BBA 45672

STUDIES ON THE FUNCTIONAL RELATIONSHIP BETWEEN THE PHOSPHOPYRUVATE SYNTHESIS AND THE SUBSTRATE LEVEL PHOSPHORYLATION IN GUINEA-PIG LIVER MITOCHONDRIA

NOBUO ISHIHARA AND GORO KIKUCHI

Department of Biochemistry, Tohoku University School of Medicine, Sendai (Japan)

(Received December 27th, 1967)

SUMMARY

The PEP synthesis in guinea-pig liver mitochondria in the presence of α-ketoglutarate and P_i was greatly increased by addition of 2,4-dinitrophenol, pentachlorophenol, dicumarol or the hexokinase–glucose–ATP system, but was hardly increased by exogenously added ATP. This result is in sharp contrast to those obtained with pigeon and chicken liver mitochondria.

By using ^{14}C-labeled substrates, it was revealed that the ratio of the amounts of PEP formed to those of α-ketoglutarate oxidized varied markedly according to the degree of stimulation of PEP synthesis, and when the PEP synthesis was maximally stimulated, the ratio reached the value of nearly 1.0. Under these conditions the oxidation of α-ketoglutarate was considerably stimulated by the addition of malate or fumarate and in turn, the metabolism of fumarate was greatly increased by the addition of α-ketoglutarate.

The nucleoside diphosphate kinase activity in guinea-pig liver mitochondria was found to be extremely low, while the activity in pigeon liver mitochondria was very high. Activities of GTP–AMP–phosphate transferase were low in both mitochondria.

It was concluded that in guinea-pig liver mitochondria guanine nucleotides involved in the substrate level phosphorylation are unable to interact easily with adenine nucleotides in the respiratory chain phosphorylation system because of the low level of nucleoside diphosphate kinase. It was also suggested that the α-ketoglutarate oxidation in guinea pig liver might be supported to a considerable degree by coupling with the PEP synthesis even under physiological conditions.

INTRODUCTION

The oxidation of α-ketoglutarate in mitochondria is unique for its requirement of guanine nucleotides[1–3], and the GTP produced at the α-ketoglutarate oxidation step may be consumed to form GDP mainly by the following four enzymic reactions in liver mitochondria.

Abbreviation: PEP, phosphopyruvate.

$$GTP + ADP \rightarrow GDP + ATP \quad (1)$$

$$Oxaloacetate + GTP \rightarrow PEP + GDP + CO_2 \quad (2)$$

$$GTP + AMP \rightarrow GDP + ADP \quad (3)$$

$$Fatty\ acid + GTP + CoA \rightarrow fatty\ acyl\text{-}CoA + GDP + P_i \quad (4)$$

Reaction (1) is catalyzed by nucleoside diphosphate kinase[4] (EC 2.7.4.6). Reaction (2) depends upon the function of mitochondrial phosphopyruvate carboxylase (EC 4.1.1.32) which is distinguishable from the phosphopyruvate carboxylase in the soluble fraction of liver cells[5]. Reaction (3) is catalyzed by a GTP–AMP–phosphate transferase which was revealed to be present in the inner compartment of liver mitochondria[6]. The reaction (4), when tested *in vitro*, is strongly inhibited by the addition of P_i (refs. 7, 8).

With respect to the reactions (1) and (2), there have been several reports indicating a special functional relationship between the PEP formation and the substrate level phosphorylation in liver mitochondria from various animals. STANBURY AND MUDGE[9] and MUDGE *et al.*[10] found that the PEP synthesis in rabbit liver mitochondria in the presence of α-ketoglutarate and P_i was greatly stimulated by 2,4-dinitrophenol and the relationship between the rate of PEP formation and the dinitrophenol concentration was polyphasic. Similar stimulation by dinitrophenol of the PEP synthesis was also observed with rat liver mitochondria by SCHELLENBERG[11] and by SCHOLTE AND TAGER[12] and with guinea-pig liver mitochondria by NORDLIE AND LARDY[13]. On the other hand, it was proposed by AZZONE AND ERNSTER[14] that the ATP formed in the substrate level phosphorylation is compartmentalized from the ATP originating from respiratory chain phosphorylation on the basis of their observations of dinitrophenol-stimulated α-ketoglutarate oxidation in rat liver mitochondria but in the presence of only trace amounts of P_i; this idea of ATP-specific compartmentation was adopted by NORDLIE AND LARDY[13] to explain their findings on the dinitrophenol-stimulated PEP synthesis in guinea-pig liver mitochondria.

The view of AZZONE AND ERNSTER, however, was questioned by CHARLES *et al.*[15], SCHOLTE AND TAGER[12] and by CHAPPELL AND GREVILLE[16]. SCHOLTE AND TAGER[12] also pointed out that with rat liver mitochondria the ratio of the amounts of PEP formed to the amounts of α-ketoglutarate oxidized was about 0.1, indicating that only about 1 in 10 of the GTP molecules reacted according to reaction (2). Moreover, recently GEVERS[17] presented evidence that in intact pigeon liver mitochondria the nucleotides are able freely to interact and there is no particular correlation between the substrate level phosphorylation and the PEP synthesis. Thus the concept of the so-called compartmentation of the substrate level phosphorylation is now very confusing.

In the present paper we wish to report the results of our investigations made with guinea-pig liver mitochondria which aimed to clarify in more detail the nature of the functional relationship between the PEP synthesis and the substrate level phosphorylation and to reveal the role of PEP carboxylase in the regulation of carboxylic acid metabolism in guinea-pig liver mitochondria. The results of some experiments made with liver mitochondria from other animals will also be included in this paper.

