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STUDIES ON SUCCINATE–TETRAZOLIUM REDUCTASE SYSTEMS

III. POINTS OF COUPLING OF FOUR DIFFERENT TETRAZOLIUM SALTS

T. F. SLATER, BARBARA SAWYER AND URSULA STRÄULI

Department of Chemical Pathology, University College Hospital Medical School, University Street, London (Great Britain)

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SUMMARY

An investigation has been made of the points of coupling between four tetrazolium salts and the respiratory chain (succinate to O_2) in rat-liver tissue suspensions. Each tetrazolium salt has been studied with four levels of tissue and under various incubation conditions. The effects of various respiratory-chain inhibitors on these reactions have been studied in order to localise where the tetrazolium salts are reacting with the respiratory chain. The reaction of nitro-blue tetrazolium with the respiratory chain is virtually insensitive to the presence of antimycin A, or to the level of O_2 in the incubation mixture. This and other evidence suggests that nitro-blue tetrazolium reacts almost completely at one site on the respiratory chain, possibly ubiquinone. *C,N*-diphenyl-*N'*-4,5-dimethylthiazol-2-yltetrazolium bromide, however, appears to react at two sites of roughly equal importance, one of which is sensitive to antimycin A; this latter site is in the region of cytochrome *c*. Of the other two tetrazolium salts investigated here, triphenyltetrazolium chloride reacts with the terminal oxidase, and 2-*p*-nitrophenyl-3-*p*-iodophenyl-5-phenyltetrazolium chloride reacts in a manner similar to *C,N*-diphenyl-*N'*-4,5-dimethylthiazol-2-yltetrazolium bromide. These results, combined with the results for neotetrazolium chloride in the preceding communication, are discussed in terms of previous reports in the literature and the applicability of using tetrazolium reduction for histochemical purposes.

INTRODUCTION

The preceding communication[1] was in part concerned with the points of interaction of NT with the respiratory chain. A similar experimental approach has been made in this paper using four other tetrazolium salts in order to establish where these couple with the respiratory chain in rat-liver suspensions*.

Abbreviations: NT = neotetrazolium chloride; MTT = *C,N*-diphenyl-*N'*-4,5-dimethylthiazol-2-yltetrazolium bromide; INT = 2-*p*-nitrophenyl-3-*p*-iodophenyl-5-phenyltetrazolium chloride; TTC = triphenyltetrazolium chloride; NBT = nitro-blue tetrazolium = 2,2'-di-*p*-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride.

* In conformity with the International Union of Biochemistry recommendation, the enzyme systems coupling succinate oxidation with tetrazolium reduction are described as succinate–

The determination of the points of coupling of tetrazolium salts with the respiratory chain has provoked several investigations in recent years[2-4] and the consequent results have raised controversial issues which have largely been resolved by the realisation that the coupling points vary with the tetrazolium salt being used. Whether the coupling points also vary from tissue to tissue in a given species, or in the same tissue from different species is as yet unclear. This paper deals only with rat-liver suspensions and provides evidence enabling the localisation of the coupling points of four different tetrazolium salts with the respiratory chain to be established.

METHODS

Adult, female, albino rats were used in this investigation. They were fed Diet 41B (ref. 5) and water *ad libitum*. Rats were killed by cervical dislocation and liver suspensions were quickly prepared in ice-cold water (1 g liver plus 9 ml water). Succinate–tetrazolium reductases were assayed by incubating buffer mixture, tetrazolium salt and various levels of tissue under aerobic conditions at 37° (the amounts of tissue used varied with the tetrazolium salt under investigation and the levels adopted are indicated in the appropriate table). The buffer mixture was a phosphate buffer–sodium succinate–EDTA mixture as described earlier[6]. To correct for the varying volume added as tissue suspension, or as additives, water was added such that the final volume of the incubation mixture was constant for all tubes in each experiment. The reaction was started by adding 0.15 ml tetrazolium salt to each tube; the concentrations of the tetrazolium salts and the method used to stop the reactions are described below.

