5. The nitroreductase is non-specific and requires reduced diphosphopyridine nucleotide as H-donor. Its activity is stimulated by flavinadenine dinucleotide and inhibited by hydroxylamine and nitrite.

6. Dialysis against ethylenediaminetetra-acetic acid, 1:10-phenanthroline, 8-hydroxyquinoline and potassium cyanide causes extensive inhibition and reveals the requirement for a bivalent metal. Mn^{2+}, Mg^{2+} and, in particular, Fe^{2+} ions are active. The effect of the last-named was also observed in experiments with metal-free media.

7. The significance in the overall metabolism of several hydroxynitrobenzoic acids isolated on chromatograms from experiments with *Nocardia* species could not be deduced.

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Studies on the Succinate–Neotetrazolium Reductase System

ACTIVATION BY VITAMIN K_3

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The formation of insoluble formazans by the enzymic reduction of tetrazolium salts has been extensively used in the histological demonstration of dehydrogenase activity. Recently several reports have been concerned with the application of this procedure to the quantitative estimation of dehydrogenase activity in tissue homogenates (Kun & Abood, 1949; Sprinz & Waldschmidt-Leitz, 1953; Zöllner & Rothemund, 1954; Shelton & Rice, 1957; Sourkes & Lagnado, 1957).

The mechanism of the reaction is still unknown, as is the point(s) at which tetrazolium couples with

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the respiratory chain. Attempts to elucidate this point have been made by Brodie & Gots (1951) and by Ling, Su & Tung (1957). In this paper the enzyme system coupling the oxidation of succinate with the reduction of neotetrazolium chloride is termed succinate–neotetrazolium reductase.

In attempts to use the procedure given by Shelton & Rice (1957) for routine assay of succinate–neotetrazolium reductase, it was found that the formazan produced did not increase proportionately with increasing amounts of tissue added; in the text this is called the non-linear response. This suggested either that the reagents of the experimental system had affected the enzyme or alternatively that there had been dilution of an essential cofactor. A cofactor requirement for succinate–neotetrazolium reductase has been indicated by the work of Bril (1954), Sprinz & Waldschmidt-Leitz (1953) and by Sugimura & Ono (1956). A similar non-linear response in tetrazolium reduction has been reported by Sourkes & Lagnado (1957) for monamine oxidation.

This paper describes preliminary work on the mechanism of the non-linear response and the stimulation of succinate–neotetrazolium reductase by vitamin K_3.

METHODS

The rats used were black-and-white adult females of the Medical Research Council strain; body wt. was 180–200 g. They were fed on a diet described by Parkes (1946). Mice were adult males (body wt. 35–40 g.) of the Tuck strain. Animals were killed by cervical dislocation and liver suspensions (1:10) were prepared in ice-cold aqueous 0·25 M-sucrose in a Potter–Elvehjem (1936) type of homogenizer with a plastic pestle (clearance 0·005 in.). For experiments with mitochondrial fractions the centrifuging scheme was as follows: the tissue suspension was spun at 600 *g* for 10 min. in a refrigerated M.S.E. Angle 13 centrifuge at 0° to remove the so-called cell debris and nuclei. This spinning was repeated on the supernatant. The supernatant from the second nuclear centrifuging was spun at 10 000 *g* for 10 min. to sediment the large-particle or mitochondrial fraction. This fraction was resuspended in the original volume of 0·25 M-sucrose. In all separations of sediment and supernatant a pipette with a bent tip was used. For experiments with mammary-gland suspensions, homogenization was carried out as described by Greenbaum & Slater (1957).

The following reagents were used: (Na_2HPO_4–NaH_2PO_4) buffer, 0·1 M, pH 7·4; disodium succinate (succinic acid was recrystallized and the pH adjusted to 7·4 with sodium hydroxide), 0·5 M, pH 7·4; disodium ethylenediaminetetraacetic acid (EDTA), 0·1 M, pH 7·4; neotetrazolium chloride, 1% (w/v) in water; 2-amino-2-hydroxymethylpropane-1·3-diol (tris, recrystallized), 0·1 M, adjusted to pH 7·4 with hydrochloric acid. Vitamin K_3 was added in suspension form prepared as follows: 20 mg. of vitamin K_3 was dissolved in 3 ml. of absolute ethanol; 12 ml. of 0·2% ox-plasma albumin in 0·1 M-Na_2HPO_4–NaH_2PO_4 buffer (pH 7·4) was then added and the resulting suspension was kept cold and protected from bright light. Other reagents are mentioned at the appropriate position in the text.