MATERIALS AND METHODS

Preparation of mitochondria

Guinea-pig liver mitochondria, as well as those from rat and rabbit, were prepared essentially by the method of SCHNEIDER AND HOGEBOOM[18]. The liver was

homogenized in 0.25 M sucrose containing 0.01 M Tris–HCl buffer (pH 7.2) and 0.5 mM EDTA with a teflon homogenizer, followed by a 10-min centrifugation at 600 × *g*. The resulting supernatant fraction was centrifuged for 10 min at 5000 × *g* and the precipitates obtained were washed once with the same solution, but by centrifuging at 8000 × *g* for 10 min. The precipitates were suspended in the same isolation medium to give a protein concentration of approximately 1.5 mg as nitrogen per ml. Protein amounts were determined by a biuret method, employing the calibration curve standardized according to the Kjeldahl method. Pigeon and chicken liver mitochondria were prepared according to the procedures described by GEVERS[17], except that the isolation medium was the same as used for guinea pig.

Incubation conditions for PEP formation

Reaction mixtures contained, in a final volume of 2.8 ml: 15 μmoles of potassium phosphate (pH 7.2) labeled or unlabeled with ^{32}P, 5 μmoles of $MgCl_2$, 60 μmoles of Tris–HCl buffer (pH 7.2), 75 μmoles of KCl, 300 μmoles of sucrose, and appropriate amounts of intact liver mitochondria and other additions as indicated in respective tables and figures. Reactions were carried out aerobically in Warburg manometric flasks or in erlenmeyer flasks of approx. 30 ml capacity for 20 min at 25° in a shaking water bath incubator, and terminated by adding 0.2 ml of 50 % trichloroacetic acid. When ^{14}C-labeled substrates were employed, the $^{14}CO_2$ formed was trapped in 0.2 ml of 20 % KOH placed in the center well of the manometric flask.

Assay of PEP

When non-radioactive P_i was employed in the reaction, the PEP formed was assayed enzymatically by the method described by CZOK AND ECKERT[19]. Each cuvette contained 0.68 ml of 0.2 M Tris–HCl buffer (pH 7.6), 0.10 ml of 2 M KCl, 0.04 ml of 0.5 M $MgSO_4$, 0.07 ml of 0.01 M ADP, 0.06 ml of 0.01 M NADH, 0.1 ml of 10 mg/ml bovine serum albumin solution and an aliquot of the neutralized deproteinized reaction mixture in a final volume of 3.05 ml. Lactate dehydrogenase (EC 1.1.1.27) was added and the reaction was started by adding pyruvate kinase (EC 2.7.1.40). An Hitachi recording spectrophotometer was used for the spectrophotometric measurements.

When the reaction mixture contained ^{32}P, the deproteinized supernatant fraction was treated twice with about 10 ml of ethyl ether to extract trichloroacetic acid, then with about 30 mg of charcoal and filtered. The filtrate was diluted with water so as to contain 1 to 2 μmoles of phosphor scintillation per ml and the P_i fraction in this solution was precipitated by the addition of a mixture of triethylamine–HCl, ammonium molybdate and perchloric acid as devised by SUGINO AND MIYOSHI[20]. PEP remaining in the supernatant fraction was cleaved to pyruvate and P_i by the method of MUNTS AND HURWITZ[21] and P_i formed was recovered after precipitation with triethylamine–HCl, and assayed on the basis of radioactivity. The validity of this assay method was ascertained by the recovery test with ^{32}P-labeled PEP which was highly purified by column chromatography on Dowex 1-X8 (*cf.* ref. 22).

Assay of activities of nucleoside diphosphate kinase and GTP–AMP–phosphate transferase

Activities of nucleoside diphosphate kinase (EC 2.7.4.6) were assayed spectrophotometrically according to the method of MOURAD AND PARKS[23], using crystalline

yeast hexokinase (EC 2.7.1.1) and yeast glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (analytical reagent grade), in the reaction systems described in legend to Fig. 3. Activities of GTP–AMP–phosphate transferase were assayed in a similar way but with GTP and AMP as the substrates, assuming the cyclic operation of the following three reactions:

$$GTP + AMP \rightarrow GDP + ADP$$
$$GTP + ADP \rightarrow GDP + ATP$$
$$2ADP \rightarrow ATP + AMP$$

Details of assay procedures will appear in appropriate places in the RESULTS section.

Assay of $^{14}CO_2$

$^{14}CO_2$ absorbed in KOH was converted into $BaCO_3$ by precipitation with $BaCl_2$. Before addition of $BaCl_2$, 100 μmoles of Na_2CO_3 were added as the carrier. Radioactivity of $Ba^{14}CO_3$ was measured by a windowless gas flow counter and the observed counts per min were corrected for background radiation and self-absorption to infinite thinness.

Reagents

ATP, ADP, $NADP^+$, NADH, GTP, α-ketoglutaric acid, oligomycin and yeast hexokinase (Type II) were obtained from Sigma Chem. Co., U.S.A.; phosphopyruvate, lactate dehydrogenase, pyruvate kinase, crystalline yeast hexokinase and yeast glucose-6-phosphate dehydrogenase (analytical reagent grade), from Böhringer and Söhne, West Germany; bovine serum albumin, from Armour Pharmaceutical Co., U.S.A. Atractyloside was the gift of Prof. T. Ajello, University of Palermo, Italy. ^{14}C-labeled compounds were obtained from Daiichi Chem. Co., Tokyo, and diluted by respective carriers so as to give a specific radioactivity of 0.01 mC/mmole before use.

RESULTS

Effects of 2,4-dinitrophenol on the rates of PEP formation and oxygen uptake

As shown in Fig. 1, the rate of PEP synthesis in the presence of α-ketoglutarate and P_i was greatly increased by the addition of dinitrophenol, and the relationship

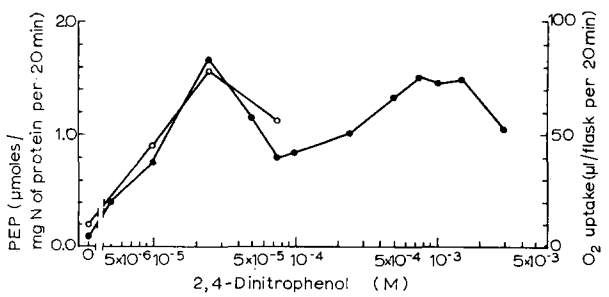

Fig. 1. Effects of addition of different concentrations of dinitrophenol on the rates of PEP synthesis and oxygen uptake. Reaction mixtures contained, in a final volume of 2.8 ml: 10 μmoles of α-ketoglutarate, 15 μmoles of $^{32}P_i$ (as potassium phosphate buffer of pH 7.2), 2.6 mg (as nitrogen) of guinea-pig liver mitochondria, dinitrophenol at indicated concentrations, and other additions as described in METHODS. Reactions were carried out in Warburg manometric flasks for 20 min at 25°. ●, PEP; ○, O_2 uptake.

