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STUDIES ON A SUCCINATE–NEOTETRAZOLIUM REDUCTASE SYSTEM OF RAT LIVER

II. POINTS OF COUPLING WITH THE RESPIRATORY CHAIN

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SUMMARY

The reduction of neotetrazolium chloride does not increase linearly with the amount of rat-liver suspension used when succinate is the substrate. This non-linear response of the succinate–neotetrazolium reductase system is partly dependent on the oxygen level in the incubating medium but is not affected by cytochrome oxidase (EC 1.9.3.1) inhibitors. It is suggested that, in this system, neotetrazolium chloride is mainly reduced via an interaction with a factor which is sensitive to O_2 and which couples neotetrazolium chloride with the respiratory chain.

The results obtained allow the sites of interaction of neotetrazolium chloride with the respiratory chain to be determined. Under aerobic conditions 90% of the neotetrazolium is reduced near cytochrome *c* via the factor described above and 10% is reduced near ubiquinone. Under anaerobic conditions the relative proportions of these two sites change so that neotetrazolium is reduced approx. 50% at each site.

The relevance of these findings to histochemical procedures for succinate–neotetrazolium reductase is discussed as is the fact that neotetrazolium reacts with the $NADH_2$-respiratory chains at three sites close to the three regions of oxidative phosphorylation.

INTRODUCTION

The pathways by which tetrazolium salts are reduced by tissue suspensions in the presence of succinate as substrate are still obscure despite an increasing number of publications on the subject. Satisfactory interpretation of the data in the literature on this subject is difficult because of widely differing conditions used by each group of investigators; major variations in approach have been the tissue under investigation, the type of tetrazolium salt used as the final acceptor and the composition of the assay medium. Examination of published results on tetrazolium reduction indicates that such variations in experimental procedure have considerable effect not only on the magnitude but even on the site of tetrazolium reduction. There is considerable evidence for rat liver, at least, that the succinate–tetrazolium reductase

Abbreviation: NT, neotetrazolium chloride.

systems* are different for each of the several classes of tetrazolium salt; this has recently been discussed by NACHLAS, MARGULIES AND SELIGMAN[1].

In the first part of this series[2] it was shown that the reduction of NT by rat-liver tissue in the presence or succinate did not increase linearly with the amount of tissue added under the conditions used. This "non-linear response" was thought to be probably due to a cofactor linking the respiratory chain with NT reduction, and which was diluted out during the preparation of the tissue suspension. This cofactor apparently served as an endogenous electron-carrier in the succinate–neotetrazolium reductase sequence; the fact that dilution of the cofactor occurred to a varying degree from animal to animal made the system unsuited for use as a routine assay for succinate–neotetrazolium reductase. However, it was later shown[3] that a suitable succinate–neotetrazolium reductase assay system could be constructed by adding an exogenous electron carrier (such as vitamin K_3 or phenazine methosulphate) together with vitamin C; under such conditions the reduction of NT coupled to succinate oxidation proceded linearly both with the amount of rat-liver tissue and with incubation time and, in this respect, was similar to the results found with unsupplemented Keilin–Hartree preparations[2], beef-heart mitochondria[4], or mouse-liver suspensions[5]. It must be emphasised that the succinate–tetrazolium reductase system measured in rat liver in the presence of added acceptor (vitamin K_3) is different from that operating in the unsupplemented rat-liver suspension; the points on the respiratory chain at which NT is reduced in the absence or presence of added vitamin K_3 are different as will be discussed in this communication.

It is of considerable theoretical importance to establish whether the non-linear response is, in fact, due to the presence of a diffusible endogenous electron-carrier linking succinate oxidation to neotetrazolium reduction and, if so, to ascertain its nature. This communication carries on the work reported in the first part of this series[2] towards this aim. Bearing in mind the properties of the endogenous carrier, many well established cofactors have been tried to see how they affect the non-linear response. The results of these studies are intended to be interpreted as effects on the non-linear response only; they are not suitable for comparison of the absolute amounts of formazan produced from experiment to experiment since, in many instances, different preparations (*i.e.*, mitochondria, frozen and thawed homogenate, etc.) were used and the extent of non-linearity varies from animal to animal. The opportunity has also been taken in this communication to study the effects on NT reduction of several substances used routinely in the histochemical demonstration of tetrazolium reduction, and for which the biochemical basis is slight or even contra-indicated.

In dealing with the non-linear response, this communication considers only one tetrazolium salt, NT, and only one type of tissue suspension, rat-liver homogenate. It is important to remember this when interpreting the results since many previous investigations on tetrazolium reduction have used other tissue preparations

* In conformity with the International Union of Biochemistry recommendation, the enzyme systems coupling succinate oxidation with tetrazolium reduction are described as the succinate–tetrazolium reductase systems. The generic term succinate–tetrazolium reductase will be used when remarks are general in character and can be applied to tetrazolium salts as a whole; when the remarks are intended to apply to a particular tetrazolium salt, the enzyme system will be described more specifically, for instance as succinate–neotetrazolium reductase.

or tetrazolium salts, under which conditions the pathways of reduction are probably different. The results presented enable the points on the respiratory chain at which neotetrazolium is reduced to be deduced with some degree of precision. This localisation of the points of reduction of neotetrazolium (and of four other tetrazolium salts described in the following communication[6]) with the respiratory chain enables the histochemical demonstrations of "succinate dehydrogenase" activity to be seen in a more realistic light and critical assessment of a complex and, to a certain extent, confused, literature becomes possible. One other major finding of this investigation which affects work aimed at comparing the absolute amounts of formazan produced in different tissue samples is the effect of O_2 on the system. It will be shown that the presence of O_2 alters not only the magnitude of neotetrazolium reduction but also changes the relative importance of the sites of reduction.

METHODS

Adult, female, albino rats were used in this investigation; they were fed Diet 41B (ref. 7) and water *ad libitum*; they were killed by cervical dislocation and liver suspensions were quickly prepared either in ice-cold water or 0.25 M sucrose (usually 1 g liver plus 9 ml water or 0.25 M sucrose). Unless otherwise stated, succinate–neotetrazolium reductase was assayed by incubation in tubes at 37° under aerobic conditions with 5–20 mg (wet wt.) tissue and 1 ml stock solution per tube. The stock solution was a phosphate buffer–sodium succinate–EDTA mixture as described by SLATER[2]. To correct for the varying volume added as tissue suspension or as additives, water or sucrose was added such that the volume of the incubation mixture was constant for all tubes in each experiment. The reaction was started by adding 0.15 ml of 1% (w/v) NT, and was stopped by the addition of 2 ml 10% (w/v) trichloroacetic acid. Where a different incubation volume was required, the composition of the stock solution was adjusted so that the concentrations of succinate, phosphate buffer, EDTA and neotetrazolium in the final incubation mixture were the same as above. Formazan was extracted by shaking with 4 ml purified ethyl acetate and the results are given as μg formazan present in the 4 ml of solvent. A calibration curve relating formazan concentration to absorption at 510 mμ was constructed by chemically reducing re-crystallised NT with $Na_2S_2O_4$.