Except where otherwise indicated in the text, incubations were carried out aerobically at 37° with 0·8–1·0 ml. of stock solution of the following composition: 20 ml. of Na_2HPO_4–NaH_2PO_4 buffer, 2 ml. of sodium succinate and 1 ml. of EDTA, A sample of tissue suspension (0–0·2 ml.) was added to each tube, giving a final pre-incubation volume of 1 ml. The mixture was pre-incubated for (usually) 2 min., after which neotetrazolium chloride (1·5 mg.) was added to each tube and the resulting reaction was finally stopped with 1 ml. of 10% trichloroacetic acid solution. The formazan was extracted with 4 ml. of ethyl acetate and the extinction at 510 mμ was obtained with a Hilger Uvispek spectrophotometer. The incubation time and other additions to the incubation mixture are given in the text.

RESULTS

Preliminary experiments. The relationship between the formazan produced (extinction at 510 mμ) and the amount of tissue added was not linear with rat liver (Fig. 1, curve *a*). Similar results

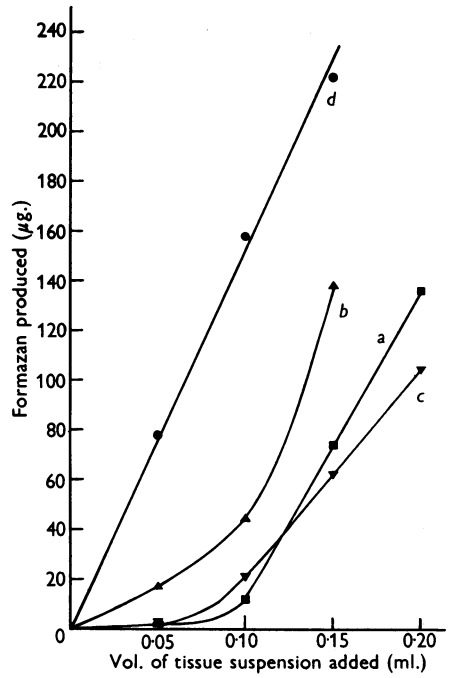

Fig. 1. Formazan production by various tissue suspensions. Assay conditions were as described in the Methods section; pre-incubation time was 2 min. ■, Rat-liver homogenate: 100 mg. (wet wt.) of tissue/ml., incubation time 15 min.; ▲, rat-mammary-gland homogenate: 200 mg. (wet wt.)/ml., incubation time 30 min.; ▼, mouse-liver homogenate: 100 mg. (wet wt.) of tissue/ml., incubation time 10 min.; ●, Keilin–Hartree-type heart-muscle preparation: 13·1 mg. of protein/ml., incubation time 3 min.

were obtained with rat-mammary-gland and mouse-liver suspensions (Fig. 1, curves *b* and *c*, respectively); linearity was obtained under identical conditions with a Keilin–Hartree-type heart-muscle preparation kindly provided by Dr M. B. Thorn (Fig. 1, curve *d*).

The non-linear response found with the above-mentioned types of tissue suspension was not affected by recrystallization of reagents, by varying the ionic strength of the phosphate buffer or by changing the buffer to tris. In attempts to obtain a linear response, tissue suspensions were prepared either in water or in 0·25M-sucrose and were then frozen and thawed five times. Such treatment did not lead to any appreciable increase in formazan production and did not induce linearity of response with increasing amounts of added tissue (Table 1). Homogenization in 0·5M-sucrose or in 0·25M-sucrose containing 0·1M-nicotinamide was also without effect on the non-linear response.

The overall production of formazan was increased by the addition of EDTA to the incubation medium, although EDTA did not affect the non-linear response (Table 1). The addition of 1 mg. or 20 mg. amounts of ox-plasma albumin did not alter the non-linear response (Table 1), although occasionally there was an increase in formazan production.

