SEDIMENTATION STUDIES ON FUMARASE

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WEBER[1] had discussed the possibility of the occurrence of such internal structural changes in a protein molecule that a certain measure of rotational freedom inside the molecule is obtained without actual dissociation into subunits. In this connection WEBER mentioned fumarase, for which studies on the rotational relaxation time, (ϱH), of the enzyme under various conditions definitely suggest the possibility (MASSEY[2]). Thus it was shown at pH 7.4, immediately after the addition of $M/10$ ammonium thiocyanate, that a decrease in the relaxation time to about half the original value occurred, without the expected change in sedimentation behaviour. On the other hand, on standing, no further change in relaxation time occurred, whilst slow dissociation of the fumarase molecule into translationally independent units was observed from sedimentation. A full report on these observations, previously unpublished apart from the short description given by WEBER[1], is now given.

EXPERIMENTAL

Sedimentation measurements were made mainly in a Phywe air-driven ultracentrifuge at a speed of 43,000 r.p.m. (*ca.* 140,000 *g*). Under these conditions, rotor temperature, as indicated by a thermistor calibrated by the use of diphenyl ether in the cell (JOHNSON[3]), rose some 4–5° C during the first hour but thereafter at the rate of about 1° per hour. The later stages of ultracentrifuge runs, for which the mean temperature was never greater than 28° C, were therefore employed in evaluating accurate sedimentation constants.

At a later stage of the work, a small number of runs were carried out for comparative purposes in a Spinco ultracentrifuge. The correction of WAUGH AND YPHANTIS[4] for the temperature of the cell was applied.

The fumarase used, for which a partial specific volume of 0.75 was assumed, was twice-recrystallized material prepared according to MASSEY[5]. Ammonium thiocyanate, sodium *trans*-aconitate, sodium fumarate and buffer salts were of analytical grade. 0.05 M phosphate buffer (0.04 M Na_2HPO_4 + 0.01 M KH_2PO_4) at pH 7.4 and 0.02 M phosphate buffer (0.004 M Na_2HPO_4 + 0.0156 M KH_2PO_4) at pH 6.3, to which various reagents were added as required, were used.

RESULTS

In 0.05 M phosphate buffer at pH 7.4, fumarase sediments as a well-defined and symmetrical single peak (Fig. 1). Determination of sedimentation constants over a range of protein concentration (0.15–0.8 g/100 ml) showed the absence of pronounced concentration effects. The data shown in Fig. 2 were obtained on two ultracentrifuges; those for the air-driven instrument were obtained on different fumarase specimens

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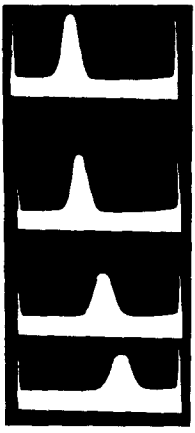

Fig. 1. Sedimentation diagrams of fumarase in 0.05 *M* phosphate buffer at pH 7.4. Fumarase concentration = 0.55 g/100 ml.

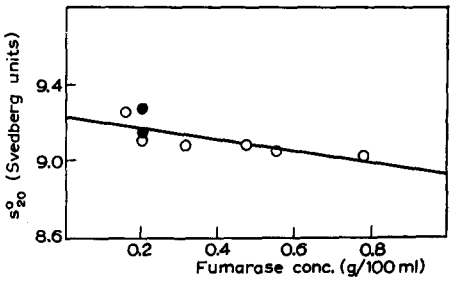

Fig. 2. Plot of sedimentation constant (s°_{20}) against concentration for fumarase in 0.05 *M* phosphate buffer at pH 7.4. ○ – from Phywe air-driven ultracentrifuge; ● – from Spinco ultracentrifuge.

as well as at different times. The least-squares line for all these results is given by

$$s^\circ_{20} = 9.24\ (\pm\ 0.04) - 0.32\ (\pm 0.10)c,$$

standard errors being given in parentheses. Whilst the value of s°_{20} extrapolated to zero protein concentration is relatively accurate, the slope of the *s* versus *c* line is very uncertain (*cf.* HARRINGTON, JOHNSON AND OTTEWILL[6]). One sample of fumarase, however, gave more variable and lower (by about 0.2 *S*) sedimentation constants, but since its sedimentation diagrams also showed anomalous features, it has not been considered in Fig. 2 or in further discussion. In no case, however, were sedimentation constants obtained as low as that ($s^\circ_{20} = 8.51$ *S*) determined by CECIL AND OGSTON[7] from the oil turbine ultracentrifuge.

