METABOLISM IN INFECTION: STUDY ON THE ENZYMATIC DAMAGE IN KIDNEY OF GUINEA PIG INFECTED WITH MYCOBACTERIUM TUBERCULOSIS*

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Experimental infections with *Mycobacterium tuberculosis*, strain H 37 Rv or BCG reduce the succinic dehydrogenase activity of the kidneys of guinea pigs, although no focal microscopic lesions occur in the organ (1, 2). This biochemical lesion is not specific for the tuberculous infection but occurs in tuberculin shock and chronic diseases of diverse microbial etiology which show the same type of host response (3). The metabolic lesion can be reversed *in vitro* by a nucleotide-like substance present in many tissues (4). A similar loss of enzyme activity occurs after the administration of the cord factor from the tubercle bacilli in the rats (5).

The present studies extend the observations to malic dehydrogenase system and electron transport. By the use of this enzyme system it is possible to localize better the defects at the level of dehydrogenation of the substrate or at another level in the electron system.

Methods and Materials[1]

Male albino guinea pigs weighing 250 to 350 gm were used in this study. The animals maintained on Purina guinea pig chow were infected intraperitoneally with 0.1 ml of 7-day-old culture of *M. tuberculosis* var. H 37Rv in Dubos media (6). Uninfected animals maintained in the same way as the infected series served as controls. At the end of the fourth week, the infected animals were killed by decapitation, the kidneys were quickly removed, chilled in isotonic potassium chloride solution, and were subsequently studied as outlined in the following.

Spectrophotometric Measurement of Malic Dehydrogenase Activity of Kidney Homogenate.— The cortical tissue of kidney was homogenized as previously described (1). The protein content of each specimen was determined by the biuret reaction (7) and the tissue concentration was adjusted to a value equivalent to 100 mg per ml homogenate. The homogenate containing 66 μg of kidney tissue was added to 0.05 M glycine buffer (pH, 10.2) containing 50 μmoles

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[1] The abbreviations used are: DPN and DPNH, the oxidized and the reduced forms of diphosphopyridine nucleotides, respectively; p-CMB, para-chloromercurobenzoate; PMC, phenazine methochloride; Tz, neotetrazolium chloride; MDH, malic dehydrogenase.

malate and 1.5 μmoles of DPN. The change in the optical densities at 340 mu of the solution at the room temperature was measured using 1 cm light path cuvettes in a Beckman DU spectrophotometer. The results were expressed as μ moles of DPN per 100 mg tissue. This was calculated from the molar extinction coefficient of reduced DPN (DPNH) at 340 mμ (8).

Colorimetric Measurement of Malic Dehydrogenase in Kidney Homogenate.—Homogenate equivalent to 20 mg of kidney tissue was added to a 1/15 M phosphate buffer (pH 7.4) containing 3 μmoles DPN, 100 μmoles malate and 0.5 μmoles neotetrazolium chloride (Bios) in a final volume of 1 ml. This was incubated in conical centrifuge tubes at 37°C. The reaction was stopped by the addition of 1 ml of glacial acetic acid and the formazan produced was extracted with 5 ml of acetone. The optical density of the clear acetone extracts was measured at 540 mμ in a Coleman 6D spectrophotometer. The results were expressed as micromoles of tetrazolium according to the method of Chatterjee *et al.* (9). A solution containing 0.5 μg of phenazine methochloride (Sigma) per ml of potassium cyanide (10^{-3} M) was used in specified experiments. Cyanide had no direct effect on the reduction rate of Tz but was necessary to maintain reproducible rates with PMC in the reacting mixtures. Niacin in amounts indicated under appropriate tables was dissolved in M/15 phosphate buffer and tested as indicated.

In measuring the DPNH dehydrogenase system in the homogenate of the kidney, 3.0 uM of DPNH in 1 ml of 1/15 M phosphate buffer was used instead of DPN and malate. The mixtures were incubated at 37°C. At the end of the incubation the procedure for the extraction of formazan described above was followed.