TTC was a 1% (w/v) aqueous solution; after stopping the reaction with 10% trichloroacetic acid and extracting with ethyl acetate, the formazan was estimated at 485 mμ. MTT (a generous gift from Dr. T. PYL) was a 0.5% (w/v) aqueous solution. Reactions using MTT were stopped with 2 ml saturated $(NH_4)_2SO_4$ and the formazan extracted with 4 ml ethyl acetate; the absorbancy was measured at 560 mμ. The absorption spectrum for MTT-formazan varies with the method used to stop the enzymic reaction; if trichloroacetic acid is used the absorption maximum is 430 mμ whereas with saturated $(NH_4)_2SO_4$ it is 560 mμ. NBT (Sigma Chemical Co.) and INT (Sigma Chemical Co.) were both used as 0.5% (w/v) aqueous solutions. Although the formazans obtained from both NBT and INT by chemical reduction are readily soluble in organic solvents (*i.e.*, ethyl acetate) they are extracted only with great difficulty when formed in the presence of tissue suspensions. Presumably they are bound very tightly to protein. Reactions using NBT and INT were stopped with a Triton X-100–formaldehyde–formate buffer mixture as given by LESTER AND SMITH[3]. Vigorous mechanical shaking was then performed to disperse the mixture and the absorbancies were measured at 560 mμ for NBT and 540 mμ for INT. Experiments on the effects of O_2 on the succinate–tetrazolium reductase systems were carried out by comparing formazan reduction under the normal unshaken aerobic incubation conditions as just described with the reduction found (a) when the

tetrazolium reductase systems. The generic name '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.

incubation mixture was vigorously shaken at 120 shakes/min in small flasks open to the air and (b) when the incubation was carried out in Thunberg tubes under anaerobic conditions.

The preparation and source of all additives are described in the preceding communication[1]; the concentrations of the various additives used in this communication are the optimal concentrations for the purposes in question. These levels have been derived from the literature and from previous work on NT (see preceding paper for details and references). For example, in experiments aimed at

TABLE I

EFFECTS OF VARIOUS INHIBITORS AND TREATMENTS ON SUCCINATE–MTT REDUCTASE IN RAT-LIVER SUSPENSIONS

The final conc. of any additive is shown in Column 2. Incubation of tissue, MTT, phosphate buffer, sodium succinate and EDTA was at 37°. The reaction was stopped by the addition of 2 ml satd. $(NH_4)_2SO_4$ soln., the formazan was extracted with 4 ml ethyl acetate and the absorbancy measured at 560 mμ. Other details as in the Methods section and the preceding communication[1]. In the experiment with dicumerol, the tissue, buffer mixture and additive was preincubated for 10 min at 0° prior to warming to 37° and starting the reaction with MTT.

Addition or treatment	*Amount added (mM)*	*Incubation vol. (ml)*	*Incubation time (min)*	*μg formazan produced at various levels of tissue*				
				2 mg	*4 mg*	*6 mg*	*8 mg*	*10 mg*
Shaking		1.45	10	30	63	94	127	—
Control		1.45	10	25	63	100	126	—
Anaerobic		1.30	10	18	46	96	146	—
Control		1.30	10	11	48	77	126	—
Antimycin A	0.001	1.3	12	13	37	—	111	—
Control		1.3	12	19	67	97	188	—
Chlorpromazine	0.8	1.3	10	14	33	61	—	—
Stelazine	0.8	1.3	10	15	31	44	—	—
Control		1.3	10	30	56	122	—	—
Antimycin A	0.001	1.4	10	16	23	31	—	—
Antimycin A + chlorpromazine	0.001 + 1.4	1.4	10	14	21	31	—	—
Cytochrome *c*	0.004%	1.4	10	18	36	60	—	—
Control		1.4	10	16	42	74	—	—
Amytal	1.3	1.5	12	11	51	93	—	—
Vitamin K_3	0.5	1.5	12	21	54	108	—	—
Amytal + vitamin K_3	1.3 + 0.5	1.5	12	14	62	114	—	—
Control		1.5	12	15	47	79	—	—
Antimycin A (aerobic)	0.0036	1.25	10	—	56	—	—	152
Antimycin A (anaerobic)	0.0036	1.25	10	—	54	—	—	154
Azide	13	1.45	10	46	77	106	134	—
Cyanide	1.3	1.45	10	22	66	94	108	—
Control		1.45	10	25	63	100	126	—
Dicumerol	1.25	1.45	10	24	53	92	—	—
Dicumerol	0.125	1.45	10	48	92	134	—	—
Control		1.45	10	27	90	124	—	—

seeing whether tetrazolium salts are affected by cytochrome oxidase (EC 1.9.3.1) inhibition, an adequate concentration of cyanide or azide is used which is known to inhibit the oxidase at the levels of tissue being studied. In all cases where the additive was added in other than a neutral aqueous solution, the controls reported contained an equivalent amount of solvent to that containing the additive. All additions with the exception of dicumerol were made in solution at pH 7.4.

