COMPOSITION AND OF NATIVE ARGINASE

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SUMMARY

A comparative study has been made of metallo-complexes of yeast arginase (L-arginine ureohydrolase, EC 3.5.3.1) prepared *in vitro* and of yeast arginase demonstrable in fresh cell-free extracts without the addition of activating cations (native arginase). Since native yeast arginase is strongly inhibited by phosphate, yeast grown with α-glycerophosphate as sole source of phosphorus has been used for this purpose.

The activation of yeast arginase by Fe^{2+} *in vivo* is stated on account of the following observations:

1. The pH–activity curves of native arginase and of Fe^{2+}–arginase were similar, showing shapes and optima different from those of Mn^{2+}–, Co^{2+}–, Ni^{2+}– and Mg^{2+}–arginases.

2. The specific activity of native arginase at pH 8.5 was hardly increased by previous incubation of the cell-free extracts with Fe^{2+} salts.

3. Both native and Fe^{2+}–arginase were inhibited by phosphate in contrast with other metallo–arginases.

4. The Michaelis constants, as well as the inhibition constants for ornithine, were equal for native and for Fe^{2+}–arginase at 4 pH values; they were significantly different from those of other metallo–arginases.

INTRODUCTION

Arginase (L-arginine ureohydrolase, EC 3.5.3.1) catalyzes the hydrolysis of L-arginine to equimolar amounts of L-ornithine and urea. Since the first demonstration of arginase in liver by KOSSELL AND DAKIN[1] in 1904, many investigations on its action have been carried out, especially after the elucidation of the role of this enzyme in the urea synthesis in the liver of ureotelic organisms by KREBS AND HENSELEIT[2] in 1932. Liver arginase is a metallo-enzyme[3], the presence of either Mn^{2+}, Co^{2+}, Fe^{2+} or

Ni^{2+} being required for its action. The apoenzyme forms stable complexes with the activating cations[4]. For activation of arginase preparations, incubation with an excess of the cations prior to arginine addition is necessary[4]. Complete activation requires incubation at elevated temperatures or for long periods[4]. The enzyme is protected against heat denaturation by the presence of the activating cations[5]. The pH–activity curve of liver arginase varies with the activating cation. Mn^{2+}–arginase shows its optimum at pH 10, Co^{2+}– and Ni^{2+}– arginases at pH 7–9.5 (ref. 4).

EDLBACHER AND BAUR[6] were the first to demonstrate the presence of arginase in baker's yeast. Afterwards, MIDDELHOVEN[7] has shown the inducibility of the enzyme by arginine and its participation in the arginine breakdown in yeast. Yeast arginase shows a requirement for bivalent cations like liver arginase[6]. It differs from the latter in being more readily inactivated by dialysis; reactivation by addition of activating cations is possible[6].

In liver homogenates and in cell-free extracts of yeast, an arginase activity was found to be demonstrable, without the addition of activating cations. This activity (to be referred to as native arginase) was strongly inhibited by chelating agents such as 8-hydroxyquinoline. Hence it must be concluded that its activity depends on bivalent cations as well. Because of the slow reaction of apoarginase with its activating cations[4], it is improbable that the native arginase activity originates from the reaction of arginase with free cations during the preparation of cell-free extracts. Hence the native arginase may be assumed to be identical with the metallo–arginase that is active *in vivo*. The question arises as to which of the cations, capable of activating the enzyme *in vitro*, takes part in the enzyme reaction *in vivo*. The answer to this question may be given by a comparative study of native arginase and of metallo–arginases, prepared *in vitro*, of known composition. During the thirties and early forties, several studies on the nature of the cation, activating liver arginase *in vivo*, have been undertaken. The results of these studies will be briefly summarized.

EDLBACHER AND ZELLER[8] suggested that either Fe^{2+} or Mn^{2+} activate the native liver arginase. They considered the inhibition of native liver arginase by KCN at pH 6–7 (ref. 9) as an argument in favour of Fe^{2+}. Nevertheless, they stated that Mn^{2+} is the activating cation in native liver arginase, on account of the excellent activation of the enzyme by Mn^{2+} *in vitro* and on account of the ability of Mn^{2+} to protect the liver arginase against inactivation by trypsine; Fe^{2+} failed to protect the enzyme under such conditions. RICHARDS AND HELLERMAN[10] purified native liver arginase 100-fold without addition of activating cations during their fractionating procedure. Analyses of their preparations by flame spectroscopy demonstrated that, of all metals capable of activating arginase *in vitro*, only Mn^{2+} and Fe^{2+} were present. They suggested that Mn^{2+} is the activating cation *in vivo*, without completely excluding the possibility of participation of Fe^{2+}, on account of the superior reactivation of an inactivated preparation by minor concentrations of Mn^{2+}.

Assays of liver arginase in Mn-deficient rats and mice[11,12] have learned that the arginase activities in Mn-deficient animals were significantly smaller than those in the control group. The Mn-deficient animals showed growth retardation and failed to reproduce. No effect on urea excretion was observed[11,12], however, even after the administration of large doses of ammonium citrate[12]. The observed decrease in liver arginase activity, caused by Mn deficiency, is generally accepted as the definite proof of Mn^{2+} being involved in the liver arginase reaction *in vivo*. This conclusion has

never been substantiated by a comparative study of native liver arginase and of metallo–arginases of known composition, however.

The aim of the present research was to study the native arginase in cell-free extracts of baker's yeast and to compare its properties with those of metallo-complexes of yeast arginase prepared *in vitro*. In this paper evidence is presented for the involvement of Fe^{2+} in the action of yeast arginase *in vivo*. The results of the present research have already been published as a preliminary report[13].

MATERIALS AND METHODS

Cultivation of yeast

A strain of *Saccharomyces cerevisiae* Hansen, isolated from "Koningsgist" of the Koninklijke Nederlandsche Gist- en Spiritusfabriek (Delft, The Netherlands) was used throughout this investigation. The growth medium contained per l: 40 g of glucose; 10 g of peptone (acid hydrolysate of casein); 1.36 g (10 mmoles) of KH_2PO_4; 400 mg of $MgCl_2 \cdot 6\ H_2O$; 100 mg of $CaCl_2 \cdot H_2O$; 100 mg of NaCl; 2 mg of $FeCl_3 \cdot 6\ H_2O$; 0.5 mg of H_3BO_3; 0.1 mg of $CuSO_4 \cdot 5\ H_2O$; 0.1 mg of KI; 0.4 mg of $MnSO_4 \cdot H_2O$; 0.2 mg of Na_2MoO_4; 0.4 mg of $ZnSO_4 \cdot 5\ H_2O$; 0.2 mg of thiamine; 0.1 mg of riboflavine; 5 mg of nicotinic amide; 0.3 mg of *p*-aminobenzoic acid; 1 mg of pyridoxine; 2 mg of calcium pantothenate; 10 mg of *myo*-inositol; 0.02 mg of biotin; pH 4.5. The yeast was grown aerobically at 30° in a Kluyver flask, was harvested in the stationary phase of growth and was washed 3 times with water at 0°. Yeast poor in phosphate was grown in the same way, using 0.63 g (2 mmoles) of disodium α-glycerophosphate per l of medium as a source of phosphorus, instead of KH_2PO_4.

Preparation of crude cell-free extracts

2-g yeast samples were sonically disintegrated for 5 min at 0° in a M.S.E. Ultrasonic Power Unit, after addition of 1 ml of a solution of 1% $NaHCO_3$ in 10 mM β-mercaptoethanol. After dilution with 3 ml of H_2O, nuclei and cell debris were removed by centrifugation (10 min, $4000 \times g$).

Preparation of metallo–arginases

Bivalent cations were removed from the crude cell-free extract by reaction with 10 mM EDTA (pH 7.5) for 10 min at 30° and by subsequent gel filtration on a column of Sephadex G-25 (10 mM Tris–HCl, pH 7.5). In this way, purified cell-free extracts were obtained which were devoid of EDTA and other low-molecular substances (*e.g.* inhibiting amino acids). In these extracts no arginase activity was observed, unless activating cations were added. Metallo–arginases of known composition were prepared from these purified cell-free extracts by incubation with 40 mM bivalent cations (as hydrochlorides of A.R. quality) and 40 mM Tris–HCl (pH 7.5). The activations with Mn^{2+} and Mg^{2+} salts were performed at 50° for 15 min, those with other cations at 45° for 15 min.

Isolation of native arginase

Cell-free extracts were prepared from yeast poor in phosphate in the way described above. Low-molecular substances were removed from the crude cell-free extracts by gel filtration on a column of Sephadex G-25 (10 mM Tris–HCl, pH 8.5,

bed dimensions 18 cm × 1.6 cm), within 10 min at 0°. Maintenance of the pH at 8–9 was found to be a requirement for obtaining active preparations. For this purpose $NaHCO_3$ was added prior to the disintegration of the yeast.

pH–activity curves

The activities of native arginase and of the metallo–arginases were determined at various pH values by incubating enzyme, 100 μmoles L-arginine · HCl (pH adjusted) and 200 μmoles of buffer in a total volume of 2 ml. The tubes were incubated at 30° for 1 h, unless otherwise indicated. No more than 5 μmoles urea were allowed to be formed. Buffers used were sodium succinate for pH 5.0, 5.5 and 6.0; sodium cacodylate for pH 6.5 and 7.0 (potassium phosphate for Ni^{2+}–arginase pH 6.5, 7.0 and 7.5); Tris–HCl for pH 7.5, 8.0, 8.5 and 8.75; and sodium glycinate for pH 9.0, 9.5, 10.0 and 11.0. The final concentrations of the activating cations were 1 mM. The reactions were stopped with $HClO_4$. The protein precipitates were removed by centrifugation, if necessary. Urea was determined spectrophotometrically; the isonitrosopropiophenone method of ARCHIBALD[14] was used. Since the presence of arginine in the aliquots was shown to decrease the absorbance, corrections were made by addition of arginine to the urea standard series. Similar corrections were made for the presence of bivalent cations, especially Mn^{2+}, which tended to increase the absorbance. The activities of arginase at various pH values were expressed as specific activities, *i.e.* units per mg protein. One unit was defined as the quantity of enzyme, catalyzing the production of 1 μmole urea per h at 30°. Protein was determined in the cell-free extracts[15], using crystalline bovine serum albumin as the standard.

Determinations of the Michaelis constant and of the inhibition constant for ornithine

Equal amounts of enzyme were added to a series of test tubes containing the same buffer as used for the pH–activity curves (final concentration 100 mM) and arginine (variable concentration). After incubation at 30° for 1 h, the amount of urea was determined[14], corrections being made for arginine and for bivalent cations. The series was run in triplicate. The substrate concentrations (S) and the calculated rate of reaction (v) were plotted reciprocally according to LINEWEAVER AND BURK[16]. From this plot the Michaelis constant (K_m) was calculated graphically (see Fig. 1).

TABLE I

SPECIFIC ACTIVITIES OF DIFFERENT METALLO-COMPLEXES OF YEAST ARGINASE

Native yeast arginase and different metallo-complexes of yeast arginase were prepared and their specific activities determined as described in MATERIALS AND METHODS. The specific activities (μmoles urea produced per h per mg protein), observed in various cell-free extracts, were expressed as per cent of the specific activity of Mn^{2+}-activated arginase at pH 9.5.

Activating cation	*pH*	*Buffer*	*% Specific activity*
Mn^{2+}	9.5	Sodium glycinate	100
Mg^{2+}	9.5	Sodium glycinate	5–15
Native	8.5	Tris–HCl	20–25
Fe^{2+}	8.5	Tris–HCl	15–25
Co^{2+}	8.5	Tris–HCl	20
Ni^{2+}	7.0	Potassium phosphate	10–20

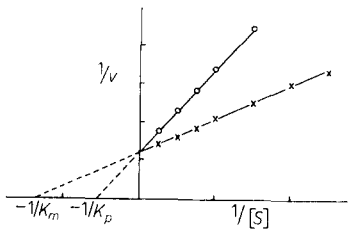

Fig. 1. Determination of the Michaelis constant and of the inhibition constant for ornithine. ×—×, no ornithine; ○—○, 5 mM L-ornithine.

The inhibition constant for ornithine (K_{Orn}) was determined by measuring the reaction rate in a series of incubation mixtures, identical with the series used for the K_m determination, except for the addition of 5 mM L-ornithine·HCl (Fluka) to each tube. These determinations were also run in triplicate. Ornithine was shown to be a competitive inhibitor of liver arginase by HUNTER AND DOWNS[17]. The Lineweaver-Burk plot gave a straight line, intersecting that of the series without ornithine at the $1/v$ axis thus demonstrating that the inhibition was of the purely competitive type[18] (see Fig. 1). The $1/[S]$ axis was intersected by this line at $-1/K_p$. K_{Orn} was calculated from K_p by the following equation (see ref. 18 for the derivation):

$$K_p = K_{Orn}(1 + [\text{Ornithine}]/K_{Orn}),$$

in which the ornithine concentration is 5 mM.

RESULTS

pH–activity curves of metallo–arginases prepared in vitro

Cell-free extracts of baker's yeast freed from bivalent cations by reaction with EDTA and subsequent gel filtration, showed no arginase activity unless activating cations had been added. Metallo–arginase preparations of known composition were obtained by reaction of cell-free extracts with 40 mM cations (as hydrochlorides) at pH 7.5. Only the addition of Mn^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+} or Mg^{2+} resulted in arginase activity. The bivalent cations: Ba^{2+}, Be^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Pd^{2+}, Sn^{2+}, Sr^{2+}, UO_2^{2+}, ZrO^{2+} and Zn^{2+}; and the trivalent cations: Al^{3+}, Ce^{3+}, Fe^{3+} and La^{3+} did not activate yeast arginase.

The various metallo–arginases showed different pH–activity curves (see Figs. 2a–2e). The Mn^{2+}– and Mg^{2+}–arginases had optima at pH 9.5 and 10.0, respectively; the optimum of Fe^{2+}–arginase was at pH 8.5–9.0. The curve of Co^{2+}–arginase was more complicated, showing a shoulder at pH 7–9 and an optimum at pH 9.5. Ni^{2+}–arginase was found to be inhibited by cacodylate and Tris buffers; it showed its optimum at pH 7.0 in phosphate buffer, and its activity decreased gradually with increasing pH.

The observed specific activities of arginase varied with the activating cation. Table I shows the specific activities of various metallo-complexes of yeast arginase. Mn^{2+} was the most potent activator, similar to the case with liver arginase[4]. Because of the great heat resistance of Mn^{2+}–arginase, the activation by this cation was found to be excellently reproducible. The activation of yeast arginase by the other cations

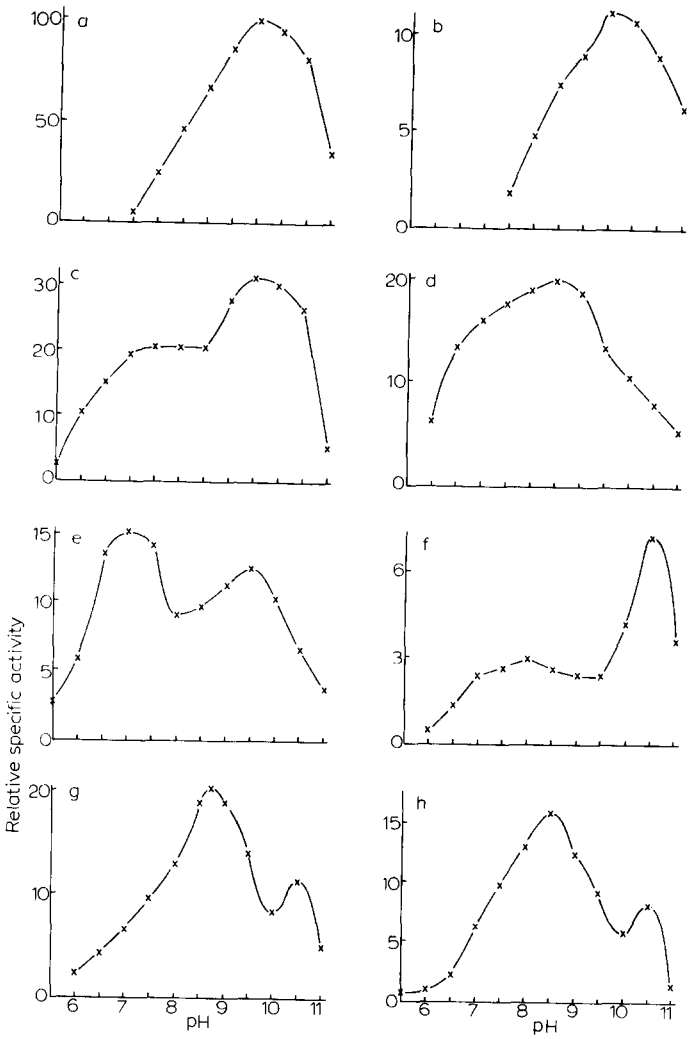

Fig. 2. pH–activity curves of various metallo-complexes of yeast arginase. Ordinate: relative specific activity, given as percent of the specific activity of Mn^{2+}–arginase (pH 9.5) (see Table I). a, Mn^{2+}; b, Mg^{2+}; c, Co^{2+}; d, Fe^{2+}; e, Ni^{2+}: f, native with phosphate; g, native phosphate-poor; h, Fe^{2+}, excess Fe^{2+} removed.

was variable. For the sake of clearness, all specific activities in Fig. 2 and in Table I have been expressed as percent of the specific activity of Mn^{2+}–arginase at pH 9.5.

Native arginase

It is possible to demonstrate an arginase activity in cell-free extracts of commercial baker's yeast, without the addition of bivalent cations prior to or during the incubation with arginine (native arginase). The specific activity of this arginase is small in comparison with that of arginase activated by bivalent cations. Its pH–activity curve shows a sharp peak at pH 10.5 and another maximum at about pH 8 (see Fig. 2f).

In early experiments it has been demonstrated that especially the native arginase activity (pH 8) is inhibited by phosphate. Since the phosphate concentration in commercial baker's yeast is sufficient for inhibition of the native arginase during the preparation of the cell-free extracts, the native arginase in cell-free extracts of yeast poor in phosphate has been studied as well. For that purpose the yeast was grown with α-glycerophosphate, instead of KH_2PO_4, as the phosphorus source. In the pH–activity curve of the native arginase of yeast poor in phosphate (Fig. 2g), the peak at pH 8.5–9.0 is predominant, in contrast with that of commercial baker's yeast; besides this optimum there is a smaller peak at pH 10.5. The specific activity at pH 8.5 approached that of Fe^{2+}–arginase (Table I). The period of incubation in the determination of this curve was 12 min, instead of 1 h as usual for the other pH–activity curves. The instability of native arginase did not allow longer periods of incubation.

Native arginase was found to be rather stable at pH 8–10. At other pH values, preparations of native arginase readily lost their activities (Table II), most probably due to dissociation of the metallo–arginase complex. For this reason it must be emphasized that the preparation of a cell-free yeast extract with strong native arginase activity is successful only if the pH during the whole procedure is above 8 and when the yeast has been grown in a medium rich in arginine, containing α-glycerophosphate as the phosphorus source.

TABLE II

STABILITY OF NATIVE ARGINASE

A cell-free extract with a strong native arginase activity was incubated for 30 min at 30° in 20 mM buffers of various pH's (see MATERIALS AND METHODS). Afterwards the specific activity of native arginase was determined at pH 8.5, as described in MATERIALS AND METHODS. The results are given as μmoles urea produced per ml of cell-free extract per h and as percent of the control (untreated cell-free extract).

pH	*Specific activity*	*% Specific activity*
6.0	400	21
7.0	1360	71.5
8.0	1730	91
8.5	1900	100
9.0	1770	93
10.0	1820	96
11.0	1520	80
Control	1900	100

The native arginase activity in the cell-free extracts was lost as soon as these extracts were submitted to protein fractionation procedures, such as precipitation with $(NH_4)_2SO_4$ or with cold acetone, unless the enzyme was protected by activating cations. In this respect it differs from native liver arginase[10]. Therefore, all the work for the elucidation of the nature of the cofactor of native yeast arginase had to be done with crude cell-free extracts; in purified enzyme preparations the native cofactor most probably would have been exchanged for added cations.

The pH–activity curve of native arginase resembles that of Fe^{2+}–arginase. Both enzyme activities are optimal at pH 8.5–9.0, quite distinct from those of Mn^{2+}–, Mg^{2+}–, Co^{2+}– and Ni^{2+}–arginases. Hence the cofactor of native yeast arginase is more

likely to be Fe^{2+} than any of the other activating cations. The activities of Fe^{2+}– arginase at unfavourable pH values were relatively greater than those of native arginase. This phenomenon might be explained by the instability of native arginase at unfavourable pH values. The excess of Fe^{2+}, present during the determination of the Fe^{2+} curve, might protect the enzyme at these pH values, thus causing apparently greater specific activities. This possibility has been investigated by the determination of the pH–activity curve of Fe^{2+}–arginase after removal of the excess of Fe^{2+} by gel filtration on a column of Sephadex G-25 (10 mM Tris–HCl, pH 8.5). Immediately before the gel filtration, 80 mM trisodium citrate was added to the Fe^{2+}–arginase solution (which contained 40 mM $FeCl_2$ and 40 mM Tris–HCl, pH 7.5). When the addition of citrate was omitted, all arginase activity was lost during the gel filtration, owing to adsorption of the enzyme by the $Fe(OH)_2$ precipitate. The pH–activity curve of Fe^{2+}–arginase, determined after removal of the excess of Fe^{2+}, is shown in Fig. 2h. It resembles that of native arginase both in shape and in optima. This resemblance points to the identity of native and Fe^{2+}–arginases. Further support for this assumption was found in studies on the behaviour of yeast metallo–arginases to inhibitors and in the determination of some kinetic constants.

Inhibition experiments with yeast arginase

Both liver and yeast arginase are strongly inhibited by reagents capable of binding bivalent cations, such as 8-hydroxyquinoline and EDTA. Native yeast arginase was also inhibited by these reagents. Many of the inhibitors behave unspecifically, reacting with all bivalent and trivalent cations. Some reagents, however, exhibit more specificity, *e.g.* Na_2S and dimethylglyoxim which react with heavy metals but not or less readily with Mn^{2+} and Mg^{2+}. A study of the sensitivity of native yeast arginase to these reagents was expected to give information about the nature of its activating cation.