Oxidative Phosphorylation by the Kidney Mitochondrial Suspension.—Mitochondrial suspension was prepared according to the technique of Hogeboom *et al.* (10). The required aliquot, equivalent to 70 to 75 mg of kidney tissue, was incubated in a reacting mixture prepared in 0.075 M tris buffer (pH 7.4). Each ml of buffer contained 3 μmoles adenosine triphosphate (ATP), 15 μmoles niacin, 10 μmoles inorganic phosphate, 25 μmoles of β-hydroxybutyric acid, 15 μmoles DPN, 5 μmoles each of potassium fluoride and magnesium sulphate, 150 μmoles of glucose and 5 mg hexokinase, type II (Sigma). The mixtures were incubated at 30°C in a Dubnoff metabolic shaker. The reactions were stopped at the specific times by the addition of chilled 10 per cent trichloroacetic acid. The inorganic phosphate contents of the protein-free supernates were estimated by the method of Taussky and Shorr (11). The decrease in the phosphate values were expressed as μmoles of inorganic phosphate esterified per 100 mg kidney tissue.

DPNase Activity of Kidney Homogenate.—The kidney cortex from the normal and the tuberculous guinea pigs were homogenized in 0.25 M sucrose solution and centrifuged at 600 × *G* for 10 minutes. The protein content of the supernate was estimated by the biuret test (7) and was adjusted to a tissue content of 200 mg per ml. All operations were carried out at 2°C. The DPNase activity of the homogenate was measured according to the method of Kaplan (12). A 5 ml reacting mixture was used. Each ml contained 6.5 μmoles DPN and 20 mg tissue in 0.06 M phosphate buffer (pH 7.2). Mixtures were incubated at 37°C for 1 hour. Aliquots removed at the beginning and the end of the incubation and were deproteinized with 0.5 M perchloric acid. The precipitated protein was sedimented by centrifugation and the supernatant was neutralized to pH 6.5 with 5 N potassium hydroxide. The clear supernatant fluid was used for the estimation of the DPN content.

Estimation of DPN.—The quantity of DPN present in the neutralized supernatant was assayed chemically (13) and enzymatically (14). In the chemical method 0.5 ml aliquots of the supernatant were added to 4.5 ml 0.2 M semicarbazide-glycine buffer (pH 10.2) containing 1.0 M potassium cyanide. After 15 minutes, 1 ml aliquots were removed and added to 2 ml of 1 M potassium cyanide solution. The absorption at 325 mμ in 1 cm light path cuvettes was measured and the values were converted to the DPN by the use of the extinction coefficient of the DPN-cyanide complex (13). In the enzymatic method, 0.5 ml aliquots of the samples were added to 4 ml 0.2 M semicarbazide-glycine buffer containing 0.1 M lactic acid.

0.5 ml of 1:20 dilution of purified lactic dehydrogenase enzyme (Worthington) was added. The tubes were allowed to stand for 30 minutes at room temperature (23–25°C). 1 ml aliquots of these reacting mixtures were taken, diluted to 3 ml with distilled water and the absorption measured at 340 mμ in 1 cm light path cuvettes using Beckman DU spectrophotometer. The blank adjustment was made with semicarbazide-glycine medium diluted 1:3 with distilled water. The optical density of the solution was expressed as μmoles of DPN using the molecular extinction coefficient of reduced DPN at 340 mμ (8).

Succinic dehydrogenase activity of the homogenate was estimated by the reduction of neotetrazolium in the presence of succinate as described earlier and the results were expressed as μmoles of Tz reduced per 100 mg tissue in 20 minutes.

RESULTS

Rate of DPN Reduction by Kidney Homogenate.—Spectrophotometric estimation of the rate of reduction of DPN was found to be linear for 30 minutes

TABLE I

Metabolic Parameters of Kidney Homogenates from Normal and Tuberculosis Guinea Pigs

