BBA 45725

INHIBITION BY ANIONS OF DINITROPHENOL-INDUCED ATPase OF MITOCHONDRIA

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(Received May 27th, 1968)

SUMMARY

1. Succinate inhibits the dinitrophenol-induced ATPase of rat-liver mitochondria without having any effect on the redox state of the NAD(P). Cyanide, on the other hand, causes an increased reduction of NAD(P), without having any effect on the ATPase activity, provided that the latter is measured over a short period with a high concentration of mitochondria. Anaerobiosis also has no effect on the ATPase under these conditions.

2. There appear to be two reasons why succinate inhibits. In the first place it provides substrate for oxidative phosphorylation. However, even when oxidative phosphorylation is inhibited by antimycin, there is a residual inhibition that is largely competitive with dinitrophenol.

3. The non-oxidizable anion malonate also inhibits competitively in the absence of antimycin and partly competitively in its presence.

4. Inhibition by the dibasic acid anions malonate and D-malate separately is about the same and no further inhibition is obtained by the addition of two anions together. The tribasic acid anion tricarballylate also inhibits and its effect is additive to that of malonate or D-malate. Further inhibition is obtained when acetate and aspartate are added in addition to malonate and tricarballylate.

5. The dinitrophenol-induced ATPase of house-fly mitochondria is not inhibited by succinate, α-oxoglutarate, isocitrate or L-malate, which are not oxidized by these mitochondria. It is also not inhibited by L-glutamate, D-malate or lactate, but is inhibited by acetate, malonate, L-aspartate, pyruvate and, especially, by tricarballylate.

6. The inhibitory effects of anions can be explained by competition with dinitrophenol for penetration of the mitochondrial matrix. It is proposed that dinitrophenol may enter the mitochondria *via* a number of carrier systems specific for different types of anion.

INTRODUCTION

According to the chemical hypothesis of respiratory-chain phosphorylation[1], the dinitrophenol-induced hydrolysis of ATP involves the coupling of the hydrolysis

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of ATP with the formation of a high-energy compound which is split under the influence of dinitrophenol. In its simplest form, this is represented by the equations

$$ATP + A + C \rightleftharpoons ADP + P_i + A\sim C \quad (1)$$

$$A\sim C \xrightarrow{\text{(dinitrophenol)}} A + C \quad (2)$$

Since, according to this formulation, A is a component of the respiratory chain, one would expect the ATPase to be inhibited under conditions in which A is reduced to AH_2. Early experiments[2,3] indicated that, in fact, the ATPase is resistant to reducing conditions, and this led MYERS AND SLATER[2] to suggest that a second high-energy intermediate (C ~ D) formed from A ~ C by Eqn. 3

$$A\sim C + D \rightleftharpoons C\sim D + A \quad (3)$$

is the dinitrophenol-sensitive intermediate. CHANCE AND WILLIAMS[4], for different reasons, had come to the same conclusion. In 1960, however, CHEFURKA[5] reported that the addition to rat-liver mitochondria of high concentrations of cyanide (1–10 mM) in the absence of added substrate, or of substrates in the presence of low concentrations of cyanide (0.1 mM), or the careful removal of oxygen, treatments which would be expected to reduce components of the chain, markedly inhibit the ATPase. WADKINS AND LEHNINGER[6] had earlier obtained similar results with digitonin particles. For this reason, it appeared correct to revert to the 1953 formulation[1] as the minimum hypothesis (see SLATER[7] for a review).

More recently, however, FONYÓ[8,9] has reported data that have cast doubt on the interpretation given by WADKINS AND LEHNINGER[6] and CHEFURKA[5]. FONYÓ found that, although in accordance with what would be predicted on the basis of the earlier observations[5,6], succinate causes an inhibition of the dinitrophenol-induced ATPase, malonate does not reverse this inhibition. Moreover, malonate inhibits on its own, and the effects of malonate and succinate are not additive. The recent demonstrations[10–12] of competition between substrate anions and uncouplers, which has been interpreted by VAN DAM[12] as occurring for entry through the mitochondrial inner membrane, has supplied an alternative explanation for the inhibition by substrate of the uncoupler-induced ATPase. It appeared desirable to test this alternative explanation and, at the same time, to reinvestigate the effects of cyanide and anaerobiosis on the ATPase, under conditions in which the effect on the redox state of the chain could be monitored.