TABLE III

INHIBITION OF NATIVE YEAST ARGINASE AND OF DIFFERENT METALLO–ARGINASES

The different arginase preparations, all derived from the same cell-free extract, were exposed to the action of inhibiting reagents for 30 min at 30° in 20 mM Tris–HCl (pH 7.5), the concentration of bivalent cations being 0.4 mM. Afterwards the specific activities of the arginases were determined as usual (final concentration of bivalent cations 0.1 mM; Fe^{2+}–, Co^{2+}–, Ni^{2+}–, native arginases in Tris–HCl (pH 8.5); Mn^{2+}– and Mg^{2+}–arginases in sodium glycinate (pH 9.5)). All data are specific activities (μmoles urea produced per h per mg protein).

Inhibitor	*Native*	Fe^{2+}	Co^{2+}	Ni^{2+}	Mn^{2+}	Mg^{2+}
Control	14	18.5	22	16	110	9
1 mM 8-hydroxyquinoline	0	0	0	0	0	0
0.4 mM Na_2S	0	0	0	0	75	8
4 mM dimethylglyoxim	3	0	0	0	60	6
20 mM potassium phosphate (pH 7.5)	3	1	22	16	110	9

Native yeast arginase and metallo–arginases of known composition, prepared *in vitro*, were exposed to the action of some inhibitors, and the activities of the various arginases were determined. As shown in Table III, native arginase resembles Fe^{2+}–, Co^{2+}– and Ni^{2+}–arginases in being inhibited by dimethylglyoxim and Na_2S. Mn^{2+}–

TABLE IV

K_m AND K_{Orn} OF NATIVE YEAST ARGINASE AND OF METALLO-ARGINASES OF KNOWN COMPOSITION

Activating cation	*pH*	*Buffer*	K_m *(mM)*	K_{Orn} *(mM)*
Co^{2+}	6.7	Potassium phosphate	6.0–7	2.7
Native	7.0	Sodium	4.3	1.5
Fe^{2+}	7.0	cacodylate	4.1–5.0	1.5–2.1
Co^{2+}	7.0		6.2	1.9
Native	7.5	Tris–HCl	4.0	1.4
Fe^{2+}	7.5		4.0	1.2
Native	8.5	Tris–HCl	3.3	0.8
Fe^{2+}	8.5		3.3	0.75
Native	9.5	Sodium	4.0	1.6
Fe^{2+}	9.5	glycinate	3.3	1.5
Co^{2+}	9.5		2.15	1.0
Ni^{2+}	9.5		1.3–1.6	1.3–2.1
Mn^{2+}	9.5		5.0–7.7	2.0
Mg^{2+}	9.5		7.0–9.0	4.5

and Mg^{2+}–arginases are rather unsensitive to these reagents. Since only native arginase and Fe^{2+}–arginase were inhibited by phosphate, it was concluded that Fe^{2+} is the activator of native yeast arginase.

Kinetic properties of yeast arginase

More evidence for the stated identity of native and of Fe^{2+}–arginase was provided by determining the Michaelis constant (K_m) and the inhibition constant for ornithine (K_{Orn}), both for native and for Fe–arginase at various pH values, and in comparison with those of other metallo-arginases. Table IV shows that the kinetic constants of native and Fe^{2+}–arginase are equal, within the error of the determination, and that they are significantly different from those of Co^{2+}–, Ni^{2+}–, Mn^{2+}– and Mg^{2+}–arginases. Ornithine was found to be a competitive inhibitor of yeast arginase under all circumstances investigated.

DISCUSSION

In the present investigation, the properties of various metallo-complexes of yeast arginase prepared *in vitro* have been studied. Only the bivalent cations: Mn^{2+}, Mg^{2+}, Co^{2+}, Fe^{2+} and Ni^{2+} were shown to activate the enzyme *in vitro*. The activation of yeast arginase[6] by Cd^{2+} was not observed. Mn^{2+}, Co^{2+}, Fe^{2+} and Ni^{2+} have been reported as activators of liver arginase *in vitro*[3,4]; the activation of an arginase by Mg^{2+} has never been described before. The various metallo-complexes of yeast arginase were readily distinguished from each other by the shapes and the optima of their pH–activity curves and by their sensitivities to various reagents that bind bivalent cations.

Carefully prepared cell-free extracts of yeast, grown in an α-glycerophosphate medium, also showed an arginase activity if no bivalent cations had been added prior to or during the incubation with arginine. This arginase activity (native arginase), which showed the characteristics of Fe^{2+}–arginase, was considered to be identical with

the metallo–arginase that is active *in vivo*. This identity is highly probable because the activation of arginase by bivalent cations is a slow process[4]. If native arginase would be an artifact, caused *e.g.* by an exchange reaction between genuine natural arginase and traces of Fe^{2+} present during the preparation of the cell-free extract, one might expect that native arginase would exhibit the properties of a mixture of different metallo–arginases. The observed pH–activity curve and especially the almost complete inhibition by phosphate demonstrated that this is not true; native arginase showed the properties of homogeneous Fe^{2+}–arginase. Another argument in favour of the identity of native arginase and genuine natural arginase is the great specific activity of native arginase. At the optimal pH this specific activity hardly increased after activation of the cell-free extract with Fe^{2+} salts (Table I). Hence most of the arginase *in vivo* must have been present as Fe^{2+}–arginase.

The assumption that the native arginase had arisen by an exchange reaction with Fe^{2+} during the preparation of the cell-free extract, is contradicted by the results of experiments reported in a subsequent paper[19]. In that investigation, a study was made of the native arginase in cell-free extracts of yeast grown in Fe^{2+}-deficient media supplied with toxic concentrations of Mn^{2+} or Co^{2+} salts. The native arginase of such yeast samples showed the properties of Fe^{2+}–arginase, as usual. Since both Mn^{2+} and Co^{2+} were accumulated from the medium and hence appeared in the cell-free extracts in concentrations largely exceeding that of Fe^{2+}, it was concluded that those ions were not able to replace Fe^{2+} in native arginase, either *in vivo* or during the preparation of the cell-free extracts. The supposed origin of Fe^{2+}–arginase from an exchange reaction proceeding during the extraction of the yeast is highly improbable.

The pH–activity curves of native and of Fe^{2+}–arginase show their optima at pH 8.5–9.0. In addition to this optimum, the curve of native arginase shows a peak at pH 10.5 (Fig. 2g), which is also present in the curve of Fe^{2+}–arginase, provided that the excess of Fe^{2+} has been removed prior to the incubation with arginine (Fig. 2h); if not, the peak at pH 10.5 is masked (Fig. 2d). The cause and significance of the latter type of arginase activity are not clear. That it is due to the presence of a different arginase protein has not to be excluded. Its presence in preparations of Fe^{2+}–arginase suggests that it depends on Fe^{2+} as well. The predominance of the peak at pH 10.5 in the pH–activity curve of native arginase extracted from commercial baker's yeast (Fig. 2f) suggests that this arginase activity is more resistant to the action of phosphate. When the peak at pH 10.5 is really caused by a distinct arginase protein, the physiological role of the latter would be of minor importance because of its small specific activity and its extreme optimal pH. No more attention has been paid to this matter.

The identity of native and of Fe^{2+}–arginase is stated on account of their pH–activity curves, both showing optima at pH 8.5–9.0. The pH–activity curves of other metallo-arginases show optima at different pH values. Another argument in favour of the identity of native and of Fe^{2+}–arginase is their susceptibility to inhibition by phosphate. This phenomenon is not shown by any of the other metallo–arginases.

Determinations of some kinetic constants demonstrate the identity of Fe^{2+}–arginase and native arginase as well. The K_m and K_{Orn} values of native arginase at various pH values were the same as those of Fe^{2+}–arginase (Table IV). At pH 9.5 the K_m values of different metallo–arginases varied considerably. A decrease in K_m was observed in order of Mg^{2+}–, Mn^{2+}–, native and Fe^{2+}–, Co^{2+}– and Ni^{2+}–arginase. The inhibition by ornithine was always found to be of the purely competitive type. The

K_{Orn} was proportional to the K_m, being approx. 30–50% of the latter. The K_m of native and of Fe^{2+}–arginase varied with the pH. Close to the optimal pH, the K_m was minimal. The K_m–pH relationship of Fe^{2+}–arginase is related to the bell-shaped K_m–pH curve observed by ROHOLT AND GREENBERG[20] in their study on Mn^{2+}-activated liver arginase. These authors also report that near the optimal pH the K_m is minimal. A comparative study[21] has shown that the K_m at pH 9.5 of Mn^{2+}-activated liver arginase of various ureotelic animals is 10–20 mM, that of uricotelic animals 100–200 mM. The inhibition by ornithine of the latter is of the purely competitive type, that of the former is both competitive and noncompetitive[22]. MORA *et al.*[22] report that the arginase of *Neurospora crassa* is of the uricotelic type, with respect to the K_m, the inhibition by ornithine and a number of other characteristics. Although in the present research only the K_m and the K_{Orn} of yeast arginase have been determined, it is concluded that the yeast arginase is distinct from that of Neurospora. Because of its low K_m it shows more resemblance to that of ureotelic animals. It differs from the latter in its behaviour to ornithine, the inhibition being of the purely competitive type.

The activation of yeast arginase by Fe^{2+} *in vivo* is rather surprising because arginase is generally believed to be a Mn^{2+}-enzyme. Fe^{2+} has been considered hitherto as only a weak activator of liver arginase[10]; activation of yeast arginase by Fe^{2+} has been described to be impossible[6]. In those studies the arginase preparations had been activated in the presence of phosphate buffers, however. Furthermore the decrease of the specific activity of liver arginase, under conditions of Mn deficiency[22,12] is generally considered as an indication of the involvement of Mn^{2+} in the liver arginase reaction *in vivo*. Since native liver arginase is inhibited by KCN[9] and since its pH optimum is 7.7–9.0 (ref. 23), a role of Fe^{2+}, Co^{2+} or Ni^{2+} in liver arginase *in vivo* cannot be ruled out, however. It must be concluded that hitherto no definite answer has been given to the question as to which cation activates the liver arginase *in vivo*. A comparative study of native liver arginase and its metallo-complexes of known composition may give the solution to this problem.

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THE FERROUS ION AS THE COFACTOR OF ARGINASE *IN VIVO*

II. EXPERIMENTS ON THE REPLACEMENT OF FERROUS IONS IN NATIVE YEAST ARGINASE BY OTHER CATIONS *IN VIVO*

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SUMMARY

The possibility of the replacement of Fe^{2+} in yeast arginase (L-arginine ureohydrolase, EC 3.5.3.1) by Mn^{2+} or Co^{2+} *in vivo* has been studied. Neither the conditions of moderate Fe deficiency nor the addition of Mn^{2+} salts to the Fe-deficient culture medium exerted any effect on the properties and on the activity of native yeast arginase. The same applies to the addition of Co^{2+} salts; only when the selected concentration ratio of Co^{2+}: Fe^{2+} was extreme, were the two effects observed: primarily the appearance of unactivated apoenzyme and secondly that of some Co^{2+}–arginase. It is dubious, however, whether the latter had arisen *in vivo* or during the preparation of the cell-free extract.

INTRODUCTION

In many organisms, yeast included[1], the pathway of arginine breakdown is initiated by the action of arginase (L-arginine ureohydrolase, EC 3.5.3.1). Especially the liver arginase of ureotelic animals has been studied by numerous investigators. Liver arginase requires the presence of bivalent cations (Mn^{2+}, Co^{2+}, Fe^{2+} or Ni^{2+}) for its action[2]. The enzyme forms stable complexes with its activating cations[3]. These complexes can be prepared *in vitro* by reaction of apoarginase and metal ions. Liver arginase has been considered to be activated by Mn^{2+} *in vivo*[4,5].

The arginase of baker's yeast is similar to that of liver[6–8]. Its apoenzyme *in vitro* forms enzymically active complexes with the same cations as does liver arginase; in addition to these cations, Mg^{2+} is activating yeast arginase, though less effectively[8]. The metallo-complexes of yeast arginase are more readily inactivated by dialysis than those of liver arginase[6]. The various metallo-complexes of yeast arginase, which are readily prepared *in vitro*, are distinguished from each other by their pH–activity curves, Michaelis constants, specific activities and sensitivities to inhibiting reagents[8].

The metallo-complex active *in vivo* (native yeast arginase) can be isolated by careful extraction of the yeast[7,8]. A comparative study of native arginase and of me-

tallo–arginases of known composition has shown that the yeast arginase is activated *in vivo* by Fe^{2+} (refs. 7, 8).

The aim of the present study was to investigate the possibility of the replacement of Fe^{2+} in native yeast arginase by other cations *in vivo*. For this purpose the yeast was grown in Fe-deficient media supplied with Co^{2+} or Mn^{2+} salts. The nature of the activating cation in the native arginase was deduced from the shape of the pH–activity curve and from the sensitivity to inhibition by phosphate.

MATERIALS AND METHODS

The yeast strain was the same as previously used[1,7,8]. The Fe-deficient medium contained per l: 20 ml of DL-lactic acid, 10 mmoles L-arginine · HCl (N.B.C.), 40 mmoles L-glutamic acid, 2 mmoles disodium α-glycerophosphate, 400 mg of $MgCl_2 \cdot 6H_2O$, 100 mg of $CaCl_2 \cdot 2H_2O$ and 100 mg of NaCl. Vitamins were added as described previously[8]. All ingredients were of analytical standard (A.R.) quality and were dissolved in glass-distilled water. Fe was removed from the medium by adding 100 mg of 8-hydroxyquinoline. After adjusting the pH to 7.0 with KOH, the medium was boiled for 10 min, left overnight at room temperature and afterwards shaken with 100 ml of chloroform. After discarding the chloroform layer, the pH was brought to 3.5 with H_2SO_4 (A.R.). The residual 8-hydroxyquinoline was removed by repeated extraction with chloroform, the last traces of which were removed by boiling the medium for 10 min. Trace elements (B, Cu, I, Mn, Mo and Zn) were added as described previously[8].

The growth of the yeast in Fe-deficient media, prepared in the described way, was found to be 30–60% of that in identical media supplied with excess of $FeSO_4$ (2 mg Fe^{2+} per l). Cell yield of the Fe-deficient medium was determined as the dry weight of the culture (10 ml of medium in a 100-ml conical flask), after shaking for 48 h at 30°. This culture was inoculated with 0.1 ml of an end-log-phase culture in the same medium, supplied with 0.5 mg Fe^{2+} per l.

For arginase experiments the yeast was grown in Fe-deficient medium supplied with small amounts of $FeSO_4$ to increase the maximal cell yield to 80% of that in the same medium, supplied with 2 mg Fe^{2+} per l. Amounts of 100 ml of Fe-deficient medium were inoculated with 1 ml of an end-log-phase culture in the same medium supplied with 0.5 mg Fe^{2+} per l. The cultures were shaken in 1000-ml conical flasks at 30°. The yeast was harvested in the log phase, before the pH of the culture rose above 6.5.

The methods concerning the determinations of the specific activity of total arginase (as Mn^{2+}-activated enzyme, pH 9.5), of the pH–activity curve of native arginase and of the protein content of the cell-free extracts were described previously[8]. All specific activities were expressed as μmoles urea produced per h per mg protein. The inhibition of the native arginase by phosphate was studied by incubating the cell-free extract for 10 min at 20° in 100 mM potassium phosphate (pH 6.0), followed by determination of the specific activity of native arginase at pH 8.75.

RESULTS

The native arginase of Fe-deficient yeast

The possibility of replacing Fe^{2+} in native arginase by other cations was in-

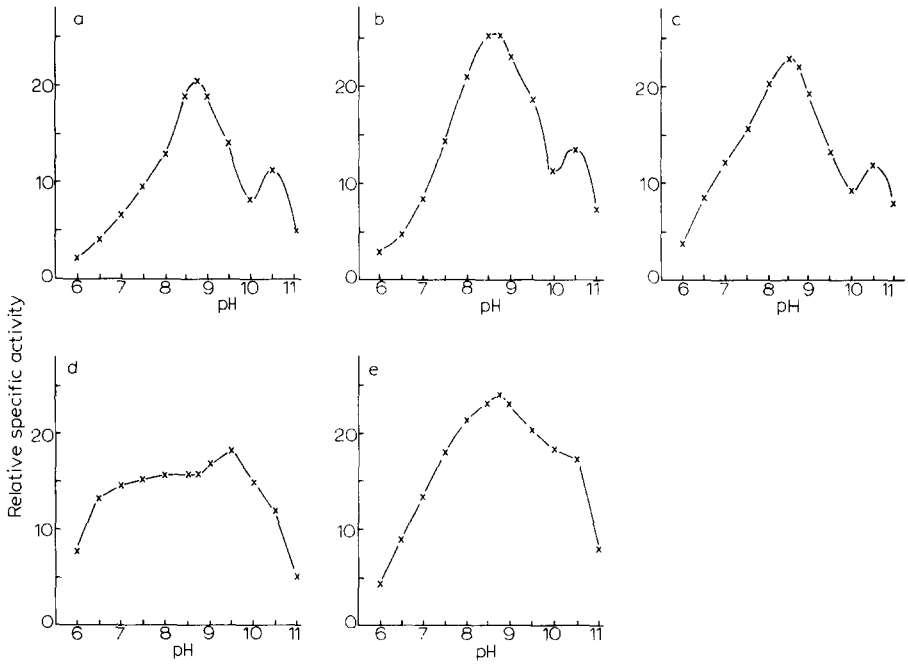

Fig. 1. pH–activity curves of native arginase. Ordinate: relative specific activity (percent of the specific activity of total arginase, determined as Mn^{2+}–arginase at pH 9.5). a, Fe-deficient; b, Fe-deficient, 600 mg Mn^{2+}/l; c, Fe-deficient, 10 mg Co^{2+}/l; d, Fe-deficient, 40 mg Co^{2+}/l; e, 2 mg Fe^{2+}/l and 40 mg Co^{2+}/l.

vestigated by studying the native arginase of yeast grown in a Fe-deficient medium supplied with a growth-retarding amount of a Mn^{2+} or a Co^{2+} salt. When replacement of Fe^{2+} in native arginase by Mn^{2+} or Co^{2+} would be possible *in vivo*, this phenomenon might be expected to occur in yeast grown in the above-mentioned medium because of the selected extreme concentration ratio of Mn^{2+} or Co^{2+} and Fe^{2+}. The other growth conditions in the lactate–arginine–glutamate medium were expected to favour the replacement of Fe^{2+} as well because of the following reasons:

(1) Lactate is metabolized by the yeast exclusively aerobically. Hence the available Fe^{2+} might be expected to be preferably incorporated in cytochromes.

(2) Arginine induces the synthesis of arginase in yeast[1]. This induction is hardly counteracted by the presence of glutamate in the medium[12]. Since glutamate is an excellent source of nitrogen, the activity of arginase in the yeast is no requirement for the growth in this medium. Even if Mn^{2+}– and Co^{2+}–arginase would be unable to degrade arginine *in vivo*, as efficiently as does Fe^{2+}–arginase, the growth of the yeast would not be retarded.

The pH–activity curve of the native arginase of yeast grown in the Fe-deficient medium (Fig. 1a) had the same shape as the curve observed earlier[8], in a study on the native arginase of yeast grown in media containing an excess of Fe (2 mg of $FeCl_3 \cdot 6H_2O$ per l). The inhibition of native yeast arginase by phosphate (Table I) demonstrated its identity with Fe^{2+}–arginase as well. The specific activity of native arginase at the

TABLE I

THE ARGINASE OF YEAST, GROWN IN MEDIA OF DIFFERENT MINERAL COMPOSITION

	Medium				
	Fe-deficient	*Fe-deficient 600 mg Mn^{2+}/l*	*Fe-deficient 10 mg Co^{2+}/l*	*Fe-deficient 40 mg Co^{2+}/l*	*Fe^{2+} 2 mg/l 40 mg Co^{2+}/l*
Growth period (h)	24	48	40	48	36
Yield (g yeast per 100 ml)	1.0	1.3	1.4	1.1	1.2
Specific activity of total arginase (units/mg protein, as Mn^{2+}–enzyme at pH 9.5)	31	30	25.5	32	28
Specific activity of native arginase (units/mg protein, at pH 8.75)	6.3	7.5	5.1	4.3	6.9
Inhibition (%) of native arginase by phosphate	85	83	80	59	70
Relative specific activity of native arginase (% of the specific activity of total arginase)	21	25	20	13.5	24.5

optimal pH 8.75 (Table I) was 25% of that of total arginase (determined at pH 9.5 after heating the cell-free extract with $MnCl_2$). Both specific activities were the same as those usually observed in yeast grown in similar media with an excess of Fe (ref. 8). These results demonstrate that the arginase of Fe-deficient yeast *in vivo* was activated completely and exclusively by Fe^{2+}. The conditions of moderate Fe deficiency obviously did not affect the total amount of arginase in the yeast. Under these conditions all native arginase of the yeast was present as Fe^{2+}–arginase; there was no evidence for the presence of unactivated apoarginase.

The effect of Mn^{2+} on yeast arginase in vivo

The yeast was grown in the Fe-deficient medium to which 600 mg of Mn^{2+} per l (as $MnSO_4 \cdot H_2O$, A.R.) had been added. As shown in Table I, the growth of the yeast was much retarded by this Mn^{2+} concentration. The native arginase of yeast, grown in the Mn^{2+} medium, was inhibited by phosphate (Table I). Its pH–activity curve (Fig. 1b) was found to be identical with that of native arginase of yeast, grown in the Fe-deficient medium (Fig. 1a). Furthermore, its specific activity at pH 8.75 was 25% of that of total arginase. These results clearly demonstrate that neither the total amount of arginase in the cell nor the specific activity of the native arginase nor the properties of the latter were influenced by the addition of Mn^{2+} to the culture medium. No evidence was presented for the incorporation of Mn^{2+} in yeast arginase *in vivo*.

It must be emphasized that the results as described above were only obtained when the yeast was harvested before the pH of the culture had risen to 7.0. The native arginase of yeast harvested from old cultures often showed the properties of Mn^{2+}–arginase. In such cultures, in which the pH may have been as high as 8.5 for several hours, the yeast was dying and its cell membranes became more permeable, as was readily demonstrated by adding methylene blue. It is evident that the observed Mn^{2+}–arginase originated from a secondary reaction, occurring in old cultures. Hence it must be considered as an artifact. The presence of Mn^{2+}–arginase as native yeast arginase

has never been demonstrated in cultures with a pH below 7.0, even when the Mn^{2+} concentration was as high as 1200 mg per l.