Animal	Malic dehydrogenase			Succinic dehydrogenase	DPNase activity	Esterification of phosphate
	DPN reduced (10 min.)	Tetrazolium reduced (60 min.)	Tetrazolium reduced with niacin and PMC (10 min.)	Tetrazolium reduced (20 min.)	DPN split/100 mg (60 min.)	Phosphate (20 min.)
	μmole	*μmole*	*μmole*	*μmole*	*μmole*	*μmole*
Normal	49.9 ± 5.8 (20)	4.5 ± 1.1 (20)	10.1 ± 1.7 (19)	2.56 ± 0.38 (12)	2.3 ± 0.4 (12)	40.7 ± 4.2 (6)
Tuberculous	51.9 ± 9.3 (10)	1.8 ± 0.8 (10)	10.0 ± 2.6 (6)	1.16 ± 0.18 (11)	10.1 ± 2.7 (11)	25.1 ± 6.6 (9)
Significance	None	$p < 0.001$	None	$p < 0.001$	$p < 0.01$	$p < 0.01$

at room temperature. Semicarbazide (0.2 M), niacin (0.05 M) and p-CMB (0.001 M) did not influence the rate. Employing the values obtained after 20 minutes incubation the rates of the activities of the homogenates of the normal and the infected animals were compared. These results are presented in the first column of Table I and show no significant difference in the enzymatic reduction of DPN with tissue from normal and infected animals.

Malic Dehydrogenase Activity of the Homogenate Using Tetrazolium as Indicator.—In the second column of the Table I are presented the respective rates of Tz reduction by the MDH system with kidney homogenates from normal and the tuberculous animals. In contrast to the spectrophotometric reduction of DPN, shown in the first column of Table I, the Tz reduction by the kidney homogenate of the infected animals was far below the normal. In addition, studies show p-CMB (0.0001 M) markedly depressed the rate of both normal and tuberculous tissue and PMC, when added to the reacting mixture, did not alter the depression of p-CMB. The addition of amytal (0.001 M) did

not alter the Tz reduction. The same tissues were used in the experiments; therefore, the inconsistency in the results obtained by the two methods is most striking. The defect in tuberculous tissue thus appears to affect some steps involved in the electron-transport beyond the initial dehydrogenation of the substrate.

Effect of Niacin and Phenazine Methchloride on the Reduction of Tetrazolium.— In order to explore further the nature of the differences between the kidneys of the normal and the tuberculous animals, mixtures of the homogenates in equal parts were prepared and the activity of the mixtures was compared with the individual preparations. Mixtures were prepared using tissue from normal and

TABLE II

Effect of Niacin and Phenazine Methochloride on Malic Dehydrogenase Activity

Reagent added during preincubation	Tetrazolium reduced per 100 mg tissue		
	Normal	Tuberculous	Mixture
	μmoles	*μmoles*	*μmoles*
DPN only	4.34	0.0	0.0 (2.17)
DPN, niacin	4.50	0.56	1.57 (2.53)
DPN, PMC	12.32	0.0	0.0 (6.16)
DPN, niacin, PMC	11.76	5.94	11.20 (8.85)
DPNH only	5.38	3.24	4.76 (4.31)

Homogenates were preincubated for 15 minutes with 3 μmoles DPN at 37°C with or without niacin (20 μmoles/ml) and phenazine methochloride (0.05 μg/ml). Tetrazolium was added after the preincubation and formazan produced was estimated after 60 minutes without phenazine and after 10 minutes with phenazine in the reacting mixture.

Results are indicated as μmoles of tetrazolium reduced per 100 mg tissue. Figures in parentheses indicate the expected level of reduction.

tuberculous animals. The resultant activity was as expected with 1:1 mixture. However, when the mixture of tissues was preincubated with DPN for 10 minutes at 37°C, the subsequent reduction of Tz was far below the summation of two constituent parts measured separately. The addition of niacin and PMC during the period of pre-incubation restored the activity of the mixtures to the normal range. Alone, neither effected this restoration. The results are represented in the Table II. A pre-incubation with DPNH had no effect on the subsequent enzyme activity. The third column in the Table I summarizes the effect of niacin and PMC on the enzymes of the normal and the tuberculous tissues in a larger number of animals.

Tetrazolium Reduction by the Kidney Homogenate with DPNH as Substrate.— The rate of reduction of Tz by kidney homogenate in the presence of DPNH was linear for 20 minutes at 37°C. Using the values obtained at 20 minutes, the activity of preparations from the normal animals (10) was 8.9 ± 0.5μmoles Tz reduced/100 mg tissue and that of the infected group (9) was 5.1 ± 1.8

μmoles Tz reduced. The DPNH dehydrogenase activity of the preparation from the infected group was significantly reduced ($t = 6.45$, $p < 0.001$) and could not be restored with niacin. Since in the presence of PMC, DPNH reduces chemically without the intervention of the enzyme (15) its effect on the DPNH dehydrogenase system from the infected animals could not be tested.