between the rates of the PEP synthesis and dinitrophenol concentration was polyphasic. These results are in good agreement with those reported by STANBURY AND MUDGE[9] for rabbit and by NORDLIE AND LARDY[13] for guinea pig. As far as examined, the rate of oxygen uptake also changed in parallel with the change of rate of PEP synthesis. Within the range of dinitrophenol concentration below 0.1 mM, the maximum stimulation of both the PEP synthesis and oxygen uptake was obtained at $2.5 \cdot 10^{-5}$ M of dinitrophenol.

Pyruvate kinase activity of mitochondrial preparation

In order to examine the possible contamination by pyruvate kinase, guinea-pig liver mitochondria were incubated with exogenously added PEP and the amounts of PEP disappearing were measured. The results are shown in Table I. Without $MgCl_2$ added, the decrease in PEP amount was negligible. The PEP disappearance was not appreciably influenced by the addition of dinitrophenol or ADP alone. This would show that the values of PEP presented in Fig. 1 actually reflect the rates of PEP synthesis. The PEP disappearance, however, was significantly increased by the addition of the hexokinase–glucose–ATP system, and in this reaction system a considerable amount of pyruvate accumulated. Probably the hexokinase system served to maintain a high level of ADP which would favor the pyruvate formation from PEP.

Relationship between the α-ketoglutarate oxidation and the PEP synthesis

As already shown by previous investigators[10,12,13], PEP formation was greatly reduced when malonate was added to the system containing α-ketoglutarate alone as the substrate, but the yield of PEP was restored nearly completely when fumarate or malate was further added (Table II). When malate or fumarate was the sole substrate added, the yield of PEP was negligibly small either in the presence or absence of dinitrophenol (*cf.* Table VIII).

In order to obtain further information on the stoichiometric relationship between the α-ketoglutarate oxidation and PEP formation, we incubated [1-^{14}C]glu-

TABLE I

DISAPPEARANCE OF EXOGENOUSLY ADDED PEP DURING INCUBATION WITH GUINEA-PIG LIVER MITOCHONDRIA UNDER VARIOUS CONDITIONS

Reaction mixtures contained, in a final volume of 2.8 ml: 4 μmoles of PEP, 5 μmoles of $MgCl_2$, 15 μmoles of non-radioactive potassium phosphate (pH 7.2), 1.1 mg (as nitrogen) of guinea-pig liver mitochondria, 60 μmoles of Tris–HCl buffer (pH 7.2), 75 μmoles of KCl, 300 μmoles of sucrose, and other additions as indicated in the table. Reactions were carried out for 20 min at 25°. PEP and pyruvate were assayed enzymatically (see METHODS).

Addition	*PEP disappearing (μmoles/flask)*	*Pyruvate found (μmole/flask)*
None	0.26	
ADP, 5 μmoles	0.53	
Dinitrophenol, $1 \cdot 10^{-5}$ M	0.40	
Dinitrophenol, $2.5 \cdot 10^{-5}$ M	0.26	
Dinitrophenol, $7.5 \cdot 10^{-5}$ M	0.66	
Hexokinase system*	1.14	0.81

* Hexokinase system was composed of 0.1 mg as protein of yeast hexokinase (Type II), 30 μmoles of glucose and 5 μmoles of ATP.

TABLE II

EFFECT OF ADDITION OF OTHER CARBOXYLIC ACIDS ON PEP SYNTHESIS IN THE PRESENCE OF α-KETOGLUTARATE AND DINITROPHENOL

5 μmoles of α-ketoglutarate, 10 μmoles each of malonate, malate and fumarate, 15 μmoles of $^{32}P_i$ (as potassium phosphate), and 1.2 mg (as nitrogen) of guinea-pig liver mitochondria were employed. All reaction mixtures contained $2.5 \cdot 10^{-5}$ M of dinitrophenol. Other additions and reaction conditions were similar to those for Fig. 1.

Substrate and addition	*PEP formed (μmoles/mg N of protein/20 min)*
α-Ketoglutarate	2.24
α-Ketoglutarate *plus* malonate	0.13
α-Ketoglutarate *plus* malonate and fumarate	1.83
α-Ketoglutarate *plus* malonate and malate	2.36

TABLE III

STOICHIOMETRIC RELATIONSHIP BETWEEN AMOUNTS OF PEP FORMED AND AMOUNTS OF α-KETOGLUTARATE METABOLIZED IN THE PRESENCE OF MALONATE, MALATE AND DIFFERENT CONCENTRATIONS OF DINITROPHENOL

Reaction mixtures for the measurement of $^{14}CO_2$ formation contained 10 μmoles each of DL-[1-^{14}C]-glutamate, malonate and malate, 15 μmoles of non-radioactive potassium phosphate, dinitrophenol of indicated concentrations, 1.1 mg (as nitrogen) of guinea-pig liver mitochondria, and other components as described in METHODS. Reaction mixtures for the measurement of PEP synthesis were of the similar composition as above, except that unlabeled DL-glutamate and $^{32}P_i$ were employed. Both series of the reaction were carried out simultaneously in Warburg manometric flasks for 20 min at 25°.