The addition of riboflavin 5′-phosphate (2 μmoles), lipoic acid (1 μmole) or formazan (0·02 μmole) did not affect the formation of formazan in the presence of fresh homogenate. Preincubation of fresh homogenate with riboflavin 5′-phosphate with or without adenosine triphosphate (ATP) for 20 min. before adding neotetrazolium chloride was without effect.

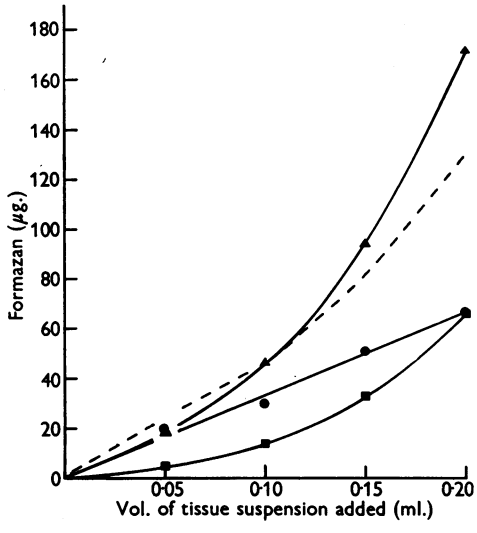

Fig. 2. Formazan production by rat-liver suspensions frozen and thawed three times. Substrates used were ■, succinate (50 μmoles); ●, DPN (2 μmoles) plus β-hydroxybutyrate (10 μmoles); ▲, succinate + DPN + β-hydroxybutyrate. Assay conditions were as described in the text; preincubation time was 2 min., incubation time 10 min. The broken curve is the arithmetical sum of curves —■— and —●—.

Effect of diphosphopyridine nucleotide. With tissue or mitochondrial suspensions, the addition of diphosphopyridine nucleotide (DPN) (0·3–2·0 μmoles) did not stimulate succinate–neotetrazolium reductase. Formazan production was that expected from the sum of the individual contributions from succinate and reduced DPN (DPNH) (Fig. 2). This conclusion applied to fresh, aged (stored overnight at 0°) or frozen and thawed suspensions with various levels of added DPNH or DPN with β-hydroxybutyrate.

Effect of reducing agents. The results of adding cysteine, ascorbic acid or glutathione to the incubation medium are shown in Table 1. Although both cysteine and ascorbic acid lead to an increase in formazan production at low concentrations of tissue, the response remained non-linear.

Effect of metal ions. The addition of trace amounts of heavy-metal ions (copper, zinc) known to form mercaptides with sulphydryl groups produced large inhibition of the succinate–neotetrazolium reductase reaction. For instance, 5 μg. of Cu^{2+} ions caused almost complete inhibition of neotetrazolium reduction (Table 1). Similar amounts of Fe^{2+} ions were without effect (Table 1).

Evidence for cofactors. As the volume of the incubation mixture was decreased, the amount of formazan produced by a given amount of tissue increased (Fig. 3). This behaviour suggested that a cofactor was being diluted out on addition of the homogenate to the incubation mixture. The formazan produced by small amounts of fresh homogenate could be greatly increased by adding small volumes of boiled homogenate (1:10 sucrose homogenates boiled for 5 min., rehomogenized and made up to the original volume with water). Such boiled homogenates possessed very little succinate–neotetrazolium reductase activity (Fig. 4, curve *a*). Boiled homogenates, however, stimulated formazan production in the presence of fresh homogenate, indicating that the boiled homogenate contained a heat-stable cofactor for the reaction (Fig. 4, curves *b* and *c*). The stimulation by boiled homogenate was not marked at low levels of fresh homogenate (see curve *c*, Fig. 4), indicating that a heat-labile factor was also involved and which is supplied by fresh homogenate. The addition of ascorbic acid together with boiled homogenate to fresh homogenate, however, produced a linear response with increasing amounts of fresh homogenate (Fig. 4, curve *d*).

