A Cytochemical Study of the Dehydrogenases of Mitochondria and Mitochondrial Particulates by a Monotetrazolium-Cobalt Chelation Method

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ABSTRACT

In one of the current histochemical methods for dehydrogenases and diaphorases the final product is a metal-formazan dye derived from reduction of an N-thiazolyl-substituted tetrazolium. Sites of enzymic activity consistently appear as intramitochondrial dots 0.2 to 0.3 μ in diameter. When applied to active particles from disrupted mitochondria (Keilin-Hartree preparation, electron transport particle, Cooper-Lehninger particle) the individual particles appear as black dots 0.1 to 0.3 μ in diameter. It is clear that formazan is deposited progressively upon the particles and the results suggest that the latter may be spatially arranged in mitochondria so that areas of activity are separated by quiescent regions.

The monotetrazolium-cobalt chelation method (19), applied to the cytochemical localization of succinic dehydrogenase in thin tissue sections and isolated mitochondria, consistently results in the deposition of a series of 2 to 5 intramitochondrial formazan granules measuring 0.2 to 0.3 μ in diameter (19, 20). Subsequent cytochemical studies of various pyridine nucleotide-linked dehydrogenases in tissue sections by Hess, Scarpelli, and Pearse (10, 11) have shown a similar pattern of enzyme localization.

These findings suggested the possibility that the metal formazan method might demonstrate units of enzyme activity related to the minute enzymically active particles derived from disrupted mitochondria by Keilin and Hartree (16), Green (8), and by Cooper and Lehninger (6). These particles, though far smaller than mitochondria, are still capable of integrated biochemical function and they are considered to be elementary units consisting of organised components of the respiratory chain (Green, 8).

In the present communication chemical and morphological evidence will be given to show that the intramitochondrial cobalt-formazan deposits could represent sites of enzymatic activity localized on the particles of the Keilin-Hartree preparation, on the electron transfer particle (ETP) described by Crane, Glenn, and Green (7), and on the particulate enzyme complex capable of oxidizing D(−)-β-hydroxybutyrate and succinate, isolated by Cooper and Lehninger (1956).

Materials and Methods

Sarcosomal and Mitochondrial Preparations:

Sarcosomes.—These were prepared from the hearts of hooded rats according to the method described by Cleland and Slater (5) using 0.32 M sucrose.

Mitochondria.—These were prepared from hooded rat livers according to the method described by Hogeboom, Schneider, and Palade (12) using 0.88 M sucrose.

Preparation of Mitochondrial Fragments:

Keilin and Hartree Preparation.—Heart muscle preparation was prepared by the method of Keilin and Hartree (16).

Electron Transfer Particle (ETP).—The ETP was

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prepared essentially by the method described by Crane, Glenn, and Green with the exception that hearts from hooded male rats weighing between 200 to 250 gm. were used instead of beef heart. Starting material consisted of 10 rat hearts weighing 0.5 to 0.8 gm. each. Since this was approximately one-hundredth the amount of tissue recommended for the preparation of ETP, all volumes of solutions were modified accordingly.

Particulate Enzyme Complex.—A particulate mitochondrial extract capable of oxidizing both D(−)-β-hydroxybutyrate and succinate was prepared from the livers of well fed hooded male rats weighing 200 to 250 gm. as described by Cooper and Lehninger (6). These preparations were used for the following biochemical and morphological studies.

Measurement of Enzyme Activities

The enzymic activities of sarcosomes, mitochondria, and their fragments were measured by the method of Kun and Abood (17).

The contents of a reaction flask were as follows: 0.1 ml. of a 10 per cent sarcosomal or mitochondrial suspension, or 0.1 ml. of a particulate preparation was added to 1 ml. of the following media:

Succinic Dehydrogenase (SD).—3,5-diphenyl-2-(4,5-dimethylthiazol-2-yl) tetrazolium MTT 0.001 M; aminotrishydroxymethylmethane (tris) buffer, 0.2 M, pH 6.8; aluminum chloride, 0.001 M; cobaltous chloride 0.005 M; succinate, 0.01 M.

Diphosphopyridine Nucleotide Diaphorase (DPND) and Triphosphopyridine Nucleotide Diaphorase (TPND).—MTT 0.001 M; tris buffer 0.2 M, pH 7.2; cobaltous chloride 0.005 M; DPNH or TPNH 0.01 M.