RESULTS

Table I gives the results for various treatments and inhibitors on succinate–MTT reductase. It can be seen that shaking the mixture during incubation had little influence on the formazan production compared to the normal aerobic incubation in unshaken tubes. Anaerobic conditions produced a small but consistent stimulation.

Antimycin A (final concentration 0.5 μg/ml) inhibited succinate–MTT reductase by 40%; under anaerobic conditions antimycin A was only very slightly less effective as an inhibitor. The phenothiazine derivatives, Chlorpromazine (2-chloro-10-(3-

TABLE II

EFFECTS OF VARIOUS TREATMENTS AND ADDITIONS ON RAT-LIVER SUCCINATE–TTC REDUCTASE

Details as in Table I and the Methods section.

Addition	*Amount added (mM)*	*Incubation vol. (ml)*	*Incubation time (min)*	*μg formazan produced at 4 levels of tissue*			
				20 mg	*40 mg*	*60 mg*	*80 mg*
Cytochrome *c*	0.004%	2.15	10	35	93	153	190
Control		2.15	10	3	53	116	178
Antimycin A	0.0015	2.15	10	2	7	13	45
Control		2.15	10	3	48	111	167
Vitamin K_3	0.38		10	26	100	181	252
Control			10	2	44	101	147
Chlorpromazine	0.85	2.35	10	1	8	15	61
Cytochrome *c*	0.004%	2.35	10	23	80	125	164
Chlorpromazine + cytochrome *c*	0.85 + 0.004%	2.35	10	2	8	48	105
Control		2.35	10	2	6	70	174
Dicumerol	0.1	2.35	10	1	4	46	105
Control		2.35	10	2	5	43	95
Shaking		2.25	10	0	0	6	7
Anaerobic		2.25	10	34	84	128	176
Control		2.25	10	1	57	120	172
Cyanide	0.85	2.35	10	1	2	6	7
Control		2.35	10	2	46	108	160
Azide	9	2.15	10	1	1	36	50
Control		2.15	10	1	39	114	200
Amytal	1.5	2.15	10	4	73	150	216
Control		2.15	10	4	62	134	200

dimethylaminopropyl)phenothiazine hydrochloride) and Stelazine (10-[3-(4-methylpiperazine-1-yl)propyl]-2-trifluoromethylphenothiazine hydrochloride) were effective inhibitors of succinate–MTT reductase. The antimycin A-insensitive reduction was not affected by Chlorpromazine indicating that the phenothiazines are inhibiting MTT reduction at a point later than the antimycin-sensitive factor.

Azide (final concentration 13 mM) slightly increased formazan production by the succinate–MTT reductase system. Sodium amytal (final concentration, 1.3 mM) an inhibitor of $NADH_2$ oxidation[7] and of succinate oxidase system[8], had an effect similar to that given by azide.

Table II gives similar results for succinate–TTC reductase. With this tetrazolium salt, however, azide, cyanide or antimycin A produced virtually complete inhibition particularly with the lower levels of tissue indicating that the major coupling point was on cytochrome oxidase. Added cytochrome *c* increased TTC reduction as did anaerobic conditions. TTC reduction was strongly inhibited by shaking the incubation mixture during the course of the reaction.

Tables III and IV give the data for succinate–NBT reductase and succinate–INT reductase. The effects of antimycin A, anaerobic conditions, cytochrome *c* and Chlorpromazine are particularly interesting when considering the coupling points of these tetrazolium salts with the respiratory chain.

TABLE III

EFFECTS OF VARIOUS TREATMENTS AND ADDITIVES ON SUCCINATE–NBT REDUCTASE IN RAT-LIVER SUSPENSIONS

Details of the incubation mixture and preparation of the additives are described in the preceding communication. The reaction was stopped with a Triton X-100–formaldehyde–formate buffer mixture and the absorbancy measured at 560 mμ. Formazan produced is given in terms of this absorbancy.