The effect of Co^{2+} on yeast arginase in vivo

Fig. 1c shows the pH–activity curve of native arginase, extracted from Fe-deficient yeast grown in the presence of 10 mg of Co^{2+} per l (as $CoCl_2 \cdot 6H_2O$, A.R.). The shape of the curve and the other properties of the native arginase (Table I) show that this Co^{2+} concentration is without effect on the nature and the specific activity of yeast arginase. Hence it is concluded that the arginase of Fe-deficient yeast, grown in the presence of a slightly growth-retarding Co^{2+} concentration, is activated *in vivo* completely and exclusively by Fe^{2+}.

When the yeast was grown in the Fe-deficient medium in the presence of 40 mg of Co^{2+} per l, a native arginase of other properties was observed. The pH–activity curve of this native arginase (Fig. 1d) resembles the curve of Co^{2+}–arginase[8] in showing an optimum at pH 9.5 and a broad shoulder at pH 7.0–9.0. The specific activity of Co^{2+}–arginase at pH 7.0–9.0 is about $^2/_3$ of that at the optimal pH (ref. 8); in the curve of Fig. 1d the shoulder is relatively more active, however. Hence the native arginase of Fe-deficient yeast, grown in the presence of 40 mg of Co^{2+} per l, is considered as a mixture of Co^{2+}– and Fe^{2+}–arginases, as is shown by its susceptibility to phosphate inhibition as well (Table I). This native arginases was inhibited by phosphate much less strongly than normal native yeast arginase. This is an indication for the involvement of a cation, distinct from Fe^{2+}, because phosphate is inhibiting only Fe^{2+}–arginase and not the other metallo–arginases[8].

The total amount of arginase in Fe-deficient yeast grown in the presence of 40 mg of Co^{2+} per l was the same as that in yeast grown in normal Fe-deficient medium (Table I). The specific activity of native arginase (pH 8.75) was only 13.5% of that of the total arginase activity, instead of 20–25% as usual (Table I). Since the specific activities of Fe^{2+}– and Co^{2+}–arginases at pH 8.75 are about the same[8], it is concluded that the native arginase of Fe-deficient yeast grown in the presence of 40 mg of Co^{2+} per l comprised, next to Co^{2+}– and Fe^{2+}–arginase a large amount (35–50% of the total) of unactivated apoenzyme. This suggests that the effect of Co^{2+} on yeast arginase *in vivo* is primarily a decreased incorporation of Fe in the enzyme. This conclusion is in accordance with that of HEALY *et al.*[9], who state that the main phenomenon caused by Co^{2+} intoxication in Neurospora is a severe Fe deficiency, resulting in decreased activities of many Fe-enzymes. This is probably not caused by an inhibition of the Fe^{2+} assimilation from the medium but by a competition of Co^{2+} and Fe^{2+} in the system that accomplishes the incorporation of Fe^{2+} in enzymes[10].

The addition of 40 mg of Co^{2+} per l culture medium, containing an excess of Fe^{2+} (2 mg/l) did not influence the specific activity and the susceptibility to phosphate inhibition of native yeast arginase (Table I). The pH–activity curve of this native yeast arginase (Fig. 1e) has its optimum at pH 8.75. It differs from the normal native yeast arginase in showing a greater activity at unfavorable pH values. It closely resembles the curve of Fe^{2+}–arginase, determined in the presence of excess Fe^{2+} (ref. 8). Possibly, the addition of 40 mg of Co^{2+} per l of Fe^{2+}-sufficient medium results in the formation of a more stable native Fe^{2+}–arginase complex.

Since the addition of large amounts of Co^{2+} to a Fe-deficient medium caused a reduced native arginase activity, the toxicity of Co^{2+} for yeast might be more severe

TABLE II

THE TOXICITY OF Co^{2+} FOR YEAST IN Fe-DEFICIENT LACTATE–ARGININE MEDIA, WITH AND WITHOUT GLUTAMATE

The growth was determined as the dry weight of the culture (expressed as mg/10 ml).

Additions	*Concn. mg/l*	*Glutamate concentration*	
		0	*40 mM*
Fe^{2+}	2	25.8	36.0
	0	21.6	30.0
Co^{2+}	10	15.7	25.2
	20	11.1	25.9
	30	9.5	18.9
	40	3.2	15.2

when the organism is grown with arginine as the sole nitrogen source than in the presence of an additional nitrogen source. In order to investigate this hypothesis, the yeast was grown with different Co^{2+} concentrations in Fe-deficient lactate–arginine media, with and without L-glutamate. The growth was measured by determining the dry weight of 10 ml of the culture after growing the yeast at 30° for $2\frac{1}{2}$ days in the usual way. As shown in Table II, Co^{2+} was significantly more toxic in the lactate–arginine medium than it was in the medium containing an additional amount of glutamate.

DISCUSSION

The influence of some variations in the mineral composition of the culture medium on the properties of native yeast arginase has been investigated. A similar study has recently been undertaken by CURDEL[11], who studied the D-lactate dehydrogenase (D-lactate:NAD^+ oxidoreductase, EC 1.1.1.28) of baker's yeast. This is a Zn^{2+}-enzyme in which the Zn^{2+} is replaceable by Co^{2+} or Mn^{2+} *in vitro* without loss of enzymic activity. When Zn^{2+} is omitted from the medium, the yeast produces unactivated apoenzyme. If $CoCl_2$ is added to the Zn-deficient medium, a Co^{2+}-enzyme is produced which, however, differs from the Co^{2+}-enzyme prepared *in vitro*.

By growing *Saccharomyces cerevisiae* under conditions of Fe deficiency and of intoxication by Mn^{2+} or Cc^{2+} salts, the possibility of incorporating these cations in the yeast arginase *in vivo* was studied. The nature of the activating cation of yeast arginase *in vivo* was deduced from the properties of the native arginase, especially the shape of the pH–activity curve and the susceptibility to phosphate inhibition were shown to be of great use for this purpose[8].

Besides a replacement of the Fe^{2+} in native yeast arginase, the conditions of Fe deficiency might change the amount of arginase in the cell. The appearance of unactivated apoarginase belonged to the possibilities as well. These phenomena were studied by the determination of the specific activities of native arginase and of total arginase (as Mn^{2+}–arginase, after heating the cell-free extracts with $MnCl_2$).

From the results obtained, it was concluded that the conditions of moderate Fe deficiency exerted no effect on the properties and the activity of the native arginase. Neither did the addition of growth-retarding amounts of $MnSO_4$ to the Fe-

deficient culture medium, provided that the yeast culture was harvested before the end of the log phase. In old cultures secondary exchange reactions may occur.

The effect to Co^{2+} on yeast arginase *in vivo* was more complex than that of Mn^{2+}. The native arginase retained the properties of Fe–arginase when 40 mg of Co^{2+} had been added per l of Fe^{2+}-sufficient medium or when a slightly growth-retarding amount of Co^{2+} (up to 10 mg per l) is added to the Fe-deficient medium. The specific activity of the arginase was not affected under these conditions. However, when more Co^{2+} (40 mg per l) had been added to the Fe-deficient medium, the native arginase had changed, both in properties and in specific activity; the total amount of arginase had not changed. Under these conditions, 35–50% of the total arginase was present as the apoenzyme; the rest showed the properties of a mixture of Co^{2+}– and Fe^{2+}–arginases, with respect to both the pH–activity curve and the inhibition by phosphate. Consistent with this finding of decreased native arginase activity was the observation that Co^{2+} is more toxic for baker's yeast when the organism is growing in a Fe-deficient lactate medium with arginine as the sole source of nitrogen than when glutamate is added to this medium at the same time.

In Neurospora, Co^{2+} intoxication brings about a severe Fe deficiency[9], probably caused by competition of Co^{2+} and Fe^{2+} in the system that achieves the incorporation of Fe^{2+} in enzymes[10]. This results in a considerable decrease in the activity of many Fe-enzymes[9]. The reduced native yeast arginase activity, caused by Co^{2+} intoxication of Fe-deficient yeast, is in agreement with the results of the Neurospora studies. It strongly suggests that the effect of Co^{2+} on yeast arginase *in vivo* is primarily an inhibition of the incorporation of Fe^{2+} in the apoarginase. Secondly, the apoarginase partially reacts with Co^{2+}. Since Co^{2+}–arginase is more resistant to heat inactivation and to storage at 0° than is Fe^{2+}–arginase (unpublished results), this activation by Co^{2+} apparently is only partial. Would it be complete, a native arginase of greater specific activity would be expected (the specific activities of Co^{2+}– and of Fe^{2+}–arginases are about the same at the pH concerned). The reaction of apoarginase and Co^{2+} may proceed directly or may be catalyzed by the same system that accomplishes the incorporation of Fe^{2+} in the arginase. This system has apparently a strong preference for Fe^{2+}, because the occurrence of Co^{2+}–arginase *in vivo* was observed only under extreme conditions. A direct reaction of apoarginase and Co^{2+}, either *in vivo* or during the preparation of the cell-free extracts, is more probable, however, because Co^{2+} was accumulated by the yeast (the cell-free extracts were pinkish).

The results of the present investigation confirm the conclusions of the previous report[8], in which it is stated that Fe^{2+} is the cofactor of yeast arginase *in vivo*.

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PORPHYRIN BIOSYNTHESIS

VI. SEPARATION AND PURIFICATION OF PORPHOBILINOGEN DEAMINASE AND UROPORPHYRINOGEN ISOMERASE FROM COW LIVER. PORPHOBILINOGENASE AN ALLOSTERIC ENZYME

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SUMMARY

1. Porphobilinogenase has been isolated and purified from cow liver and its components, porphobilinogen deaminase and uroporphyrinogen isomerase, have been separated from each other and purified.

2. The effect of NH_4^+ was studied. The deaminase exhibited classical Michaelis–Menten kinetics in the absence or presence of NH_4^+, which at high concentrations behaved as a noncompetitive inhibitor of the deaminase. As expected from Hill plots, $n = 1$ both in the absence or presence of NH_4^+. Instead, when activity of porphobilinogenase is plotted *versus* porphobilinogen concentration, sigmoid curves are obtained; but the presence of NH_4^+ at different concentrations altered the kinetic parameters of this enzymic system, again showing normal kinetics. In addition, n values were found to be 2 for porphobilinogen per porphobilinogenase molecule and 1 in the presence of NH_4^+ which behaves as a competitive inhibitor of the isomerase. Results are discussed in relation to the allosteric theories of MONOD *et al.*[1,2] and liver porphobilinogenase is proposed to be an allosteric protein.

3. The presence of an ultrafiltrable factor which stimulates uroporphyrinogen formation from porphobilinogen has been revealed.

INTRODUCTION

FALK *et al.*[3] demonstrated that porphobilinogen could be used for the enzymic synthesis of porphyrins, and the conversion of porphobilinogen into uroporphyrinogens by the enzymic system called porphobilinogenase[4] has been studied in preparations from various sources, including bacteria, algae, higher plants and avian and mammalian red cells[5–12]. Evidences[3–12] show that porphobilinogenase contains at least two enzymes: the relatively heat-stable porphobilinogen deaminase (porphobilinogen ammonia-lyase) which catalyzes the synthesis of uroporphyrinogen I from porpho-

bilinogen and uroporphyrinogen isomerase or uroporphyrinogen III cosynthetase which directs the formation of uroporphyrinogen III from porphobilinogen when deaminase is also present in the system. The mechanism of the enzymic condensation of porphobilinogen to uroporphyrinogen is still unknown, as it is one of the substrates for the isomerase. We intend to study the reaction using purified preparations of the porphobilinogenase as well as those of deaminase and isomerase. Either the isolation or purification of porphobilinogenase or the separation of deaminase from isomerase have been previously reported[5–12]. The present work describes the isolation and purification from cow liver of the porphobilinogenase, that is the deaminase–isomerase complex which converts porphobilinogen into uroporphyrinogen III and the separation and purification of the deaminase and isomerase from each other. The presence of an ultrafiltrable factor which stimulates uroporphyrinogen formation from porphobilinogen has been revealed. Kinetic analyses have been conducted on the deaminase and the porphobilinogenase preparations both in the absence and presence of NH_4^+. This paper reports that porphobilinogenase displays cooperative interactions, suggesting that it is an allosteric protein.

MATERIALS AND METHODS

Porphobilinogen was obtained according to SANCOVICH *et al.*[13] and was assayed as described by MAUZERALL AND GRANICK[14]. Fresh cow liver was kindly supplied by Lab. Asoc. I.F.F.A., Estrella (Buenos Aires) and was stored frozen in solid CO_2. Sephadex was obtained from Pharmacia (London) (N.13), and Bio-Gel P was a generous gift from Bio-Rad Laboratories (Richmond, Calif.). Calcium phosphate gel was prepared using the method of KEILIN AND HARTREE[15].

Estimation of enzyme activity

The standard incubation system, unless stated otherwise, contained the enzyme preparation (usually 5 ml) together with 0.05 M Tris buffer (pH 7.4), 120 μg of porphobilinogen, 0.5 ml of 0.6 M NaCl and 0.5 ml of 0.12 M $MgCl_2$ in a final volume of 10 ml (pH 7.4). Incubations were carried out aerobically in conical 25-ml flasks in the dark with mechanical shaking at 38° for 3–4 h. For kinetic studies, the incubation system contained, in the same final volume and at the same pH, only enzyme, substrate and buffer, with or without the addition of other reagents such as NH_4^+. Blanks were run with porphobilinogen and without enzyme preparation. After incubation, 1.2 ml of concentrated HCl were added to each flask to precipitate the protein; the mixture was then exposed to light and air for 25–30 min to oxidize porphyrinogens to porphyrins. The precipitated protein was removed by filtration, and total porphyrins were determined in a 5% solution[16]. Porphyrins were then fractionated and esterified using the usual procedures[17]. Identification and quantitative determination of porphyrins formed were made using the method of BATLLE AND GRINSTEIN[18], and isomeric composition of uroporphyrin fractions was determined using the method of CORNFORD AND BENSON[19]. Porphobilinogen was estimated by the usual procedure[14]. Protein content was calculated by the method of LOWRY *et al.*[20]. An enzymic unit was defined as the amount of enzyme that catalyzes the formation of 1 nmole of uroporphyrinogen per h, under the standard conditions, the activity being units of enzymes per mg of protein.

For the estimation of isomerase activity, the reaction mixture was the same as described above, except that an excess of deaminase was also added along with varying amounts of the isomerase preparation. NH_4^+ is known to inhibit the porphobilinogenase[21,22]; therefore, they must be removed from the enzyme preparation before estimating enzymic activity. This was carried out using the method of molecular sieving with 1.8 cm × 30 cm columns of Sephadex G-25 or Bio-Gel P-20, using 0.05 M Tris buffer (pH 7.4) as eluent. This method was chosen because recovery of enzyme units was total and because it was more rapid than dialysis. When Sephadex G-100 columns were used, protein was eluted with 0.05 M Tris buffer (pH 7.4) containing 0.1 M NaCl, unless otherwise stated. Fractions were automatically collected with an LKB collector (Stockholm). Protein content and enzymic activity were determined in each fraction, and those fractions containing activity were pooled.

Sephadex columns were prepared as described by BATLLE *et al.*[23].

RESULTS

Preparations of enzymes

All operations were carried out in the cold room at 4°, unless otherwise stated.

Preparation of porphobilinogenase (deaminase–isomerase complex)

Homogenate (10%, w/v) of liver was prepared in 0.25 M sucrose (Table I, Step 1); and the homogenate thus obtained was centrifuged at 11 000 × *g* for 10 min and the sediment was discarded (Step 2). Glacial acetic acid was added to the supernatant, to adjust it to pH 5.0; and after 20 min, the material was centrifuged for 10 min at 11 000 × *g*, and again the sediment was discarded (Step 3). The supernatant solution was then fractionated with solid $(NH_4)_2SO_4$; the fraction precipitating at 35–50% satn. was collected by centrifugation, dissolved in a small volume of 0.05 M Tris buffer (pH 7.4) and passed through a Sephadex G-25 or Bio-Gel P-20 column (Step 4).

TABLE I

ISOLATION AND PURIFICATION OF PORPHOBILINOGENASE FROM COW LIVER

Incubation conditions were as described in the MATERIALS AND METHODS section. Identification and quantitative determination of porphyrins formed and isomer analysis of uroporphyrin fraction were carried out by usual methods[18,19].

Fraction	*Units/mg* × 10^{-3}	*Purification*	*Porphyrin formed (%)*		
			Uroporphyrin III	*Uroporphyrin I*	*Others*
1. Homogenate	28.3	1	15	—	85
2. 11 000 × *g* (supernatant)	59.4	2.1	15	—	85
3. Treatment with glacial acetic acid to pH 5.0 (supernatant)	102.0	3.6	35	—	65
4. 35–50% satd. $(NH_4)_2SO_4$ fraction	234.9	8.3	75	—	25
5. $Ca_3(PO_4)_2$-gel treatment	469.8	16.6	75	25	—
6. Second 50% satd. $(NH_4)_2SO_4$ fraction	736.0	26.0	75	25	—
7. Sephadex G-100 column (pooled fractions)	3090.4	109.2	100	—	—
7a. Sephadex G-100 column (peak activity)	5162.0	182.4	100	—	—

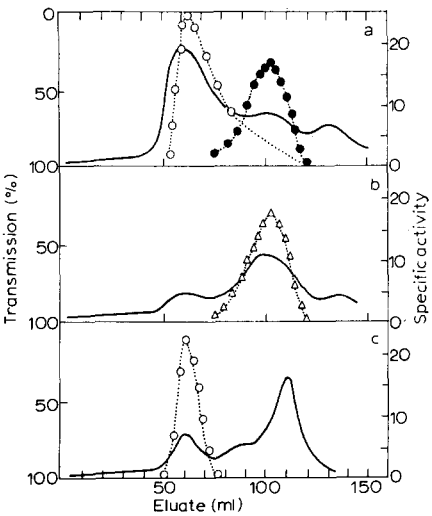

Fig. 1. Elution diagram in a 2 cm × 40 cm column of Sephadex G-100 of (a) porphobilinogenase, (b) deaminase and (c) isomerase preparations. ———, ultraviolet absorption as recorded, with a Uvicord I; ○·····○, isomerase activity; △·····△, porphobilinogenase activity; ●—●, deaminase activity. Activities were determined as described in MATERIALS AND METHODS.

The protein eluted was then treated with calcium phosphate gel (1 mg protein: 2 mg gel); after being stirred for 10 min, the mixture was centrifuged at 1000 × *g* for 5 min and the sediment was discarded (Step 5). $(NH_4)_2SO_4$ was added to the supernatant to 50% satn., and the protein precipitate was dissolved in a small volume of Tris buffer (Step 6). The enzyme preparation from the previous stage was then applied to a Sephadex G-100 column (Step 7). Fig. 1 shows a typical elution diagram where porphobilinogenase activity was associated with the protein shoulder and the specific activity of the peak of activity was 7 times higher than that of the preparation from the previous

TABLE II

ISOLATION AND PURIFICATION OF DEAMINASE FROM COW LIVER

Incubation conditions were as described in the MATERIALS AND METHODS section. Identification and quantitative determination of porphyrins formed and isomeric analysis of uroporphyrins fraction were carried out by usual methods[18,19].

Fraction	*Units/mg* × 10^{-3}	*Purification*	*Porphyrin formed (%)*		
			Uroporphyrin III	*Uroporphyrin I*	*Others*
1. Homogenate	28.3	1	15	—	85
2. 11 000 × *g* (supernatant)	59.4	2.1	15	—	85
3. Supernatant from dialysis	141.5	5.0	45	55	—
4. 45–70% satd. $(NH_4)_2SO_4$	325.5	11.5	40	60	—
5. $Ca_3(PO_4)_2$-gel treatment	750.0	26.5	30	70	—
6. Heat treatment	2549.8	90.1	20	80	—
7. Second 70% satd. $(NH_4)_2SO_4$ fraction	3056.4	108.0	20	80	—
8. Sephadex G-100 column (pooled fractions)	9474.8	334.8	—	100	—

stage. It must be emphasized, however, that the main protein peak, as we suspected, had very high isomerase activity, which was extended towards the shoulder, where, in fact, both activities were measured. It has also been observed, by using approximate values of molecular weights (unpublished results), that the isomerase is a relatively more active enzyme than the deaminase, small amounts of the former being sufficient to restore the uroporphyrinogen III forming activity of deaminase.

Preparation of deaminase

Steps 1 and 2 (Table II) were the same as those described for porphobilinogenase. Dialysis of the supernatant against glass-distilled water for 24 h caused a heavy flocculent precipitate to form, which was centrifuged and discarded (Step 3). This supernatant was then fractionated with $(NH_4)_2SO_4$; the fraction precipitating at 45–70% satn. was collected by centrifugation, dissolved in a small volume of 0.05 M Tris buffer (pH 7.4) and passed through a Sephadex G-25 or Bio-Gel P-20 column (Step 4). The protein eluted was treated with calcium phosphate gel (1 mg protein: 2 mg gel), and the sediment was discarded (Step 5). The supernatant was heated at 65–70° for 20 min, was immediately cooled in an ice bath and was centrifuged; the precipitate was discarded (Step 6). The supernatant was made to 70% satn. with $(NH_4)_2SO_4$, and the precipitate was dissolved in 0.05 M Tris buffer (pH 7.4) (Step 7). The protein solution was applied to a Sephadex G-100 column (Step 8). Fig. 1b shows a typical elution diagram where deaminase activity was associated with the second protein band.

Preparation of isomerase

Steps 1, 2 and 3 (Table III) were the same as those described for porphobilinogenase. In Step 4, the supernatent was fractionated with $(NH_4)_2SO_4$ to 70–90% satn., and this precipitate was dissolved in 0.05 M Tris buffer (pH 7.4). In Step 5 the protein solution was then applied to a Sephadex G-100 column. Fig. 1c shows a typical elution

TABLE III

ISOLATION OF ISOMERASE FROM COW LIVER

For the estimation of isomerase activity (Steps 4a and 5a), incubation conditions were the same as described in MATERIALS AND METHODS section, except that purified deaminase was used (1 mg of deaminase: 3–6 mg isomerase). As the exact isomerase/deaminase ratio in the homogenate and supernatant is not known as yet, results cannot be expressed in terms of specific activity of isomerase. Identification and quantitative determination of porphyrins formed and isomeric analysis of uroporphyrin fraction were carried out by usual methods[18,19].