Dinitrophenol (M)	*PEP formed (μmoles/mg N/20 min)*	*$^{14}CO_2$ formed (μmoles/mg N/20 min)*	*PEP/$^{14}CO_2$*	*O_2 uptake (μl/mg N/20 min)*
0	0.12	0.41	0.29	9
$1 \cdot 10^{-5}$	0.86	1.33	0.65	41
$2.5 \cdot 10^{-5}$	2.16	2.22	0.97	71
$7.5 \cdot 10^{-5}$	0.85	1.62	0.53	51

tamate with mitochondria in the presence of malonate and malate, and measured the amounts of $^{14}CO_2$ and PEP produced. As shown in Table III, the $^{14}CO_2$ production was also greatly increased by the addition of dinitrophenol, and the maximum production of $^{14}CO_2$ was again obtained at $2.5 \cdot 10^{-5}$ M dinitrophenol. Under these conditions the $^{14}CO_2$ formation increased more than five times, and the yield of PEP increased about 20 times over the control value without dinitrophenol. It is also noteworthy (Table III) that the ratio of the amounts of PEP formed to the amounts of $^{14}CO_2$ evolved varied significantly according to the degree of stimulation of PEP synthesis; at $2.5 \cdot 10^{-5}$ M dinitrophenol, the ratio was found to be nearly 1.0, while the value obtained without dinitrophenol was as low as 0.29. Essentially similar relations were also observed with [1,5-$^{14}C_2$]citrate as the labeled substrate (Table IV). These results suggest that when large amounts of GTP were produced along with the increased oxidation of α-ketoglutarate, the GTP thus formed is utilized almost stoi-

TABLE IV

STOICHIOMETRIC RELATIONSHIP BETWEEN PEP SYNTHESIS AND α-KETOGLUTARATE OXIDATION IN THE PRESENCE OF CITRATE, MALONATE, MALATE AND DINITROPHENOL

Reaction systems as well as the procedures of experiments were similar to those for Table III, except that [1,5-$^{14}C_2$]citrate and unlabeled citrate were employed in place of [1-^{14}C]glutamate and unlabeled glutamate, respectively, and $2.5 \cdot 10^{-5}$ M of dinitrophenol was added to all reaction systems.

Reaction system	*PEP formed (μmoles/mg N/20 min)*	*$^{14}CO_2$ formed (μmoles/mg N/20 min)*	*PEP/$^{14}CO_2$*	*O_2 uptake (μl/mg N/20 min)*
[1,5-$^{14}C_2$]Citrate *plus* malonate	0.05	1.16	0.04	19
[1,5-$^{14}C_2$]Citrate *plus* malonate and malate	1.40	1.53	0.91	56

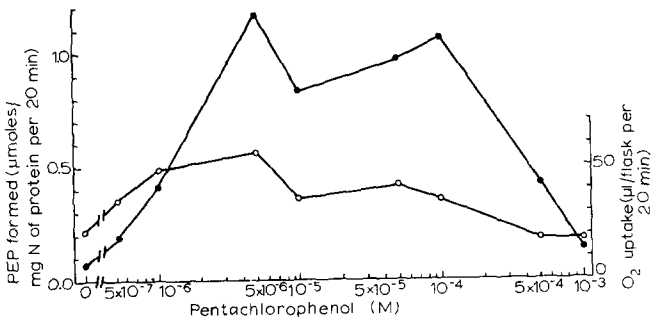

Fig. 2. Effects of addition of different concentrations of pentachlorophenol on the rates of PEP synthesis and oxygen uptake. Reaction mixtures contained 10 μmoles each of DL-[1-^{14}C]glutamate, malonate and malate, 15 μmoles of non-radioactive potassium phosphate, 1.2 mg (as nitrogen) of guinea-pig liver mitochondria, pentachlorophenol of indicated concentrations and other components as described in METHODS. Other reaction conditions were similar to those for Fig. 1. ●, PEP; ○, O_2 uptake.

chiometrically to form PEP; the supply of oxaloacetate may also be increased as a consequence of respiratory stimulation by dinitrophenol.

The stimulation of PEP formation is not specific to dinitrophenol; similar patterns of stimulation could also be observed with other uncouplers such as pentachlorophenol (Fig. 2) and dicumarol (Table V). The concentration of pentachlorophenol which brought about the maximum stimulation of PEP synthesis was far lower than that observed for dinitrophenol, and this concentration of pentachlorophenol had been shown to be sufficient for complete uncoupling of the oxidative phosphorylation[24].

The PEP synthesis was also stimulated by the addition of hexokinase–glucose–ATP system, confirming the observations of NORDLIE AND LARDY[13] (Table VI). The values of PEP found in the reaction systems with the hexokinase system were somewhat lower than those obtained in the reaction systems with dinitrophenol. This is probably due to the fact that the hexokinase system also stimulated the conversion of the once formed PEP into pyruvate through the pyruvate kinase activity contaminating the mitochondrial preparation as was discussed in the previous section (*cf.* Table I). The stimulation of PEP synthesis by the hexokinase system was com-

TABLE V

STOICHIOMETRIC RELATIONSHIP BETWEEN PEP SYNTHESIS AND α-KETOGLUTARATE METABOLISM IN THE PRESENCE OF VARIOUS EFFECTORS OF RESPIRATORY STIMULATION

Reaction mixtures contained 10 μmoles each of DL-[1-^{14}C]glutamate, malonate and malate, 15 μmoles of non-radioactive potassium phosphate, indicated amounts of uncouplers or hexokinase system, appropriate amounts of guinea-pig liver mitochondria, and other additions as described in METHODS.

Addition	*PEP formed (μmoles/mg N/20 min)*	*$^{14}CO_2$ formed (μmoles/mg N/20 min)*	*PEP/$^{14}CO_2$*
None	0.17	0.35	0.49
Dinitrophenol, $2.5 \cdot 10^{-5}$ M	1.97	2.06	0.96
Pentachlorophenol, $5 \cdot 10^{-6}$ M	1.16	1.16	1.00
Dicumarol, $1 \cdot 10^{-5}$ M	1.80	1.72	1.08
Hexokinase system*	1.13	1.12	1.01

* Hexokinase system employed was the same as described in Table I.

TABLE VI

EFFECT OF ADDITION OF THE HEXOKINASE–GLUCOSE–ATP SYSTEM ON PEP SYNTHESIS FROM α-KETOGLUTARATE

Each reaction mixture contained 5 μmoles of α-ketoglutarate, 15 μmoles of radioactive potassium phosphate, 1.2 mg (as nitrogen) of guinea-pig liver mitochondria, indicated additions and other components as described in METHODS.