Addition or treatment	*Amount added (mM)*	*Incubation vol. (ml)*	*Incubation time (min)*	*Formazan produced at various levels of tissue*					
				2 mg	*4 mg*	*5 mg*	*6 mg*	*8 mg*	*10 mg*
Antimycin A	0.0015	1.35	15	—	—	0.107	—	—	0.242
Chlorpromazine	0.35	1.35	15	—	—	0.192	—	—	0.282
Cytochrome *c*	0.006%	1.35	15	—	—	0.124	—	—	0.242
Azide	7.4	1.35	15	—	—	0.164	—	—	0.285
Control		1.35	15	—	—	0.155	—	—	0.289
Anaerobic		1.35	10	0.099	0.190	—	0.227	0.283	—
Shaking		1.35	10	0.047	0.086	—	0.125	0.207	—
Control		1.35	10	0.045	0.106	—	0.145	0.200	—
Anaerobic		1.25	10	0.107	—	—	0.222	0.275	—
Control		1.25	10	0.072	0.118	—	0.175	0.215	—
Antimycin A	0.0015	1.25	10	0.074	0.117	—	0.167	0.212	—
Control		1.25	10	0.073	0.124	—	0.171	0.210	—

DISCUSSION

The preceding paper[1] has very briefly outlined present views on the basic structure of the respiratory chain and dealt with the coupling points of NT. Much of the general

TABLE IV

EFFECTS OF VARIOUS TREATMENTS AND ADDITIVES ON SUCCINATE-INT REDUCTASE IN RAT-LIVER SUSPENSIONS

Details of the incubation mixture and preparation of the additives are described in the Methods section and in the preceding communication. The reaction was stopped with a Triton X-100–formaldehyde–formic acid buffer mixture and the absorbancy measured at 490 mμ. Formazan production is given in terms of this absorbancy.

Treatment or addition	*Amount added (mM)*	*Incubation vol. (ml)*	*Incubation time (min)*	*Formazan production at various levels of tissue*					
				2 mg	*4 mg*	*5 mg*	*6 mg*	*8 mg*	*10 mg*
Anaerobic		1.25	5	0.282	0.520	—	0.844	1.135	—
Control		1.25	5	0.278	0.664	—	0.935	1.230	—
Antimycin A	0.0015	1.25	5	0.147	0.330	—	0.500	0.666	—
Control		1.25	5	0.318	0.520	—	0.895	1.214	—
Antimycin A	0.0015	1.25	5	—		0.365	—	—	0.700
Cytochrome *c*	0.007%	1.25	5	—		0.452	—	—	0.850
Chlorpromazine	0.38	1.25	5	—		0.255	—	—	0.560
Azide	8.0	1.25	5	—		0.560	—	—	1.000
Anaerobic		1.25	5	—		0.543	—	—	1.028
Shaking		1.25	5	—		0.552	—	—	1.000
Control		1.25	5	—		0.546	—	—	1.000

discussion in that communication is relevant here and will not be repeated *in extenso*. Important points discussed in the preceding paper[1] and which are applicable to this communication were the site of action of antimycin A, the varying effects of cyanide or azide, the effects of O_2 on the system, and the locus of action of Chlorpromazine. In this and the preceding communication, the reduction of tetrazolium salts which occurs in the presence of antimycin A will be taken to occur via an interaction with ubiquinone. It is realised that the evidence for this is, at the present, not substantial, being based on the two findings (a) that reduced quinones, ubiquinone in particular, will react spontaneously with tetrazolium salts[9,10] and (b) that none of the tetrazolium salts so far tested has reacted with soluble succinate dehydrogenase (EC 1.3.99.1) preparations[2,3]. However, until good evidence to the contrary is available, the above working hypothesis that antimycin A-insensitive tetrazolium reduction occurs via ubiquinone will be used. Fig. 1 shows the respiratory chain structure which will be used for the purposes of this discussion and the points of action of the various inhibitors studied. It is realised that views on the structure of the respiratory chain are at present in a state of flux and that Fig. 1 leaves out many components for whose inclusion there is a certain amount of evidence. However, it is believed that Fig. 1 represents in a general fashion the main oxidation–reduction framework involved in respiration and tetrazolium reduction. It will be convenient to discuss the results taking each tetrazolium salt in turn.