Fraction	*Porphyrin formed (%)*		
	Uropor-phyrin III	*Uropor-phyrin I*	*Others*
1. Homogenate	15	—	85
2. 11 000 × *g* (supernatant)	15	—	85
3. Treatment with glacial acetic acid to pH 5.0 (supernatant)	35	—	65
4. 70–90% satd. $(NH_4)_2SO_4$ fraction	—	—	—
4a. 70–90% satd. $(NH_4)_2SO_4$ fraction + deaminase (from Stage 6 of purification)	90–100	10–0	—
5. Sephadex G-100 column (pooled fractions)	—	—	—
5a. Sephadex G-100 column (pooled fractions) + deaminase (from Stage 6 of purification)	90–100	10–0	—

diagram, isomerase activity was associated entirely with the first protein peak, the main protein band being hemoglobin.

Properties

Optimum activities of all preparations were observed at pH 7.4 in 0.05 M Tris buffer or 0.067 M phosphate buffer, under the standard conditions described. The rate of disappearance of porphobilinogen is constant for at least 6 h and shows that about 4 moles of substrate are consumed for each mole of uroporphyrinogen which appears and is proportional to enzyme concentration.

Isomer analysis of reaction products

Preparations from different stages of purification of porphobilinogenase predominantly formed uroporphyrinogen III. The only product of the deaminase preparation obtained after treatment on a Sephadex G-100 column was uroporphyrinogen I, and the effect of dialysis was similar to the effect of heating, as to the isomeric type of uroporphyrinogens formed. Isomerase obtained either after 70–90% satn. with $(NH_4)_2SO_4$ or after treatment on a Sephadex G-100 column, consumed no porphobilinogen, and only uroporphyrinogen III was formed when deaminase was added to the system.

Effect of temperature

As previously shown in other tissues, liver isomerase is a heat-labile enzyme[3–6,9–12,14,24,26] and loses 80% of its activity when heated for 20 min at 65–70° in the presence of deaminase; heat inactivation is slightly greater when isomerase is heated in the absence of deaminase.

TABLE IV

EFFECT OF ANAEROBIOSIS ,AEROBIOSIS AND CYSTEINE ON UROPORPHYRINOGEN RATE

Assay conditions were as described in MATERIALS AND METHODS section, except that incubations were anaerobic in 1, 2 and 3, and aerobic in 4, 5 and 6; cysteine at concentrations indicated was included in 2, 3, 5 and 6 at the time incubation. 15–20-Fold purified porphobilinogenase was used as enzyme preparation.

Conditions	*Uroporphyrin formed (nmoles/h)*
1. Anaerobic	5.9
2. Anaerobic + cysteine (0.1 mM)	6.1
3. Anaerobic + cysteine (1 mM)	5.9
4. Aerobic	5.7
5. Aerobic + cysteine (0.1 mM)	6.0
6. Aerobic + cysteine (1 mM)	6.0

Effect of anaerobiosis, aerobiosis and cysteine

The rate of uroporphyrinogens formed was the same whether the reaction mixture was incubated in air or anaerobically (Table IV). In either case, only uroporphyrinogens were present at the end of incubation; the isomer composition of the reaction product was unchanged. Cysteine at 1 and 0.1 mM did not modify either the isomer type or the porphyrinogen yield.

Effect of dialysis

Dialysis of the supernatant, obtained after Step 2 of all preparations, against

glass-distilled water for 24 h produced a heavy precipitate, which was separated by centrifugation. Activity was determined in both the supernatant and the precipitate. As already stated, the supernatant had deaminase activity (Step 3, Table II), while the precipitate had neither porphobilinogenase nor deaminase activity. As we suspected, however, it had isomerase activity; thus, on addition of deaminase to different amounts of precipitate dissolved in 0.05 M Tris buffer (pH 7.4), porphobilinogenase activity was completely restored. Therefore, dissociation of isomerase from deaminase by dialysis is a reversible process different from heat treatment, which is not reversible. Moreover, other activities were investigated in such a precipitate, which also was shown to have δ-aminolaevulinic acid dehydratase and decarboxylase(s) activities.

Stimulating factor for uroporphyrinogen formation

We have evidence for the presence of a factor which seems to be necessary and also stimulates uroporphyrinogen formation from porphobilinogen (Table V). When supernatant of 11 000 × *g* (A) was subjected to ultrafiltration for 3 h using an LKB ultrafilter, the ultrafiltrate residue (C) had less or no porphobilinogenase activity,

TABLE V

ULTRAFILTRATION EXPERIMENT

Activity of system A was taken as 100%. Incubation conditions were as those described in the MATERIALS AND METHODS section; that is 5 ml of A were used as "Enzyme preparation", but volumes of B and C, either incubated alone or mixed, were equivalent to 5 ml of A.

Homogenate
↓
Supernatant (A)
↓
Ultrafiltration
↓ ↓
Ultrafiltrate (B) Ultrafiltered residue (C)

Test system	*Deaminase–isomerase activity (%)*
A	100
B	0
C	0–50
B + C	110–130

while the ultrafiltrate (B) did not have activity; on addition of the ultrafiltrate to the ultrafiltrate residue, porphobilinogenase activity was not only restored but also slightly stimulated. A similar stimulating effect was reported to be found by HEATH AND HOARE[7,8] in extracts of various tissues.

Kinetics of deaminase

Plots of velocity against porphobilinogen concentration and their reciprocal, in the absence and presence of NH_4^+ followed classical Michaelis–Menten kinetics. Concentration of NH_4^+ was varied from 0.001 to 0.1 M; at 0.1 M deaminase was inhibited about 30% under the assay conditions described above. Nonetheless, the Michaelis constant, $K_m = 5$ μM, for porphobilinogen was found to be essentially independent of the NH_4^+ concentration (Table VI), which behaved as a noncompe-

TABLE VI

HILL COEFFICIENTS AND APPARENT K_m FOR PORPHOBILINOGEN

Activities were assayed as described in MATERIALS AND METHODS. Deaminase preparation used was 300-fold purified and porphobilinogenase preparation was 180-fold purified. "Heated enzyme" was porphobilinogenase heated 20 min at 65–70° and "dialyzed enzyme" was the supernatant obtained from a preparation of porphobilinogenase dialyzed 24 h against glass-distilled water, as described in Step 5 for purification of deaminase.

Enzyme and addition	*n**	*Apparent K_m or $[S_{\frac{1}{2}}]$ (μM)*
1. Deaminase		
None	1.0	5.0
0.01 M NH_4^+	1.0	5.0
0.1 M NH_4^+	0.9	5.0
2. Porphobilinogenase		
None	1.7–1.95	11–14
0.0001 M NH_4^+	2.0	10
0.001 M NH_4^+	1.85	10
0.01 M NH_4^+	1.77	7
0.1 M NH_4^+	1.0	5
Heated enzyme	1.0	5
Dialysed enzyme	1.0	5.5

* n was calculated from the slopes of the plot of log $(v/v_{max} - v)$ against log [porphobilinogen].

titive inhibitor of the deaminase. The estimated K_i for NH_4^+ obtained from the Lineweaver–Burk plots and by using the graphical method of DIXON[27] was 0.172 M. However, it is interesting to add that NH_4^+ at concentrations that inhibited formation of uroporphyrinogen I has only a very slight effect upon the rate of porphobilinogen consumption (Table VII); that is, porphobilinogen was consumed without the corresponding production of porphyrinogen as if NH_4^+ in some way produce an accumulation of one intermediate between porphobilinogen and uroporphyrinogen which, under normal conditions, could be identified with the substrate for the isomerase. Similar observations in plant tissues were reported by BOGORAD[21,22].

TABLE VII

EFFECT OF NH_4^+ ON THE DEAMINASE

Porphobilinogen consumption of control was taken as 100%. Deaminase 150-fold purified was used.

Addition	*Porphobilinogen consumption (%)*	*Porphyrin yield (%)*
None	100	100
0.01 M NH_4^+	100	78
0.05 M NH_4^+	95	70
0.1 M NH_4^+	92	66
0.2 M NH_4^+	85	57

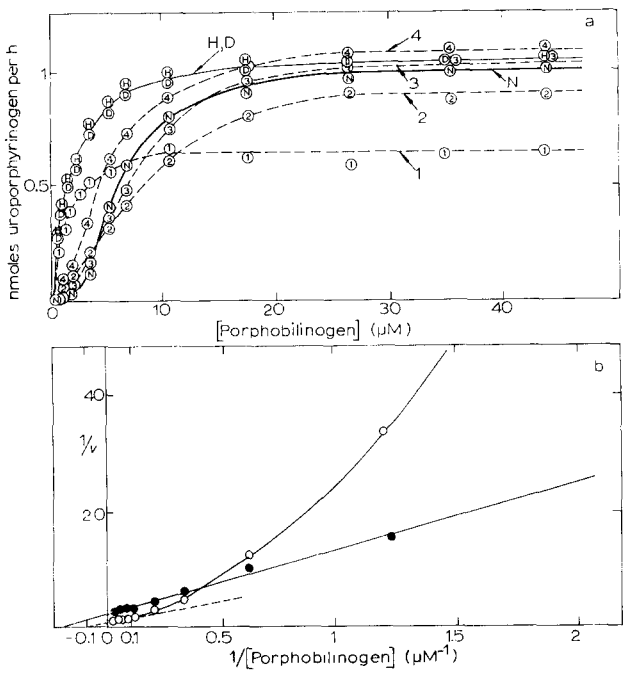

Fig. 2. Effect of porphobilinogen concentration on the activity of the porphobilinogenase under various conditions. Activities were measured as described. (a) With different concentrations of porphobilinogen: alone (Curve N); in the presence of NH_4^+, 0.1 M (Curve 1); 0.01 M (Curve 2); 0.001 M (Curve 3) and 0.0001 M (Curve 4); with porphobilinogenase heated 20 min at 65–70° (Curve H) and with supernatant of porphobilinogenase dialyzed against glass-distilled water (Curve D) (as described in Step 5 of deaminase purification). (b) Double reciprocal plots of velocity against porphobilinogen concentration without additions (○—○) ;in the presence of 0.1 M NH_4^+ or with heated or dialyzed porphobilinogenase (●—●).

Kinetics of porphobilinogenase

Plots of activity of porphobilinogenase *versus* porphobilinogen concentration at all enzyme concentrations studied were sigmoid, and the reciprocal plots were not linear (Fig. 2). The $[S_{\frac{1}{2}}]$ values for porphobilinogen, calculated from several different experiments, were between 11 and 14 μM (Table VI). It can also be seen from Fig. 2 and Table VI that heat treatment or dialysis against glass-distilled water, or addition of different concentrations of NH_4^+, alters the shape of the substrate saturation curve from a sigmoid to an hyperbolic pattern and also increases the affinity of the enzyme system for porphobilinogen; thus the apparent K_m values for porphobilinogen in the presence of high concentrations of NH_4^+, or with the heated or dialyzed enzyme, was again 5 μM.

It is also very interesting to note that if porphobilinogen consumption was taken as a measure of enzyme activity, plots of velocity of porphobilinogenase against porphobilinogen concentration showed normal kinetics pattern.

It is well known that nonlinear double reciprocal plots or sigmoid substrate saturation curves can result from a variety of causes such as allosterism[1,2] and random substrate–enzyme interactions. The possibility of a random mechanism, according to the description of CLELAND[28], cannot be completely excluded on the basis

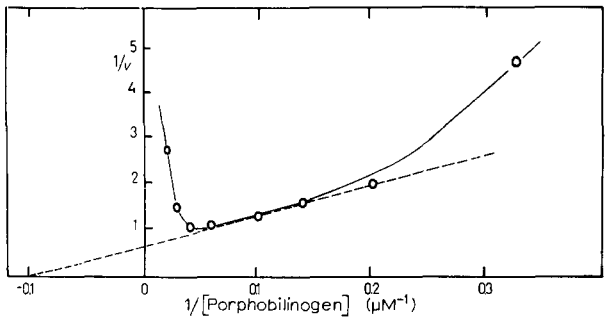

Fig. 3. Effect of porphobilinogen concentration on the formation of uroporphyrinogen III. Assay conditions were as described. Enzyme preparation was porphobilinogenase 150-fold purified. Velocity was measured as nmoles of uroporphyrinogen III formed per h.

of the kinetic results obtained, and further experiments will have to be performed to resolve this issue.

Velocity plots of any such allosteric reaction can be accounted for by the relation empirically deduced by HILL[29]. A plot of log $(v/v_{max}-v)$ against the log $[S]$ (Hill plots) gives a straight line with a slope of n. The value of n is an approximate[30] measure of the probable binding sites for the substrate. When such manipulations were carried out (Table VI), n values of about 2 were obtained for the porphobilinogenase, while either the addition of high concentrations of NH_4^+, heat treatment or dialysis appreciably changes the slope of the Hill plot to $n = 1$, which was the value obtained for the deaminase both in the absence or presence of NH_4^+.

The effect of porphobilinogen concentration

The action of substrate concentration on the activity of porphobilinogenase was also studied, and inhibition of isomerase by high substrate concentration was found,

TABLE VIII

EFFECT OF NH_4^+ ON THE ENZYMIC SYNTHESIS OF UROPORPHYRINOGEN III

Assay conditions were as described in MATERIALS AND METHODS. Porphobilinogenase preparation was 150-fold purified. Identification and quantitative determination of porphyrins formed and isomer analysis of uroporphyrin fraction were carried out using usual methods[18,19].

Concn. of inhibitor NH_4^+ (M)	*Total porphyrinogen formed*		*Uroporphyrinogen III formed*	
	nmoles/h	*Inhibition (%)*	*nmoles/h*	*Inhibition (%)*
None	0.530	0	0.530	0
0.2	0.298	44.0	0.060	90.0
0.1	0.349	34.3	0.087	83.5
0.05	0.369	30.5	0.143	63.0
0.01	0.465	12.4	0.278	47.5
0.005	0.520		0.360	30.0
0.001	0.536		0.428	20.0
0.0005	0.537		0.537	0
0.0001	0.575		0.575	0
0.00001	0.530		0.530	0

as increased amounts of uroporphyrinogen I were found with increased porphobilinogen concentration, while total porphyrin formed remained practically unchanged. Similar observations were reported by CORNFORD[10].

The effect of NH_4^+

Concentrations ranging from 0.00001 to 0.2 M were tested on the porphobilinogenase at constant concentrations of substrate (Table VIII); it was found that NH_4^+ at concentrations up to 0.2 M inhibited 90% the formation of uroporphyrinogen III and only 40% that of uroporphyrinogen I. These values were taken as a measure of the effect of NH_4^+ on the isomerase and deaminase activities, respectively. From plots of activity of isomerase against NH_4^+ concentration, typical inhibition curves were obtained (Fig. 4). Moreover, it was found using the method of DIXON[27] for graphical determination of K_i that NH_4^+ inhibited competitively the isomerase, showing a $K_i = 0.01$ M. Either Sephadex G-25 or Bio-Gel P-20, using 0.05 M Tris buffer (pH 7.4) as eluent, removed inhibition of NH_4^+ both with the deaminase and the isomerase. It has been found that low concentrations of NH_4^+ (0.0001–0.001 M) slightly activited the enzyme (Fig. 2 and Table VIII). It has also been observed, working with partially purified preparations of porphobilinogenase, that the addition of sodium or magnesium salts at certain concentrations to the incubation mixtures does not appreciably change total porphyrins formed but that it seems to increase the amount of uroporphyrinogen III. Moreover, different behaviors of purified isomerase and porphobilinogenase preparations on calibrated Sephadex columns in the absence or presence of salts have

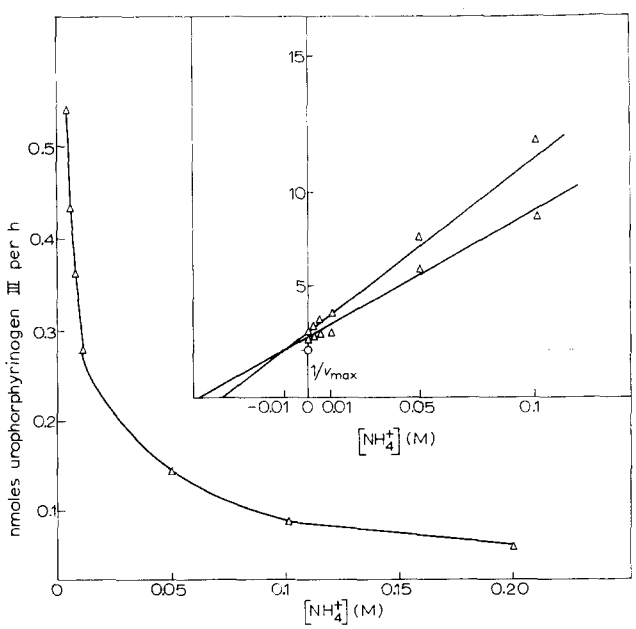

Fig. 4. Effect of NH_4^+ on porphobilinogenase. Assay conditions were as described. Velocity of porphobilinogenase as nmoles of uroporphyrinogen III per h was plotted against inhibitor concentration. Porphobilinogen concentration was held at a constant saturating level (5 times $[S_{\frac{1}{2}}]$. The enzyme preparation used was 150-fold purified. Inset: plots of reciprocal of velocity against inhibitor concentration for graphical determination of inhibitor at two porphobilinogen concentrations.

also been observed, and it has been found that protein fractions identified with deaminase and isomerase activities have very different molecular weights. These findings are being investigated further.

Evidences have also been found indicating that in crude liver homogenates there is a great excess of isomerase.

DISCUSSION

The formation of uroporphyrinogen III from porphobilinogen is one of the most complex steps in porphyrin metabolism. In some metabolic diseases, such as hepatic and erythropoietic porphyria, large amounts of Type I porphyrins are formed; accumulation of I isomers could then be attributed to a failure or absence of the isomerase[22] or perhaps to an inhibition of this enzyme by an excess of substrate; therefore it seemed worthwhile examining the conversion of porphobilinogen into uroporphyrinogens in liver at the enzymic level.

Procedures described here for the isolation and purification of porphobilinogenase, that is the system normally converting porphobilinogen into uroporphyrinogen III, and the separation of deaminase and isomerase activities from each other are very reproducible. Although both activities have been separated from various sources, with or without previous destruction or inactivation of one of them[4–12], we cannot be completely sure that they are, in fact, two distinct enzymes and not part of a complex protein formed by two or more different subunits having two activities. Thus, most of the methods described to separate deaminase from isomerase involved either inactivation of the isomerase by heating or reversible dissociation by dialysis against glass-distilled water, as in the present paper. On the other hand, isomerase free of deaminase has been obtained by treatment at high ionic strength, and indeed we have observed that high ionic strength greatly influences the behavior of isomerase on calibrated gel columns.

The kinetics of porphobilinogenase as a function of porphobilinogen concentration has been found to exhibit a sigmoid dependence as shown in Fig. 2. This type of relationship suggest that cooperative effects act to facilitate the binding of porphobilinogen to the isomerase as no cooperative effects were observed with the deaminase. The values of n from the Hill plots (Table VI) ranging from 1.7 to 1.95 for different porphobilinogenase preparations, and $n = 1$ for deaminase, would suggest the existence of two binding sites for porphobilinogen per porphobilinogenase molecule, one being on the deaminase unit and the other in the isomerase unit. The loss of homotropic interactions when ammonium ions were added to the system or when porphobilinogenase was heated or dialyzed against glass-distilled water was clearly shown (Fig. 2). The curve for reaction velocity against porphobilinogen concentrations in these conditions has now a Michaelis–Menten form; the reciprocal plot is linear, the Hill plot has a slope of unity and there is an increase in the affinity of the enzyme for porphobilinogen, so the enzyme system is converted to a form which is still fully active catalytically, but uroporphyrinogen I is formed instead of uroporphyrinogen III. We identified this form with the deaminase.

The action of NH_4^+ on deaminase and isomerase activities showed that they have a separate effect on the two activities as already suggested by BOGORAD[22], acting noncompetitively on the deaminase and competitively on the isomerase; moreover, excess

porphobilinogen also acts inhibiting the isomerase (Fig. 3), and then it appears as if there is some kind of competition between the porphobilinogen–isomerase complex and porphobilinogen (or NH_4^+) for the deaminase–polypyrrol complex, as it was in some way proposed by CORNFORD[10]. Perhaps NH_4^+ could inhibit by binding at the same site of porphobilinogen on the isomerase or probably at a different site which could be that of the polypyrrolic substrate on the isomerase or, still, they could produce dissociation of the deaminase–isomerase complex. The inhibition of isomerase by an excess of porphobilinogen could explain the abnormal formation of uroporphyrinogen I in some porphyria. The deaminase–isomerase complex is thought to have special kinetic properties because it binds porphobilinogen at least at two sites which are able to interact. For porphobilinogenase, the affinity for porphobilinogen is variable, being poor when porphobilinogen is very low and good when saturation is approached. This explanation would account for the sigmoidal saturation curve and would fit the model proposed by MONOD *et al.*[1,2]. Some evidences, shortly to be published, have been obtained showing that excess of isomerase exists in a crude preparation of porphobilinogenase, which would suggest that probably under normal conditions several units of isomerase are associated with one unit of deaminase in an active complex, in which the excess of isomerase assures the formation of uroporphyrinogen III and presumably reversibly modifies the properties of the complex by altering its enzymic specificity as suggested by LEVIN AND COLEMAN[11]. Either destruction or dissociation by dialysis, heating, high ionic strength or deficiency of the isomerase would remove such control, the regulatory properties of the system being lost. In any case, the existence of co-operative effects produced by some kind of protein–protein interactions are suggested and, although speculative in many respects, one could also think that porphobilinogen could induce a conformational change in the isomerase, forming the isomerase–porphobilinogen complex, which in turn, assumed to be bound to the deaminase trypyrrol complex, could induce a change in the deaminase, thus reducing its binding with another porphobilinogen molecule. Although the mechanism of the enzymic synthesis of uroporphyrinogens from porphobilinogen is still unknown, our data would suggest that, at least in liver, the mechanism of the porphobilinogenase enzyme would be "allosteric" in nature.