RESULTS

Effect of redox state on the dinitrophenol-induced ATPase

Table I shows that cyanide, although bringing about a substantially increased reduction of both NAD and NADP (presumably by endogenous substrate), does not inhibit the dinitrophenol-induced ATPase under the conditions of this experiment. Succinate, on the other hand, strongly inhibits (*cf.* refs. 8, 9) without having any significant effect on the redox state of the nicotinamide nucleotides. Succinate and cyanide together had a variable effect, inhibiting in Expt. 2, but not in Expt. 1. The results of Expt. 2 are in agreement with those of FONYÓ[9].

The lack of effect of cyanide is in contrast with the reports of WADKINS AND

TABLE I

THE EFFECT OF SUCCINATE AND CYANIDE ON THE DINITROPHENOL-INDUCED ATPase OF RAT-LIVER MITOCHONDRIA AND ON THE REDOX STATE OF NAD(P)

2.7 and 3.0 mg protein/ml in Expts. 1 and 2, respectively. 3 mM ATP used.

Expt.	*Dinitrophenol (mM)*	*Cyanide (mM)*	*Succinate (mM)*	*ATPase (nmoles P_i/min per mg protein)*	*NAD^+ (nmoles/mg protein)*	*$NADP^+$ (nmoles/mg protein)*
1	0.1	—	—	220	2.5	1.0
	0.1	1	—	233	0.8	0.1
	0.1	—	7	80	2.5	1.0
	0.1	1	7	232	1.6	0.2
2	—	—	—	—	3.2	0.9
	0.1	—	—	262	5.8	3.1
	0.1	1	—	282	1.3	0.1
	0.1	—	7	153	5.6	2.6
	0.1	1	7	178	1.4	0.2

LEHNINGER[6] and of CHEFURKA[5], but in agreement with that of FONYÓ[9]. However, the conditions of the experiment described in Table I were very different from those used by these authors. In order to have sufficient material for the NAD(P) assays a concentrated mitochondrial suspension (3–4 mg protein/ml) was used and, in order to avoid excessive lowering of substrate (ATP) concentration and too high concentration of the inhibitory ADP, a short reaction time was used. Table II illustrates the effect of the experimental conditions on the inhibition of ATPase activity by cyanide and by anaerobiosis (*cf.* refs. 5, 6). Under the conditions used by the earlier authors[5,6] (dilute mitochondrial suspension, long reaction time) the inhibition by anaerobiosis or by 1 mM KCN is confirmed. However, with more concentrated preparations and a shorter reaction time no such inhibition was observed. CHEFURKA[5] also found that cyanide has little effect with high enzyme concentrations and a relatively short (5 min) reaction time.

TABLE II

INFLUENCE OF CYANIDE AND ANAEROBIOSIS ON THE DINITROPHENOL-INDUCED ATPase OF RAT-LIVER MITOCHONDRIA MEASURED UNDER DIFFERENT CONDITIONS

In these experiments the reaction was started by adding the mitochondria to the otherwise complete reaction mixture. 3 mM ATP used.

Expt.	*Protein (mg)*	*Reaction time (min)*	*KCN (mM)*	*Conditions*	*ATPase (nmoles P_i/min per mg protein)*
1	5.2	1	—	Aerobic	228
	5.2	1	1	Aerobic	235
	0.3	15	—	Aerobic	222
	0.3	15	1	Aerobic	130
2	4.0	1	—	Aerobic	210
	4.0	1	—	Anaerobic	198
	0.23	15	—	Aerobic	238
	0.23	15	—	Anaerobic	135

It seems then that both 1 mM KCN and anaerobiosis inactivate the ATPase when this is measured over a period of 15 min with dilute preparations. The reason is not known. It appears from the control experiments of CHEFURKA[5] that the inactivation due to anaerobiosis is reversible. Even with a high concentration of mitochondria, preincubation with 1 mM KCN for only 1 min before adding the dinitrophenol caused 45 % inhibition of the ATPase, measured over a period of 1 min after adding the dinitrophenol. With 5-sec preincubation, the inactivation was only 3 %.

In any case, when conditions are chosen so as to avoid this inactivation, cyanide, although substantially increasing the redox state of the chain, does not inhibit the ATPase. These experiments give no support to the idea that the redox state of the chain affects the dinitrophenol-induced ATPase[5,6] (*cf.* ref. 9).