MTT

It can be seen that azide slightly stimulates the succinate–MTT reductase system (Table I); the main aerobic coupling site between MTT and the respiratory

chain cannot, therefore, involve cytochrome oxidase. The stimulation produced by azide is probably due to the inhibition of cytochrome oxidase resulting in an increased channelling of electrons into the MTT pathway; however, it is surprising that cyanide does not produce a similar effect. Anaerobic conditions also produce an increase in MTT reduction probably for the same reason. Unlike the case found[1] for NT, O_2 *per se* does not appear to have any significantly deletarious action on MTT reduction since vigorously shaking the incubation mixture does not decrease formazan production.

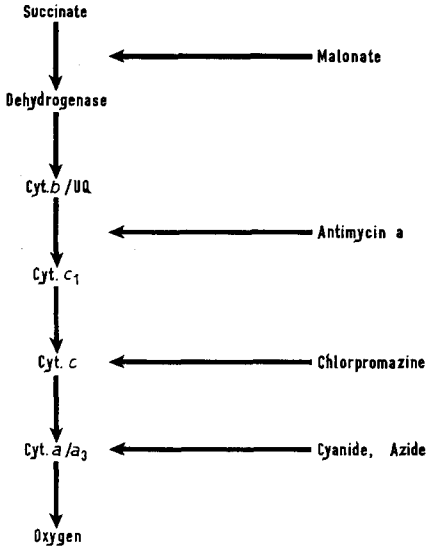

Fig. 1. Diagrammatic representation of the rat-liver respiratory chain from succinate to O_2. On the right-hand side opposite the horizontal lines are shown the positions at which the various inhibitors are taken to interact with the respiratory chain for the purposes of the discussion.

Antimycin A has much less effect on succinate–MTT reductase than on NT reduction. The results obtained with MTT show that only 40% of the reduction is antimycin A sensitive under both aerobic and anaerobic conditions. Thus, approximately one-half of the total reduction of MTT occurs via an interaction with the respiratory chain prior to the antimycin A-sensitive factor. For reasons mentioned above[1] it is likely that this interaction is at the level of ubiquinone.

Surprisingly, added cytochrome *c* produced a small reduction in the aerobic succinate–MTT reductase activity (Table I); this might be due to cytochrome *c* establishing a new pathway shunting out the region of ubiquinone and so eliminating the 60% reduction of MTT which occurs at this point.

Chlorpromazine and Stelazine inhibit succinate–MTT reductase in a manner similar to that described for NT in the previous communication[1]. It would appear, therefore, that the antimycin A-sensitive reduction of MTT occurs via a coupling point in the region of cytochrome *c* as was the case for NT. If MTT couples in this region via the same oxygen-sensitive factor as found for NT, then it would not be

so easily demonstrable since only 60% of the aerobic MTT reduction occurs in this region. Even so, it would be expected that shaking the incubation mixture during the reaction would cause a demonstrable decrease in MTT reduction. And anaerobicity should cause an appreciable increase in MTT reduction. However, the results of Table I show that shaking has no effect and anaerobicity only a small stimulatory effect. Thus, it is possible that the antimycin A-sensitive MTT reduction, although occurring between cytochrome *c* and cytochrome oxidase, does not occur via a coupling with the same factor as is involved in NT reduction, and which is so dependent on the oxygen level in the incubation mixture.

TTC

Cyanide (and azide providing the tissue level:azide concentration is not too high) completely inhibits succinate–TTC reductase at final concentrations of 1 and 10 mM respectively. This result indicates that, alone of the tetrazolium salts investigated, TTC couples with the respiratory chain via cytochrome oxidase. This singular behaviour could be the result of the very negative electrode potential possessed by TTC (E_0' for TTC has been given as -415 mV[11] and -240 mV[12]; JERCHEL AND MOHLE[13], however, reported the value as -83 mV) compared with the other tetrazolium salts tried, the most electronegative of which was NT (-170 mV[11]). As might be expected from these results, antimycin A almost completely abolishes TTC reduction in this system.

Probably as a result of its highly negative standard electrode potential, TTC is a very inefficient electron acceptor compared with cytochrome oxidase. Thus, anaerobic conditions greatly stimulate, and shaking vigorously in air strongly suppresses the reduction of TTC. These effects are even more striking than found for NT reported previously[1].