Vitamins K_1, K_3 and E together with ubiquinone (UQ_{10}) and duroquinone (DQ) were freshly prepared on each occasion and were added as a suspension in phosphate buffer, ethanol and bovine-plasma albumin as described by DEUL, SLATER AND VELDSTRA[8] (ubiquinone and duroquinone were very kindly supplied by Dr. E. R. REDFEARN). Aqueous solutions of *p*-benzoquinone and hydroquinone were freshly prepared before use; 1,4-napthoquinone was dissolved in 25% (v/v) aqueous ethanol. Antimycin A (obtained from the Wisconsin Alumni Foundation) was prepared as an ethanol solution; the amount of ethanol added per tube never exceeded 0.02 ml, this level of ethanol having no significant effect on NT reduction at the lowest concentration of tissue studied. Even so, in most experiments with antimycin A, and particularly where preincubation of enzyme and inhibitor was required, the solution of antimycin A was added to the tubes first of all and then ethanol was removed by gently warming. Dicumerol was freshly prepared on each occasion as follows: 80 mg dicumerol were dissolved in the minimum amount of 0.1 N NaOH

and the pH was then adjusted to approx. 9.0 with dilute HCl and the solution diluted to volume with water. Stelazine and Librium were gifts from Smith, Kline and French, and Roche Products, respectively; both tranquillisers, as well as Chlorpromazine, were freshly prepared in aqueous solution before use*. Where appropriate, additives were adjusted to pH 7.4 before mixing with the incubation medium; this was not done with dicumerol, for example, as a result of its low solubility at this pH.

NADP, cytochrome *c*, ATP and coenzyme A were the purest grades commercially supplied by Sigma Chemical Co.; desamino-coenzyme A was prepared from coenzyme A as described by MARTIN *et al.*[9]. Sodium amytal was obtained from Eli Lilley and Co. In all instances where an additive was added in other than a neutral aqueous solution the controls reported contained an equivalent amount of solvent to that containing the additive (with dicumerol for example the controls contained an aqueous solution of alkaline NaCl (pH 9.0) and, where appropriate, for antimycin A, the controls contained ethanol). Experiments performed under anaerobic conditions were done in Thunberg tubes; the incubation mixture and tissue suspension were placed in the tube and the tetrazolium salt in the side-arm. During the evacuation period the lower part of the tube was cooled in iced-water. After a 1-min incubation at 37° the reaction was started by tipping.

RESULTS

Evidence will be presented below showing that the succinate–neotetrazolium reductase system is an enzymic system operating via succinate dehydrogenase (EC 1.3.99.1). For instance, the system was strongly inhibited by malonate (50% inhibition was obtained with a final malonate concentration of 1.1 mM, and 90% inhibition with 7.8 mM). The system was also strongly inhibited by heating such that immersion of the tissue suspension in a boiling water bath for 30 sec inhibited the subsequent reduction of neotetrazolium by 90%.

Each result in Tables I–V is a typical result taken from a set of comparisons done at incubation times varying between 5–20 min. In no instance did varying the incubation time have any influence on the comparisons reported for the effects of additives on the shape of the non-linear response. It is important to remember that the effects being studied were primarily on the nature of the non-linear response and the absolute value of NT reduction was of secondary importance.

SLATER[2] has reported on the stimulation of succinate–neotetrazolium reductase by vitamin K_3; various other substances have been found to act in a similar manner, *i.e.*, phenazine methosulphate[10] and methylene blue[11]. Table I shows the effects of various quinones on succinate–neotetrazolium reductase and it can be seen that UQ_{10} like vitamin K_3 stimulated succinate–neotetrazolium reduction. The response with UQ_{10}, however, was linear with increasing amounts of tissue unlike the case with vitamin K_3. Another tetra-substituted benzoquinone, duroquinone, slightly inhibited the succinate–neotetrazolium reductase system. The effects of 1,4-naphthoquinone, benzoquinone and hydroquinone are also shown in Table I. Benzo-

* Stelazine, Librium and Chlorpromazine are trade-names respectively for 10-[3-(4-methylpiperazine-1-yl)propyl]-2-trifluoromethylphenothiazine hydrochloride; 7-chloro-2-methylamino-5-phenyl-3-hydro-1,4-benzodiazepine-4-oxide hydrochloride; 2-chloro-10-(3-dimethylaminopropyl)phenothiazine hydrochloride.

TABLE I

EFFECTS OF VARIOUS QUINONES AND DICUMEROL ON THE SUCCINATE-NEOTETRAZOLIUM REDUCTASE SYSTEM OF RAT-LIVER HOMOGENATES UNDER AEROBIC ASSAY CONDITIONS

The effects of several of these substances on the anaerobic system are given in Table IV. The following remarks refer not only to Table I but to all of the tables in this paper. The amount of each substance present in the incubation mixture is shown as its final concn. (mM). Pre-incubation of tissue, buffer mixture and additive was carried out at 0°; incubation, after the addition of NT, was at 37°. The amount of tissue present in the incubation mixture was either 5, 10, 15 or 20 mg (wt wt.) tissue which, unless stated to the contrary, was added as a 1:10 tissue suspension in water. The amount of formazan produced with each level of tissue is shown as μg formazan calculated from a calibration curve described in the Methods section. For details regarding preparation of the additives see the Methods section. The control tubes contained in each case the same amount of solvent as was included with the additive.

Addition	*Amount added (mM)*	*Incubation vol. (ml)*	*Pre-incubation time (min)*	*Incubation time (min)*	*Formazan produced at 4 tissue concentrations*			
					5 mg	*10 mg*	*15 mg*	*20 mg*
Quinol	0.7	1.45		20	12	37	83	150
Benzoquinone	0.7	1.45		20	3	4	6	6
Control		1.45		20	5	35	99	149
Naphthoquinone	0.32	1.55		20	6	12	46	200
Control		1.55		20	24	40	58	110
Naphthoquinone	0.64	1.55		20	9	8	10	59
Control		1.55		20	21	35	55	95
Vitamin K_3	0.54	1.40		7	13	44	157	—
Ubiquinone	0.54	1.40		7	13	21	28	—
Control		1.40		7	6	13	21	—
Dicumerol	1.29	1.55	30	15	3	14	55	70
Dicumerol	2.58	1.55	30	15	4	11	9	18
Control		1.55	30	15	7	27	55	87
Phenazine methosulphate	0.25	1.45		6	7	44	264	—
Control		1.45		6	6	8	23	—

quinone (final concentration 0.7 mM) was very inhibitory; hydroquinone (0.7 mM) had no consistent effect; 1,4-naphthoquinone (0.3 mM) was inhibitory with small amounts of tissue but stimulated neotetrazolium reduction with higher concentrations of tissue. The effect could be changed to an overall stimulation of the neotetrazolium reduction by adding vitamin C to the incubation mixture (Fig. 1). Vitamins K_1 and E (final concentration 0.2–0.9 mM) had no effect on the succinate–neotetrazolium reductase system either with or without preincubation with tissue prior to the addition of NT.