Addition	*PEP formed (μmoles/mg N/20 min)*	*P_i recovered (μmoles/flask)*
None	0.06	14.0
Hexokinase system*	1.21	1.96
Hexokinase system *plus* atractyloside, $5 \cdot 10^{-5}$ M	0.11	14.4
Dinitrophenol, $2.5 \cdot 10^{-5}$ M	2.02	13.6
Hexokinase system *plus* dinitrophenol	1.19	10.0
Hexokinase system *plus* dinitrophenol and atractyloside	1.22	13.8

* Hexokinase system employed was the same as described in Table I.

pletely abolished by atractyloside, a specific inhibitor of translocation of adenine nucleotides across the mitochondrial membrane[25–27]. Atractyloside, however, had no effect on the dinitrophenol-stimulated PEP synthesis either in the presence or absence of the hexokinase system added. Also it should be noted in Table VI that the amount of P_i recovered in the reaction system with the hexokinase system alone was as small as about 2 μmoles, while 10 μmoles of P_i were recovered in the reaction system which contained both the hexokinase system and dinitrophenol. These data clearly indicate that our mitochondrial preparation performed an active oxidative phosphorylation and this phosphorylation was effectively uncoupled by the concentration of dinitrophenol employed.

In Table V are compared the effects of uncouplers and the hexokinase system on the reaction of PEP synthesis in the presence of [1-^{14}C]glutamate, malonate and malate. Apparently the PEP/$^{14}CO_2$ ratios became close to 1.0 in all of the reaction

systems tested, provided that the respective reactions were carried out under conditions which maximally stimulated the PEP synthesis.

Relationship between fumarate metabolism and PEP formation

Guinea-pig liver mitochondria metabolized fumarate fairly well even in the presence of malonate, as can be seen from the $^{14}CO_2$ formation from [1,4-$^{14}C_2$]fumarate shown in Table VII. With fumarate alone as the substrate, however, the yield of PEP was very small and the value of PEP/$^{14}CO_2$ ratio was only 0.22. The $^{14}CO_2$ formation in this reaction system was considerably increased by the addition of dinitrophenol, but the yield of PEP was not appreciably increased by dinitrophenol, and therefore the ratio of PEP/$^{14}CO_2$ remained low. The addition of α-ketoglutarate alone also failed to increase the yield of PEP in the reaction system with fumarate *plus* malonate. The $^{14}CO_2$ formation in this reaction system was rather suppressed by the addition of α-ketoglutarate, probably because fumarate and α-ketoglutarate competed at a terminal oxidation step for the oxidation; in fact, the amounts of oxygen consumed were not much different in either system with or without added α-ketoglutarate. When both α-ketoglutarate and dinitrophenol were added to the system containing [1,4-$^{14}C_2$]fumarate and malonate, the production of both PEP and $^{14}CO_2$ increased markedly, and the ratio of PEP/$^{14}CO_2$ again approached 1.0. These observations would indicate that the metabolism of 4-carbon dicarboxylic acids is highly dependent upon the amount of GTP available to the synthesis of PEP under these reaction conditions.

In turn, the $^{14}CO_2$ formation from [1,5-$^{14}C_2$]citrate in the presence of dinitrophenol was considerably increased by the addition of fumarate (Expt. 2 in Table VII). The PEP synthesis was also increased when fumarate was added to the reaction system containing citrate, malonate and dinitrophenol, and the PEP/$^{14}CO_2$ ratio

TABLE VII

RELATIONSHIPS BETWEEN THE METABOLISM OF α-KETOGLUTARATE AND FUMARATE AND THE PEP SYNTHESIS

In Expt. 1, 10 μmoles each of [1,4-$^{14}C_2$]fumarate and α-ketoglutarate, 15 μmoles of non-radioactive potassium phosphate and 1.2 mg (as nitrogen) of guinea-pig liver mitochondria were used. In Expt. 2, [1,5-$^{14}C_2$]citrate was used in place of [^{14}C]fumarate. All reaction systems contained also 10 μmoles of malonate.

Expt. No.	*Reaction system*	*PEP formed (μmoles/mg N/20 min)*	*$^{14}CO_2$ formed (μmoles/mg N/20 min)*	*PEP/$^{14}CO_2$*	*O_2 uptake (μl/mg N/20 min)*
1	[1,4-$^{14}C_2$]Fumarate	0.13	0.60	0.22	23
	[1,4-$^{14}C_2$]Fumarate *plus* dinitrophenol, $2.5 \cdot 10^{-5}$ M	0.17	0.85	0.20	28
	[1,4-$^{14}C_2$]Fumarate *plus* α-ketoglutarate	0.14	0.13	1.08	22
	[1,4-$^{14}C_2$]Fumarate *plus* dinitrophenol and α-ketoglutarate	1.19	1.38	0.87	44
2	[1,5-$^{14}C_2$]Citrate	—	0.27	—	10
	[1,5-$^{14}C_2$]Citrate *plus* dinitrophenol	0.05	0.89	0.06	27
	[1,5-$^{14}C_2$]Citrate *plus* fumarate	0.05	0.26	0.19	19
	[1,5-$^{14}C_2$]Citrate *plus* dinitrophenol and fumarate	1.07	1.31	0.82	51

increased to 0.82. Apparently the metabolism of α-ketoglutarate, too, is stimulated under conditions which allow an active PEP synthesis to proceed.

Comparison of utilization of ATP for PEP synthesis by liver mitochondria from guinea pig, pigeon and chicken

With guinea-pig liver mitochondria, the PEP synthesis was very small when malate alone was the substrate; the yield of PEP from malate was increased, though only slightly, by the addition of ATP, and the stimulation by ATP was completely abolished by atractyloside (Table VIII). ATP also slightly stimulated the PEP synthesis from α-ketoglutarate in the absence of dinitrophenol. Apparently ATP, either

TABLE VIII

COMPARISON OF PEP SYNTHESIS BY LIVER MITOCHONDRIA FROM GUINEA PIG, PIGEON AND CHICKEN

10 μmoles each of α-ketoglutarate and malate, 15 μmoles of non-radioactive potassium phosphate, 5 μmoles of ATP, $2.5 \cdot 10^{-5}$ M of dinitrophenol and $5 \cdot 10^{-5}$ M of atractyloside were employed in these reactions. Amounts of liver mitochondria used were 1.2 mg, 0.9 mg, and 1 mg (as nitrogen), respectively, for guinea pig, pigeon and chicken. Other conditions were similar to those described in METHODS.