Effect of vitamin K_3. Menadione (vitamin K_3) produced very marked stimulation of the succinate–

neotetrazolium reductase reaction at low final concentrations (mM); the response remained non-linear with increasing amounts of fresh tissue added (Table 1). The stimulation by vitamin K_3 was virtually unaffected by the pre-incubation of homogenate and vitamin K_3 in the absence of neotetrazolium chloride. Evidence indicated a slight but variable increase in formazan production as the time of preincubation was increased up to 40 min. Formazan production by 10 mg. wet wt. of liver tissue was followed with varying preincubation periods and with mM-vitamin K_3 and an incubation time of 10 min. Formazan production after preincubation for 0, 20, 40 and 85 min. was 222, 240, 286, and 187 μg. of formazan, respectively.

The finding that preincubation with added vitamin K_3 did not greatly affect formazan production indicated that no permeability barrier was present between the added vitamin K_3 and the enzyme. This was further indicated by studying the effect of freezing and thawing and of high-speed blending on mitochondrial fractions. Disrupting the mitochondria by these methods did not increase the stimulation of the enzyme reaction by added vitamin K_3 (Table 1). Although vitamin K_3 had little influence on formazan production with small amounts of tissue, the addition of ascorbic acid produced a synergistic effect in that region. Ascorbic acid (1 μmole) and vitamin K_3 (1 μmole) in an incubation volume of 1 ml. led to a linear relationship between the extinction at 510 mμ and the amount of tissue added. For this effect, ascorbic acid was replaceable by cysteine in equivalent concentration. The addition of higher levels of ascorbic acid obscures the synergistic effect in homogenates as a result of the high blanks produced by both an enzymic and a non-enzymic interaction of neotetrazolium chloride and ascorbic acid; such blanks are very much smaller when the enzyme sample used is a mitochondrial suspension.

DISCUSSION

Since the succinate–neotetrazolium reductase enzyme sequence is associated with the mitochondrial fraction in rat-liver and rat-mammary-gland suspensions (Slater & Planterose, 1960) it is surprising that disruption of the mitochondria by a

Table 1. *Effects of various treatments and additives on formazan production by tissue suspensions*

Control samples were assayed as described in the Methods section except for results with EDTA and ox-plasma albumin; in these instances EDTA was omitted from the stock mixture used in the controls. All additions were made in 0·1 ml.; pre-incubation time was 2 min. Total volume of incubation mixture was 1·25 ml.

Tissue	Addition or treatment	Amount added	Incubation time (min.)	Formazan produced at three tissue concentrations (μg.) 5 mg.	10 mg.	15 mg.
Mouse liver	Tris buffer	—	10	4	23	64
	Untreated	—	10	4	17	51
	Freezing and thawing	—	10	12	26	55
	Untreated	—	10	15	26	78
Rat liver	EDTA	20 μmoles	10	15	39	67
	EDTA	150 μmoles	10	18	50	108
	Ox-plasma albumin	2 mg.	10	15	35	75
	Untreated	—	10	15	40	64
Rat liver	0·5 M-Sucrose	—	10	8	28	78
	0·25 M-Sucrose + 0·1 M-nicotinamide	—	10	7	29	54
	Untreated	—	10	7	27	59
Rat liver	Vitamin C	1 μmole	5	14	29	57
	Cysteine	1 μmole	5	10	21	43
	Glutathione	1 μmole	5	3	15	49
	Untreated	—	5	3	9	42
Rat liver	Cu^{2+} ions	0·5 μg.	10	7	23	48
	Cu^{2+} ions	5·0 μg.	10	4	7	12
	Fe^{2+} ions	5·0 μg.	10	8	28	40
	Untreated	—	10	11	26	45
Rat-liver mitochondria	Vitamin K_3	1 μmole	10	32	240	504
	Vitamin K_3 with freezing and thawing	1 μmole	10	19	209	360
	Untreated	—	10	10	23	37
Rat liver	Vitamin K_3	1 μmole	5	4	155	326
	Vitamin C	1 μmole	5	14	29	54
	Cysteine	1 μmole	5	10	21	44
	Vitamin K_3 + vitamin C	1 + 1 μmole	5	94	235	348
	Vitamin K_3 + cysteine	1 + 1 μmole	5	115	187	312
	Untreated	—	5	3	9	42