Since vitamin K_3 produced marked stimulation of succinate–neotetrazolium reductase it was thought worth while to check the effect of dicumerol on the unsupplemented homogenate. The effect of dicumerol on neotetrazolium reduction is shown in Table I. No inhibition of succinate–neotetrazolium reductase could be found with concentration of dicumerol known to inhibit various diaphorases[12,13] with either fresh or frozen and thawed tissue suspensions. In fact, inhibition of neo-

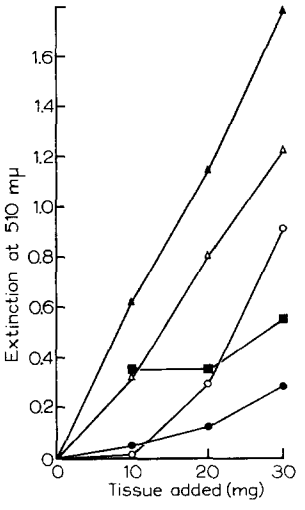

Fig. 1. Effect of 1,4-naphthoquinone (with and without vitamin C) on rat-liver succinate–neotetrazolium reductase. Assay conditions as in the Methods section; incubation 5 min at 37°. The naphthoquinone was dissolved in 25% aqueous ethanol, final concentration in incubation mixture 0.32 mM; vitamin C was an aqueous neutral solution, final concentration, 1.30 mM. ●, no additions to homogenate; ○, plus naphthoquinone; ▲, plus naphthoquinone and vitamin C; ■, boiled homogenate with naphthoquinone and vitamin C; △, fresh homogenate with naphthoquinone and vitamin C corrected for the non-enzymic activity shown in ■.

tetrazolium reduction was only obtained at a very high dicumerol concentration (0.6 mM), a concentration high enough to greatly depress succinate oxidase activity measured manometrically. This effect was probably related to the fact that dicumerol was present as a suspension at that concentration.

Table II gives the effects of various uncouplers of oxidative phosphorylation, and tranquillisers on the succinate–neotetrazolium reductase system. Since both neotetrazolium reduction and oxidative phosphorylation appear to involve diversions away from the main respiratory chain into side reaction (see Discussion) and since the major site of neotetrazolium reduction occurs near one of the sites of oxidative phosphorylation (*i.e.*, between cytochrome *c* and oxygen, see ref. 14) it was thought possible that neotetrazolium might be reduced by reaction with a component of the oxidative phosphorylation sequence even though the system studied was a non-phosphorylating one. Further support for this idea is provided by our findings that NT is a potent uncoupler of oxidative phosphorylation in rat-liver mitochondria[15]. Table II shows that dinitrophenol inhibits the reaction particularly with high tissue concentrations where neotetrazolium reduction per mg tissue is more efficient. $MnCl_2$ had little influence on the reduction; sodium salicylate and urethane (final concentration, 9 mM) were similarly without effect.

Recently, the phenothiazines and other tranquillisers have been shown to uncouple oxidative phosphorylation and also, under certain conditions, to affect respiration[16]. The results shown in Table II indicate that Chlorpromazine (final concentration 0.7 mM) inhibits succinate–neotetrazolium reductase by approx. 40%. Stelazine and Librium, two potent tranquillisers, inhibit neotetrazolium reduction

TABLE II

EFFECTS OF UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION ON SUCCINATE–NEOTETRAZOLIUM REDUCTASE IN RAT-LIVER HOMOGENATES

Other details as in Table I and the Methods section. For the experiment with $MnCl_2$, EDTA was omitted from the incubation mixture.

Addition	*Amount added (mM)*	*Incubation volume (ml)*	*Pre-incubation time (min)*	*Incubation time (min)*	*Formazan produced at 4 tissue concentrations*			
					5 mg	*10 mg*	*15 mg*	*20 mg*
Dinitrophenol	0.12	1.65		20	10	21	37	57
Dinitrophenol	0.36	1.65		20	11	20	32	49
Control		1.65		20	10	23	43	78
$MnCl_2$	0.12	1.65		25	6	14	38	110
$MnCl_2$	0.36	1.65		25	7	13	35	100
Control		1.65		25	11	24	45	100
Urethane	9.0	1.45		15	9	19	34	43
Salicylate	0.7	1.45		15	7	18	29	40
Control		1.45		15	8	20	34	47
Chlorpromazine	0.7	1.45	10	15	8	15	28	51
Chlorpromazine	0.07	1.45	10	15	18	18	41	54
Librium	0.7	1.45	10	15	—	17	33	48
Librium	0.07	1.45	10	15	11	25	34	64
Control		1.45	10	15	14	39	45	59
Stelazine	0.78	1.55	25	15	7	16	34	48
Stelazine	0.15	1.55	25	15	8	23	37	54
Control		1.55	25	15	12	22	44	69

to the same extent as Chlorpromazine. The inhibition by Chlorpromazine was virtually independent of the neotetrazolium concentration over a large range (final concentration 0.0125–0.20%, w/v). The inhibition produced by the tranquilliser(s) does not, therefore, appear to be the result of a competition for electrons between it and neotetrazolium. As can be seen from Table II, none of the tranquillisers studied had any effect on the basic non-linearity of the system despite in some cases an overall inhibition of the reaction.

Table III gives the results obtained by adding cytochrome *c* ,various steroids, *p*-chloromercuribenzoate, amytal and other respiratory inhibitors on the succinate–neotetrazolium reductase system. The importance of sulfhydryl-groups for the succinate–neotetrazolium reductase system is shown by the almost complete inhibition produced by *p*-chloromercuribenzoate. Another respiratory chain inhibitor which can be conveniently discussed in this section is sodium amytal which blocks the oxidation of $NADH_2$ at the level of the flavoprotein. Recent reports have indicated that electron transfer can take place from succinate via a part of the respiratory chain and back to NAD[17]. Amytal blocks this process. It can be seen from the results of Table III that sodium amytal has very little effect on succinate–neotetrazolium reductase indicating that the final stages of the above mentioned electron pathway are not involved in the succinate–neotetrazolium reductase system. It was essential to check this point since $NADH_2$–neotetrazolium reductase is an extremely active enzyme system in rat liver[18].