Substrate and addition	*PEP formed (μmoles/mg N/20 min)*		
	Guinea pig	*Pigeon*	*Chicken*
α-Ketoglutarate	0.05	0.51	0.11
α-Ketoglutarate *plus* dinitrophenol	2.24	0.30	0.06
α-Ketoglutarate *plus* ATP	0.19	0.78	0.23
Malate	0.23	2.26	1.36
Malate *plus* dinitrophenol	0.35	0.12	0.06
Malate *plus* ATP	0.4	3.60	1.23
Malate *plus* ATP and atractyloside	0.21	1.91	1.45
Malate *plus* α-ketoglutarate and dinitrophenol	2.36	—	—

exogenously added or endogenously generated by the respiratory chain phosphorylation, is able to support the PEP synthesis in intact guinea-pig liver mitochondria, but the efficiency of ATP utilization for the PEP synthesis seems to be very low. In an independent experiment, we also examined the effect of addition of GTP on the PEP synthesis from malate in the absence of dinitrophenol, but in no case could we observe the stimulation of PEP synthesis by exogenously added GTP (5 μmoles per flask). It was shown by HELDT[28] that endogenous guanine nucleotides do not directly communicate with extramitochondrial guanine nucleotides.

In sharp contrast to the case of guinea pig mitochondria, it was demonstrated by GEVERS[17] that pigeon liver mitochondria produced a large amount of PEP from malate alone, and the exogenously added ATP significantly increased the yield of PEP from malate. As shown in Table VIII, we obtained results similar to those reported by GEVERS, *i.e.*, with pigeon liver mitochondria, the yield of PEP from α-ketoglutarate was far smaller than that obtained with malate, and the yields of PEP were markedly reduced when dinitrophenol was added, irrespective of whether α-ketoglutarate or malate was the substrate. We also performed similar experiments with chicken liver mitochondria. As can be seen from Table VIII, the features of the reaction with chicken liver mitochondria were essentially similar to those observed

for pigeon, although the activities in chicken were lower in general than in pigeon. It is apparent that the interaction of ATP and GTP is not appreciably restricted in the matrix of pigeon and chicken liver mitochondria.

Phosphopyruvate carboxylase activities in liver mitochondria from guinea pig, pigeon and rat

The activities of phosphopyruvate carboxylase in liver mitochondria from guinea pig, pigeon and rat were compared by using sonic extracts of respective mitochondrial preparations. Mitochondrial suspensions were diluted by addition of 5 times the volume of 0.15 M KCl, and sonicated for 3 min at 10 kcycles/sec, followed by 60 min centrifugation at 105000 × *g*. Proteins in the supernatant fractions were precipitated with ammonium sulfate (80 % saturation), removed in 0.02 M Tris–HCl buffer (pH 7.2) and dialyzed for 3 h against the same buffer with the buffer being renewed every hour. As shown in Table IX, the enzyme activity in pigeon liver mitochondria was very high. Guinea-pig liver mitochondria showed also quite a high activity, while the activity in rat liver mitochondria was relatively low.

TABLE IX

PHOSPHOPYRUVATE CARBOXYLASE ACTIVITY IN SONIC EXTRACTS OF LIVER MITOCHONDRIA FROM PIGEON, GUINEA PIG AND RAT

Reaction mixtures contained 10 μmoles of oxaloacetate, 10 μmoles of GTP, 120 μmoles of Tris–HCl buffer (pH 7.2), 75 μmoles of KCl, 200 μmoles of sucrose, 3 μmoles of reduced glutathione, 5 μmoles of $MnCl_2$ and respective extracts in a final volume of 2.8 ml. Amounts of mitochondrial extracts used were 0.27 mg, 0.96 mg and 0.6 mg (as nitrogen), respectively, for pigeon, guinea pig and rat. Reactions were carried out in test tubes at 25° for 10 min. Amounts of PEP formed were assayed enzymatically.

Species of animals	*PEP formed (μmoles/mg N/10 min)*
Pigeon	12.1
Guinea pig	4.6
Rat	1.4

Nucleoside diphosphate kinase and GTP–AMP–phosphate transferase

The activities of nucleoside diphosphate kinase and GTP–AMP–phosphate transferase in sonic extracts of liver mitochondria from guinea pig, pigeon, rat and rabbit were compared. The extracts were dialyzed against 0.05 M Tris–acetate buffer (pH 7.5) before being used. The nucleoside diphosphate kinase activities in these extracts were assayed spectrophotometrically by following the increase in absorbance at 340 mμ due to the reduction of NADP in the reaction systems, as described in the legend to Fig. 3. The crystalline hexokinase preparation employed in these experiments exhibited a slight activity with GTP, too. Therefore, blank activities due to both adenylate kinase in mitochondrial extracts and the hexokinase preparation were subtracted from the values obtained in the complete system with GTP and ADP. Blank values were similarly subtracted in the assay of GTP–AMP–phosphate transferase activity.