β-Hydroxybutyric Dehydrogenase.—MTT 0.001 M; tris buffer 0.2 M, pH 6.8; cobaltous chloride 0.005 M; DPN 0.01 M; β-hydroxybutyric acid sodium salt 0.01. The final pH of this solution was readjusted to pH 6.8 by additional tris buffer.

To each flask 0.01 M sodium azide was added prior to incubation at 37.5°. The reaction was stopped, and the protein precipitated, by addition of 0.5 ml. of saturated ammonium sulfate. The formazan was extracted with ethyl acetate and the optical density measured in a Unicam spectrophotometer model S.P. 500 at a wave length of 660 mμ where maximum extinction of the cobalt-formazan compound was observed. A calibration curve was prepared by reducing various concentrations of cobalt-tetrazolium medium with an excess of 0.1 M ascorbic acid. Optical density is directly proportional to the concentration of formazan (Fig. 1). Duplicate samples of sarcosomal, mitochondrial, and particulate suspensions were used for nitrogen determination from which the specific activities of the various dehydrogenase preparations were calculated. The specific activity was expressed as micrograms of formazan formed per minute per milligram of protein.

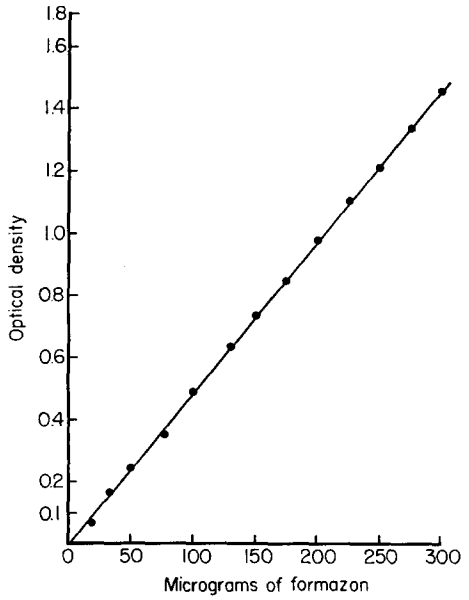

FIG. 1. Concentration curve of an ethyl acetate solution of cobalt-formazan chelate showing a linear relation between concentration of formazan and optical density.

Morphological Studies

Duplicate samples of these preparations were incubated in identical media and mounted in 15 per cent gelatin for microscopic study. These were examined under oil immersion with a Leitz dialux microscope at an initial magnification of × 1895 and enlarged photographically thereafter.

Measurement of Particles.—The size of particles in the various sarcosomal and mitochondrial preparations was measured from the original photographic negatives following enlargement to a total magnification of 10,000 in an enlarger. Since there was marked aggregation of the particles, only a few in each photograph were resolvable as single particles and were therefore suitable for measurement. Sufficient photographs were taken so that 100 individual particles were measured from each of the preparations. Duplicate unreacted samples from each preparation were also examined under the phase contrast microscope. To determine the particle size of formazan produced by non-enzymic reduction of monotetrazolium salt, a control experiment was performed as follows: A 0.001 M solution of MTT in 15 per cent gelatin was allowed to gel in a Petri dish. This was reduced by the addition of a 0.01 M solution of ascorbic acid buffered at pH 8.5. The formazan suspension in gelatin was allowed to gel and the resulting formazan particles were measured as described above.

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RESULTS

Enzyme Activities of Sarcosomes and Liver Mitochondria:

In most instances sarcosomes and liver mitochondria oxidized the substrates tested at similar rates as shown in Table I. Liver mitochondria, however, oxidized D(−)-β-hydroxybutyrate at twice the rate and TPNH at four times the rate observed for sarcosomes.

Enzyme Activities of Sarcosomal and Mitochondrial Fragments:

In most instances the enzymic activity of sarcosomal and mitochondrial fragments was increased as compared to the intact organelles from which they were derived. Table II shows the specific activities of the various sarcosomal and mitochondrial particle preparations, with reference to the oxidation of succinate, the D(−)-β-hydroxybutyrate, and DPNH and TPNH.