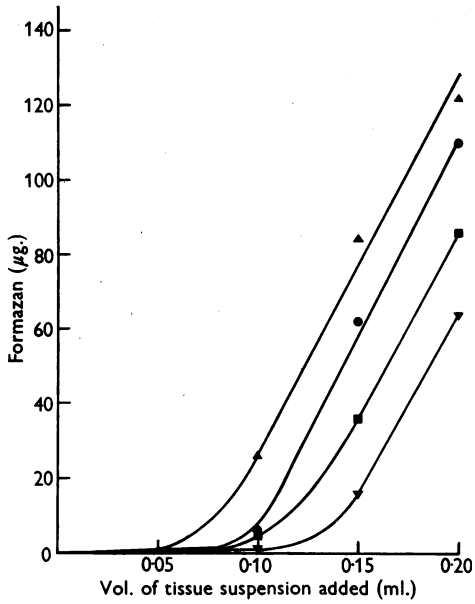

Fig. 3. Formazan production in the presence of varying volumes of stock mixture. Preincubation time was 2 min., incubation time 10 min.; 1·5 mg. of neotetrazolium chloride was present in each tube; rat-liver homogenate (1:10) was made in 0·25 M-sucrose. Curves ▲, ●, ■, ▼ were obtained with 1, 2, 3 and 4 ml. of stock solution, respectively.

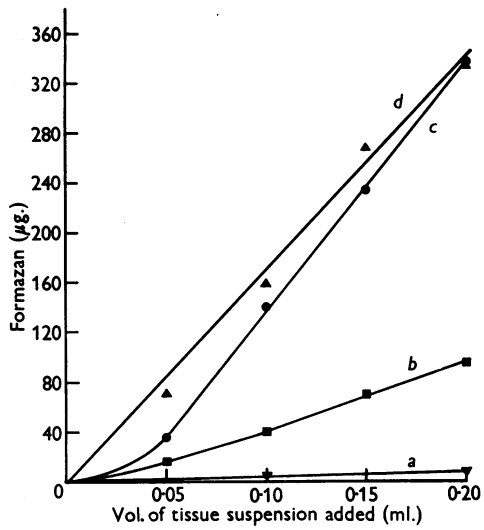

Fig. 4. Formazan production by rat-liver suspension (1:5) in 0·25 M-sucrose. Assay conditions were as described in the text; preincubation time was 2 min., incubation time 15 min. ▼, Activity of boiled homogenate; ■, activity of fresh homogenate; ●, activity of fresh homogenate + 67 mg. wet wt. of boiled tissue; ▲, as for ● but with 2 μmoles of vitamin C.

variety of treatments does not increase formazan production. This suggests that there is no permeability barrier to neotetrazolium chloride. Disruption of the mitochondrial fraction does not affect the manometric assay of succinic oxidase, or the reduction of neotetrazolium chloride with succinate as substrate (Shelton & Rice, 1957).

It was considered possible that the non-linear response might be overcome by adding large quantities of an enzymically inactive protein, i.e. ox-plasma albumin. This could fulfil three functions: (*a*) as a general remover of contaminants such as metal ions by adsorption; (*b*) as a stabilizer for small amounts of homogenate in relatively large volumes of stock solution; (*c*) as a source of trace quantities of certain cofactors (see e.g. Nason, Averbach & Terrell, 1956). As indicated by the results of this investigation, however, none of these factors appears responsible for the non-linear response. Similarly, the addition of EDTA was ineffective in improving the non-linear response. EDTA did, however, increase formazan production and this effect may be ascribed to the removal of metal ions (i.e. copper) which were shown to inhibit the reaction.

The non-linear response could be the result of contaminants in the experimental system used or the effect produced by diluting an essential cofactor. The latter alternative would appear to be the operative one for the following reasons. First, recrystallization of reagents, change of buffer, change in ionic strength of the incubating solution and addition of EDTA did not produce a linear response, which was, however, obtained with a Keilin–Hartree preparation under identical experimental conditions. Secondly, decreasing the volume of the incubation medium in the presence of a constant amount of tissue increased the amount of formazan produced. This increase was most marked when small amounts of tissue were used. This indicates that the final dilution of the tissue suspension is important, and contrasts with the behaviour of similar tissue suspensions used in the manometric assay of succinic oxidase where oxygen uptake increases linearly with increasing amounts of tissue. Thirdly, boiled homogenate, which was relatively inactive itself in the experimental system, greatly stimulated the activity of small quantities of fresh homogenate. This evidence suggests that the succinate–neotetrazolium reductase system is sensitive to dilution and contains a heat-stable cofactor essential for maximum activity.