TABLE III

EFFECTS OF MISCELLANEOUS SUBSTANCES ON THE SUCCINATE–NEOTETRAZOLIUM REDUCTASE SYSTEM UNDER AEROBIC CONDITIONS

Other details as for Table I and the Methods section. For the experiments with $CoCl_2$, EDTA was omitted from the incubation mixture and for the experiments with stilbestrol there was a pre-incubation period at 0° of 90 min.

Addition	*Amount added (mM)*	*Incubation volume (ml)*	*Incubation time (min)*	*Formazan production at 4 tissue concentrations*			
				5 mg	*10 mg*	*15 mg*	*20 mg*
Cytochrome *c*	0.0005%	1.65	15	10	—	34	52
Control		1.65	15	9	18	30	42
PCMB*	0.37	1.8	20	2	1	2	—
Control		1.8	20	3	17	34	—
Amytal	0.37	1.55	20	11	24	44	—
Amytal	1.50	1.55	20	10	22	38	64
Control		1.55	20	11	26	37	76
Co^{2+}	1.19	1.45	10	—	2	3	5
Control		1.45	10	—	10	21	29
Stilbestrol	1.29	1.45	30	2	5	10	18
Control		1.45	30	10	26	48	77

* PCMB = *p*-chloromercuribenzoate.

It is well-known that a certain amount of mitochondrial cytochrome *c* can be readily removed by washing and it can be seen that added cytochrome *c* stimulates the succinate–neotetrazolium reductase system particularly in the regions of higher tissue concentration. The response with added cytochrome *c*, however, remains non-linear and resembles the effect found with added vitamin K_3 or boiled homogenate[2]. In these latter cases it was found possible to produce a linear response by the concurrent addition of a reducing agent such as vitamin C or cysteine but this was not found to occur with cytochrome *c*. In this connection it is worth mentioning that the increased reduction of neotetrazolium due to added cytochrome *c* is sensitive to antimycin A (Table V) so that it would appear that cytochrome *c* is forming a different electron pathway from that studied in the presence of vitamin K_3 which is antimycin A insensitive (Table V). Despite reports that coenzyme A[19] or desamino-coenzyme A[9,20] stimulated tetrazolium reduction in pigeon-breast muscle and kidney homogenates respectively, neither substance (final concentration 0.42 mM) had any effect on the succinate–neotetrazolium reductase system studied here. Similar negative effects on the system were obtained on adding ATP (final concentration 0.6 mM), NADP (final concentration 0.17 mM) and vitamin B_1 (final concentration 0.76 mM).

Table III also shows the inhibitory effect of Co^{2+} on the succinate–neotetrazolium reductase system; in this respect Co^{2+} closely resembles the effect of Cu^{2+} ions previously reported[2]. The use of Co^{2+} in histochemical demonstrations of dehydrogenase activity with a thiazolyl-tetrazolium salt has been widely accepted following

TABLE IV

EFFECTS OF ANAEROBIC CONDITIONS AND OF INHIBITORS OF CYTOCHROME OXIDASE ON THE SUCCINATE–NEOTETRAZOLIUM REDUCTASE SYSTEM

The effects of various other inhibitors, discussed in other tables, on the anaerobic reaction are also shown. Other details as for Table I and the Methods section. In the experiment contrasting aerobic and anaerobic conditions with an incubation vol. of 3.95 ml, the liver had been perfused with cold 0.25 M sucrose prior to killing the animal and preparing a tissue suspension.

Addition	*Aerobic (A) or anaerobic (An)*	*Amount added (mM)*	*Incubation volume (ml)*	*Pre-incubation time (min)*	*Incubation time (min)*	*Formazan production at different levels of tissue*						
						5 mg	*10 mg*	*15 mg*	*20 mg*	*25 mg*	*30 mg*	*40 mg*
None	A		1.65		15	9	18	30	40	—	—	—
None	An		1.65		15	26	60	90	127	—	—	—
None	A		3.95		10	1	2	5	10	15	—	—
None	An		3.95		10	3	15	27	48	74	—	—
Vitamin K_3	An	0.54	1.4		7	85	202	274	—	—	—	—
Ubiquinone	An	0.54	1.4		7	23	47	67	—	—	—	—
Control	An		1.4		7	14	30	47	—	—	—	—
Azide	An	12.5	1.65		15	14	64	78	118	—	—	—
Control	An		1.65		15	26	60	91	128	—	—	—
Dicumerol	An	0.053	1.4		10	26	62	83	—	—	—	—
Control	An		1.4		10	22	53	78	—	—	—	—
Cytochrome c	An	0.0005%	1.65		15	29	59	100	145	—	—	—
Control	An		1.65		15	26	60	91	128	—	—	—
NaN_3	A	11.4	1.75		10	—	19	—	33	—	50	68
Control	A		1.75		10	—	17	—	26	—	41	69
Hydroxylamine	A	6.5	1.55		15	9	22	39	61	—	—	—
Control	A		1.55		15	7	18	39	56	—	—	—
NaCN	A	2.2	1.35	20	20	—	34	—	—	—	—	—
	A	1.5	1.35	20	20	—	34	—	—	—	—	—
	A	0.70	1.35	20	20	—	34	—	—	—	—	—
	A	0.30	1.35	20	20	—	31	—	—	—	—	—
	A	0.15	1.35	20	20	—	33	—	—	—	—	—
	A	0.07	1.35	20	20	—	37	—	—	—	—	—
Control	A		1.35	20	20	—	38	—	—	—	—	—

TABLE V

EFFECTS OF ANTIMYCIN A ON THE SUCCINATE-NEOTETRAZOLIUM REDUCTASE SYSTEM UNDER AEROBIC AND ANAEROBIC CONDITIONS AND ALSO IN THE PRESENCE OF ADDED VITAMIN K_3 AND CYTOCHROME *c*

Other details as for Table I and the Methods section.