Keilin-Hartree Preparation.—Both succinate and DPNH were oxidized at considerable rates by the Keilin-Hartree heart muscle preparations. The more rapid oxidation of DPNH, as measured by the reduction of tetrazolium salt, compares favourably with the manometric data published by Slater (22). TPNH was oxidized at approximately one-half the rate of succinate and at one-fourth the rate of DPNH oxidation. D(−)-β-Hydroxybutyrate was oxidized very slowly. Aliquots of the preparations inactivated by boiling and incubated with the D(−) isomer, failed to reduce tetrazolium salt.

Electron Transfer Particle (ETP).—ETP preparations oxidized DPNH at approximately four times and TPNH at two and one-half times the rate observed for succinate.

D(−)-β-hydroxybutyrate was oxidized at one-half the rate of succinate oxidation.

Cooper-Lehninger Particle.—These preparations showed a high enzyme activity for each of the enzymes tested and were the only ones capable of rapidly oxidizing D(−)-β-hydroxybutyrate. TPNH was oxidized only slightly less rapidly than DPNH.

Particle Size and Morphology of Sarcosomes and Liver Mitochondria:

Sarcosomes examined under the phase contrast microscope appeared as spheres which measured 1 to 2 μ in diameter. When these were treated with osmium tetroxide they appeared as black granules of the same size, though many smaller granules measuring less than 1 μ were also seen. Sarcosomes incubated for 10 minutes in media containing tetrazolium salt measured 1 to 2 μ, while incubation periods of 30 minutes or more resulted in marked agglutination and swelling of the organelles, some of which measured as much as 4 μ.

Liver mitochondria appeared as short rods measuring 2 to 5 μ in length under the phase contrast microscope. Osmium tetroxide treatment stained these rods black, did not precisely change the size or shape, but caused considerable agglutination. Incubation of mitochondria with tetrazolium salt and various substrates localized sites of enzyme activity as 3 to 5 formazan deposits measuring 0.2 to 0.3 μ in diameter distributed along the course of the organelle, and these were separated from each other by intervals of 0.2 to 0.4 μ. Incubation periods longer than 25 minutes resulted in extensive swelling so that some

TABLE I

Specific Activities of Sarcosomes and Liver Mitochondria

(Specific activity was measured spectrophotometrically and is expressed as micrograms of formazan formed per minute per milligram of protein. For experimental conditions, see text.)

Substrate	Sarcosomes	Liver mitochondria
Succinate (10^{-1} M)	2.2	3.7
D(−)-β-hydroxybutyrate (10^{-1} M)	1.8	4.2
DPNH (10^{-2} M)	5.4	6.1
TPNH (10^{-2} M)	0.9	4.8

TABLE II

Specific Activities of Sarcosomal and Mitochondrial Particulates

(Specific activity was measured spectrophotometrically and is expressed as micrograms of formazan formed per minute per milligram of protein. The results of two experiments are given.)

Substrate	Keilin-Hartree		ETP		Cooper-Lehninger	
Succinate	13.9	11.0	7.2	5.7	14.9	12.5
D(−)-β-hydroxybutyrate	0.1	0.2	3.0	2.4	31.6	35.7
DPNH	24.4	19.2	31.2	23.5	62.5	66.6
TPNH	6.0	5.0	18.0	13.3	55.2	54.6