Sedimentation constants were also obtained in 0.02 *M* phosphate buffer at pH 6.3, and in the presence of 0.02 *M* *trans*-aconitate and 0.017 *M* fumarate; no significant difference from the more accurate values available for 0.2 *M* phosphate buffer at pH 7.4 was noted.

On adding NH_4CNS to a final concentration of 0.1 *M* to a 0.45% solution of fumarase in 0.05 *M* phosphate buffer at pH 7.4, the solution was divided into two portions. One portion was stored at 4°C and examined after 96 hours; the other, contained in the ultracentrifuge cell at 17–20°C, was examined at various times. Summarized results are contained in Table I. At 17–20°C and very short times (*e.g.* 2 hours) from the addition of NH_4CNS, the protein sedimented as a well-defined symmetrical peak with $s^\circ_{20} = 8.6 \pm 0.2$ *S*. After seven hours and very clearly after 25 hours, more slowly sedimenting material occurred. After 49 hours, the slower sedimenting material with $s^\circ_{20} = 5.9 \pm 0.2$ *S*, was in excess and continued to increase in amount at the expense of the more rapidly sedimenting component. Examination of the solution stored at 4°C for 96 hours showed the almost complete transformation into the slower sedimenting component and, as shown in Table I, the sedimentation pattern fits well into the sequence of patterns obtained from solutions stored at room temperature. It was further shown, on thoroughly dialyzing away CNS ions against 0.05 *M* phosphate buffer, that no recovery of the original sedimenting component occurred, though traces of a component sedimenting more rapidly than the dissociation product were observed.

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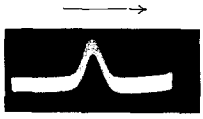
TABLE I

THE DISSOCIATION OF FUMARASE IN THE PRESENCE OF 0.1 *M* NH_4CNS

Protein concentration: 0.45 g/100 ml. 0.04 *M* Na_2HPO_4:0.01 *M* KH_2PO_4 pH 7.4

Sedimentation diagrams	*Time after adding NH_4CNS (hours)*	*Storage temperature 0° C*	*s°_{20} of main component*
	2	17–20	8.64
	7	17–20	8.63
	25	17–20	8.66
	49	17–20	5.88
	73	17–20	5.98
	96	4	5.80

In 0.02 *M* phosphate buffer at pH 6.3, the sedimentation pattern obtained as soon as possible (*ca.* 1 hour) after adding thiocyanate to 0.1 *M* concentration, indicated almost complete dissociation. A main component of $s^\circ_{20} = 5.2$ *S* with traces of aggregated components (see later) occurred, indicating that much more rapid dissociation occurred under these conditions than in 0.05 *M* phosphate buffer at pH 7.4. Whilst the dissociation of fumarase seemed to be the chief process occurring in the presence of *M*/10 CNS, it was noted after 25 hours room temperature incubation in 0.05 *M* phosphate buffer at pH 7.4 containing *M*/10 CNS, that aggregation products also were visible in ultracentrifuge patterns. Fig. 3, containing such patterns at comparable stages of sedimentation, shows the presence of these products in solutions stored at room temperature but their absence after storage at 4°C. Peak areas of the main components, given in arbitrary units alongside, show their progressive fall in concentration at room temperature, which runs parallel with the appearance of the aggregation products. The unchanged area value for the solution stored at 4°C for 96 hours is also apparent. Nor did aggregation products appear in this solution on further storage at room temperature, or in fumarase solutions (with or without added fumarate or *trans*-aconitate) in absence of thiocyanate. On the other hand, the mixture of enzyme plus NH_4CNS in 0.02 *M* phosphate buffer at pH 6.3, in which dissociation was rapid, gave appreciable aggregation products on standing at room temperature. Even more pronounced aggregation than that described above was observed with

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fumarase in 1 M thiocyanate at pH 7.4 and room temperature. It was shown that the conjugation of fumarase with 1-dimethylamino-naphthalene-5-sulphonyl chloride (used in the polarisation experiments) did not affect the sedimentation constant of the enzyme, its aggregation or its dissociation in the presence of $M/10$ thiocyanate.

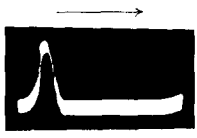
Sedimentation diagrams	*Storage time (hours) at 17–20°C*	*Total peak area of main components (arbitrary units)*
	7	380
	25	310
	49	320
	73	205
	96 at 4°C	370

Fig. 3. Sedimentation patterns, at comparable stages, showing the increase in amount of the aggregation products on storage at room temperature (and at 4°C) of thiocyanate-treated fumarase solution. Buffer: 0.05 M phosphate at pH 7.4 + 0.1 M NH_4SCN. Initial fumarase concentration = 0.43 g/100 ml.