The above-mentioned evidence for a cofactor in succinate–neotetrazolium reductase is in agreement with previous reports on the coupling of succinate oxidation with tetrazolium reduction. Bril (1954) showed that the activity of a homogenate decreased on prolonged dialysis and that after differential

centrifuging full activity could be obtained only by recombining the mitochondrial and soluble fractions. Bril's work therefore suggests that the enzyme system contains a water-soluble cofactor which, on homogenizing and centrifuging, is largely found in the soluble fraction. Sprinz & Waldschmidt-Leitz (1953) showed that boiled homogenate stimulated tetrazolium reduction by fresh tissue although they did not report on the activity of the boiled extract by itself. Sugimura & Ono (1956) have reported the presence of a cofactor in hot-water extracts of pigeon-breast muscle.

In contrast with these reports several investigations have indicated that succinate–neotetrazolium reductase was insensitive to dilution (Shelton & Rice, 1957; Kun & Abood, 1949). These workers reported a linear increase in formazan production with increasing amounts of tissue added; Kun & Abood (1949), however, obtained a very inefficient production of formazan. It was found possible to obtain a similar linear response by using a very dilute homogenate (0–0·5 ml. of 1:50 liver or mammary-gland homogenate) and a long incubation time; in these instances, also, formazan production was extremely inefficient. This suggests the possibility that neotetrazolium chloride can be reduced by two pathways, one of which is inefficient and insensitive to dilution, the other being much more efficient in terms of μg. of formazan produced/mg. of tissue, but is correspondingly affected by dilution. This paper is concerned solely with the second possible pathway of reduction.

Various attempts to elucidate the general nature of the heat-stable cofactor are reported in the Results section. An obvious possibility was flavine mononucleotide (FMN) since several reports have shown that tetrazolium reduction occurs in many systems via a flavoprotein enzyme (Brodie & Gots, 1951; Kun, 1951; Shelton & Schneider, 1952). However, FMN with or without preincubation with ATP and homogenate was without effect on the non-linear response. Similarly, low concentrations of iron and copper which are often found in essential association with flavoenzymes (Nicholas, 1957) did not induce a linear response, but copper, on the contrary, caused intense inhibition. Metal-ion inhibition is often the result of combination between the metal ion and essential sulphydryl groups. Both ascorbic acid and cysteine, which are effective in protecting sulphydryl groups against metal-ion inhibition and oxidation, caused considerable increase in formazan production; glutathione, very surprisingly, did not produce this effect. The combination of reducing agent and boiled homogenate produced a linear response with increasing amounts of fresh homogenate. This suggests the possibility that the non-linear response is the result of a dilution of a heat-stable cofactor found in boiled homogenate and a heat-labile cofactor which protects the sulphydryl groups of the enzyme system. The heat-labile factor can apparently be replaced by ascorbic acid or cysteine.

Vitamin K_3 stimulates the succinate–neotetrazolium reductase system above a certain limiting concentration of tissue below which it has little effect. This again suggests the possibility that two factors are operative in producing the non-linear response. Since the stimulation produced by vitamin K_3 is remarkably similar to that produced by boiled homogenate, it is possible that vitamin K_3 is acting in a similar fashion to the heat-stable factor mentioned previously. It is also possible that vitamin K_3 is creating an entirely artificial electron pathway of its own. Quinones have become increasingly associated with pathways of electron transfer. In particular, several reports have indicated a role for a quinone in the respiratory chain (Colpa-Boonstra & Slater, 1958; Martius, 1954). Investigations on the pharmacological activity of several quinones (Herz, 1954) have shown that some possess a stimulatory effect on tetrazolium reduction. Vitamin K_3 is heat-stable, and although it is concentrated in the particulate fractions of the cell (Martius, 1956) it does not appear to be strongly bound to the mitochondria as does vitamin K_1 (Martius & Nitz-Litzow, 1954; Schulz & Goss, 1956). As the effects produced by boiled homogenate and vitamin K_3 are similar, and as these effects are similarly affected by cysteine or ascorbic acid, it seems possible that the heat-stable cofactor present in boiled homogenates is a quinone replaceable by vitamin K_3. From the indications that a quinone group and sulphydryl groups are essential for an efficient production of formazan, it is worth noting that 2-substituted naphthaquinones like vitamin K_3 react readily with sulphydryl compounds to give 2:3-disubstituted naphthaquinones (Thomson, 1957). The natural 2:3-disubstituted naphthaquinone vitamin K_1, which does not react with sulphydryl compounds, is inactive in stimulating succinate–neotetrazolium reductase (unpublished results).