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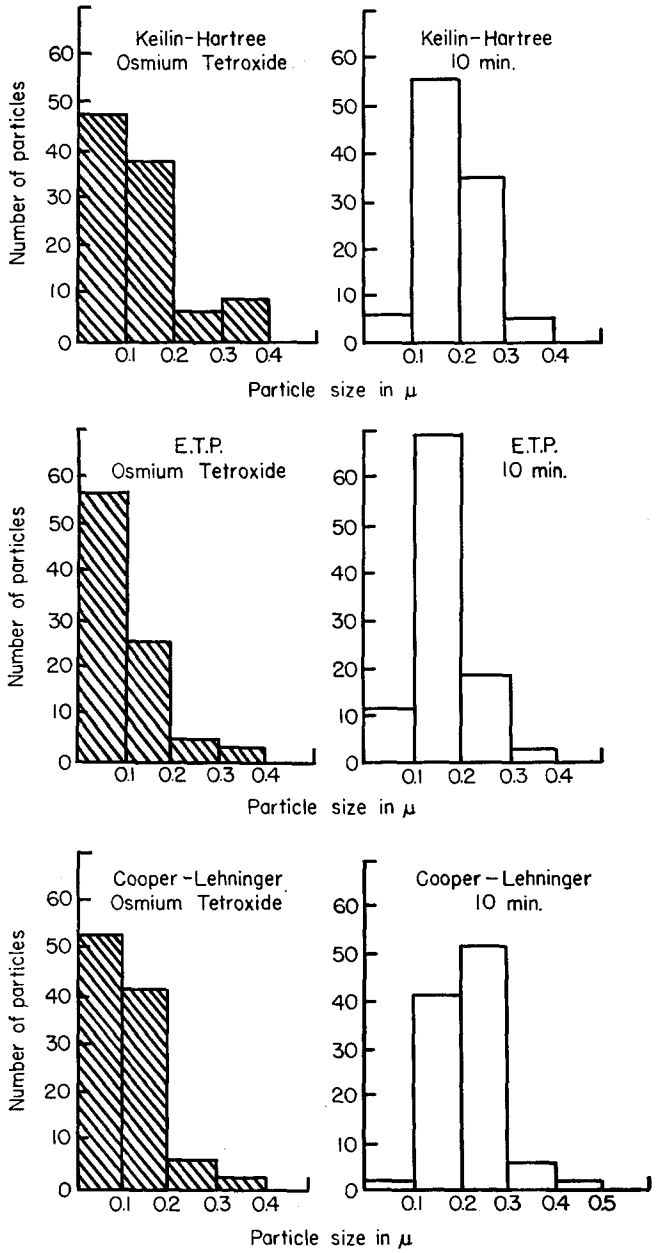

FIG. 2. Particle size of various sarcosomal and mitochondrial fragments. 0.1 ml. of a particle preparation was added to 1 ml. of either 1 per cent osmium tetroxide or DPNH-containing media as described in text. Osmium tetroxide (cross-hatched areas) and DPND reaction (clear areas).

of the organelles became globose bodies measuring 3 to 6 μ in diameter. This process causes a reduction in the absolute number of formazan particles present.

Particle Size and Morphology of Sarcosomal and Mitochondrial Fragments:

The majority of sarcosomal and mitochondrial fragments were not resolvable as single particles

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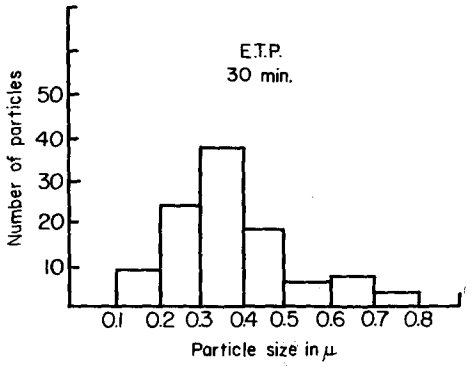

FIG. 3. The effect of incubation time on the particle size of ETP following incubation with tetrazolium and DPNH.

when examined under the phase contrast microscope, although not infrequently particles measuring up to 0.1 to 0.2 μ were observed. These stained readily with osmium tetroxide and tended to agglutinate, although numerous individual spherical particles could be seen. The majority of these (Fig. 2) measured 0.1 to 0.2 μ and were just within the limit of resolving power of the microscope. Occasionally, particles measuring up to 0.5 μ in diameter were observed. After 10 minutes incubation with tetrazolium salt and various substrates the majority of particles measured 0.2 to 0.3 μ. When the incubation time of the ETP (Fig. 3) was lengthened to 30 minutes the average size increased to 0.3 to 0.6 μ with some particles as large as 0.8 μ. [The formazan resulting from the non-enzymic reduction of tetrazolium appeared as a diffuse purple-black suspension.] Individual deposits were not resolvable under the light microscope. This suggests that if such deposits were present they measured less than 0.1 μ.

DISCUSSION

The experiments indicate that sites of oxidative enzyme activity can be localized in or on sarcosomes and mitochondria when an N-thiazolyl–substituted monotetrazolium salt is used as the electron acceptor, followed by simultaneous chelation of cobalt by the resulting formazan. The localization of enzyme activity on the various submitochondrial particulates supports the view expressed by Cleland and Slater (5) that some of the oxidative enzymes are intimately associated with the membranous components of the mitochondrion. Recent electron microscopic studies by Watson and Siekevitz (23) have shown that portions of cristae mitochondriales are present in submitochondrial fragments isolated from deoxycholate-treated mitochondria.