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INHIBITION OF MAMMALIAN URIDINEDIPHOSPHOGLUCOSE 4-EPIMERASE BY THE DITHIOTHREITOL-STIMULATED FORMATION OF NADH

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SUMMARY

1. Human fibroblast lysates preincubated with dithiothreitol showed inhibition of UDPGlc 4-epimerase (EC 5.1.3.2) activity at endogenous levels of NAD^+. This effect is probably due to NADH that is generated by the dithiothreitol stimulation of an NAD^+-reducing system. Inhibition was reversed by the addition of (at least) 0.2 mM NAD^+. A concentration-dependent inhibition of epimerase was also observed by NADH.

2. Several components of human fibroblasts and erythrocytes separable by gel electrophoresis are capable of NAD^+ but not of $NADP^+$ reduction in the absence of other added substrates. Another separate component is capable of NAD^+ and $NADP^+$ reduction only in the presence of dithiothreitol. These systems appear enzymatic in nature.

INTRODUCTION

UDPGlc 4-epimerase (EC 5.1.3.2) catalyzes the reversible conversion of UDPGlc to UDPGal. The dependence of epimerase activity on NAD^+ (refs. 1, 2) and its inhibition by NADH[3,4] have been documented. More specially, the effect of particular ratios of NAD^+/NADH on epimerase activity have been demonstrated with L and HeLa cell cultures[3]. It has also been reported that incubation of human fibroblast preparations with dithiothreitol caused inhibition of UDPGlc consumption at endogenous levels of NAD^+ (ref. 5).

This report will show that the inhibition of epimerase produced by dithiothreitol is due to stimulation of an NAD^+-reducing system endogenous to human fibroblast preparations.

METHODS AND MATERIALS

Maintenance and preparation of human diploid cell cultures

Human diploid fibroblast cultures were derived from skin biopsies and were

cultivated as monolayers by the method of HAYFLICK AND MOORHEAD[6]. Protein was determined by the method of LOWRY *et al.*[7]. Cells were harvested, and DNA was determined as previously described[8]. Fibroblast lysates were prepared by suspending $10 \cdot 10^6$–$20 \cdot 10^6$ cells/ml water. The suspensions were rapidly freeze-thawed 5 times and were centrifuged at 20 000 $\times$ *g* for 15 min. All centrifugations were performed at 4°. The supernatants were used in experimental procedures. Several lysates were homogenized according to KOBAYASHI *et al.*[9] for the preparation of mitochondria-free lysates, except that proteolytic treatment with nagase was omitted and that centrifugation was at 20 000 $\times g$. In the particular experiments indicated, lyophilized whole fibroblasts which had been stored below 0° were reconstituted at 20 or 40 mg dry wt./ml water and were centrifuged at 20 000 $\times g$ just before use.

Human erythrocyte preparations

Washed packed red cells were lysed by the addition of an equal volume of cold water followed by freezing and thawing. Hemolysates were mixed with an equal volume of 50% DEAE-cellulose suspension which had been previously equilibrated with 0.005 M phosphate buffer (pH 7.0). The mixture was stirred for 1 h at 4° and was poured onto a chromatographic column. Hemoglobin was removed from the column with 5 mM phosphate buffer (pH 7.0) until 280 mμ absorption of the eluate was below 0.05. The cellular components capable of NAD^+ and $NADP^+$ reduction were eluted from the column by supplementing the phosphate buffer with 50 mM $(NH_4)_2SO_4$. Crystalline $(NH_4)_2SO_4$ was added to the eluate to a final concentration of 20 g/100 ml, and the precipitate was centrifuged and discarded. A precipitate obtained (20–42 g/100 ml) was collected by centrifugation, was redissolved and was lyophilized. The dry powder which contained NAD^+- and $NADP^+$-reducing activity was stored below 0°. This material, which will be referred to as "semipurified erythrocyte preparation", was reconstituted at 20 or 40 mg dry wt./ml water and was centrifuged at 20 000 $\times g$ just before use.

Epimerase activity (spectrophotometric)

Spectrophotometric determinations were performed with either a Hitachi Perkin–Elmer Model 139 or a Gilford-converted Beckman. Epimerase activity in both directions was assayed by a modification of the two-step procedure described by MAXWELL[10]. The reaction system in a final volume of 0.25 ml contained 0.1 M glycylglycine (pH 8.7), fibroblast lysate equivalent to 10^6 cells, 20 mM dithiothreitol where indicated (33 mM in the preincubation system before the addition of substrate) varying concentrations of NAD^+, NADH and NADPH as indicated in particular experiments and 1 mM UDPGlc or UDPGal. Either UDPGlc or UDPGal was added to start reactions at 37°, and reactions were stopped by placing tubes in a boiling-water bath and by adding 0.05 ml of 0.35 M HCl. After boiling for 5 min, tubes were cooled, and UDPGlc formation or UDPGlc disappearance was measured with UDPGlc dehydrogenase as previously described[5].

In the experiments involving preincubation with dithiothreitol, 0.05 ml of 0.5 M glycylglycine (pH 8.7), 0.05 ml of lysate (10^6 cells) and 0.05 ml of 0.1 M dithiothreitol in a volume of 0.15 ml were incubated at 37° before the addition of UDPGlc or UDPGal.

In the experiments involving treatment of fibroblast lysate with NAD dinucleo-

tidase, 0.05 ml of fibroblast lysate equivalent to 10^6 cells was mixed with 0.05 ml of NAD dinucleotidase (0.1 unit). After 5 min at 37°, the mixture was used for epimerase assay as described above.

Epimerase activity (fluorimetric)

Epimerase activity was measured fluorimetrically at 27° by coupling the reaction with UDPGlc dehydrogenase in the presence of 1 mM NAD^+. Increasing fluorescence was recorded as a measure of UDPGlc formation. The reaction system in a final 0.5-ml vol. contained 0.1 M glycylglycine (pH 8.7), fibroblast lysate equivalent to $0.2 \cdot 10^6$ cells, 1 mM NAD^+, 100 units of UDPGlc dehydrogenase and 1 mM UDPGal. The reaction was started by the addition of UDPGal. The effect of 1–20 mM dithiothreitol was tested in this reaction. Control reactions were identical except that UDPGal was deleted.

Fluorimetric determinations were performed with an Eppendorff fluorimeter containing a 313 + 366 nm excitation filter and a 400–3000 nm output filter. The fluorimeter was equipped with a zero suppression scale expander unit (registrier-adapter 2134) and a Honeywell recorder.

UDPGlc dehydrogenase activity (fluorimetric)

UDPGlc dehydrogenase was assayed at 27° by recording increasing fluorescence as a measure of NADH formation. The reaction system in a final 0.5-ml vol. contained 0.1 M glycylglycine (pH 8.7), fibroblast lysate equivalent to $0.2 \cdot 10^6$ cells, 1 mM NAD^+ and 1 mM UDPGlc. UDPGlc was added to start the reaction.

Measurement of NAD^+ reduction (fluorimetric)

The reaction system used to measure the conversion of NAD^+ to NADH in the absence of other added substrates in a final 0.5-ml vol. contained 0.1 M glycylglycine (pH 8.7), fibroblast lysate equivalent to 10^6 cells or 1 mg dry wt. of semipurified erythrocyte preparation and varying concentrations of NAD^+ from 0.2 to 1.0 mM. Reactions were started by the addition of NAD^+, and the increase in fluorescence was recorded as a measure of NADH formation at 27°.

$NADP^+$ was tested in this system by substituting $NADP^+$ for NAD^+ at equimolar concentrations.

Measurement of dithiothreitol-dependent NAD^+ and $NADP^+$ reduction (fluorimetric)

The reaction system in a final 0.5-ml vol. contained 0.1 M glycylglycine (pH 8.7), lyophilized whole fibroblasts or semipurified erythrocyte preparation in the amounts indicated in individual experiments, 10 mM dithiothreitol and 1 mM NAD^+ or $NADP^+$. Reactions were started with either dithiothreitol or coenzyme, and the increase in fluorescence was recorded at 27°.

In experiments to determine the effect of pH, 0.2 M Tris–HCl was substituted for glycylglycine buffer at pH's 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5.

K_m values for dithiothreitol, comparing erythrocyte and fibroblast preparations, were obtained by varying the dithiothreitol concentration between 0.01 and 10 mM.

The effect of 10 mM dithioerythritol, reduced glutathione, cysteine, mercaptoethanol, 2,3-dimercaptopropanol, erythritol and xylitol was tested. Each was substituted for dithiothreitol in the reaction using $NADP^+$ as coenzyme.

The effect of 1 mM Cu, Mn, Mg, Zn and $CaCl_2$ and of 1 mM Na_2EDTA was each tested in the reaction with 10 mM dithiothreitol and 1 mM $NADP^+$.

Vertical polyacrylamide gel electrophoresis

Electrophoresis was performed using 6.6% polyacrylamide gel in a continuous buffer system with 0.1 M Tris–borate buffer (pH 9.0). The vertical polyacrylamide gel electrophoresis apparatus was from E.C. Apparatus Co. Semipurified erythrocyte and lyophilized whole fibroblasts preparations were reconstituted at 40 mg dry wt./ml water and were centrifuged at 20 000 × *g* for 15 min. 0.05 ml of the supernatants were electrophoresed at 4° for 16 h with 100 V (30 mA) applied across the gel. Reducing activity in the gel was located with longwave ultraviolet light after the gels had been divided into strips and had been incubated at 27° in 0.2 M glycylglycine buffer (pH 8.7), containing 2 mM NAD^+ or $NADP^+$ in combination with 10 mM dithiothreitol or other substrates tested.

RESULTS

Epimerase activity (spectrophotometric)

The influence of substrate concentration on human fibroblast epimerase activity was tested by the two-step method at endogenous levels of NAD^+ and supplemented with 1 mM NAD^+. At endogenous NAD^+, the K_m for UDPGlc was 0.3 mM and that for UDPGal was 0.12 mM. When supplemented with 1 mM NAD^+, the K_m for UDPGlc was 0.21 mM and that for UDPGal was 0.27 mM. After treatment of lysates with NAD nucleosidase no epimerase activity could be detected.

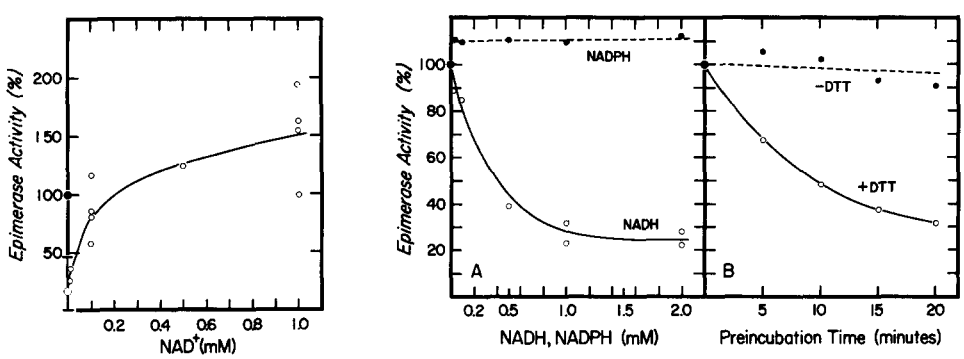


Fig. 1. The effect of adding NAD^+ to dithiothreitol-inactivated epimerase systems in fibroblast lysates. Lysates were preincubated with 33 mM dithiothreitol for 20 min at pH 8.7. Epimerase activity (UDPGlc to UDPGal) was assayed by the two-step assay. Activity without dithiothreitol is arbitrarily at 100% (solid dot). At endogenous levels of NAD^+ the range for dithiothreitol inhibition is 52–100% with a mean of 81% (large circle), based on 40 lysates. Upon adding increasing levels of NAD^+ to duplicate systems after dithiothreitol inactivation, reactivation of epimerase is observed. The line represents the mean of four separate experiments.

Fig. 2. The effect of increasing concentrations of NADH and NADPH on the epimerase activity of fibroblast lysates compared with the inhibitory effect of dithiothreitol (DTT) as a function of preincubation time at endogenous levels of NAD^+. A. Replicate tubes containing buffered lysate were treated with increasing concentrations of NADH and of NADPH before starting the reactions with UDPGlc. B. Replicate tubes containing buffered lysate preincubated for the times indicated in the presence and absence of 33 mM dithiothreitol before starting the reactions with UDPGlc.

Preincubation of fibroblast lysates buffered at pH 8.7 with 33 mM dithiothreitol for 20 min before the addition of epimerase substrate caused 52–100% inhibition in both directions. This inhibition could not be demonstrated if reaction systems were supplemented with 1 mM NAD^+. In 40 lysates tested, UDPGlc consumption after preincubation with dithiothreitol showed a mean inhibition of 81% with only endogenous NAD^+ present. As seen in Fig. 1, epimerase activity inhibited by dithiothreitol could be restored by supplementing inactivated systems with NAD^+, and the NAD^+ response was concentration dependent. The mean specific activity for epimerase (UDPGlc to UDPGal) at endogenous levels of NAD^+ was 40 nmoles/min per mg DNA* (n = 28, range 13 – 88), and that for epimerase (UDPGal to UDPGlc) was 35 nmoles/min per mg DNA* (n = 18, range 13 – 88).

Epimerase activity (fluorimetric)

The presence of 20 mM dithiothreitol in the fluorimetric rate reaction for epimerase activity had no effect. It is evident from the data in Fig. 1 and from coupled rate reaction studies that assay procedures which contain more than 0.2 mM NAD^+ are not inhibitable by dithiothreitol and that dithiothreitol has no direct effect on epimerase. The mean specific activity of epimerase in the coupled fluorimetric assay with 1 mM supplemental NAD^+ was 580 nmoles/min per mg DNA ($n = 7$), range 240 – 1050).

Comparison of epimerase inhibition by dithiothreitol and by NADH

UDPGlc consumption by epimerase was assayed by the two-step method after varying periods of preincubation in the presence and absence of dithiothreitol. Fig. 2B shows that dithiothreitol inactivation of epimerase is a function of preincubation time. Similar assays without preincubation were performed with increasing concentrations of NADH and NADPH. Fig. 2A demonstrates that NADH exerts a concentration-dependent inhibition on epimerase, while NADPH in equimolar concentrations has no effect.

This combined evidence suggested the hypothesis that preincubation of fibroblast preparations with dithiothreitol-stimulated generation of NADH from endogenous NAD^+ and that inhibition of epimerase by dithiothreitol is mediated by this reaction.

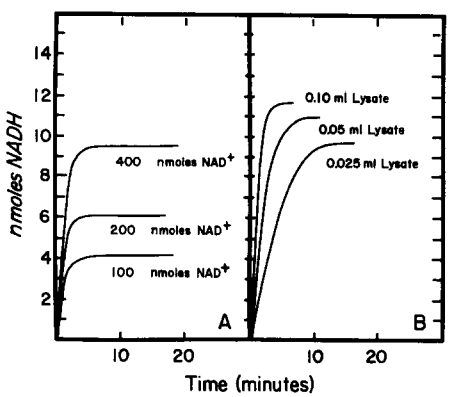
UDPGlc dehydrogenase activity

In order to eliminate the possibility that endogenous UDPGlc dehydrogenase activity was introducing significant error in epimerase assays, 5 lysates were tested in the fluorimetric rate reaction assay for both epimerase and UDPGlc dehydrogenase using equivalent amounts of lysate. It was found that UDPGlc dehydrogenase activity was not detectable at endogenous levels of NAD^+ under the conditions of our assay. UDPGlc dehydrogenase was barely detectable when 0.2 mM NAD^+ was added. In the presence of 1 mM NAD^+, the measurable UDPGlc dehydrogenase activity was 14–34% of the epimerase activity (mean 26.7%).

NAD^+ reduction by fibroblast and erythrocyte preparations

The addition of NAD^+ to fibroblast lysates buffered at pH 8.7 resulted in a rapid

* 10^6 fibroblasts = 13 μg DNA = 300 μg total cell protein.



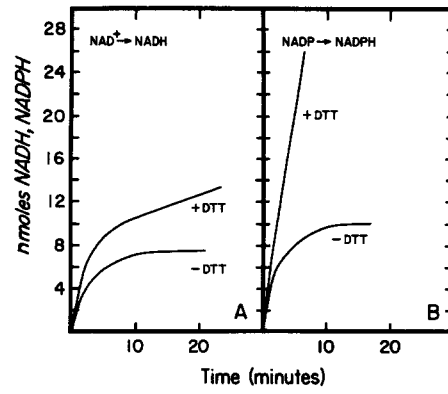


Fig. 3. Fluorimetric determination of NAD^+-reducing activity. A. NADH formation after the addition of 0.1, 0.2 or 0.4 μmole NAD^+ to cuvettes containing 0.1 M glycylglycine buffer (pH 8.7) and fibroblast lysate equivalent to 10^6 cells, in a final reaction volume of 0.5 ml. B. NAD^+ reduction after the addition of 0.4 μmole NAD^+ to cuvettes containing 0.1 M glycylglycine buffer (pH 8.7): cell culture lysate equivalent to $0.5 \cdot 10^6$, $1.0 \cdot 10^6$ or $2.0 \cdot 10^6$ cells (0.025-, 0.05-, and 0.1-ml lysates) in a final reaction volume of 0.5 ml.

Fig. 4. The effect of dithiothreitol on the conversion of NAD^+ to NADH and $NADP^+$ to NADPH by fibroblast lysates. The assay system contained 0.1 M glycylglycine buffer (pH 8.7); fibroblast lysate equivalent to $0.4 \cdot 10^6$ cells; 20 mM dithiothreitol where indicated in a final volume of 0.5 ml. Reactions were started by adding 0.5 μmole NAD^+ or $NADP^+$. A. NAD^+ to NADH reactions and the effect of dithiothreitol stimulation. B. The same reactions for $NADP^+$ to NADPH.

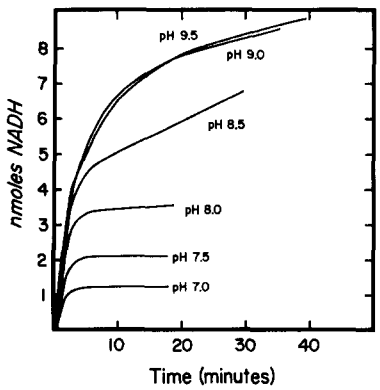
increase in fluorescence that quickly reached a maximal level. This level represented the reduction of 2–5% of the total NAD^+ in the cuvette, approx. 10 nmoles NADH per mg fibroblast DNA. Fig. 3A shows that the maximal level of this response is proportional to the amount of NAD^+ in the cuvette. If a second aliquot of NAD^+ is added to a system which has reached its maximal level, the reaction is repeated suggesting that the initial reaction was not due to exhaustion of endogenous substrates for NAD^+-linked reactions. Fig. 3B shows that the rate of this reaction is proportional to lysate concentration. Similar activity was observed with semipurified erythrocyte preparations.

When $NADP^+$ is substituted for NAD^+ in fibroblast systems, a reaction similar to those shown in Fig. 3A occurs. This reaction is independent of $NADP^+$ concentration and does not show proportionality between rate of reaction and lysate concentration. The maximal level of fluorescence obtained with $NADP^+$ is proportional to lysate concentration suggesting the utilization of endogenous substrates for $NADP^+$-linked reactions. Electrophoresed material did not show any activity with $NADP^+$ (see below) supporting the suggestion that endogenous substrates are needed for this reaction.

Semipurified erythrocyte preparations had no detectable activity with $NADP^+$ alone.

Dithiothreitol-stimulated NAD^+ and $NADP^+$ reduction

The addition of dithiothreitol to both NAD^+- and $NADP^+$-containing systems caused a linear increase in fluorescence. Fig. 4 shows the effect of dithiothreitol on NAD^+ and $NADP^+$ reduction for a fibroblast lysate. Similar reactions were observed



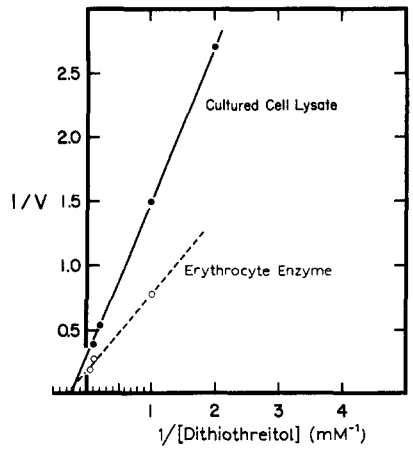


Fig. 5. The effect of pH on $NAD^+ \rightarrow NADH$ reactions in the presence of dithiothreitol. Reaction systems contained 0.2 M Tris–HCl buffer at the pH's indicated, fibroblast lysate equivalent to 10^6 cells; and 10 mM dithiothreitol in a final volume of 0.5 ml. Reactions were started with 0.2 μmole NAD^+. The effect of dithiothreitol on the rate of NADH formation is maximal at pH 8.5.

Fig. 6. Lineweaver–Burk plots comparing the effect of dithiothreitol concentration on the rate of $NADP^+ \rightarrow NADPH$ activity for a lyophilized whole fibroblast and a semipurified erythrocyte preparation. Reaction systems contained 0.1 M glycylglycine buffer (pH 8.7), 1 mg dry wt. of lyophilized whole fibroblast or 1 mg dry wt. of semipurified erythrocyte preparation, 1 mM $NADP^+$ in a final vol. of 0.5 ml. Varying concentrations of dithiothreitol were added to start the reactions. 1/[dithiothreitol] (mM^{-1}) final concentration was plotted *vs.* $1/v$ (expressed as 1/the number of recorder scale divisions per 10 min $\times$ 10). The points obtained at 1/[dithiothreitol] (mM^{-1}) = 10 are not shown in the plot.

for a semipurified erythrocyte preparation. These activities were found in the supernatant fraction after fibroblasts were homogenized and centrifuged at 20 000 $\times g$.