In the reaction with sonic extracts of guinea-pig liver mitochondria and with GTP and AMP as substrates, the absorbance at 340 mμ increased soon after a short

lag period, and continued to increase linearly, as shown in Fig. 3. In contrast, when GTP and ADP were the substrates, the rate of increase of absorbance in the initial phase of the reaction was rather lower than those in the reaction system with ADP alone (probably GTP was inhibiting the adenylate kinase activity in the extracts), so that a large lag period was observed before the absorbance increase above blank values

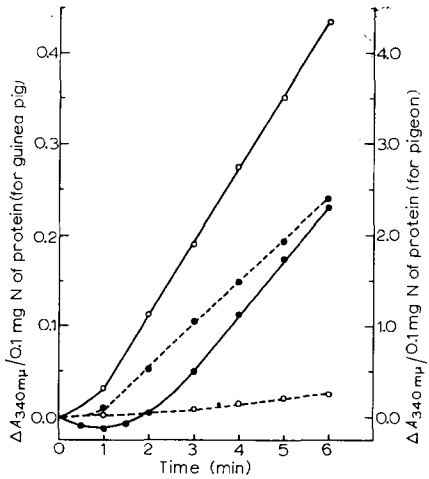

Fig. 3. Apparent activities of nucleoside diphosphate kinase and GTP–AMP–phosphate transferase in sonic extracts of liver mitochondria from guinea pig and pigeon. Assay systems for nucleoside diphosphate kinase activities contained, in a final volume of 3.0 ml: 3 μmoles of GTP, 0.25 μmole of ADP, 30 μg protein of crystalline yeast hexokinase, 17 μg protein of glucose-6-phosphate dehydrogenase, 60 μmoles of glucose, 0.6 μmole of $NADP^+$, 5 μmoles of $MgCl_2$, 30 μmoles of KCl, 450 μmoles of Tris–acetate buffer (pH 7.5), and appropriate amounts of mitochondrial extracts. Assay systems for GTP–AMP–phosphate transferase were the same as above except that 0.5 μmole of AMP was used instead of ADP. Reactions were carried out at 20° in a cuvette (light path, 1 cm) of the recording spectrophotometer. Values of blank activities were subtracted from the readings in the complete systems as described in text. Solid lines: activities observed with GTP and ADP; broken lines: activities observed with GTP and AMP. ●, guinea pig; ○, pigeon.

became apparent (Fig. 3). Moreover, the rate of increase of absorbance in the subsequent period of experiment in this reaction system was very close to those observed in the reaction system with GTP and AMP. These results suggest that the nucleoside diphosphate kinase activity in guinea-pig liver mitochondria is very low, if not nil, and the apparent activity observed in the reaction system with GTP and ADP may reflect instead the function of GTP–AMP–phosphate transferase which results from the combined and cyclic operation of the following two reactions:

$$2\ ADP \rightarrow ATP + AMP$$

$$AMP + GTP \rightarrow ADP + GDP$$

In contrast, the nucleoside diphosphate kinase activity in extracts of pigeon liver mitochondria was exceedingly high (Fig. 3). This is in agreement with the observation by GEVERS[17]. The activity of GTP–AMP–phosphate transferase was very low in extracts from either pigeon or guinea pig (Fig. 3). The activity of GTP–AMP–

phosphate transferase in sonic extracts of rabbit liver mitochondria was even lower than that of guinea pig, and in the reaction system with GTP and ADP, the rate of reduction of NADP was again found to be far lower than that in the reaction system with ADP alone, indicating that the nucleoside diphosphate kinase activity in rabbit liver mitochondria is extremely low. The extracts from rat, however, contained quite a high activity of nucleoside diphosphate kinase, while the activity of GTP–AMP–phosphate transferase was very low, similarly to those for guinea pig and other animals. The observed activities of these two enzymes in rat liver mitochondria are almost comparable to those reported by HELDT AND SCHWALBACH[6] when calculated on the protein basis of mitochondrial extracts. In Table X are compared the apparent activities of the two enzymes in respective mitochondrial extracts from the animals tested, although the activities observed in the reaction system with GTP and ADP may not always represent the actual activities of nucleoside diphosphate kinase.

TABLE X

COMPARISON OF APPARENT ACTIVITIES OF NUCLEOSIDE DIPHOSPHATE KINASE AND GTP–AMP–PHOSPHATE TRANSFERASE IN SONIC EXTRACTS OF LIVER MITOCHONDRIA FROM VARIOUS ANIMALS

The values in the table were calculated from the data presented in Fig. 3.

Species of animals	$\Delta A_{340m\mu}$/0.1 *mg N of protein/min*	
	With GTP and ADP	*With GTP and AMP*
Guinea pig	0.065	0.05
Pigeon	0.85	0.07
Rabbit	0.03	0.03
Rat	0.28	0.09

The nucleoside diphosphate kinase activities in the precipitates obtained after the centrifugation of sonicates of guinea pig mitochondria were also examined. In these experiments, oligomycin was also added to the reaction system with the aim of preventing the hydrolysis of ATP. The results obtained varied considerably from preparation to preparation, but the activities recovered in the precipitated insoluble fractions were always less than 20 % of those in the soluble fractions of mitochondria.

DISCUSSION

It is conceivable that the observed stimulation of the PEP formation by dinitrophenol, pentachlorophenol, dicumarol and by the hexokinase system may be due mainly to the stimulation of the mitochondrial respiration by these uncouplers and the phosphate acceptor system which would give rise to the increased production of GTP and oxaloacetate from the substrates added. The results of the present investigation also suggest that in guinea-pig liver mitochondria, nucleotides involved in the substrate level phosphorylation cannot easily interact with the adenine nucleotides of respiratory chain phosphorylation system and with exogenously added adenine nucleotides, while the GTP formed at the step of α-ketoglutarate oxidation is freely accessible to the site of PEP synthesis. This is in sharp contrast to the situations

in pigeon and chicken liver mitochondria where the nucleotides appeared to be able to interact freely and no particular correlation could be observed between the PEP synthesis and the substrate level phosphorylation. The differences observed between guinea-pig liver mitochondria and avian liver mitochondria seem to be due principally to the difference in the level of nucleoside diphosphate kinase rather than the so-called "compartmentation" of nucleotides as proposed first by AZZONE AND ERNSTER[14], since it was revealed that (1) the level of nucleoside diphosphate kinase in guinea-pig liver mitochondria was extremely low, while the level in pigeon liver mitochondria was very high, and (2) the phosphopyruvate carboxylase activity was quite high in mitochondria from both pigeon and guinea pig liver. Note that rat liver mitochondria contained high activities of nucleoside diphosphate kinase but showed relatively low activities of phosphopyruvate carboxylase. This satisfactorily accounts for the finding that the PEP/α-ketoglutarate ratio obtained with rat liver mitochondria in the presence of dinitrophenol was only about 0.1 (ref. 12). With respect to rabbit, the situation seems to be very similar to that for guinea pig; in fact, intimate functional relationships have been observed between the PEP synthesis and α-ketoglutarate oxidation in rabbit liver mitochondria[9,10]. In view of these circumstances, it seems plausible to assume that the so-called "compartmentation" of the substrate-linked phosphorylation in liver mitochondria may in reality be only functional than substantial and that the difference in the apparent tightness of the so-called "compartmentation" in liver mitochondria from various sources would reflect mainly, if not exclusively, the difference in the activity of mitochondrial nucleoside diphosphate kinase.