Added cytochrome *c* stimulates TTC reduction at low levels of tissue, a response which is different from that found for NT where cytochrome *c* tended to stimulate only the reduction obtained with the higher levels of tissue. Chlorpromazine inhibits TTC reduction and this inhibition is removed by adding cytochrome *c* to the system, thus giving further support to the conclusions stated previously that Chlorpromazine is acting on these tetrazolium systems at the level of cytochrome *c*.

Although added vitamin K_3 stimulated TTC reduction the stimulation is not so marked as found with NT[6]. Since it is probable that vitamin K_3 couples efficiently with succinate dehydrogenase, the different degrees of vitamin K_3-activation found with the various tetrazolium salts are probably reflections of the ease of coupling of the reduced quinone with the tetrazolium salts. Differences in reactivity between reduced ubiquinone and the various tetrazolium salts are further indicated by the results found with antimycin A under aerobic and anaerobic conditions. The antimycin A-insensitive reduction of NT is greatly increased by anaerobic conditions and the suggestion has been made that this is due to an increased coupling of reduced ubiquinone with NT in the absence of O_2 (ref. 1). However, this effect is not seen with MTT, where antimycin A sensitivity is approximately the same under aerobic and anaerobic conditions.

The reduction of TTC in rat-liver suspensions would appear from the above discussion to proceed via a coupling with cytochrome oxidase. Histochemical demon-

stration of the succinate–TTC reductase system thus involves a demonstration of the entire respiratory chain with the exception of the final reaction between the oxidase and O_2.

NBT

The results found for succinate–NBT reductase are markedly different from those discussed for TTC. NBT reduction is virtually insensitive to antimycin A, to Chlorpromazine, to azide and to added cytochrome *c*. Although anaerobic conditions produce a small stimulation of the reaction this could be due to the consequent decrease in the autoxidation of reduced ubiquinone through which the majority of the reduction apparently occurs.

If the working hypothesis that the antimycin A-insensitive NBT reduction occurs via an interaction with ubiquinone is accepted then NBT can be seen to be virtually a specific electron acceptor for the ubiquinone region. Further, since reduced ubiquinone reduces tetrazolium salts directly, then the succinate–NBT reductase system involves the same enzymic steps as does succinate–ubiquinone reductase.

INT

Succinate–INT reductase resembles in many of its properties succinate–MTT reductase. INT reduction is inhibited about 40% by antimycin A, is inhibited by Chlorpromazine and cytochrome *c*, and is virtually unaffected by anaerobic conditions or by shaking. Arguments similar to those already outlined enable the points of interaction of INT and the respiratory chain to be established as approximately two-thirds on ubiquinone and one-third near cytochrome *c*, probably via the same factor as for MTT.

The conclusions reached above have been combined with those already reached for NT in the preceding communication[1] in constructing Fig. 2. It can be seen that the tetrazolium salts studied fall into two main groups (a) where the tetrazolium salt is reduced almost exclusively at one site on the respiratory chain as found for NBT, TTC and under aerobic conditions for NT and (b) where the tetrazolium salts show (at least) two interactions with the respiratory chain of more or less equal importance. In this latter group are MTT, INT, and, under anaerobic conditions, NT.

In conclusion, it can be seen that succinate–tetrazolium reductases are in general complex systems affected in many cases by the oxygen level in the incubation mixture, a property which makes satisfactory routine assay procedures cumbersome. From the results presented here it would appear that if a routine assay procedure is required for the succinate dehydrogenase end of the respiratory chain, then NBT would be the best tetrazolium salt of the five tested here for this purpose. This conclusion is reached because NBT is reduced almost completely at one point of interaction with the respiratory chain, near to the primary dehydrogenase, and O_2 has little effect on the NBT system. However, if a tetrazolium assay is required to demonstrate succinate dehydrogenase more rigorously than the succinate–NBT reductase system, it is essential to study suspensions supplemented with an electron

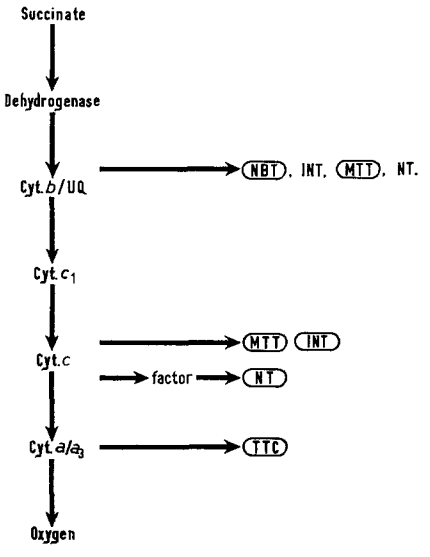

Fig. 2. The postulated points of interaction of the five tetrazolium salts studied in this and the preceding communication. The major site of reduction, under aerobic conditions, is shown encircled.