Effect of substrate on dinitrophenol-induced ATPase

Table I shows that, in agreement with FONYÓ[8,9], 7 mM succinate strongly inhibits the dinitrophenol-induced ATPase. The effect of other anions is summarized in Table III. FONYÓ[9] has reported inhibition by succinate or L-malate and little inhibition by fumarate or DL-β-hydroxybutyrate. In our hands, all oxidizable substrates tested proved to be inhibitory. The lack of effect of fumarate and α-oxoglutarate is understandable, since CHAPPELL AND HAARHOFF[13] have found that liver mitochondria are impermeable to fumarate, and DE HAAN AND TAGER[14] have shown that, in the absence of malate or malonate, they are also impermeable to α-oxoglutarate. In agreement with FONYÓ[8,9], malonate was also found to inhibit.

TABLE III

EFFECT OF VARIOUS ANIONS ON DINITROPHENOL-INDUCED ATPase OF RAT-LIVER MITOCHONDRIA

1.8 mg/ml mitochondria.

Anion	*Inhibition (%) of ATPase*
Succinate (7 mM)	37
L-Glutamate (7 mM)	26
L-Malate (7 mM)	25
L-Glutamate (7 mM) + L-malate (7 mM)	34
DL-β-Hydroxybutyrate (7 mM)	18
Acetate (7 mM)	18
Malonate (7 mM)	15
Fluoride (7 mM)	10
Oxalate (7 mM)	8
Fumarate (7 mM)	4
α-Oxoglutarate (7 mM)	0

The effect of varying the dinitrophenol concentration with a fixed succinate concentration is shown in Fig. 1. At high dinitrophenol concentrations the inhibition by succinate is competitive with respect to dinitrophenol, but at lower dinitrophenol concentrations, the inhibition is greater than would be expected from simple competition. The probable explanation is that succinate has two effects: (i) it competes with dinitrophenol for entry into the mitochondria, as proposed by VAN DAM[12]; (ii) it provides substrate to the respiratory-chain phosphorylation mechanism with the result that high-energy intermediates are formed, which, at concentrations of dinitro-

phenol less than 0.05 mM, are not completely dissipated. In terms of the 'chemical' theory[1] sufficient A~C is present to drive Reaction 1 towards ATP synthesis.

In order to eliminate the synthesis of high-energy intermediates, the experiment was repeated in the presence of antimycin. Fig. 2 shows that the inhibition by succinate under these conditions is considerably less than in the absence of respiratory inhibitor. The inhibition is largely competitive with respect to dinitrophenol. Subsequent experiments were carried out with non-oxidizable anions.

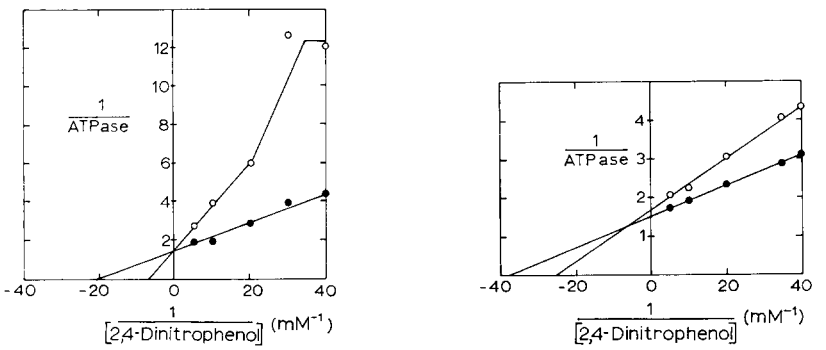

Fig. 1. The effect of succinate on the dinitrophenol-induced ATPase. 2.5 mg/ml rat-liver mitochondria were used. ●—●, no further addition; ○—○, 3 mM sodium succinate present. ATPase in arbitrary units.

Fig. 2. The effect of succinate in the presence of antimycin on the dinitrophenol-induced ATPase. 1.1 mg/ml rat-liver mitochondria were used. 0.2 μg antimycin present. ●—●, no further addition; ○—○, 10 mM sodium succinate present. ATPase in arbitrary units.