The mean activity measured in 8 fibroblast lysates for dithiothreitol-stimulated NAD^+ reduction was 11.1 nmoles NADH per min per mg. DNA (range: 3.8–26.8), and

TABLE 1

RELATIVE REDUCTION OF NAD^+ AND $NADP^+$ BY ERYTHROCYTE AND FIBROBLAST PREPARATIONS WITH DITHIOTHREITOL AND WITH OTHER THIOLS AND ALCOHOLS

Substrate (10 mM)	*Cofactor (1 mM)*	*Relative activity* (%)*	
		Erythrocyte	*Fibroblast*
Dithiothreitol	$NADP^+$	100	100
Dithiothreitol	NAD^+	130	27
Dithioerythreitol	$NADP^+$	42	81
GSH	$NADP^+$	173	3.9
Mercaptoethanol	$NADP^+$	15.4	6.9
2,3-Dimercaptopropanol	$NADP^+$	4.8	4.9
Cysteine	$NADP^+$	0	2.9
Erythreitol	$NADP^+$	2	4
Xylitol	$NADP^+$	2	0

* Activities related to that with dithiothreitol and $NADP^+$.

the mean activity of 4 lysates for dithiothreitol-stimulated $NADP^+$ reduction was 62.7 nmoles NADPH per min per mg DNA (range: 24–101).

None of these activities were present in heat-inactivated lysates, and dithiothreitol had no direct effect on either NAD^+ or $NADP^+$ reduction in the absence of lysates.

The effect of pH on the reactions converting NAD^+ to NADH in the presence of dithiothreitol is shown in Fig. 5 for a fibroblast lysate. Maximal levels of NADH increased with increasing pH, but the initial rate of reaction did not appear to change significantly. A dithiothreitol-stimulated response was evident at pH 8.5. Dithiothreitol-stimulated $NADP^+$ reduction of fibroblast lysate was maximal at pH 8.0. At pH's 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5, the relative activities were 26, 65, 100, 73, 60 and 53%, respectively.

The effect of dithiothreitol concentration on the rate of $NADP^+$ reduction was tested with a fibroblast lysate and a semiperified erythrocyte preparation. Fig. 6 shows Lineweaver–Burk plots comparing the two cell types. At 1 mM $NADP^+$ the K_m for dithiothreitol was 4.0 mM for both the erythrocyte and fibroblast preparation.

Cu^{2+} and Zn^{2+} completely inhibited NAD^+ and $NADP^+$ reduction in the presence of dithiothreitol, and no reactivation was observed with the addition of EDTA to inactivated systems. Ca^{2+}, Mn^{2+}, Mg^{2+} or EDTA alone had no effect.

Other thiols and alcohols that were tested for their effect on $NADP^+$ reduction are listed in Table I with their relative activities compared to the activity of dithiothreitol. Erythrocyte and fibroblast preparations were compared as well as the relative activities of NAD^+ and $NADP^+$ with dithiothreitol. As seen in Table I, dithioerythritol

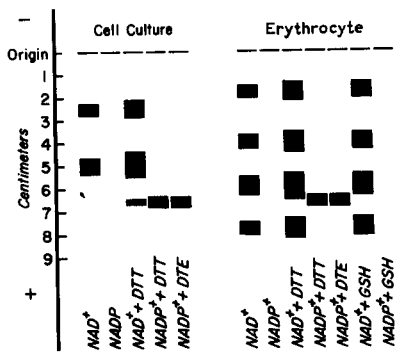


Fig. 7. Vertical polyacrylamide gel electrophoresis comparing lyophilized whole fibroblast and semipurified erythrocyte preparations. 6.6% polyacrylamide gel in 0.1 M Tris–borate (pH 9.0) was used in a continuous buffer system. Semipurified erythrocyte and lyophilized whole fibroblast preparations were reconstituted at 40 mg dry wt./ml water and were centrifuged at 20 000 × *g* for 15 min. 0.05 ml of the supernatants was used in each slot for electrophoresis. Preparations were electrophoresed at 4° for 16 h with 100 V (30 mA) applied across the gel. Gels were then divided into strips corresponding to the width of each slot and the length of the gel. Each strip was individually incubated in a test tube with its specific staining media. Various staining media in 0.2 M glycylglycine buffer (pH 8.7) contained 2 mM NAD^+ or $NADP^+$ alone or in combination with 20 mM dithiothreitol (DTT), dithioerythritol (DTE) or GSH as indicated in the illustration at the bottom of each strip. After the bands had developed (2–4 h) the strips incubated under different conditions were reassembled so that individual bands could be compared in terms of intensity of fluorescence and distance they had migrated toward the anode. The bands were located by their fluorescence with longwave ultraviolet light.

shows significant $NADP^+$ reducing activity with both erythrocyte and fibroblast preparations. Reduced glutathione (GSH) has significant activity only in the erythrocyte preparation.

Polyacrylamide gel electrophoresis

Reconstituted lyophilized whole fibroblast and semipurified erythrocyte preparations were electrophoresed in 6.6% gel. After incubation in 0.2 M glycylglycine buffer (pH 8.7) containing 2.0 mM NAD, 2 fluorescent bands appeared in the lyophilized whole fibroblast channels and 4 bands appeared in the semipurified erythrocyte channels. (This semipurified erythrocyte preparation represents pooled erythrocytes from four different individuals.) When incubated in 2.0 mM $NADP^+$, no fluorescence was observed in either preparation. Dithiothreitol alone gave no fluorescence, but dithiothreitol in the presence of $NADP^+$ gave a single band in both semipurified erythrocyte and lyophilized whole fibroblast preparations (see Fig. 7). In the lyophilized whole fibroblast channels a band appeared in the dithiothreitol + NAD^+ staining mix with the same mobility as the dithiothreitol + $NADP^+$ band. The dithiothreitol-dependent band was less intense with NAD^+ than with $NADP^+$. The 4 semipurified erythrocyte bands that appeared with NAD^+ alone obscured any dithiothreitol-dependent band that might be present. Dithioerythritol when substituted for dithiothreitol gave the same band (see Fig. 7). GSH, which caused significant $NADP^+$ reduction in the fluorescence assay with semipurified erythrocyte preparations, produced no obvious bands in the gel. Fig. 7 illustrates the fluorescent bands observed due to reducing activity in the gel and compares semipurified erythrocyte and lyophilised whole fibroblast preparations.

DISCUSSION

The dependence of uridinediphosphogalactose 4-epimerase on NAD^+ and its inhibition by NADH was demonstrated with lysates of cultured human diploid cells. Inhibition of epimerase activity was also shown to be a function of preincubation time in the presence of dithiothreitol at endogenous levels of NAD^+. Since high levels of dithiothreitol had no effect on epimerase activity in the presence of supplemental NAD^+, it is most probably that dithiothreitol has no direct effect on the epimerase enzyme.

Endogenous levels of NAD^+ in human fibroblast lysates were estimated at 1–10 μM in preparations containing $20 \cdot 10^6$–$50 \cdot 10^6$ cells/ml lysate, and NAD nucleosidase actively was not detectable*.

In order to link together the observations that (1) dithiothreitol preincubation, and (2) NADH concentration both inhibit epimerase activity, it was necessary to first identify NAD^+-reducing systems in fibroblast cultures. Such systems were indeed found to be present in fibroblast lysates. As supportive evidence for the presence of endogenous NAD^+-reducing systems, it was found that intact fibroblasts grown on glass cover slips, then washed and air dried, showed formazan staining after incubation with NAD^+ alone in the presence of nitro blue tetrazolium*, while incubation with

* W. J. MELLMAN AND T. A. TEDESCO, unpublished observations.

$NADP^+$ under similar conditions did not produce staining. Having established the presence of such systems, their response to dithiothreitol was investigated.

Dithiothreitol, since its description by CLELAND[11], has become a common sulfhydryl group protector in enzyme reactions and protein purification procedures and has recently been employed by VACQUIER AND MAZIA[12,13] to promote twinning of sand dollar and sea urchin embryos. Our observations show that dithiothreitol may play a more active role than sulfhydryl group protection in biological systems.

The present study indicates that dithiothreitol inhibition of epimerase activity is in fact mediated by dithiothreitol stimulation of NAD^+ reduction. Human erythrocyte and cultured diploid fibroblasts not only contain endogenous activities that reduce NAD^+ but also a discrete activity, separable by gel electrophoresis, that reduces NAD^+ and $NADP^+$ only in the presence of dithiothreitol. Furthermore, since dithioerythritol serves as a substitute for dithiothreitol in this reaction while erythritol does not, it appears that free SH groups are necessary for this activity. Dithiothreitol was shown to be capable of saturation in this reaction and to obey Michaelis–Menten kinetics. Other thiols and alcohols tested were either inactive or considerably less active.

The activity observed with GSH in erythrocyte preparations is most likely a separate system, since no activity was observed with GSH in fibroblast lysates.

Although the reducing activities described in this paper appear to function as enzymatic systems, they cannot be clearly defined as enzymatic at this time; nor can any significance be placed on their presence as endogenous systems in human erythrocytes and cultured diploid fibroblasts.

The use of dithiothreitol in the UDPGlc consumption assay of α-D-galactose-1-phosphate uridyltransferase (EC 2.7.7.12) demonstrates a useful application of this system, since dithiothreitol not only provides the sulfhydryl requirement for transferase activity, but also inhibits UDPGlc consumption by epimerase[5]. Furthermore, the dithiothreitol-stimulated reduction of NAD^+ and of $NADP^+$ described here should be recognized by investigators as a possible source of error in a variety of enzyme reactions.

ACKNOWLEDGEMENTS

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SHORT COMMUNICATIONS

BBA 63408

Analytical method for the estimation of enzyme kinetic parameters

The existing graphical methods[1] for the determination of the Michaelis constant, K_m, and the maximal rate, V, require experimental estimations of the rate of enzyme reactions, v, at the broad range of substrate concentrations, $[S]$, beginning with the smallest ones up to at least twice the K_m values. While studying the cholinesterase hydrolysis of indophenyl acetates which are hardly soluble in water these methods have proved to be unreliable in this case, and a new method of computation has been suggested by us[2].

From the Michaelis–Menten equation

$$v = \frac{V\,[S]}{K_m + [S]} \tag{1}$$

it derives that

$$\left(\frac{dv}{d[S]}\right)_{[S]=0} = \frac{V}{K_m} \tag{2}$$

According to Eqn. 2, the tangent to the experimental curve in coordinates v against $[S]$ at $[S] = 0$ forms an angle α, of which the cotangent is equal to K_m/V (see Fig. 1). Introducing value K_m/V into the modified Eqn. 1

$$\frac{1}{V} = \frac{1}{v} - \frac{K_m}{V}\cdot\frac{1}{[S]} \tag{3}$$

we compute the value V for all experimental values v at corresponding $[S]$'s. We determine the value K_m out of Eqn. 2, taking the mean value of V.

The aim of the present paper is to improve this method, making it universal and more precise.

It is impossible to draw exactly the tangent to the curve, and the received value $(K_m/V)_{exp}$ will always differ from the true one $(K_m/V)_t$ to the value $\Delta\,(K_m/V)$:

$$(K_m/V)_t = (K_m/V)_{exp} \pm \Delta\,(K_m/V) \tag{4}$$

Due to this fact the values V computed with the help of Eqn. 3 change regulary with the increase of $[S]$ (see Table I, 3rd column). One can estimate the systematic error $\Delta\,(K_m/V)$ with the help of the algebraic method.

For any two values, $[S]_1$ and $[S]_2$, we have

$$\frac{1}{V_1} = \frac{1}{v_1} - \left(\frac{K_m}{V}\right)_{exp}\cdot\frac{1}{[S]_1} \tag{5}$$

$$\frac{1}{V_2} = \frac{1}{v_2} - \left(\frac{K_m}{V}\right)_{exp}\cdot\frac{1}{[S]_2} \tag{6}$$

TABLE I

THE RESULTS OF THE COMPUTATION OF VALUES K_m AND V FOR THE CHOLINESTERASE HYDROLYSIS OF DICHLOROINDOPHENYL ACETATE

0.05 M phosphate buffer (pH 8.0); temp., 25°; $[E]_0 = 2 \cdot 10^{-11}$ M; Ctg$\alpha = K_m/V = 0.023$ from Fig. 1.

$(S) \times 10^4$ (M)	$v \times 10^3$ (enzyme units/sec)	$V \times 10^3$ from $(K_m/V)_{exp}$ (enzyme units/sec)	$\Delta(K_m/V) \times 10^3$	$V \times 10^3$ from $(K_m/V)_t$ (enzyme units/sec)	$K_m \times 10^4$ (M)
0.2	0.77	3.0	3.30	6.80	1.56
0.6	1.90	5.2	3.22	7.00	1.62
1.0	2.70	5.9	3.25	7.15	1.64
1.5	3.30	6.0	3.05	6.85	1.57
2.0	3.85	6.2	3.11	6.90	1.58

Substracting Eqn. 6 from Eqn. 5 we get

$$\Delta\left(\frac{1}{V}\right) = \frac{1}{v_1} - \frac{1}{v_2} - \left(\frac{K_m}{V}\right)_{exp} \cdot \left(\frac{1}{[S]_1} - \frac{1}{[S]_2}\right) \tag{7}$$

The error of the mean values of v_1 and v_2 being negligible, according to Eqn. 3 we find

$$\frac{1}{v_1} - \frac{1}{v_2} = \left(\frac{K_m}{V}\right)_t \cdot \left(\frac{1}{[S]_1} - \frac{1}{[S]_2}\right) \tag{8}$$

Introducing Eqn. 8 into Eqn. 7 we get

$$\Delta\left(\frac{1}{V}\right) = \Delta\left(\frac{K_m}{V}\right) \cdot \left(\frac{1}{[S]_1} - \frac{1}{[S]_2}\right) \tag{9}$$

The mean values $\Delta(K_m/V)$ may be computed from Eqn. 9 using the possible combinations of the experimental values $[S]$ and v. According to the value $\Delta(K_m/V)$ (see Table I, 4th column), we find $(K_m/V)_t$ with the help of Eqn. 4. Knowing the value $(K_m/V)_t$, one can compute the values K_m and V with the help of Eqn. 3 (see Table I, 5th and 6th column).

The computed values K_m and V for cholinesterase hydrolysis of 2,6-dichloro-

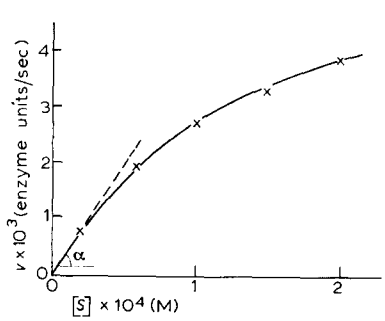

Fig. 1. The dependence of the hydrolysis rate (v) of dichloroindophenyl acetate under the action of serum cholinesterase upon the dichloroindophenyl acetate concentration ($[S]$); 0.05 M phosphate buffer (pH 8.0); temp., 25°.

TABLE II

KINETIC PARAMETERS OF THE CHOLINESTERASE HYDROLYSIS OF 2,6-DICHLOROINDOPHENYL ACETATE AND ACETYLCHOLINE

Experimental conditions see text.

Substrate	*Method*	*Serum cholinesterase*		*Bovine acetylcholinesterase*	
		$K_m \times 10^4$ *(M)*	$V \times 10^6$ *(moles/min)*	$K_m \times 10^4$ *(M)*	$V \times 10^7$ *(moles/min)*
2,6-Dichloroindophenyl acetate	Analytical	1.6	18	1.8	9.6
	Graphical[5]	12	1.2	1.9	8.2
Acetylcholine	Analytical	11	1.0	2.1	8.0

indophenyl acetate are given in Table II. As enzymes the preparations of horse serum cholinesterase (EC 3.1.1.8) (Mechnikov Institute, Moscow) and bovine erythrocyte acetylcholinesterase (EC 3.1.1.7)[3] are used. The hydrolysis of 2,6-dichloroindophenyl acetate was measured with the help of the differential photometrical method[2] in 0.05 M phosphate buffer (pH 8.0) at 20° at the range of $[S]$ $4 \cdot 10^{-5}$–$1.5 \cdot 10^{-4}$ M. Besides this, the hydrolysis of acetylcholine under the action of these enzymes was studied. The rate of the cholinesterase hydrolysis of acetylcholine was measured using the continuous potentiometric titration[4] in 0.007 M phosphate buffer (pH 7.5) at 25°. The values K_m and V were computed graphically[5] in the range of $[S]$ $1 \cdot 10^{-4}$–$2 \cdot 10^{-3}$ M and with the help of the described analytical method in the much narrower range of $[S]$ $1 \cdot 10^{-4}$–$3 \cdot 10^{-4}$ M. These values given in Table II have shown a good coincidence.

This method may be used in all cases when use of high substrate concentrations is impossible, for example, when the substrates are hardly soluble or when the inhibition by high substrate concentrations takes place.

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A new assay for phosphodeoxyribomutase: Surface localisation of the enzyme

Phosphodeoxyribomutase, which catalyses the interconversion of deoxyribose 1-phosphate (dRib-1-*P*) and deoxyribose 5-phosphate (dRib-5-*P*) (together with thymidine phosphorylase, purine phosphorylase and deoxyriboaldolase) participates in the degradation of deoxynucleosides[1,2]. The latter three enzymes are all localised near the cell surface of *Escherichia coli*[3–5]. No continuous assay has been reported for phosphodeoxyribomutase; sampling assays based on chemical differences between dRib-1-*P* and dRib-5-*P* have so far been used[6–8]. In this paper a sensitive continuous assay for phosphodeoxyribomutase is described and used to show that this enzyme also is localised near the cell surface of *E. coli*.

The assay is based on the formation of a hydrazone of dRib-5-*P* with phenylhydrazine, a principle used in assays involving ketoacids[9]. When dRib-5-*P* is mixed with phenylhydrazine a compound is formed with a characteristic absorption spectrum which has a peak at 269 nm (Fig. 1). However 300 nm was chosen for the assay due to the high absorption of phenylhydrazine itself, and the bacterial extract, at lower wavelengths. The absorption at 300 nm is proportional to the quantity of dRib-5-*P* used (Fig. 2). dRib-1-*P* and ribose 1-phosphate were found not to react with phenyl-

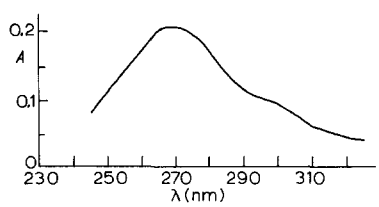

Fig. 1. Absorption spectrum of the hydrazone of dRib-5-*P*. dRib-5-*P* (0.05 μmole) and phenylhydrazine (0.5 μmole) were mixed in a total volume of 1.0 ml of 0.1 M cacodylate buffer (pH 6.8) and the absorption spectrum recorded with an SP800 spectrophotometer. The reference cell was identical but dRib-5-*P* was omitted.

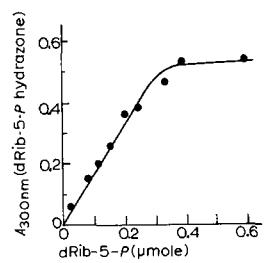

Fig. 2. Reaction of dRib-5-*P* with phenylhydrazine. dRib-5-*P* and phenylhydrazine (0.5 μmole) were mixed in a total of 1.0 ml of 0.1 M cacodylate buffer (pH 6.8) and the absorbance at 300 nm measured after 2–3 min against phenylhydrazine (0.5 μmole). From this curve a molar extinction coefficient of $1.69 \cdot 10^3$ was determined .

hydrazine since the aldehyde group is esterified. Ribose 5-phosphate also did not react under the assay conditions. In strains of *E. coli* containing deoxyriboaldolase dRib-5-*P* is cleaved to give two other aldehydes, acetaldehyde and glyceraldehyde 3-phosphate[10], which also form hydrazones with phenylhydrazine and could possibly be formed under the conditions of mutase assay. However, identical results are obtained both in the presence and absence of deoxyriboaldolase in the reaction mixture (osmotic shock extracts from a phosphodeoxyribomutase negative (*drm*⁻) strain of *Salmonella typhimurium* induced with deoxyribose were used as a source of deoxyriboaldolase[11]). This indicates that even in the presence of aldolase the dRib-5-*P* is trapped to form the

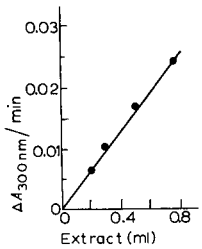

Fig. 3. Proportionality of rate of reaction and enzyme concentration. Cells of *E. coli* CR34 were grown in supplemented glucose minimal medium containing 30 μM thymine, harvested in exponential phase, and washed with 0.01 M Tris buffer (pH 7.2). The cells were resuspended in 0.1 M cacodylate buffer (pH 6.8) and disrupted by sonication. The sonicate was spun at 20 000 $\times$ *g* for 15 min before use and contained 1.5 mg/ml of protein. The reaction was followed for at least 6 min at 37° in an SP800 spectrophotometer.

hydrazone, and acetaldehyde and glyceraldehyde-3-*P* are not formed. This is presumably due to the large excess of phenylhydrazine present.

The assay mixture contains 0.5 μmole of phenylhydrazine, 2.0 μmoles of dRib-1-*P*, 75 μmoles of buffer such as cacodylate (pH 6.8) and 1–2 mg of bacterial protein in a total volume of 1.0 ml. All assays were performed at 37°. The use of phosphate buffer was avoided since phosphate inhibits the enzyme[12]. pH 6.8 was chosen, although the reported pH optimum for the enzyme is about pH 8.5 (ref. 2), since the rate of the nonenzymic formation of the hydrazone of dRib-5-*P* rapidly decreases as the pH is raised. Fig. 3 shows proportionality of initial velocity with enzyme concentration. Table I shows the results of assays performed on a variety of strains of *E. coli* and *S. typhimurium*. It can be seen that in strains known to be *drm*$^-$ no phosphodeoxyribomutase activity can be detected.

The results in Table II show that phosphodeoxyribomutase is released by the osmotic shock procedure of NEU AND HEPPEL[14] indicating that it is localised near the

TABLE I

PHOSPHODEOXYRIBOMUTASE ACTIVITIES IN SONIC EXTRACTS OF *E. coli* AND *S. typhimurium*

Strain 2006 and its derivatives are *S. typhimurium* KSU. Strain C600, CR34 and P152 are *E. coli* strains requiring threonine, leucine and thiamine for growth. All these strains have previously been assayed for phosphodeoxyribomutase[8,11] and were grown in supplemented glucose minimal medium. 30 μM thymine was used for *drm*$^-$ or *dra*$^-$ strains and 300 μM thymine for strain 2006. Cells were grown, harvested and sonicated as described in Fig. 3. Note that *thy*$^-$ (thymidylate synthetase negative) *dra*$^-$ (deoxyriboaldolase negative) strains have higher mutase activities than nonmutant strains. This is due to endogenous induction by dRib-5-*P*[7,8,13]. n.d. = not detectable.