The effect of the enzyme inhibitor *trans*-aconitate and the substrate (fumarate in equilibrium with malate) on the thiocyanate dissociation reaction was also investigated. It was shown that the presence of these compounds does not give any immediate or delayed effect on the sedimentation constant of fumarase at the protein concentrations used. However at pH = 7.4 (0.05 M phosphate), on the addition of fumarate to a final concentration of 0.017 M followed by thiocyanate to 0.1 M, a symmetrical peak of sedimentation constant 8.5 S was immediately obtained. Thus the presence of substrate did not prevent the early lowering of sedimentation constant caused by thiocyanate (*cf.* Table I). On storage at 4°C, dissociation of some kind occurred but, in view of the somewhat complex sedimentation patterns obtained compared with those in absence of fumarate, further investigation of the process is required.

In 0.02 M phosphate at pH 6.3, a considerable measure of protection by 0.023 M fumarate against the action of $M/10$ thiocyanate was observed. Thus a main component of sedimentation constant 8.5 S was obtained immediately after mixing compared with one of 5.2 S obtained under identical conditions in the absence of fumarate. On the other hand, the addition of 0.018 M *trans*-aconitate under the same conditions

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had little or no protective action, the main sedimenting component now possessing a sedimentation constant of 5.6 *S*.

DISCUSSION

The sedimentation data reported here are approximately 1½% higher than reported by FRIEDEN, BOCK AND ALBERTY[8] but some 8% higher than the single sedimentation constant reported by CECIL AND OGSTON[7]. In support of the present data, it should be recalled that they were obtained at different times, on different samples and on two different ultracentrifuges employing differently-based temperature correction factors. Further FRIEDEN *et al.* quote an s°_{20} value for bovine serum albumin at 0.74% concentration of 4.10 *S* but this is some 2% lower than is given by other Spinco workers[9,10] after proper temperature correction has been applied[6]. If the fumarase value of FRIEDEN *et al.* is corrected upwards on this basis, any remaining difference is attributable to instrumental errors and especially to slight inaccuracy in temperature calibration procedures. A rounded value for s°_{20} at zero concentration of 9.20 (± 0.10) *S* would therefore appear reasonable. Whilst this value is not supported by the single sedimentation constant of CECIL AND OGSTON[7], later values obtained by OGSTON[10a] are in fair agreement with the data of Fig. 2.

Assuming a partial specific volume of 0.75 and CECIL AND OGSTON's value for the diffusion coefficient ($4.05 \cdot 10^{-7}$), the molecular weight of fumarase is calculated to be 220,000. No change in the molecular weight occurs in the presence of fumarate or *trans*-aconitate. In spite of the discrepancy in sedimentation constants, the internal consistency of the present results is such that the immediate lowering in sedimentation constant in the presence of thiocyanate is considered outside the range of experimental error.

It is interesting to compare the effect of thiocyanate on the catalytic and physical properties of fumarase. Thiocyanate has been shown to be a powerful non-competitive inhibitor of fumarase, especially at low pH values (MASSEY[11]). This inhibition like the physical changes described above, has been found to occur in two distinct phases. The immediate effect is to cause a non-competitive inhibition, which is freely reversible with dilution, but the reversibility of the inhibition is slowly lost on standing at room temperature in phosphate buffer at pH 7.4. The time taken for complete irreversible inactivation is of the same order as that for the gradual dissociation of the enzyme described above. Hence it is likely that the irreversible loss of activity of the enzyme on incubation with thiocyanate is due to, or at least accompanied by, the splitting of the enzyme into smaller molecular weight units. It seems probable, from the sedimentation constant of the dissociation product, that its molecular weight is about 100,000 so that dissociation into *two similar* subunits apparently occurs.