Addition	Aerobic (A) or anaerobic (An)	Amount added (mM)	Incubation volume (ml)	Incubation time (min)	Formazan produced at different tissue concentrations				
					5 mg	10 mg	15 mg	20 mg	40 mg
Antimycin A	A	0.001	1.37	12	1	2	4	8	—
Control	A		1.37	12	8	20	44	40	—
Antimycin A	An	0.0035	1.35	10	13	21	33	—	—
Control	An		1.35	10	19	42	66	—	—
Antimycin A	A	0.0035	1.40	7	0	1	1	1	—
Vitamin K_3	A	0.55	1.40	7	10	53	170	277	—
Antimycin A + vitamin K_3	A	0.0035 + 0.55	1.40	7	7	42	148	277	—
Control	A		1.40	7	2	11	18	25	—
Cytochrome *c*	A	0.0048%	1.65	10	7	13	—	37	107
Antimycin A	A	0.003	1.65	10	2	2	—	3	8
Antimycin A + cytochrome *c*	A	0.0048% + 0.003	1.65	10	2	2		8	11
Control	A		1.65	10	6	11		33	55

the claims made in its favour by PEARSE[21]. It would seem from the present results, however, that the activity measured in the presence of Co^{2+} is largely the residual activity of a strongly inhibited enzyme complex. Several recent reports have indicated that steroids can act as electron carriers in a variety of systems[22]. It was thought possible that the natural cofactor responsible for the non-linear response might be a steroid and, as a consequence, several steroids were tested to see if they relieved the non-linear response. Estradiol-17 (final concentration 3–30 μM), dehydro-*iso*-androsterone (final concentration 0.15–30 μM), pregnanolone (final concentration 0.15–15 μM) and progesterone (final concentration 0.15–15 μM) had no effect either on the absolute magnitude of NT reduction or on the non-linear response. On the other hand, the synthetic estrogen, stilbestrol, was markedly inhibitory but only at a high concentration (mM) presumably unrelated to its estrogenic activity *in vivo* (Table II).

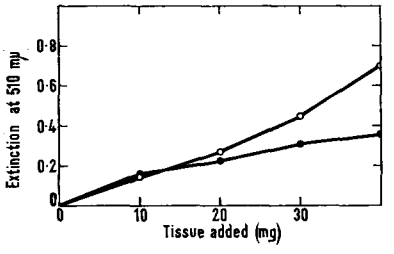

Fig. 2. Effect of shaking the reaction mixture during the incubation period on the rat-liver succinate–neotetrazolium reductase system. The incubation time was 10 min at 37°; the incubation volume was 1.45 ml. ○—○, normal unshaken procedure; ●—●, incubation carried out in conical flasks with shaking in air at 120 shakes/min. The effect was studied at four levels of tissue; the reaction was stopped with 2 ml trichloroacetic acid and the formazan was extracted into 4 ml ethyl acetate. Other details as in the Methods section.

Table IV shows that the succinate–neotetrazolium reductase system is considerably stimulated by anaerobic conditions; anaerobicity does not always, however, relieve the non-linear response. Anaerobic conditions also stimulated the succinate–neotetrazolium reductase system in the presence of added vitamin K_3 (Table IV). The opposite effect to anaerobic conditions is shown in Fig. 2 where it can be seen that shaking the incubation mixture so that the solution is kept saturated with oxygen[23] causes a decrease in the production of formazan compared with the normal procedure of aerobic incubation in unshaken tubes.

The effects of several inhibitors of cytochrome oxidase on the succinate–neotetrazolium reductase system are also included in Table IV. It can be seen that both cyanide and azide have little effect on the activity under aerobic conditions. This effect of cyanide and azide has been found at various concentrations of the inhibitors, at four levels of tissue suspension (both homogenate and mitochondrial) and at various times of incubation up to 20 min at 37°. Only when the tissue suspension was pre-incubated with cyanide in the absence of NT or when the incubation time was longer than 20 min was any marked inhibition of the succinate–neotetrazolium reductase system found. In the average aerobic incubation, as illustrated by most of the experiments reported in this communication, inhibition of cytochrome oxidase had no inhibitory effect on the succinate–neotetrazolium reductase system. Thus,

since inhibition of cytochrome oxidase by cyanide or azide has virtually no effect on the aerobic system, the effects of the many inhibitors and coenzymes tried (for their effects on the non-linear response) are unlikely to be due to action on cytochrome oxidase with consequent alteration in the oxygen level in the medium. Experiments in which azide was pre-incubated with tissue at 0° prior to adding substrate and NT showed that maximal inhibition was obtained after 40 min pre-incubation. After such a pre-incubation period, 20 mM azide inhibited the succinate–neotetrazolium reductase system by 40%. Such an inhibition is of a different order

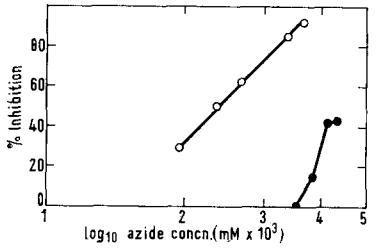

Fig. 3. The effect of NaN_3 on the rat-liver cytochrome *c* oxidase activity (○—○) and rat-liver succinate–neotetrazolium activity (●—●). The percentage inhibition produced at various levels of azide is plotted against the $\log_{10}$ (concentration of azide, mM) · 10^3. The cytochrome *c* oxidase reaction was carried out by incubating 4 mg (wet wt.) liver tissue; 1.0 ml 0.1 M Tris buffer (pH 7.2); 0.2 ml of 0.04% cytochrome *c*; varying amounts of 0.1 M NaN_3, and water to give an incubation volume of 1.5 ml for 1 min at 37°. The reaction was started with 0.7 ml of 0.11% *N,N'-p*-dimethyl-*p*-phenylenediamine oxalate. After 5 min the reaction was stopped by the addition of 2 ml cold ethanol. After mixing, the absorbancy at 540 mμ was measured. Values were corrected for endogenous colour formation by subtracting the values obtained by incubating all components except tissue which was added after the ethanol stage. The succinate–neotetrazolium reductase reaction was carried out using 10 mg (wet wt.) liver tissue per tube; the incubation volume was 1.35 ml; the tissue and buffer mixture was pre-incubated for 20 min at 0° with varying amounts of 0.1 M NaN_3 after which the mixture was transferred to a bath at 37° for 1 min. The reaction was started by the addition of 0.15 ml NT and was for 15 min. Other details as in the Methods section.

of magnitude from the inhibition of cytochrome oxidase by azide. Fig. 3 shows that cytochrome oxidase is inhibited 40% by 0.1 mM azide without any need for a pre-incubation period.

Table V gives the results for the effects of antimycin A on the rat liver succinate–neotetrazolium reductase system. It can be seen that antimycin A is strongly inhibitory when the reaction is carried out under aerobic conditions; antimycin A at a final concentration in the medium of 0.5 μg/ml gave a mean inhibition of 90 ± 1% at tissue concentrations of 5–20 mg (wet wt.) per 1.35 ml. The reduction of neotetrazolium in the presence of added vitamin K_3 was not sensitive to antimycin A indicating that vitamin K_3 is reacting with the respiratory chain prior to the antimycin A sensitive region. On the other hand, the stimulatory activity of cytochrome *c* on succinate–neotetrazolium reductase system is sensitive to antimycin A (Table V). The effect of antimycin A on the succinate–neotetrazolium reductase system is decreased if the reaction is carried out anaerobically (Table V); under such conditions, neotetrazolium reduction is only reduced approx. 50% by the addition of antimycin A. In agreement with LESTER AND SMITH[4], it was found that chemically reduced

cytochrome *c* did not directly reduce NT under neutral conditions at 37°. Using either vitamin C or *N,N'*-dimethyl-*p*-phenylene diamine as substrate, and with 1.25 μg antimycin A present, a slow enzymic reduction of NT occurred under anaerobic conditions. Under the normal aerobic conditions no such enzymic reduction occurred.