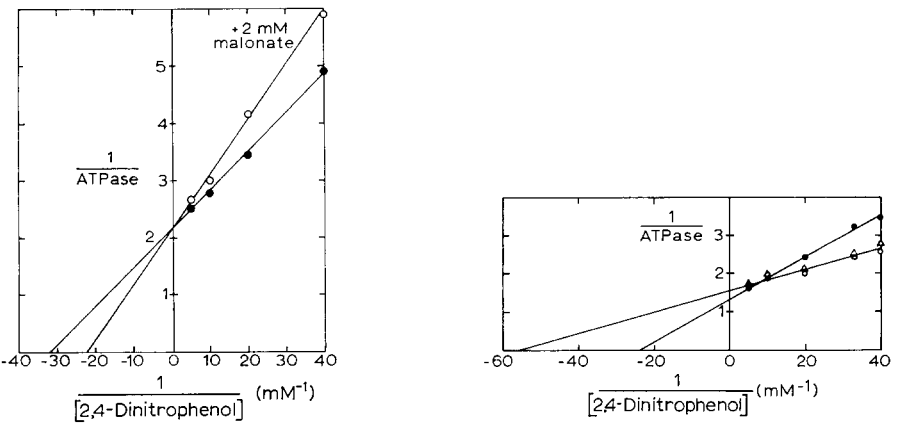

Fig. 3. Inhibition by malonate of the dinitrophenol-induced ATPase, measured at different concentrations of dinitrophenol. 1.1 mg/ml rat-liver mitochondria were used. ●—●, no further addition; ○—○, 2 mM potassium malonate present. ATPase in arbitrary units.

Fig. 4. The effect of the respiratory-chain inhibitors antimycin and cyanide on the dinitrophenol-induced ATPase. 1.8 mg/ml rat-liver mitochondria were used. ●—●, no cyanide or antimycin; △—△, 1 mM KCN present; ○—○, 1 μg antimycin present. ATPase in arbitrary units.

Effect of non-oxidizable anions on dinitrophenol-induced ATPase

Malonate inhibits the dinitrophenol-induced ATPase competitively with respect to dinitrophenol (Fig. 3), with a K_i of 5 mM. However, this value has limited sig-

nificance since, when the malonate concentration was varied between 5 and 30 mM at fixed dinitrophenol concentration, maximum inhibition (about 25 %) of the ATPase was already obtained with 5 mM malonate.

Cyanide and antimycin (Fig. 4) and rotenone (not shown) were found to lower the K_m for dinitrophenol from 0.04 mM to about 0.02 mM. At low concentrations of dinitrophenol, cyanide and antimycin stimulate the ATPase; at high concentrations there is no effect. If the lines in Fig. 4 are extrapolated to infinite dinitrophenol concentration, it would appear that cyanide and antimycin inhibit the ATPase by about 20 %, but it is doubtful if this has any significance. The stimulation by respiratory inhibitors at low dinitrophenol concentrations is probably due to the presence of oxidizable endogenous substrates. In the presence of antimycin, malonate still inhibits the ATPase, but the inhibition is now not completely competitive (Fig. 5). The reason for the difference in inhibition type in the presence and absence of antimycin is not known.

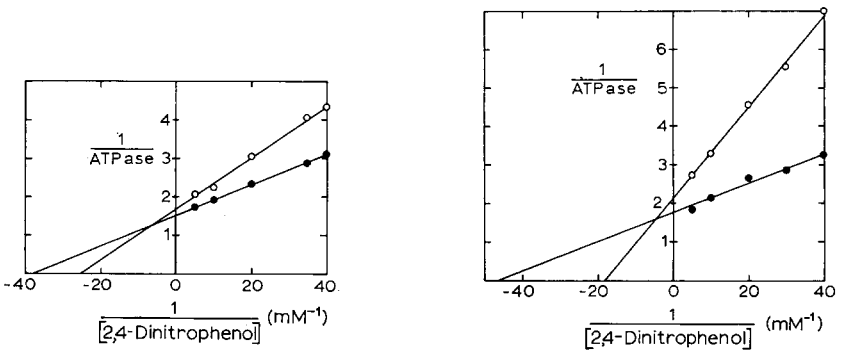

Fig. 5. The effect of malonate in the presence of antimycin on the dinitrophenol-induced ATPase. 1.3 mg/ml rat-liver mitochondria were used. 1 μg antimycin was present. ●—●, no further addition; ○—○, 10 mM potassium malonate was present. ATPase in arbitrary units.

Fig. 6. Inhibition of the dinitrophenol-induced ATPase by a combination of anions in the presence of antimycin. 1.4 mg/ml rat-liver mitochondria were used. 1 μg antimycin was present. ●—●, no further addition; ○—○, 10 mM acetate, 10 mM potassium malonate, 10 mM potassium tricarballylate, 0.5 mM potassium L-malate, 10 mM potassium L-aspartate were present. ATPase in arbitrary units.