The ratios of the specific activities of the various submitochondrial particles in the oxidation of succinate, β-hydroxybutyrate, and DPNH and TPNH compare favourably with biochemical data published by Green (8) and Cooper and Lehninger (6). A comparison of individual values, however, shows that those obtained with the tetrazolium reaction indicate approximately one-tenth of the activity obtained when oxygen uptake is measured. This discrepancy may be due to a direct effect of the tetrazolium salt on the various enzyme systems, since it has been shown by Lettré and Albrecht (18) and by Jerchel and Fischer (13) that some tetrazolium salts are highly toxic, both to fibroblasts in tissue culture and to intact animals.

Recently, Guiditta and Singer (9) have shown, in a variety of particulate preparations from heart muscle, that the relative activities for succinate oxidation vary considerably with different electron acceptors. They tested methylene blue, brilliant cresyl blue, ferricyanide, indophenol, and phenazine methosulphate, but not tetrazolium salts.

The slight β-hydroxybutyrate dehydrogenase activity found in the Keilin-Hartree preparation was less than 1 per cent of that of succinic dehydrogenase. This magnitude of activity is near the limit of sensitivity of the Warburg manometer and may account for the failure to demonstrate β-hydroxybutyric dehydrogenase activity in Keilin-Hartree preparations by manometric techniques. In addition, since the modern tetrazolium salts used in histochemistry can accept electrons directly from flavoproteins (2, 3), damage to the terminal components of the electron chain, if it occurs, is of no consequence. Our results show that monotetrazolium salts may be used for comparative though not for absolute quantitative measurements, as well as for qualitative microscopical studies of dehydrogenase activity.

Following treatment with osmium tetroxide submicroscopical particles were similar in size, regardless of source or mode of preparation. However, following incubation with the monotetrazolium salt and appropriate substrates, individual particles which were not resolvable with the light microscope prior to the reaction

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showed an average increase in size to approximately 0.1 μ and became visible. When incubation was lengthened to 30 minutes individual ETP particles increased in size up to 0.8 μ. This increase probably represents the combination of progressive deposition of formazan and of swelling of the particle. The increase from 0.1 to 0.8 μ represents a 512-fold increase in volume, since the effects of aggregation of particles can be excluded with reasonable certainty. From the figures given in Table II it can be calculated that, under the conditions used, deposition of dye could not be expected to increase the average particle size to more than 0.2 μ.

The dimensions of various submitochondrial particles suggest that they may be basically similar regardless of source or mode of preparation. This conforms to the views held by Keilin and Hartree (15), Cleland and Slater (5), and Green (8) that submitochondrial particles represent an elementary respiratory particle consisting of an integrated system capable of electron transfer from various substrates to molecular oxygen. At present little is known about the intramitochondrial distribution of these particles. Whether the intramitochondrial distribution of formazan deposits which we constantly observe represents a spatial organization of active foci of enzymes or is an artifact of the method remains to be determined. If the latter, the regularity of the artifact requires explanation.

Our results suggest that the intramitochondrial formazan deposits observed in mitochondria following enzymic reduction of the thiazolyl monotetrazolium salt could correspond to submitochondrial particles in or on which oxidative enzyme systems are localized, and that in the intact mitochondrion, therefore, active and inactive areas may alternate. However, the absolute establishment of the identity of the deposits must await further investigations. It is clear from our results that in performing cytochemical reactions for the demonstration of submicroscopic foci of enzyme activity, any incubation period which results in the formation of a particle visible under the light microscope is excessive. This is because the reaction product, when it becomes sufficiently large to be visible microscopically, necessarily constitutes a gross artifact. This particular difficulty is ultimately surmountable only by the development of techniques whose reaction product has sufficient electron opacity to be distinguishable under the electron microscope. Recently Sedar and Rosa (21) have reported encouraging results in the sarcosomal localization of succinic dehydrogenase activity within the electron microscope using 2,2′-di-(*p*-nitrophenyl)-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene) ditetrazolium chloride (nitro blue tetrazolium) as the electron acceptor and similar studies have been reported by Karmarkar, Barrnett, Nachlas, and Seligman (14). Studies which we carried out with *E. coli* and *B. subtilis* showed that the cobalt formazan from 3,5-diphenyl-2-(4,5-dimethylthiazol-2-yl) tetrazolium bromide was visible with the electron microscope after a few seconds incubation in the substrate medium.

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