DISCUSSION

The effects of various classes of substance on the succinate–neotetrazolium reductase system will be discussed first prior to an analysis of the non-linear response and the points of reduction of NT on the respiratory chain.

Quinones

The results obtained with the limited number of quinones tried (Table I) indicate that some specificity is required to increase formazan production. The redox potential of the quinone does not appear to be of primary importance since quinones which do not increase neotetrazolium reduction differ greatly in their standard electrode potentials (*i.e.*, hydroquinone + 316 mV, vitamin K_1 −53 mV, TRENNER AND BACHER[24]), and quinones which stimulate neotetrazolium reduction also have widely differing standard electrode potentials (vitamin K_3 −5 mV, ubiquinone + 122 mV, 1,4-naphthoquinone + 71 mV; TRENNER AND BACHER[24] and JOEL, KARNOVSKY, BALL AND COOPER[25]). Although vitamin K_3 greatly stimulates neotetrazolium reduction[2] the response is still non-linear under aerobic conditions but becomes linear when the reaction is carried out anaerobically. SLATER[2] suggested that this non-linearity could be the result of the oxidation of sulfhydryl groups during the incubation with vitamin K_3. Sulfhydryl-groups are known to be involved in succinate dehydrogenase and cytochrome oxidase activity[26]; *p*-chloromercuribenzoate destroys the succinate–neotetrazolium reaction (Table III). In fact, concurrent addition of cysteine or vitamin C with vitamin K_3 produces a linear aerobic reduction of neotetrazolium with increasing amounts of tissue[3]. Since vitamin K_3 can react directly within the neighbourhood of succinate dehydrogenase[27] it is probable that the neotetrazolium reaction in the presence of added vitamin K_3 takes place close to if not on the dehydrogenase itself; the vitamin K_3-activated reaction is insensitive to antimycin A (Table V) as is the reaction coupling succinate, vitamin K_3 and added cytochrome *c*[28]. It is interesting to note that the aerobic stimulation of the succinate–neotetrazolium reductase system by vitamin K_3 or UQ_{10} is further raised by anaerobicity (Table IV). This is probably the result of both hydroquinones being rapidly autoxidisable. Even under anaerobic conditions vitamin K_3 is more stimulatory than UQ_{10} on a molar basis.

Under certain experimental conditions, the succinate–neotetrazolium reaction is inhibited by dicumerol (Table I). Dicumerol is known to be a potent quinone antagonist and also has recently been reported to be a competitive inhibitor against NAD and NADP in the so-called DT-diaphorase system[29]. Dicumerol also strongly inhibits the $NADH_2$–neotetrazolium reductase system of rat liver[18]. O_2 uptake with succinate or β-hydroxybutyrate as substrate is little affected by dicumerol in concentrations (10 μM) which uncouple oxidative phosphorylation[30] although it might be expected that dicumerol would strongly inhibit the chain by interaction with

UQ_{10}; perhaps the answer lies in the relative inaccessibility of UQ_{10} in the lipid regions of the chain. The importance of lipid solubility in the inhibitory action of antimycin A and naphthoquinone derivatives has been stressed by TAPPEL[31]. The concentration of dicumerol required to inhibit succinate–neotetrazolium reductase is much greater than that required to uncouple phosphorylation and is, in fact, high enough to considerably depress O_2 uptake by the succinate oxidase system measured manometrically. The inhibition of neotetrazolium reduction produced by dicumerol would thus appear to be at least in part the result of an interference with the respiratory chain *per se* rather than with any auxiliary system associated with phosphorylation.

Tranquillisers

The effects of the three tranquillisers reported in Table II are of interest in view of the results of DAWKINS, JUDAH AND REES[16]. These authors showed that Chlorpromazine at a final concentration of 0.2 mM inhibited cytochrome *c* oxidase by 89% by a competition with cytochrome *c*. Table II, in fact, shows that the inhibition of the succinate–neotetrazolium reductase system produced by Chlorpromazine is relieved by increasing the amount of cytochrome *c* present so that it seems likely that Chlorpromazine affects cytochrome *c* oxidase and succinate–neotetrazolium reductase in a similar manner. If this assumption is correct then it follows that the major site of coupling of neotetrazolium with the respiratory chain is between cytochrome *c* and oxygen. Table II also shows that two other tranquillisers, Stelazine and Librium, also inhibit the succinate–neotetrazolium reductase system in a similar degree to Chlorpromazine even though Librium has a completely different chemical structure.

Antimycin A

Antimycin A has been shown to be a potent inhibitor of the respiratory chain[32]; its site of action has been placed at the level of the reoxidation of ubiquinone[33,34]. Table V shows that the rat-liver succinate–neotetrazolium reductase system is inhibited by over 90% by antimycin A, final concentration 0.5 g/ml. The mean of four separate experiments using four levels of tissue showed that 0.5 μg antimycin A per ml inhibited succinate–neotetrazolium reductase by 90 $\pm$ 1%. In the presence of 0.7 mM vitamin K_3 the antimycin A effect was practically abolished (Table V) indicating that the electron pathway operating in the presence of added vitamin K_3 is coupled to the respiratory chain prior to the antimycin A-sensitive factor. KAMIN, GIBBS AND MERRITT[35] have previously shown that neotetrazolium reduction is sensitive to antimycin A in kidney suspensions; LING, SU AND TUNG[36] showed that antimycin A inhibits triphenyltetrazolium reduction in rat-kidney and rat-heart muscle preparations; and both NACHLAS, MARGULIES AND SELIGMAN[1] and LESTER AND SMITH[4] have reported the antimycin A sensitivity of neotetrazolium reduction in rat-liver and beef-heart suspensions respectively. An interesting result shown in Table IV is that under anaerobic conditions antimycin A only inhibits the succinate–neotetrazolium reductase system by approx. 50%. Thus, the increase in the reaction which occurs under anaerobic conditions is largely due to an increase in reduction