Table IV summarizes experiments in which a combination of non-oxidizable anions was used. Expt. 1 shows that the inhibition by dibasic acid anions malonate and D-malate separately is about the same and that no further inhibition is obtained by the addition of the two anions together. FONYÓ[9] found no additivity with succinate and malonate or malate. The tribasic acid anion tricarballylate, used at the suggestion of Mr. A. MEIJER, is less effective as an inhibitor unless a trace of L-malate (*cf.* ref. 15) is added to facilitate its entry into the mitochondrion (Expt. 2). The effect of tricarballylate is additive to that of the dibasic anions. In Expt. 3, the effects of acetate, malonate, tricarballylate and aspartate were all partly additive and 55 % inhibition was obtained when all four anions were added. The inhibition is not an osmotic or ionic-strength effect since the addition of up to 80 mM malonate had no effect on the inhibition by 20 mM D-malate. The inhibition by a mixture of acetate, malonate, tricarballylate and L-aspartate is very largely competitive (Fig. 6).

TABLE IV

INHIBITION OF DINITROPHENOL-INDUCED ATPase OF RAT-LIVER MITOCHONDRIA BY A COMBINATION OF NON-OXIDIZABLE ANIONS

1.33, 0.8 and 1.73 mg/ml mitochondria in Expts. 1, 2, and 3, respectively.

Expt.	*Anion*	*Inhibition (%) of ATPase*
1	D-Malate (20 mM)	29
	Malonate (20 mM)	23
	Tricarballylate (20 mM)	12
	D-Malate + malonate	26
	D-Malate + tricarballylate	34
	Malonate + tricarballylate	34
2	Tricarballylate (20 mM)	15 (26*)
	L-Malate (0.5 mM)	0
	Tricarballylate + L-malate	36 (37*)
3	Acetate (20 mM)	17
	Malonate (20 mM)	27
	Tricarballylate (20 mM) + L-malate (0.5 mM)	36
	L-Aspartate (20 mM)	26
	Acetate + malonate	40
	Acetate + malonate + tricarballylate + L-malate	48
	Acetate + malonate + tricarballylate + L-malate + L-Aspartate	55

* Measured in the presence of 1 μg antimycin.

TABLE V

EFFECT OF ANIONS ON DINITROPHENOL-INDUCED ATPase OF HOUSE-FLY MITOCHONDRIA

In this experiment 0.03 mM dinitrophenol, and a reaction time of 2 min were used. 0.28, 0.20 and 0.30 mg/ml mitochondrial protein were used in Expts. 1–3, respectively.

Expt.	*Anion*	*Inhibition (%) of ATPase*
1	Succinate (20 mM)	0
	L-Glutamate (10 mM)	0
	α-Oxoglutarate (10 mM)	0
	Threo-D_s-isocitrate (10 mM)	0
	L-Malate (10 mM)	0
	D-Malate (10 mM)	0
2	Acetate (20 mM)	21
	L-Lactate (20 mM)	0
	Malonate (20 mM)	26
	D-Malate (10 mM)	0
	L-Aspartate (10 mM)	29
	Tricarballylate (20 mM) + L-malate (0.5 mM)	49
3	Succinate (10 mM)	4
	Pyruvate (10 mM)	22
	L-Malate (10 mM)	0

Table V shows an experiment with mitochondria isolated from the thoracic muscle of the house-fly. These mitochondria are exceptional in being impermeable to intermediates of the Krebs cycle, although permeable to pyruvate[16]. Malonate slowly penetrates these mitochondria[17]. In agreement with these observations no inhibition of the ATPase was found with succinate, α-oxoglutarate, isocitrate or L-malate. Inhibition was also not found with L-glutamate, D-malate or lactate. The ATPase was, however, inhibited by acetate, malonate, L-aspartate, pyruvate, and, especially by tricarballylate. Inhibition by acetate, L-aspartate, tricarballylate and pyruvate was also found in an experiment in which oxidation was inhibited by rotenone.

DISCUSSION

The inhibitory effects of anions on the dinitrophenol-induced ATPase of rat-liver and housefly-muscle mitochondria is not specific for those anions that can act as hydrogen donor to the respiratory chain. In agreement with FONYÓ[8,9], it must be concluded that the inhibition is not due to an effect on the redox state of the mitochondrial respiratory chain. This conclusion is supported by the lack of inhibition of the ATPase by cyanide (*cf.* ref. 9), under conditions in which it could be shown that the nicotinamide nucleotides were highly reduced.