carrier such as vitamin K_3 (ref. 15) or phenazine methosulphate[16] which couple with the dehydrogenase and transfer directly and non-enzymically to all of the tetrazolium salts so far tested[9,10]. Thus, in such supplemented suspensions, the differential coupling reactions of tetrazolium salts disappear and the choice of a particular one for a specific purpose can be made on the basis of requirements other than the composition of the enzyme system being measured. Providing the conclusions reached here on the coupling reactions of tetrazolium salts in tissue suspensions can be applied to tissue sections, then such other requirements mentioned above could be the nature of the formazan deposit or the extent of diffusion of formazan from the site of production.

Since none of the tetrazolium salts so far tested reacts with succinate dehydrogenase, then it is legitimate to object to results obtained with unsupplemented succinate–tetrazolium reductase systems being interpreted in terms of the primary dehydrogenase alone. This objection is more pertinent with those tetrazolium salts which couple with the respiratory chain at more than one point or which couple at the oxygen end of the chain. However, such disadvantages in the use of tetrazolium salts, can conceivably be turned to advantage in another direction. In theory by studying aliquots of the same unsupplemented tissue sample with different tetrazolium salts it should be possible to follow changes in segments of the respiratory chain of different lengths and to follow changes in these segments under various pathological disturbances. An indication that this idea can be applied comes from work on mammary gland. It was found that from early to late lactation, succinic oxidase (measured manometrically) increased 1.9-fold[17] whereas succinate–neotetrazolium reductase (in the presence of added vitamin K_3) increased 6-fold[18]. This indicates that the initial part of the respiratory chain increases faster than some

more distal rate-limiting factor. This rate-limiting factor might be ubiquinone, which has been suggested to be rate-limiting in normal respiratory chain preparations.

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REFERENCES

1 T. F. SLATER, *Biochim. Biophys. Acta*, 77 (1963) 365.
2 M. M. NACHLAS, S. I. MARGULIES AND A. M. SELIGMAN, *J. Biol. Chem.*, 235 (1960) 2739.
3 R. L. LESTER AND A. SMITH, *Biochim. Biophys. Acta*, 47 (1961) 475.
4 T. ODA AND H. OKAZAKI, *Acta Med. Okayama*, 12 (1958) 193.
5 A. S. PARKES, *J. Hyg.*, 44 (1946) 491.
6 T. F. SLATER, *Biochem. J.*, 73 (1959) 314.
7 L. ERNSTER, O. JALLING, H. LOW AND O. LINDBERG, *Exptl. Cell Res. Suppl.*, 3 (1955) 124.
8 A. M. PUMPHREY AND E. F. REDFEARN, *Biochem. Biophys. Res. Commun.*, 8 (1962) 92.
9 T. F. SLATER, *Nature*, 183 (1959) 50.
10 R. L. LESTER AND E. M. RAMASARMA, *J. Biol. Chem.*, 234 (1959) 672.
11 S. S. KARMARKAN, A. G. E. PEARSE AND A. M. SELIGMAN, *J. Org. Chem.*, 25 (1960) 575.
12 W. RIED AND M. WILK, *Ann. Chem.*, 581 (1953) 49.
13 D. JERCHEL AND W. MOHLE, *Ber.*, 77 (1944) 591.
14 Y. MIURA, Y. ASO, Y. MASHIMA AND I. NAGATA, *J. Biochem. Tokyo*, 52 (1962) 43.
15 T. F. SLATER AND D. N. PLANTEROSE, *Biochem. J.*, 74 (1960) 591.
16 E. FARBER AND E. BUEDING, *J. Histochem. Cytochem.*, 4 (1956) 359.
17 A. L. GREENBAUM AND T. F. SLATER, *Biochem. J.*, 66 (1957) 161.
18 T. F. SLATER AND D. N. PLANTEROSE, *Biochem. J.*, 74 (1960) 584.