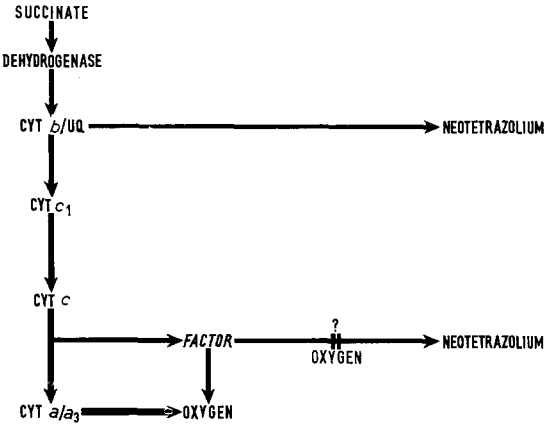

Fig. 4. Diagram of the rat-liver respiratory chain used for the purposes of the Discussion. The two suggested sites of interaction with neotetrazolium are as indicated at the level of ubiquinone (UQ) and cytochrome *c*. The possible interactions of the latter site with O_2 are shown and are discussed in the text.

occurring prior to the antimycin A-sensitive factor. With the evidence at present to hand, on the structure of this part of the respiratory chain (Fig. 4), it would appear that NT is probably coupling with ubiquinone since reduced quinones are not only rapidly autoxidisable but also react spontaneously with NT[37,38]. It is known that none of the tetrazolium salts so far tested reacts with soluble succinate dehydrogenase itself[1,4].

The non-linear response

One of the most striking yet puzzling results presented here is the inhibitory effect of O_2 on the succinate–neotetrazolium reductase system (see Table IV). The results shown in Fig. 2 and in Table IV suggest that this oxygen effect is largely responsible for the non-linear response observed under normal aerobic unshaken conditions. Examination of the conditions under which O_2 exerts its inhibitory action shows, however, that what at first sight appears to be a relatively simple effect is, in fact, a complex phenomenon.

The inhibition of NT reduction by O_2 does not appear to be the result of a direct competition between these two acceptors since increasing the concentration of NT had no effect on the non-linear response. Furthermore the oxygen effect appears to have little in common with the partial pressure of O_2 in the incubation over a wide range of O_2 concentration as shown by the contrasting effects found with anaerobic conditions and after adding cyanide or azide (Table III). Using the O_2 uptake figures given by UMBREIT, BURRIS AND STAUFFER[23] for rat-liver succinate oxidase, it can be expected that an "aerobic" unshaken incubation mixture, as used in the succinate–neotetrazolium reductase assay, would become virtually anaerobic (except near the surface of the liquid) within a few minutes in the presence of 10 mg (wet wt.) liver tissue. Yet the addition of cyanide (or azide) in concentration sufficient to inhibit cytochrome oxidase and, as a consequence, largely preventing the develop-

ment of anaerobic conditions with the higher quantities of tissue, had little effect on the succinate–neotetrazolium reductase system. If the partial pressure of O_2 in the incubation mixture were the major (or sole) factor involved in the mechanism of the non-linear response then the addition of cyanide or azide, by preserving aerobic conditions, would have led to a marked inhibition in NT reduction similar to the results found on shaking the incubation mixture in air (Fig. 2).

The previous discussion has shown that the major part of NT reduction under normal conditions occurs via an interaction with the respiratory chain later than the antimycin A-sensitive region (for discussion of this region see ref. 39). Further, neither cytochrome *c*, nor c_1, can be coupled directly to NT as already outlined and the results obtained in the presence of Chlorpromazine indicate that NT is mainly reduced at further along the chain (towards O_2) than cytochrome *c*. Cytochrome oxidase itself is ruled out as a direct participant in NT reduction since neither hydroxylamine, nor cyanide, partially let alone completely inhibited the reaction (Table IV). Thus, on the basis of such exclusions, it would appear that NT is either reduced by an interaction with an unknown, previously unrecognised, component of the respiratory chain in the cytochrome *c* region, or it is reduced by a factor (or grouping) which is not itself a component of the respiratory chain but forms a separate branch pathway. All of the available evidence suggests that the latter mechanism is the operative one. Fig. 4 shows a diagrammatic representation of the respiratory chain on which further discussion is based; the hypothetical factor (or grouping) mentioned above is shown in the cytochrome *c* region.

The assumption of two plausible properties for this factor (or grouping) allow an explanation to be made of these anomalous results and also of the non-linear response. Firstly, if the factor were sensitive to traces of O_2 then anaerobic conditions would favour NT reduction and excess O_2 would tend to reduce NT reduction compared with the normal assay conditions. Sensitivity of the factor to O_2 could take two forms as shown in Fig. 4. In the first instance (a) O_2 could inhibit the reaction between the factor and NT perhaps by oxidation of some essential group; (b) the factor could be readily autoxidisable so that in the presence of O_2 electrons would pass preferentially to oxygen rather than to NT. If this were true then the route through the factor to oxygen must be a minor route of O_2 uptake since, in rat liver, cytochrome oxidase accounts for the major part of the respiratory activity. However, even such a minor route would be sufficient to satisfy the normal rate of reduction of NT which is reduced at approx. 0.01 the rate of O_2 uptake by the succinate oxidase system. Further, the sudden availability of an excess number of electrons following inhibition of cytochrome oxidase by cyanide does not result in any appreciable increase in NT reduction. Thus, the side-path to NT would appear to be virtually saturated under normal conditions. However, this point draws attention once more to the complex character of the system for not only does cyanide divert extra electrons into the NT side-path but, by preserving aerobic conditions, acts as an inhibitor of the overall process.

Secondly, if the factor were capable of being dissociated from the respiratory chain by relatively mild procedures (*e.g.*, dilution of tissue suspensions with water) then this would explain not only previous results[2] which indicated the presence of a water-soluble cofactor, but also the observations illustrated in Table IV that a non-linear response can also be obtained under anaerobic conditions where the

oxygen effect is necessarily absent. Thus, on the basis of these two assumptions, it can be seen that the non-linear response results from the combination of two effects. The major effect under normal conditions appears to be due to oxygen inhibition of the side-path leading to NT reduction. Anaerobic conditions increase NT reduction so that formazan production increases more sharply in incubation mixtures where the O_2 level is being depleted fastest, *i.e.*, where most tissue is present. A further contribution to the non-linear response would appear to be that originally proposed[2], *i.e.*, dissociation of the factor mediating electron transfer between the respiratory chain and NT. It is not yet possible to give a definite answer as to the nature of this mediating factor. However, some indirect evidence relevant to this point will be given later.