It seems probable also that the reversible inhibition of the enzyme observed immediately on the addition of CNS is associated with the lowering in sedimentation constant (from *ca.* 9.2 *S* to 8.6 *S*) observed immediately on the addition of thiocyanate. Further, in studies of the effect of thiocyanate on the fluorescence polarization of fumarase conjugated with 1-dimethylaminonaphthalene-5-sulphonyl chloride, it was shown (MASSEY[12]) that a very large decrease in the rotational relaxation time of fumarase occurred immediately on the addition of thiocyanate. Although the smallness of the fumarase samples prevented parallel molecular weight determinations, it is unlikely that the small change in sedimentation constant is due to a rapid dissocia-

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tion into equal subunits. Further, since only one protein component was observed immediately after the addition of CNS, the splitting off of an appreciable fragment from the fumarase molecule cannot be occurring. Hence it is suggested that the immediate effect of thiocyanate is to cause a loosening of protein structure which would permit internal rotation of large fragments of the molecule, without actual dissociation occurring. This would account for the decreased rotational relaxation time reported previously[12]. It would also be consistent with the small decrease in sedimentation constant described in this paper, as such a change at constant molecular weight could well result in a slightly increased frictional constant. The occurrence of a similar structural change in the bovine serum albumin molecule at acid pH's has recently been described[6].

A detailed study of the fumarase system, involving a comparison of physical and catalytic properties at different pH values and the effect of substrate, might be expected to yield much useful information about the catalytic properties of this enzyme. As a preliminary to such an investigation, the effect of CNS was also studied in 0.02 *M* phosphate buffer at pH 6.3, where the dissociation reaction was very much more rapid than at pH 7.4 in 0.05 *M* phosphate. No appreciable quantity of material with a sedimentation constant of *ca.* 8.6 was observed; only the fully dissociated material with a sedimentation constant of 5.3.*S*. The presence of substrate in the buffer at pH 6.3 slowed down the reaction very appreciably; under these conditions, the main component observed immediately was that with the intermediate sedimentation constant of 8.6 *S*.

On the other hand the presence of the powerful competitive inhibitor, *trans*-aconitate (MASSEY[13]), did not slow down the dissociation. This is surprising since the affinity of the enzyme for *trans*-aconitate under these conditions is very much greater than that for the substrates[13]. Hence it is unlikely that the protective effect of the substrates observed at pH 6.3 can be due to combination at the active centre. This conclusion is supported by the observation that no appreciable protection from dissociation could be observed at pH 7.4, since under these conditions the affinity of enzyme for substrate is not very different from that at pH 6.3.

As has already been demonstrated, the irreversible inactivation of fumarase runs parallel with the dissociation into subunits. However, since aggregation apparently accompanies the dissociation process at room temperature, pH 7.4, the possibility exists that it is also a mechanism of irreversible inactivation. It is interesting to note that incubation at room temperature and pH 7.4 of the fumarase dissociated by CNS at 4°, gives no aggregation products. Thus aggregation under these conditions is apparently a temperature-dependent side reaction of the dissociation process, rather than a consecutive one. However, it should be noted that at pH 6.3 a somewhat different phenomenon occurs. Thus apparently fully dissociated fumarase, on further incubation at room temperature leads to the production of considerable amounts of aggregated material. However, in no experiment involving incubation at 0°C were aggregation products observed, so that the formation of aggregated as distinct from dissociated material is very much temperature-dependent.

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SUMMARY

The effect of thiocyanate on the sedimentation of fumarase has been studied over a range of experimental conditions. It was found that the action of this anion occurred in two distinct phases, an immediate lowering of the sedimentation constant from 9.28 to 8.6 *S*, followed by a slower dissociation into two similar subunits. Under certain conditions aggregation also accompanied dissociation. These physical changes are discussed in the light of fluorescence polarization studies already reported and of changes in the catalytic properties of the enzyme under similar conditions.

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INVESTIGATIONS ON ORGANIC FUNGICIDES

XI. THE ROLE OF METALS AND CHELATING AGENTS IN THE FUNGITOXIC ACTION OF SODIUM DIMETHYLDITHIOCARBAMATE (NaDDC)

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In previous publications[1,2] we reported that apart from histidine various other imidazole derivatives could antagonize on agar media the fungitoxic action of sodium dimethyldithiocarbamate (NaDDC) in the first zone of inhibition. It was suggested that the imidazoles could in some way or other prevent the fungicide from interacting with some essential enzyme system. Since imidazoles are known to form complexes with metals, and metals might well play a role in the action of the strongly metal-binding dithiocarbamates, we investigated whether there is any correlation between antagonistic activity and metal-binding ability of various imidazole derivatives. A close parallel was found for Cu; moreover, other chelating compounds (ethylenediaminetetraacetic acid and mercaptobenzothiazol) were found to have antagonistic activity[3]. Thus there was a strong indication that metals have a function in the fungitoxic action of NaDDC. The role of metals was now further investigated, especially with regard to the work of GOKSØYR.

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