The inhibitory effects of anions can be explained by competition with dinitrophenol for penetration of the mitochondrial matrix, as proposed by VAN DAM[12]. VAN DAM AND SLATER[18] have proposed that uncouplers such as dinitrophenol enter the mitochondria as the anion *via* an energy-linked non-specific anion carrier, and leave the mitochondria as uncharged acid. According to this hypothesis, Eqn. 2 represents the utilization of A~C for the transport of dinitrophenol into the mitochondria.

The incomplete inhibition of the ATPase activity by a single anion can be explained only by postulating that dinitrophenol enters *via* more than one carrier system. Indeed, the data presented in Tables III and IV suggest that at least four anion carriers are present in rat-liver mitochondria, *viz.* for (i) acetate, (ii) malonate, L-malate, and D-malate (and succinate[8,9]), (iii) tricarballylate, (iv) aspartate. Possibly, these may be equated with carriers for monobasic[15], dibasic[15], tribasic[15] carboxylate anions, and aspartate[19], respectively. It is significant that inhibition by tricarballylate was much greater in the presence of a small amount of L-malate, which activates a tricarboxylic acid anion carrier[15]. The maximum inhibition reached in Table IV, with all four carriers blocked, was still only 55 %. It is apparent, then, that dinitrophenol can also enter *via* other pathways. Possible candidates are the carriers for ATP, P_i and other amino acids, in particular glutamate (*cf.* AZZI, CHAPPELL AND ROBINSON[19]).

Thus, the suggestion by VAN DAM AND SLATER[18] that dinitrophenol enters the mitochondria *via* a non-specific anion carrier should be changed to: dinitrophenol enters non-specifically *via* a number of specific anion carriers.

METHODS

Rat-liver mitochondria were isolated according to the method of HOGEBOOM[20], exactly as described by MYERS AND SLATER[21].

House-fly (Musca domestica) mitochondria, prepared as described by VAN DEN BERGH[22], were kindly made available by Mr. A. TULP.

NAD+ and NADP+ concentrations were determined by the methods of KLINGENBERG[23,24] as described by VAN DAM[25].

ATPase activity. Unless otherwise stated ATPase activity was measured in a reaction mixture (1.5 ml) containing 75 mM KCl, 50 mM sucrose, 1 mM EDTA, 50 mM Tris–HCl buffer, 2 mM $MgCl_2$, 6 mM ATP and 0.1 mM 2,4-dinitrophenol. The final pH was 7.4. The reaction was carried out in a small vessel kept at 25° and provided with rapid magnetic mixing. The mitochondria were added to the reaction mixture 1 min before addition of dinitrophenol. When cyanide was present, this was added 5 sec before the dinitrophenol. When antimycin was present it was added to the reaction mixture before the mitochondria. Exactly 1 min after addition of the dinitrophenol the reaction was stopped by the addition of 1.5 ml 10 % (w/v) trichloroacetic acid. The protein was separated by centrifugation and the inorganic phosphate in the supernatant fluid determined by the method of SUMNER[26]. The same supernatant fluid was used for measurement of the NAD^+ and $NADP^+$.

In experiments where the effect of anaerobiosis was studied, the reactions were carried out in Thunberg tubes using the precautions described by CHEFURKA[5] to avoid damage to the mitochondria. The nitrogen was freed from oxygen by passage through alkaline pyrogallol.

Protein was determined by the biuret method as described by CLELAND AND SLATER[27].

Chemicals. 2,4-Dinitrophenol, sodium L-glutamate, L-malic acid, sodium DL-β-hydroxybutyrate, sodium fumarate, tricarballylic acid (propane-1,2,3-tricarboxylic acid), sodium pyruvate, sodium acetate, and L-aspartic acid were obtained from British Drug Houses; ATP, sodium succinate, sodium α-oxoglutarate from Boehringer und Soehne; malonic acid from E. Merck AG., Darmstadt; rotenone from S. B. Penick and Co., New York; antimycin from the Sigma Chemical Co.; D-malic acid from Nutritional Biochemical Corp. Potassium *threo*-D_s-isocitrate was kindly provided by Mr. A. MEIJER. The acids were neutralized with KOH.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the U.S. Public Health Service (Grant No. A.M. 08690) and the Life Insurance Medical Research Fund.

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