Points of reduction of NT

On the basis of the above discussion it is possible to summarise the points of interaction of NT with the respiratory chain. Firstly, under the normal aerobic unshaken conditions, 90% of the total reduction occurs via a factor or grouping mediating electron transfer between the cytochrome *c* region and NT; the remaining 10% of NT reduction is antimycin A insensitive and probably results from an interaction with ubiquinone. In the presence of added vitamin K_3, the reduction of NT which occurs is also antimycin A insensitive and probably involves an interaction between vitamin K_3 and either succinate dehydrogenase or ubiquinone. Secondly, under anaerobic conditions, NT reduction is increased and is roughly equal in extent both at the site involving the factor and at the region of ubiquinone. The ease with which reduced quinones reduce tetrazolium salts raises the possibility that the interaction of NT at the cytochrome oxidase end of the chain is also via a quinone-like structure. Indirect evidence favouring this possibility is that a quinone-like material has been indicated in the terminal span of the respiratory chain by BEYER[40]; all phosphorylation stages are sensitive to dicumerol[30] and one such stage occurs at or near the major aerobic site of NT reduction. Neotetrazolium also reacts with the respiratory chain near to or on ubiquinone and, when $NADH_2$ is substrate, near to or on the NAD-flavoprotein[18]. Thus, the three sites of interaction of NT with the respiratory chain are suggestively close to the three sites of oxidative phosphorylation. Although the systems studied in these investigations have been non-phosphorylating systems, it is possible that part of the complex sequence of events associated with oxidative phosphorylation is involved in NT reduction. If indeed NT couples with the respiratory chain at points on the locus of oxidative phosphorylation by interaction with reduced quinones then tetrazolium reduction would become a useful tool in following the fractionation and localisation of these important free-energy conserving mechanisms.

It is obvious from these results that the reduction of NT coupled to succinate oxidation is complex. It is accompanied in tissue extracts by many spontaneous and non-enzymic interactions. Further, the presence of O_2 not only alters the extent of the reduction but alters the relative magnitudes of the sites of reduction. Thus, direct quantitative histochemical comparison of NT reduction in tissue sections can be seen to be fraught with many difficulties not always fully appreciated.

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REFERENCES

1 M. M. NACHLAS, S. I. MARGULIES AND A. M. SELIGMAN, *J. Biol. Chem.*, 235 (1960) 2739.
2 T. F. SLATER, *Biochem. J.*, 73 (1959) 314.
3 T. F. SLATER AND D. N. PLANTEROSE, *Biochem. J.*, 74 (1960) 591.
4 R. L. LESTER AND A. SMITH, *Biochim. Biophys. Acta*, 47 (1961) 475.
5 E. SHELTON AND M. E. RICE, *J. Natl. Cancer Inst.*, 18 (1957) 117.
6 T. F. SLATER, B. SAWYER AND U. D. STRÄULI, *Biochim. Biophys. Acta*, 77 (1963) 383.
7 A. G. PARKES, *J. Hyg.*, 44 (1946) 491.
8 D. H. DEUL, E. C. SLATER AND L. VELSTRA, *Biochim. Biophys. Acta*, 27 (1958) 133.
9 S. P. MARTIN, C. D. COOPER, S. N. CHAUDHURI AND R. GREEN, *J. Exptl. Med.*, 101 (1955) 639.
10 E. FARBER AND E. BUEDING, *J. Histochem. Cytochem.*, 4 (1945) 357.
11 N. ZOLLNER AND E. ROTHEMUND, *Z. Physiol. Chem.*, 298 (1954) 97.
12 L. ERNSTER, M. LJUNGGREN AND L. DANIELSON, *Biochem. Biophys. Res. Commun.*, 2 (1960) 88.
13 A. GUIDITTA AND H. J. STRECKER, *Biochem. Biophys. Res. Commun.*, 2 (1960) 159.
14 E. C. SLATER, *Advan. Enzymol.*, 20 (1958) 147.
15 T. F. SLATER, A. L. GREENBAUM AND J. CLARK, to be published.
16 M. J. R. DAWKINS, J. D. JUDAH AND K. R. REES, *Biochem. J.*, 72 (1959) 204.
17 B. CHANCE AND G. HOLLUNGER, *Nature*, 185 (1960) 666.
18 T. F. SLATER, *Nature*, 183 (1959) 1679.
19 T. SUGIMURA AND T. ONO, *Gann*, 48 (1957) 169.
20 C. D. COOPER, S. P. MARTIN AND S. KORKES, *Federation Proc.*, 14 (1955) 196.
21 A. G. E. PEARSE, *Histochemistry, Theoretical and Applied*, Churchill, London, 1960.
22 L. L. ENGEL AND L. J. LANGER, *Ann. Rev. Biochem.*, 30 (1961) 499.
23 W. W. UMBREIT, R. H. BURRIS AND J. F. STAUFFER, *Mannometric Techniques*, Burgess, Minneapolis, 1959, p. 174.
24 N. R. TRENNER AND F. A. BACHER, *J. Biol. Chem.*, 137 (1941) 745.
25 C. D. JOEL, M. L. KARNOVSKY, E. G. BALL AND O. COOPER, *J. Biol. Chem.*, 233 (1958) 1565.
26 T. P. SINGER, E. B. KEARNEY AND V. MASSEY, *Advan. Enzymol.*, 18 (1957) 92.
27 J. P. COLPA-BOONSTRA AND E. C. SLATER, *Biochim. Biophys. Acta*, 27 (1958) 122.
28 K. S. AMBE AND F. L. CRANE, *Biochim. Biophys. Acta*, 43 (1960) 30.
29 L. DANIELSON, T. E. CONOVER AND L. ERNSTER, *Proc. Intern. Congr. Biochem., 5th, Moscow, 1962*, section 23.
30 L. COOPER AND A. L. LEHNINGER, *J. Biol. Chem.*, 219 (1956) 519.
31 A. L. TAPPEL, *Biochem. Pharmacol.*, 3 (1960) 289.
32 V. R. POTTER AND A. E. REIF, *J. Biol. Chem.*, 194 (1952) 287.
33 D. E. GREEN, Y. HATEFI AND W. F. FECHNIER, *Biochem. Biophys. Res. Commun.*, 1 (1959) 45.
34 A. PUMPHREY AND E. R. REDFEARN, *Biochem. J.*, 72 (1959) 2 P.
35 H. KAMIN, R. H. GIBBS AND A. D. MERRITT, *Federation Proc.*, 16 (1957) 202.
36 K. H. LING, T. C. SU AND T-C. TUNG, *Arch. Biochem. Biophys.*, 71 (1957) 126.
37 R. L. LESTER AND E. M. RAMASARMA, *J. Biol. Chem.*, 234 (1959) 672.
38 T. F. SLATER, *Nature*, 183 (1959) 50.
39 D. H. DEUL AND M. B. THORN, *Biochim. Biophys. Acta*, 59 (1962) 427.
40 R. E. BEYER, *J. Biol. Chem.*, 234 (1959) 688.