Strain	*Genotype*			*Specific activity (nmoles/min per mg)*
	thy	*drm*	*dra*	
2006	−	+	+	4.0
2006-1	−	+	−	11.0
2006-6	−	−	+	n.d.
C600	+	+	+	1.31
CR34	−	+	−	9.84
P152	−	−	+	n.d.

TABLE II

RELEASE OF PHOSPHODEOXYRIBOMUTASE BY OSMOTIC SHOCK

Exponential cells at 10^{10}/ml were washed, resuspended in 5 ml of cacodylate buffer pH 6.8 (0.1 M) and split into two portions of 2.5 ml. One was sonicated (as described in Fig. 3) to give Fraction 4, and the other was subjected to osmotic shock by the procedure of NEU AND HEPPEL[14] to give Fractions 1–3. Osmotic shock involved treatment of the cells with 20% sucrose–0.03 M Tris (pH 8.0)–1 mM EDTA followed by rapid dispersion in cold water. The supernatants from both these treatments were retained for assay. The cell pellet left after the cold-water treatment was resuspended in 2.5 ml of 0.1 M cacodylate buffer (pH 6.8) and a sonic extract was prepared. Thymidine phosphorylase and β-galactosidase assays were used as controls, the former being a known surface enzyme[3,4], and the latter an intracellular enzyme[15].

Fraction	*Phosphodeoxyribomutase*		*Thymidine phosphorylase*		*β-Galactosidase*	
	Units/ml	*% Total*	*Units/ml*	*% Total*	*Units/ml*	*% Total*
1. Sucrose–Tris–EDTA	1.89	5.1	81.4	5.3	5.6	0.2
2. Cold water	27.6	74.4	1144	73.8	17.4	0.7
3. Cell pellet	7.6	20.4	325	20.9	2400	99.1
4. Sonicate	41	—	1372	—	—	—

cell surface (see also ref. 5). It has been found that whole cells of *E. coli* have a greater capacity to catabolise the deoxyribose moiety of thymidine than sonic extracts, and it was suggested that phosphodeoxyribomutase was sensitive to sonic disruption of the cells[5]. It can be seen from Table II that the yield of enzyme released by osmotic shock is similar to that obtained by sonication. Furthermore, the specific activity of phosphodeoxyribomutase when the cells are mechanically disrupted is almost identical to that obtained in sonic extracts. Thus it appears that phosphodeoxyribomutase itself is not sensitive to the sonication procedure used here.

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Induction of tyrosine aminotransferase in isolated liver cells

Isolated liver cells are an attractive system for studying liver functions *in vitro* provided they exhibit normal cellular functions. Previous studies have shown that tyrosine aminotransferase (L-tyrosine–2-oxoglutarate aminotransferase, EC 2.6.1.5) was not induced by cortisol in isolated rat-liver cells and the lack of enzyme induction was attributed to the fact that such cells had damaged cell membranes. Recently, HOWARD *et al.*[2] have prepared isolated liver cells by incubating rat livers with a mixture of collagenase (EC 3.4.4.19) and hyaluronidase (EC 3.2.1.35 and 3.2.1.36). Such cells appear to have normal intact cellular membranes and have a high endogenous respiration rate[3]. Indeed, BURTON *et al.*[4] have shown that cells prepared by this method incorporate [^{14}C]leucine into the fatty acid synthetase complex. RAPPAPORT AND HOWZE[5,6] have prepared cell suspensions from mouse liver using sodium tetraphenylboron, a potassium chelating agent, as the dispersing agent. Recently, GERSCHENSON AND CASANELLO[7] have prepared isolated rat-liver cells by this method and have shown that such cells respond to insulin and glucagon. These data show that cells prepared by either method exhibit normal liver function. The purpose of this report is to demonstrate that isolated rat-liver cells prepared by either method are able to induce tyrosine aminotransferase in the presence of dexamethasone phosphate, a synthetic glucocorticoid. Previous studies by THOMPSON *et al.*[8] have shown that minimum deviation hepatoma cells may be induced by dexamethasone phosphate to form tyrosine aminotransferase.

Liver cells were prepared from rats obtained from the Holtzman Co. (Madison, Wisc.). Two procedures were used for the preparation of the liver cells. The first procedure was essentially the one described by HOWARD *et al.*[2,3] where the liver from 100–200-g rats was dispersed with a freshly prepared solution of 0.05% collagenase (Schwarz) and 0.10% hyaluronidase (Sigma) in Ca^{2+}-free Hanks salt solution. About 50% of the cells did not take up the vital stains, eosin Y and trypan blue, which is less than that reported by HOWARD *et al.*[2,3]. These cells had a good endogenous O_2 uptake which was linear for at least 2 h.

In the second procedure, sodium tetraphenylboron (Sigma) was used to disperse cells from the livers of 7–14-day-old rats according to the procedure described by GERSCHENSON AND CASANELLO[7] except that a Ca^{2+}- and glucose-free Hanks salt solution was used as the washing and incubation solution. In general, this procedure provided a better yield of isolated liver cells, and these cells had less tendency to reaggregate in solution than the cells prepared by the enzymatic method. However,

the tetraphenylboron method was not very effective in preparing cells from the larger rats (100–200 g) and did not aid in preparing more cells when added to the enzymatic solution. The liver cells prepared by the tetraphenylboron procedure took up the vital stains, whereas about 50% of the cells prepared by the enzymatic method did not take up the vital stain. This would suggest that the cells prepared by the tetraphenylboron procedure had a more permeable membrane.

The isolated liver cells were incubated at 37° with slow shaking in an atmosphere of O_2–CO_2 (95:5, v/v). Dexamethasone phosphate (Dex), a synthetic glucocorticoid was used in the hormonal induction experiments and was a gift from Dr. W. B. Gall of the Merck, Sharp and Dohme Research Laboratories. Liver cells were disrupted in a Sorvall Omnimixer (microattachment, 0°) at 10 000 rev./min, and the broken suspensions were centrifuged at 5000 × *g* and the supernatant solution was used for the enzymatic assays. Tyrosine aminotransferase was assayed according to the method described by LIN AND KNOX[9]. Cycloheximide was from Sigma Chemical Co. and actinomycin D was from Mann Biochemicals.

The data presented in Fig. 1 show that tyrosine aminotransferase is induced by 10 μM dexamethasone phosphate in the isolated liver cells prepared by the enzymatic method and that the extent of induction is less in the presence of actinomycin D and cycloheximide. In this experiment the inhibitors were added 1.5 h after the initial addition of Dex. Other experiments have shown similar patterns when the inhibitors are added at the same time as Dex. In 8 experiments there was a 2.4–7.5-fold induction (av. 4.4) of tyrosine aminotransferase which occurred between 3.5 and 7.0 h (av. 4.7). These results are similar to those obtained with whole animals[9] and hepatoma cells[7]. Both cycloheximide and actinomycin D inhibited the hormonal induction though at the concentration used the inhibition is not complete and indeed the results are somewhat variable. In 5 experiments the inhibition of cycloheximide ranged from 16 to 76% (av. 55) and that by actinomycin ranged from 22 to 55% (av. 32).

Fig. 1. Induction of tyrosine aminotransferase in isolated rat-liver cells prepared by the enzymatic method and the inhibition of induction by cycloheximide and actinomycin D. Total units represent the amount of enzyme present in cells prepared from 2 g of liver. The cells were prepared and incubated as described in the text. Each experimental point represents the cells from 0.1 g of liver suspended in 1 ml of incubation media. ○— — —○, control; ●—●, 10 μM dexamethasone phosphate added at zero time; □—□, 0.2 mM cycloheximide added after 1.5-h incubation with 10 μM Dex; ■—■, 0.5 μg actinomycin D per ml added after 1.5-h incubation with Dex.

Fig. 2. Induction of tyrosine aminotransferase in isolated rat-liver cells prepared by the tetraphenylboron method and the inhibition of induction by cycloheximide and actinomycin D. Total units represent the amount of enzyme present in cells prepared from 2 g of liver and each experimental point represents cells from 0.05 g liver suspended in 1 ml of incubation media (text). The other conditions and legends are identical to Fig. 1 except that the inhibitors were added at the same time as Dex.

The variability observed in the extent of induction and degree of inhibition may reflect differences in the preparation and population of the cells. Further experiments are necessary to clarify the effects of cycloheximide and actinomycin D.

The experiments presented in Fig. 2 are similar to those in Fig. 1 except that the liver cells were isolated by the tetraphenylboron technique. In this case, actinomycin D and cycloheximide were added at zero time, and both inhibitors were effective in preventing induction of tyrosine aminotransferase. Other experiments (9, from 7–18-day-old rats) have shown that the -fold induction varied from 1.3 to 4.6 (av. 3.0) in the presence of Dex. Also, the time to reach maximum induction was usually between 2-5 h (av. 3.0) which is somewhat earlier than the time observed with cells prepared by the enzymatic method. The experiment presented in Fig. 2 (tetraphenylboron) shows a larger induction time than the normal 3 h. The presence or absence of 10% calf serum had no apparent influence on the extent of induction and Medium 199 was as effective as Ca^{2+}- and glucose-free Hanks salt solution as the incubating medium.

These studies demonstrated that isolated liver cells may be induced to form tyrosine aminotransferase in the presence of dexamethasone phosphate, a synthetic glucocorticoid. The response in the liver cells isolated by the enzymatic cells is very similar to that observed with isolated hepatoma cells where it has been shown that the induction is due to enzyme–protein synthesis[10].

It would appear that the cells produced by the enzymatic method are somewhat superior to those obtained by the tetraphenylboron method, since the extent of induction was usually higher though enzyme induction occurs in both. The observation that cells prepared by the tetraphenylboron method take up vital stains would suggest that their membranes were different than the membranes of the cells prepared by the enzymatic method where the major portion of the cells did not take up the stains. Also, the protein inhibitors were more effective in the tetraphenylboron cells which may reflect differences in the cellular membranes.

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Properties of the xanthine oxidase from human liver

The xanthine oxidase (xanthine:O_2 oxidoreductase, EC 1.2.3.2) of mammalian tissues has been considered to be an oxidase due to its capacity to use O_2 as electron acceptor. However, in some tissues, including human liver[1,2], its activity is stimulated by methylene blue; this was interpreted as an indication of the existence of a dehydrogenase, as well as of an oxidase[2].

It was reported that the oxidation of xanthine by freshly prepared rat-liver supernatant occurred mainly through a dehydrogenation reaction requiring an electron acceptor such as NAD^+ or methylene blue. The reaction became an oxidase-type reaction, *i.e.* O_2 was used as acceptor, if the supernatant was kept at −20° for some hours[3] or if it was preincubated either with proteolytic enzymes[4] or in the presence of any subcellular fraction or in anaerobiosis[5]. The presence of xanthine accelerated the effect of proteolytic enzymes or of anaerobiosis. It was postulated that these changes might be consequent to conversion of the enzyme from dehydrogenase (named type D) to oxidase (named type O)[5].

TABLE I

CONVERSION OF HUMAN-LIVER XANTHINE OXIDASE FROM TYPE D TO TYPE O

The enzyme assays were performed at 25° in a mixture containing, in 1 ml: 100 μmoles of Tris–HCl buffer (pH 8.1), 60 nmoles of xanthine, 0.67 μmole of NAD^+ or 0.33 μmole of methylene blue (when present) and 0.05 ml of liver supernatant. The supernatant was preincubated in the presence of trypsin (100 μg/ml) and/or of xanthine (1 mM), before dialysis.

Case No.	*Xanthine oxidase activity (nmoles uric acid formed per min per 100 mg protein)*					
	Assayed immediately			*24 h at −20°*		
	O_2	NAD^+	*Methylene blue*	O_2	NAD^+	*Methylene blue*
1	23	134	132	133	116	228
2	25	129	—	78*	81*	—
3	21	134	211	139	127	—
4	15	127	216	105	117	—
5	24	165	245	180	183	—
6	18	113	198	91	94	—

	5 min at 37° with trypsin		*5 min at 37° with trypsin and xanthine*		*5 min at 37° in anaerobiosis with xanthine*	
	O_2	NAD^+	O_2	NAD^+	O_2	NAD^+
2	—	—	—	—	73	73
3	55	136	79	79	89	73
4	38	48	53	48	66	73
5	55	158	114	114	114	114
6	—	—	—	—	51	49

* 14 h instead of 24.

The present study was undertaken to investigate the characteristics of the xanthine oxidase of human liver.

Samples of human liver weighing about 1 g were obtained from surgical biopsies performed under pentothal/NO_2–O_2 anaesthesia from patients (4 women and 2 men, age 40–72 years) operated on for cholecystitis (No. 1, 2, 3 and 5) or for peptic ulcer (No. 4 and 6). The samples were immediately put in an ice-cold 0.1 M Tris–HCl buffer (pH 8.1) and were homogenized in the same buffer (1 g of liver *plus* 5 ml of buffer) within 30 min of excision. The homogenate was centrifuged at 600–800 $\times g$ for 20 min and then at 100 000 $\times g$ for 1 h, and the resulting supernatant was dialyzed for 3 h against a continuous flow of 300 vol. of 0.1 M Tris buffer (pH 8.1). Enzyme activity was assayed by the method of ROWE AND WYNGAARDEN[6] with or without NAD^+ or methylene blue, as reported previously[3], except that the volume of the reaction mixture was reduced to one third and that the assay was performed in 1-ml cells. Protein was determined using the method of GORNALL *et al.*[7].

The xanthine oxidase activity of human-liver supernatant had the same general characteristics as those of rat liver, although the specific activity of human supernatant was lower. The xanthine oxidase activity of the freshly prepared supernatant was very weak with O_2 as acceptor; the activity was about 7-fold higher with NAD^+ (which was reduced to NADH during the reaction) and about 10-fold higher with methylene blue (Table I). Storage at $-20°$ for 24 h brought about changes similar to those observed in rat-liver supernatant[3], *i.e.* the rate of uric acid formation with O_2 reached the rate observed with NAD^+ as acceptor. The activity in the presence of methylene blue appeared unchanged in the single assay allowed by the available material.

The possibility of changes induced by proteolysis or by anaerobiosis was investigated initially by preincubating the supernatant for 60 min, since this length of time ensured complete conversion of the enzyme activity of rat-liver supernatant[5]. The first results showed that after preincubation the xanthine oxidase activity was very low either with O_2 or with NAD^+, as compared with the activity of the non-preincubated supernatant, thus indicating that the human enzyme was inactivated at 37°. Consequently the length of the preincubation was reduced to 5 min; although some loss of activity was still observed under these conditions, it was ascertained that the human xanthine oxidase was partially converted from type D to type O after trypsinization in the absence of xanthine and that the conversion was complete after trypsinization in the presence of xanthine or after preincubation in anaerobiosis in the presence of xanthine. As it was observed with rat-liver supernatant[3–5], the reduction of NAD^+ was diminished or abolished after the human xanthine oxidase had been converted to type O, regardless of how this was obtained.

The inactivation of xanthine oxidase at 37° was investigated further. When the preincubation was performed in air, 50% of the oxidase activity was lost in 30 min, and the inactivation was even more marked for the dehydrogenase activity (Fig. 1). The preincubation in anaerobiosis in the presence of xanthine brought about a very rapid loss of the dehydrogenase activity during the first few minutes. The inactivation became much slower after the conversion of the enzyme from dehydrogenase to oxidase (Fig. 2), thus indicating that the latter form is more stable than the dehydrogenase.

These results indicate that most of the human-liver xanthine oxidase in its native state is a dehydrogenase, for which NAD^+ is probably the physiological acceptor,

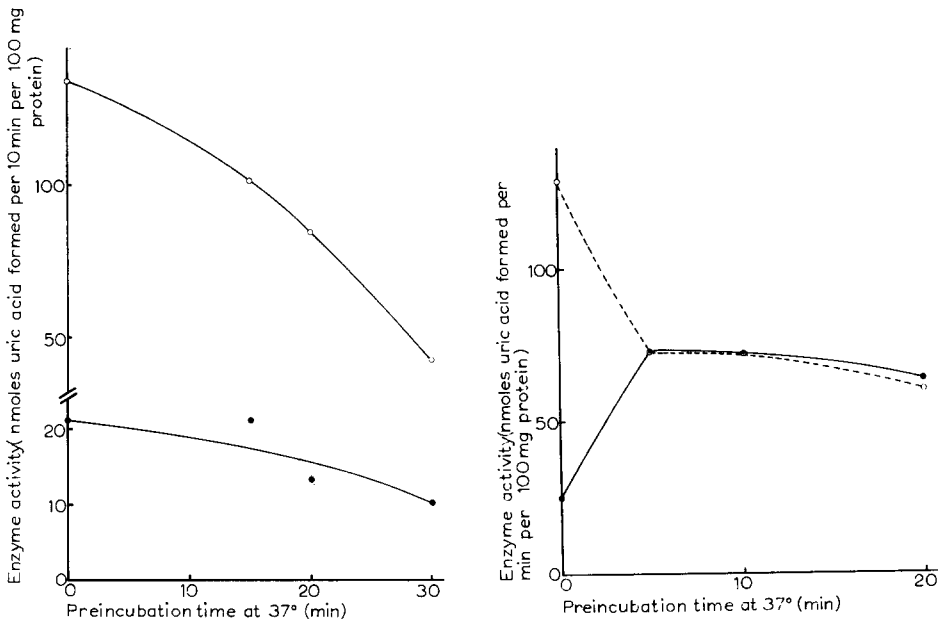

Fig. 1. The effect of preincubation on the xanthine oxidase activity. Liver supernatant was preincubated in air and then assayed with O_2 (●—●) or NAD^+ (○—○).

Fig. 2. The effect of preincubation in anaerobiosis in the presence of xanthine on the xanthine oxidase activity. Liver supernatant was preincubated at 37° under N_2 in the presence of 1 mM xanthine and then was dialyzed and assayed with O_2 (●—●) or with NAD^+ (○— — —○).

and becomes an oxidase after the treatments described above. It has been observed with rat liver that at least the changes caused by preincubation in anaerobiosis are reversible[5]; consequently, it is possible that the interconversion dehydrogenase–oxidase may have a role in the physiological regulation of xanthine oxidase activity.

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Inhibition of glucose-6-phosphate dehydrogenase from Ehrlich ascites carcinoma by ATP

ATP has been shown to inhibit the glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ oxidoreductase, EC 1.1.1.49) from both yeast[1-4] and brain[3]. This inhibition apparently arises from a competition with glucose 6-phosphate[1] and $NADP^+$ (refs. 2, 3) for the active sites and from a separate allosteric inhibition by ATP[2]. The inhibitory effects of ATP are diminished by the presence of Mg^{2+} (refs. 1, 3, 4). The work described below extends the finding of an ATP inhibition to the dehydrogenase from Ehrlich ascites tumor cells and indicates that glucose 6-phosphate as well as ATP may be involved in an allosteric inhibition of the enzyme.

Preparation of extract. A hyperdiploid strain of Ehrlich ascites carcinoma was grown for 7 days in Swiss White or Strong A mice, harvested, and washed in a medium containing 100 mM KCl, 48 mM NaCl, 2.25 mM $MgCl_2$, 5 mM Tricine buffer (pH 7.3) and 2 g/100 ml bovine serum albumin. Packed cell volume was determined on an aliquot of the final suspension[5], and the bulk of the suspension, about 30 ml containing 20–40% (v/v) cells, was exposed to 820 lb/inch^2 N_2 in an Artesan Industries pressure homogenizer (as described in refs. 6 and 7) for 20 min and then suddenly released to atmospheric pressure through a narrow aperture to rupture the cells. Nuclei and cellular debris were removed by centrifugation at 1500 $\times g$ for 15 min, and the supernatant was used directly in the assays for enzyme activity. Other studies have indicated that the glucose-6-phosphate dehydrogenase activity is entirely contained in the 150 000 $\times g$ supernatant (W. V. V. GREENHOUSE, unpublished observations).

Assay of enzyme activity. Reaction mixtures containing 1.0 ml 0.10 M Tricine buffer (pH 7.5), 0.10 ml 2.5 mg/ml (approx. 3 mM) $NADP^+$, and standard solutions of glucose 6-phosphate to give final concentrations of 0.05–10.0 mM and ATP to give final concentrations of 0.0–4.0 mM were made up to 2.60 ml in 3-ml cuvettes with a 1.0-cm light path. The reaction was followed by observing the extent of reduction of the $NADP^+$ at 340 nm with time in a Beckman model DU spectrophotometer equipped with 38° thermospacers, ERA adapter, and Sargeant model SRLG recorder set for linear absorption reading. Cuvettes were pre-warmed to approx. 38°, and the reaction was initiated by addition of 0.05 ml tumor cell extract. The velocity was estimated from the initial slopes of the recorded curves. These velocities were converted to μmoles/ml packed cells per min, using the extinction coefficient of NADPH, the determined packed cell volume, and the appropriate dilution factor. When compared on the basis of packed cell volumes, the velocities were highly consistent from one preparation to another; for example, in 5 different preparations at 0.5 mM glucose 6-phosphate and no ATP, the velocities averaged 3.60 and ranged from 3.24 to 3.95 μmoles/ml cells per min.