DPNase Activity of Spleen and Kidney Homogenate.—DPNase activity in kidney and spleen homogenate has been found to be associated with cytoplasmic fraction containing microsomes and sedimenting with the microsomal particles. The DPNase activity of the normal kidney was 2.3 ± 0.4 μmoles DPN split/100 mg tissue/hr. In comparing the DPNase activity of the tissue of normal and the infected animals the kidneys were chosen in preference to other organs

TABLE III

DPNase Activity of Spleen and Kidney from Tuberculous Guinea Pigs Estimated by the Cyanide and the LDH Methods

Tissue employed	DPNase activity DPN split/100 mg tissue	
	LDH method	Cyanide method
	μmoles	*μmoles*
Spleen	39.5	40.0
	62.0	64.8
	22.0	23.6
Kidneys	9.0	9.8
	7.0	6.5

Results are expressed as micromoles DPN split by 100 mg tissue, in 15 minutes with spleen and 1 hour with kidney.

because this organ is free of gross tuberculous lesions, and inflammatory responses hereby present a truer picture of the damage created by the systemic effect of infection. In column five of Table I the results are presented. The DPNase activity of the tissue of the infected group was increased fourfold. In the table the succinic dehydrogenase value of the same organs is charted in order to compare two metabolic parameters. The dehydrogenase activity and the DPNase activity in each experimental animal was altered, but the correlation was not always direct. Animals with the lowest dehydrogenase did not always have the highest DPNase activity. The DPNase activity of the spleens of tuberculous animals showed more DPNase activity on a weight basis than that of normal animals. The measurement of DPNase activity by cyanide and LDH method is shown in Table III. Since the measurements of DPN by both colorimetric and enzymatic methods are equal the splitting is directed toward the niacin riboside linkage. Niacin inhibited the DPNase activity of kidneys indicating the specific nature of the reaction (Table IV).

Esterification of Inorganic Phosphate by the Mitochondria.—Comparative

rates of phosphorylation by the mitochondrial preparations from the normal and the tuberculous guinea pigs with β-hydroxybutyric acid as substrate are presented in Table I. A significant depression of phosphorylation ($p < 0.01$) with the infected group was observed and the diminution in the rate seemed to be in proportion to the degree of the defect in the Tz reduction by the mitochondria. PMC which corrected the defect in the electron transport system did not, however, effect any change in the depressed rate of phosphorylation obtained with the infected tissues.

TABLE IV

Inhibition of DPNase Activity of Kidney from Tuberculous Guinea Pigs by Niacin (0.05 M)

Animal	DPNase activity DPN split/100 mg tissue	
	Without niacin	With niacin
	μmoles	*μmoles*
1	3.8	0.9
2	12.3	0.6
3	8.3	2.9
4	2.6	0.0
5	27.5	10.7
6	13.1	2.6

Results expressed as μmoles DPN split per 100 mg tissue. Time of incubation, 1 hour.

DISCUSSION

In previous reports a lowered succinic dehydrogenase activity in the kidney of tuberculous guinea pigs was describe (1,2). Activity was restored to control levels with a soluble cofactor (4, 5) indicating the basic damage in question was not in the quantity of the enzyme but due to the deficiency of a factor or factors necessary for the optimum reduction of Tz by the specific enzyme system. The data in this paper strengthen this supposition. Malic dehydrogenase activity measured by DPN reduction was unaltered in infected animals; however, when measured with tetrazolium there was a difference in the activity. This defect thus appears to be in the subsequent metabolism of the reduced DPN by the electron transport chain.