The influence of added DPN is of special interest in view of several reports about its possible participation in succinate–neotetrazolium reductase. Sprinz & Waldschmidt-Leitz (1953) showed that liver homogenates could catalyse formazan production in the presence of relatively large amounts of DPN (approximately 3 mM); similarly, both Barker (1953) and Zöllner & Rothemund (1954) showed that formazan production in homogenates was stimulated by added DPN. Bril (1954) reported that succinate–neotetrazolium reductase decreased on dialysis but that formazan production was restored at least partly by added DPN. Sourkes & Lagnado (1957), using washed mitochondria or

solubilized enzyme systems from rat liver, have shown that certain purines including DPN act as cofactors in succinate–neotetrazolium reductase; small amounts of added DPN (0·1–0·3 μmole) stimulated formazan production in the presence of succinate some five-fold. Zöllner & Rothemund (1954), however, reported no synergistic stimulation of formazan production when DPN was incubated in the presence of succinate anaerobically.

In the experiments reported in this paper there was no synergistic stimulation of succinate–neotetrazolium reductase by added DPN. DPN in the presence of suitable substrate (endogenous or added β-hydroxybutyrate) produces DPNH which then led to formazan production. DPNH does not reduce neotetrazolium chloride directly (Slater, 1959*a*). The joint addition of DPNH and succinate did not lead to any significant extra formazan formation over that expected from the sum of the individual contributions alone (Fig. 2). This conclusion applied to fresh homogenate and fresh mitochondrial suspensions, to homogenates and mitochondrial suspensions which had been frozen and thawed, and to homogenates and mitochondrial suspensions which had been aged at 0° or at 37° in the absence of added substrate, i.e. succinate or DPN under aerobic conditions.

It appears therefore that DPNH does not stimulate succinate–neotetrazolium reductase under aerobic conditions. It seems likely that the formazan produced by DPNH is formed by DPNH oxidation via an enzyme of a type similar to, if not identical with, the quinone reductase described by Martius & Strufe (1954); this type of enzyme is known to be associated with the particulate material of the cell (Slater, 1959*b*).

SUMMARY

1. The enzyme system linking succinate oxidation with neotetrazolium reduction has been investigated in rat-liver homogenates. In unsupplemented homogenates the production of formazan did not increase proportionately with increasing amounts of tissue. This non-linear response with succinate as substrate was unaffected by: freezing and thawing the suspension; high-speed blending; homogenizing in hyper-osmotic sucrose; homogenizing in the presence of nicotinamide; addition of EDTA, ox-plasma albumin, lipoic acid or riboflavin 5′-phosphate to the incubation medium.

2. The variations produced in formazan production (*a*) on varying the volume of the incubation medium and (*b*) on adding boiled homogenate to the incubation medium suggested that a water-soluble cofactor was involved.

3. Formazan production was stimulated by the addition of vitamin K_3 to the incubation medium; this stimulation was, however, small with low final concentrations of tissue.

4. Ascorbic acid or cysteine synergistically increased formazan production in the presence of vitamin K_3 especially with low final concentrations of tissue.

5. It is suggested that for optimum formazan production a heat-stable cofactor and reduced sulphydryl groups are required. The heat-stable cofactor appears to resemble vitamin K_3 but not vitamin K_1 in its properties; ascorbic acid and cysteine appear to function in the system by maintaining sylphydryl groups in the reduced form.

6. Diphosphopyridine nucleotide does not appear to be directly involved in the succinate–neotetrazolium reductase system.

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