Results. Fig. 1 presents the velocity as a function of glucose 6-phosphate concentration up to 1.5 mM in 0.0, 2.0, and 4.0 mM ATP. Superficially, the curves appear to follow the expected Michaelis–Menten relationship, the half maximal velocity occurring at 0.03–0.04 mM glucose 6-phosphate at 0 mM ATP. Since the intracellular ATP concentration ranges from 2 to 4 mM and glucose 6-phosphate from 0.1 to 0.5 mM[8,9] in these cells, the ATP inhibition evident in Fig. 1 could play a physiological role. It

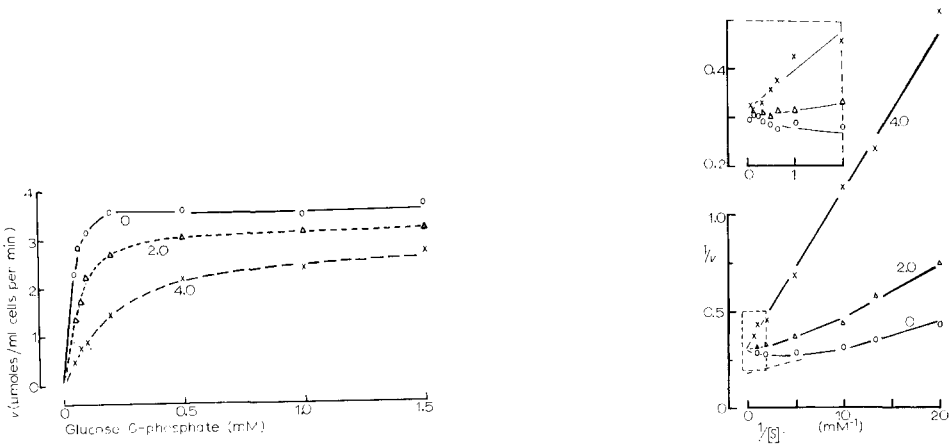

Fig. 1. Velocity of $NADP^+$ reduction as a function of glucose 6-phosphate concentration. The reaction mixture contained 38 mM Tricine buffer (pH 7.5), 0.1 mM $NADP^+$, 0.05 ml tumor extract and the indicated concentrations of glucose 6-phosphate and ATP at 37° (see text). ○—○, rate in absence of ATP; △- - -△, in 2.0 mM ATP; ×— —×, in 4.0 mM ATP.

Fig. 2. Double reciprocal plot of velocity *versus* substrate concentration. [S] = glucose 6-phosphate concentration. ○—○, in absence of ATP; △—△, in 2.0 mM ATP; ×—×, in 4.0 mM ATP. Inset at upper left shows boxed region around low $1/[S]$ values on an expanded scale. Values for 0 mM ATP represent averages of from 3 to 5 preparations; values for 2.0 and 4.0 mM ATP are averages from 2 or 3 preparations.

should be noted, however, that these curves were obtained at a low Mg^{2+} concentration (approx. 0.04 mM final concentration in the assay mixture, contributed by the cell extract) and that the intracellular levels of Mg^{2+} (10 mM or more) may be sufficient to reverse much of the ATP inhibition[1,3,4].

Substantial deviations from the simple Michaelis–Menten relationship are evident when a double reciprocal plot of velocity and glucose 6-phosphate concentration is examined (Fig. 2). Only the enzyme inhibited by 4.0 mM ATP approximates the expected linear relationship; the others show a distinct upward curvature as $1/[S]$ approaches zero. The inset in Fig. 2, giving the low values of $1/[S]$ on an expanded scale, illustrates that all three curves converge toward the same velocity at high glucose 6-phosphate concentrations. The curvature in Fig. 2 would imply a decrease in v at high concentrations in Fig. 1, although this is not apparent because the higher concentrations of glucose 6-phosphate which show a definite decrease are beyond the range covered by this figure. Linear extrapolation of the low $1/[S]$ values at 0.0 mM ATP in Fig. 2 indicates a v_{max} of about 5.4 μmoles/ml cells per min and a K_m of 0.07 mM. Since the ATP inhibition is not a simple competitive type, a K_i value cannot be calculated.

These results differ from those of AVIGAD[1], who found a simple competitive inhibition by ATP for the glucose 6-phosphate site on yeast glucose-6-phosphate dehydrogenase and agree with the finding of BONSIGNORE *et al.*[2] that an allosteric as well as a competitive inhibition may be involved. In the absence of Mg^{2+}, the K_m for yeast glucose 6-phosphate is about 0.04 mM[1,2], whereas in 5 mM $MgSO_4$ it is increased to 0.07 mM[1]. ATP appears to have a stronger effect on the yeast enzyme, since 2.0 mM ATP causes a 70% inhibition at 0.05 mM glucose 6-phosphate in the absence of Mg^{2+}

(ref. 1), whereas it causes only about 40% inhibition with the tumor enzyme under comparable conditions. It should be noted, however, that the tumor "enzyme" was actually a crude extract and probably contained 6-phosphogluconate dehydrogenase (EC 1.1.1.44). Although initial rates of $NADP^+$ reduction were used to minimize the possible contribution of this enzyme, some contribution cannot be completely excluded.

The relationship between velocity and substrate concentration seen in Fig. 2 could be explained on the basis of two assumptions: (a) there are two different types of sites on the enzyme, the active sites and separate inhibitory sites which modify the properties of the active sites, as proposed by BONSIGNORE *et al.*[2]; and (b) both types of sites are capable of binding either glucose 6-phosphate or ATP. At high concentrations of ATP, the inhibitory sites would be permanently saturated with ATP or a combination of ATP and glucose 6-phosphate, and linear double reciprocal plots reflecting a simple competitive inhibition at the active site would be obtained. At low ATP concentrations, the inhibitory sites would be largely unsaturated until higher levels of glucose 6-phosphate were attained, and the double reciprocal plot would consequently show an upward curvature at low values of $1/[S]$ and approach the same intercept on the $1/v$ axis as the enzyme in the presence of a high ATP concentration. Deductions more elaborate than this should be postponed until information from a purified ascites tumor dehydrogenase is available.

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Comparative studies of bacterial hydrogenase

Comparative studies of the physical properties of hydrogenase (H_2:ferredoxin oxidoreductase, EC 1.12.1.1) enzymes have been virtually precluded because of their general instability and sensitivity to oxygen. Recently, we have studied[1] these enzymes by using acrylamide-gel electrophoresis, density-gradient centrifugation and gel filtration. These procedures do not require prior purification of the enzyme under study, and their application to the hydrogenase enzymes has shown that most of these enzymes are extracted as mixtures of several distinct molecular species[2]. The three main forms of hydrogenase from *Clostridium pasteurianum* have been shown to be of the same molecular weight.

We wish to report in this communication an extension of this work to the measurement of the size and shape of hydrogenase enzymes from a variety of representative microorganisms.

The sources and culture conditions of the microorganisms have been reported[2]. The cells were harvested in the log phase of growth. The extracts were obtained either by autolysis of dried cells according to MORTENSON[3] or by sonic treatment of cell pastes. All nonsedimentable material at 144 000 $\times g$ for 30 min was deemed soluble in this investigation. One preparation of *Clostridium butylicum* hydrogenase was purified 400-fold by a modification of the procedure of PECK AND GEST[4]. The method of KONDO *et al.*[5] was used to solubilize membrane-bound hydrogenases.

Hydrogenase was detected in fractions after gel filtration or sucrose density-gradient centrifugation experiments either by a quantitative manometric assay using the redox dye methyl viologen to catalyze H_2 evolution[6], or methylene blue for H_2 uptake. Alternatively, a more sensitive qualitative assay using acrylamide gels has been reported[1,2]. In this latter assay, the location of the maximum hydrogenase activity in column eluates was taken as the central fraction of a consecutive series of fractions, all showing hydrogenase activity on the acrylamide gels. Comparison of the two assays in the same experiment showed the activity peak to be present in the same fraction. The presence of aggregate material possessing hydrogenase activity was deduced from the appearance of hydrogenase activity at the top of the acrylamide-gel column, indicating the presence of material of molecular weight greater than $1 \cdot 10^6$ which could not enter the gel.

Sucrose density-gradient centrifugation was carried out as previously described[1,8]. Bovine-heart cytochrome *c*, rabbit Hb, bovine-liver catalase, rat-liver ribosomes and feritin were used as standards. Gel columns using Sephadex G-75, G-100, G-150, G-200 and Bio-Gel A5 were prepared using the method of ANDREWS[7]. The columns were calibrated for molecular weight and Stokes radius by determining the elution volume of the following standards: bovine thyroglobulin, fibrinogen, bovine-heart lactate dehydrogenase, apoferitin, α-globulin, bovine serum albumin, ovalbumin, chymotrypsinogen A (bovine pancreas), trypsin and cytochrome *c*. These protein standards were obtained from commercial sources and were used without further purification. Blue dextran 2000 was used to determine the void volume of the Sephadex gels. *Escherichia coli* was used for this purpose in the Bio-Gel A5 column. The molecular weights of the hydrogenase enzymes were initially estimated from

density-gradient sedimentation analysis by assuming the proteins to be spherical and of partial specific volume 0.725 (refs. 8 and 9). An improved procedure was used which did not require the first assumption and which enabled the frictional ratio (f/f_0) to be estimated in addition to the molecular weight. In this procedure, the gel filtration columns were calibrated according to the Stokes radii of standard proteins. The value of the Stokes radius for each hydrogenase enzyme was then used in combination with the corresponding sedimentation coefficient to obtain the molecular weight and f/f_0. The equations of SIEGEL AND MONTY[10] and of LAURENT AND KILLANDER[11] were used in these calculations.

Isoelectric point determinations were carried out using acrylamide gels of different pH values. The pH of the gel in the ORNSTEIN AND DAVIS[12] formulation was 9.5; the buffer system of WILLIAMS AND REISFELD[13] was used to obtain a pH of 8.0. Continuous buffer systems were employed to obtain pH's 7.0 and 6.0[14,15]. The isoelectric points of the hydrogenase enzymes were obtained by plotting pH *versus* R_F of the active species in the gel and by extrapolating to zero mobility.

In Table I are shown molecular weights and frictional ratios of a series of hydrogenase enzymes together with the distribution of activity between three identifiable forms of the enzyme sedimenting quite differently: (i) the free or unassociated forms, (ii) the aggregate, (iii) the particulate or membrane bound preparation that does not occur in the previously defined soluble fraction. Table I is divided into three sections; grouping organisms according to common metabolic patterns as suggested by GREY AND GEST[16]. Group I, strict anaerobes, contained the clostridia which have hydrogenases of similar molecular weight (approx. 55 000) except for *C. butylicum* hydro-

TABLE I

SIZE AND SHAPE OF HYDROGENASE ENZYMES FROM VARIOUS SOURCES

The values are for the unassociated or free form of the soluble enzyme. The number of determinations is shown in parentheses and the S.D. is quoted for *C. pasteurianum*; for the other species, the maximum deviation of each determination from the mean values reported never exceeded 10%.

Group	*Bacterial source*	*Mean* $s_{20,w} \times 10^{13}$	*Mean Stokes radius* $\times 10^8$ *(cm)*	*Mol. wt.* $\times 10^{-4}$	*Frictional ratio* (f/f_0)	*% Total activity* as soluble*	*% Soluble activity as aggregate after gel filtration*
I	*C. pasteurianum*	4.0 ± 0.35 (10)	30 ± 3 (14)	5.0	1.2	100	0
	C. felsineum	4.3 (2)	33 (2)	5.9	1.3	100	0
	C. butyricum	3.9 (2)	33 (2)	5.3	1.3	100	0
	C. butylicum	5.8 (3)	44 (3)	10	1.4	100	0
	C. butylicum (400-fold purified)	5.8 (2)	44 (2)	10	1.4	100	0
II	*P. vulgaris*	8.4 (3)	53 (3)	18	1.4	40	80
	E. coli	9.7 (3)	54 (3)	21	1.3	10	70
III	*D. desulfuricans*	4.6 (2)	30 (3)	5.6	1.1	20	5
	*A. vinelandii***	6.5 (2)	48 (2)	13	1.3	20	90

* This refers to the nonsedimentable hydrogenase remaining in solution after cellular disruption and centrifugation at 144 000 × *g* for 30 min.

** A strict aerobe not classified by GREY AND GEST[16].

genase which appears to have a molecular weight about twice that of the other clostridia and may represent a case of gene doubling[17]. These bacteria did not contain a membrane-bound hydrogenase, and the enzyme preparations did not form aggregates.

Group II contained facultative anaerobes. The soluble component of these preparations was resolved into an aggregate and unassociated form. The unassociated form had a much higher molecular weight than that of Group I (approx. 200 000). However, after gel filtration, most of the activity was recovered in the aggregate form which could represent self-association of the enzymes or their association with other cell components present in the extract.

The characteristics of the hydrogenase of *Desulfovibrio desulfuricans* were intermediate between Groups I and II. The soluble preparation contained hydrogenase which existed mainly as an enzyme of mol. wt. 56 000. After gel filtration, 95% of the recovered activity occurred in this form. However, a small amount of the enzyme was just resolved by the Bio-Gel A5 (exclusion mol. wt. $5 \cdot 10^6$), indicating the formation of some aggregate enzyme.

The soluble hydrogenase enzymes of each microorganism studied were composed of several distinct molecular species[2]. These species were not separated in our experiments and often several were recovered in the single hydrogenase activity peak. The finding that the three main hydrogenase species of *C. pasteurianum* were of the same molecular weight[1] may then hold true for many of the hydrogenase forms of other organisms as well. The frictional ratio results indicated that all the hydrogenases examined were approximately spherical proteins.

In the case of *A. vinelandii*, it is possible to solubilize a major portion of the membrane-bound enzyme. The sedimentation coefficients and isoelectric points of the unassociated soluble and solubilized particulate hydrogenase were compared (Table II) and found to be similar. The aggregate form contained most of the enzymatic activity, as found for the facultative anaerobes, and is apparently more stable than the unassociated material. The *A. vinelandii* aggregate can be separated into two particles of 51 and 77 S.

Although the results of these studies have been obtained with crude enzyme preparations, identical molecular weight values were obtained with an unpurified and 400-fold purified preparation of *C. butylicum*.

TABLE II

PHYSICAL PROPERTIES OF *A. vinelandii* HYDROGENASE

The unassociated form of the soluble and solubilized particulate preparations was characterized. The number of determinations is shown in parenthesis and the maximum deviation of each determination from the mean never exceeded 10%. The isoelectric points were obtained graphically and the R_F's by direct assay on acrylamide disc gels[1,2].

	R_F of Species	*$s_{20,w} \times 10^{13}$*	*Isoelectric point*	*$s_{20,w} \times 10^{13}$ aggregate*
A. vinelandii (soluble)	0.42, 0.47	6.5 (2)	5	
A. vinelandii (solubilized)	0.46	6.7 (3)	5	51, 77 (5)

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The subunits of porcine heart TPN-linked isocitrate dehydrogenase

The molecular weight of porcine heart TPN-linked isocitrate dehydrogenase (*threo*-D_s-isocitrate:TPN oxidoreductase (decarboxylating) EC 1.1.1.42) has been measured by MOYLE AND DIXON[1] and was found to be 64 000. SEIBERT *et al.*[2] computed a molecular weight of 61 000 from sedimentation and diffusion studies. Those authors obtained a sedimentation coefficient of 4.6 S and a diffusion coefficient of $7.3 \cdot 10^{-7}$ cm^2/sec^{-1}. To compute the above molecular weight, the authors assumed a partial specific volume of 0.75 ml/g. According to SEIBERT *et al.*[2], their preparation had a specific activity 8 times higher than that of MOYLE AND DIXON. The preparation of SEIBERT *et al.* was purified further by COLEMAN[3], and she obtained a molecular weight of 58 000. The fact that this enzyme exhibits conformational changes on addition of folate[4] and the fact that it displays two coenzyme binding sites as shown from fluo-

rescence and equilibrium dialysis studies[5,6] has led us to investigate its subunit structure.

The enzyme was obtained from Sigma and was purified further using the method given in our previous paper[6]. This method of preparation differs from that of COLEMAN[3] in two small respects. Instead of using CM-cellulose, we used C-50 (CM-cellulose Sephadex); and, instead of using Sephadex G-150, we used Sephadex G-200. Our final preparation had a specific activity comparable to that of COLEMAN. Basing our estimates on ultracentrifugal studies, we believe that our starting material (essentially the preparation of SEIBERT *et al.*[2]), did not contain more than 35% isocitrate dehydrogenase.

Molecular weights were determined by the highspeed sedimentation equilibrium method of YPHANTIS[7]. Interference optics were employed, and the optical components were carefully aligned (R. B. BALDWIN AND E. G. RICHARDS, personal communication). The temperature was maintained at 5° for the 6.5 M urea run and at 10° for the native enzyme runs. The interference patterns were photographed on Kodak spectroscopic glass-plate Type II G. The data were obtained by measurements of fringe heights using both Gaertner and Nikon microcomparators. Data thus obtained were then graphed on desk-size graph paper to insure best accuracy in extrapolation. Weight-average molecular weights, M_w, were calculated for many points throughout the solution column (approx. 3 mm). The M_w values of greatest interest are obtained by extrapolating to zero concentrations of protein (*i.e.* where $\Delta y = 0$). The molecular weights were computed according to the equation

$$M_w = \frac{2RT}{(1 - \bar{\nu}\varrho)\omega^2} \cdot \frac{\mathrm{d}\ln\Delta y}{\mathrm{d}(r^2)} \qquad (1)$$

Sedimentation velocity experiments were run in 1% sodium dodecyl sulfate which was obtained from Mann Laboratories. For those preparations, the enzyme was dialyzed against 0.05 M Tris–HCl (pH 7.5), and an equal volume of 2% sodium dodecyl sulfate was added to the dialyzed solution. Sedimentation coefficients, $s_{20,w}$, were computed from the following equation[8]

$$s_{20,w} = s_{obs} \cdot \frac{\eta_t}{\eta_{20}} \cdot \frac{\eta}{\eta_0} \cdot \frac{(1 - \bar{\nu}\varrho_{\,20,w})}{(1 - \bar{\nu}\varrho)} \qquad (2)$$

where s_{obs} is the observed sedimentation coefficient, η_t/η_{20} is the ratio of the viscosity of water at the given temperature, t, to the viscosity of water at 20° and η/η_0 is the relative viscosity of the solvent to water at the same temperature. The last factor in Eqn. 2 was taken as unity.

Protein concentrations were determined spectrophotometrically on the purified preparation using a value of $E^{1\%}_{280m\mu}$ 9.10, as estimated by COLEMAN[3].

The molecular weight of the native enzyme, as obtained by the meniscus depletion method, was found to be 60 000 in a 0.05 M potassium phosphate buffer (pH 6.86). To stabilize the enzyme, the runs were made in 0.1 M NaCl. The initial protein concentration was 0.3 mg/ml in one run and 0.2 mg/ml in another. Our value of 60 000, obtained by assuming $\bar{\nu} = 0.75$ ml/g, is in close agreement with the result of COLEMAN. The native enzyme at 6.5 M urea in 0.05 M potassium phosphate buffer

(pH 6.86) at 5° exhibited a molecular weight of 32 000. In this run equilibrium was reached after 47 h at a speed of 40 000 rev./min. The initial protein concentration was 0.8 mg/ml, and the partial specific volume was assumed to be 0.70 (ref. 9). The density was taken to be 1.103 g/ml.

With respect to the sedimentation velocity results, the sedimentation coefficient of the native enzyme extrapolated to zero concentration was found to be 4.75 S, as shown in Fig. 1. In the presence of 1% sodium dodecyl sulfate, the sedimentation coefficient of the subunit extrapolated to zero concentration is 2.3 S. At this concentration of sodium dodecyl sulfate, the detergent aggregates and sediments as a single peak having a sedimentation coefficient of 0.95 S (ref. 10 and unpublished observations) and is the slower peak depicted in the Fig. 1.

A value of 2.3 S for the sedimentation coefficient for the subunit of isocitrate dehydrogenase argues for the presence of two hydrodynamically identical subunits, particularly when coupled with the fact that the molecular weight of the subunits in the urea is 32 000. However, it must be noted in the case of the detergent, that we

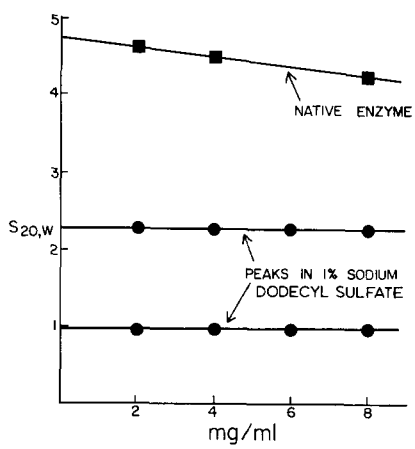

Fig. 1. Sedimentation of isocitrate dehydrogenase in detergent. The slowest peak is the sedimentation of detergent aggregates.

have only a value for the sedimentation coefficient and not for the molecular weight.

It has been our experience that, while sodium dodecyl sulfate will invariably disaggregate proteins into their subunits, the sedimentation coefficient of those subunits is only a rough indication of the molecular weight. Thus, in the case of glyceraldehyde-3-phosphate dehydrogenase, we have found a sedimentation coefficient of 2.1 S for a subunit of molecular weight 35 000 (ref. 11), while in the case of porcine heart supernatant malate dehydrogenase, we have found a sedimentation coefficient of 2.65 S for a subunit having the same molecular weight as the subunit of glyceraldehyde-3-phosphate dehydrogenase (unpublished observations).

The general conclusion of this communication is that, within the limits of experimental error, porcine heart TPN-linked isocitrate dehydrogenase has two hydrodynamically identical subunits. The result is straightforward, provides a reasonable explanation for the presence of two binding sites per 60 000 molecular

weight[5,6] and is in marked contrast to the complicated subunit structure of the DPN-linked enzyme[12].

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Purification and properties of a fungal β-glucosidase acting on α-tomatine

Crude mycelial extracts or culture filtrates from the fungus *Septoria lycopersici* convert α-tomatine, a steroidal glycoalkaloid, to β_2-tomatine[1]. This removal of a β-1,2-linked glycosyl unit markedly reduces fungitoxicity. Since *S. lycopersici* is parasitic on tomato leaves, organs which normally contain between 0.8 and 2.5% α-tomatine on a dry weight basis[2], we were interested in studying the reaction in more detail and determining whether it has any significance *in vivo*. This paper describes (1) a partial purification of the enzyme responsible for hydrolysis of α-tomatine and (2) some of the properties of the enzyme.

Enzyme activity was assayed spectrophotometrically at 460 nm in a reaction mixture containing 10 mM sodium phosphate buffer (pH 5.6), 10 units of glucose oxidase (Worthington GOP), 2 units of horseradish peroxidase (Worthington HPOD), 500 μM *o*-dianisidine, 250 μM α-tomatine and 0.2 unit or less of enzyme in a total volume of 2.0 ml. A unit of enzyme activity is defined as the amount of enzyme that would catalyze the formation of 1 μmole of glucose per min at 40°. Protein was determined by the method of Lowry *et al.*[3].