The tetrazolium salt used in this study appears to have a redox potential between the flavoprotein diaphorase system and the amytal-sensitive site because the rate of reduction was not affected by amytal but was markedly depressed by p-CMB, a potent inhibitor for the diaphorase system (16). In addition, p-CMB did not have an effect on the rate of reduction of DPN in the presence of malate. PMC, an agent which could bypass the electron transport at the level of the flavoprotein-diaphorase system, did not correct the depressed enzymic activity of the p-CMB–inhibited system, but did bypass

the rate-limiting deficiency in the kidney homogenate from tuberculous guinea pigs. This would tend to indicate the defect of the tuberculous tissue to be beyond the level of flavoprotein-diaphorase system.

The depression of oxidative phosphorylation in tissues of infected animals points to a lesion that may have significance in the response to the disease. This group of diseases is characterized by wasting which is frequently out of proportion to the degree of fever or extent of the lesion. A metabolic lesion of this nature could contribute to the picture by creating a lesion not unlike that seen with dinitrophenol. The over-all measurement of the oxidative phosphorylation in this study only indicates the total deficiency and not the locus or degree of involvements of the individual components at the different levels of the electron transport chain. Attempts are being directed to analyze the nature of this disorganization in the tuberculous tissue.

The niacin effect on the tetrazolium reduction in the tuberculous system tends to point towards another defect existing in the DPN-dependent dehydrogenase function as in the MDH system. Niacin is known to block DPNase activity of tissues (17). The elevated DPNase activity in the kidney homogenate of the infected group of guinea pigs confirms the possible mechanism of the niacin effect reported. In its absence, the high DPNase activity in the tuberculous system could have degraded the concentration of DPN in the reacting mixture to a rate-limiting level. No effect of niacin on the DPNH dehydrogenase system in the infected group could be demonstrated, probably because of DPNH not being susceptible to the DPNase activity. The dual nature of the defect affecting the MDH activity could be better brought out by the depressive effect of the infected system on the normal, and the induced defect could only be corrected when niacin and PMC were both present in the reacting mixtures (Table II).

From Table I, it will be evident that there was a wide variation from animal to animal in the DPNase activity of the infected group. This wide variation could be related to the variability in the response of different animals to the infection. However, the correlation between the depressed succinic dehydrogenase activity and the high DPNase activity was poor. The lack of correlation between two expressions of metabolic derangement in the tuberculous tissue may indicate that these two lesions may not be directly related metabolic defects but may be the manifestations of separate injuries located at the different sites in the cell. The activation of DPNase in the spleen of the tuberculous animals as observed did not appear to be due to the activation of a preexisting pool of enzyme by a diffusible cofactor. The high activity in the spleen could be caused by the inflammatory reaction with new cells containing higher DPNase enzyme than that of the cells under the normal state. Whether the elevated DPNase activity of the kidneys in the tuberculous group was mediated through some change in the kinetics of the enzymes present in the cells or

through the migration of enzyme liberated from other organs to the kidneys is under investigation. The lower activity of the DPN-dependent enzyme systems in the *in vivo* grown tubercle bacilli and the involvement of a DPNase absorbed on such cells reported in the literature (18, 19) favor the latter possibility.

Isonicotinic hydrazide, like niacin, had an inhibitory effect on the elevated DPNase activity in the tuberculous tissue but its efficiency was only half that of niacin. The effect of systemic therapy with isonicotinic hydrazide and niacin on the state of elevated DPNase activity in the organs of tuberculous animals is being investigated. Speculation on this *in vitro* effect brings up a point during the therapy of tuberculosis with isonicotinic hydrazide of a direct therapy of the host as well as the antibacterial effects on the microbe. The dramatic immediate clinical response could be directed towards host's metabolic alterations.

SUMMARY

Malic dehydrogenase activity of the kidney homogenate from the normal and the tuberculous guinea pigs has been estimated. A defect in the electron transport chain has been detected at the level of flavoprotein-diaphorase system. A significantly high DPNase activity of kidney homogenate has also been found in the infected group. Niacin and phenazine methochloride could correct both the defects and improve the tetrazolium reduction of the homogenate in the infected group to the level of the normal activity. Oxidative phosphorylation by the kidney mitochondria from the tuberculous guinea pigs was found to be low and could not be improved by niacin and phenazine methochloride, unlike their effects on the reduction of tetrazolium. Results have been discussed in the light of the over-all intercellular economy and its relation to the symptom complexes in tuberculosis.

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