PEP seems to be able to move freely across the mitochondrial membrane, for it was observed that when mitochondria were centrifuged off from the reaction mixture after 20 min of incubation but without adding trichloroacetic acid, almost all of the PEP formed was recovered in the supernatant fraction.

The interaction of guanine and adenine nucleotides, however, is not wholly abolished in guinea-pig liver mitochondria; nucleotides are still able to interact, though only to a limited extent. It was shown by experiments with ^{14}C-labeled substrates that when the reaction mixtures did not contain dinitrophenol or other uncouplers or phosphate acceptor system, the absolute values of the yield of PEP as well as the amounts of α-ketoglutarate oxidized were very small, and under these conditions the molar ratios of PEP formed/α-ketoglutarate oxidized were also low. This would indicate that when yields of GTP are relatively small, considerable portions of these GTP may be consumed in other ways than the PEP synthesis, most probably by reacting with adenine nucleotides through the function of nucleoside diphosphate kinase and GTP–AMP–phosphate transferase.

However, when the respiration was stimulated by addition of dinitrophenol or other uncouplers or the hexokinase system, it would give rise to a production of large amounts of GTP which would far exceed the capacity of the nucleotide interaction and therefore practically all of the GTP formed would become available to the PEP synthesis. If this were the case, one could easily understand why the ratio of PEP formed/α-ketoglutarate oxidized became almost 1.0 when dinitrophenol or other uncouplers were added to the reaction mixtures containing α-ketoglutarate or its precursors. Consistent with this idea, it was observed that, in the presence of dinitrophenol or other uncouplers or the hexokinase system, the oxidation of α-keto-

glutarate was considerably stimulated by the addition of malate or fumarate which could serve to consume GTP to regenerate GDP, and in turn, the metabolism of fumarate was greatly increased by the addition of α-ketoglutarate.

The observed intimate functional coupling between α-ketoglutarate oxidation and PEP synthesis points to the possibility that PEP carboxylase may play a significant role in the regulation of the citric acid cycle activity in guinea-pig liver mitochondria and possibly in rabbit liver mitochondria as well. It should be noted that the concentration of guanine nucleotides in liver mitochondria is far lower than that of adenine nucleotides[2,3] and hence the balance of concentrations of GDP and GTP may be significant in the smooth operation of the citric acid cycle. Moreover, the value of the PEP/α-ketoglutarate ratio was found to be nearly 1.0 even when the hexokinase system was employed for stimulating the respiration. This suggests the possibility that even under physiological conditions *in vivo* the oxidation of α-ketoglutarate may be supported to a considerable degree by the coupling with the PEP synthesis. In pigeon and chicken liver mitochondria, however, this type of functional coupling may have practically no meaning, and the physiological role of mitochondrial PEP synthesis in avian livers may consist mainly in its contribution to gluconeogenesis in liver, as discussed by GEVERS[17]. It is also expected that the physiological significance of the functional coupling of PEP synthesis and substrate level phosphorylation may be relatively small in rat liver mitochondria. In guinea-pig liver mitochondria, PEP may also play a role in draining energy from the site of substrate level phosphorylation to extramitochondrial cytoplasmic space, since PEP appeared to be able to pass the mitochondrial membrane easily.

In the dinitrophenol-stimulated PEP synthesis, the relationship between the rate of PEP synthesis and the dinitrophenol concentration was polyphasic. We also observed a similar polyphasic relationship with pentachlorophenol. The low rate of PEP formation, at 10^{-4} M dinitrophenol, was interpreted by NORDLIE AND LARDY[13] to coincide with the maintenance of the adenine nucleotide mainly in the form of ADP (due to the stimulation of ATPase activity by dinitrophenol) which acts as an acceptor for the P_i fixed in GTP, thus rendering it unavailable for PEP formation. However, as shown in Figs. 1 and 2, the rate of oxygen uptake also changed, apparently in parallel with the change in the activity of PEP synthesis. This is hardly explained by simply assuming the change in ADP level. In addition, the nucleoside diphosphate kinase activity in guinea-pig liver mitochondria was revealed to be extremely low. Further investigations are required to explain the unique polyphasic relationships between the rates of oxygen uptake, PEP synthesis and the concentrations of uncouplers.

ACKNOWLEDGEMENTS

We are indebted to Prof. T. AJELLO for kindly supplying atractyloside. This investigation was supported in part by Public Health Research Grant AM 08016-03 and -04, from the National Institute of Arthritis and Metabolic Diseases, U.S.A.

This work was taken from a thesis of N. ISHIHARA submitted to the Tohoku University in partial fulfillment of the requirements for the degree of Doctor of Medical Science.

REFERENCES

1 L. P. HAGER, in P. D. BOYER, H. A. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 6, Academic Press, New York, 1962, p. 387.
2 H. W. HELDT, H. JACOB AND M. KLINGENBERG, *Biochem. Biophys. Res. Commun.*, 17 (1962) 130.
3 H. W. HELDT AND M. KLINGENBERG, *Biochem. Z.*, 343 (1965) 433.
4 R. H. WEAVER, in P. D. BOYER, H. A. LARDY AND K. MYBÄCK, *The Enzymes*, Vol. 6, Academic Press, New York, 1962, p. 151.
5 R. C. NORDLIE AND H. A. LARDY, *J. Biol. Chem.*, 238 (1963) 2259.
6 H. W. HELDT AND K. SCHWALBACH, *European J. Biochem.*, 1 (1967) 199.
7 C. R. ROSSI AND D. M. GIBSON, *J. Biol. Chem.*, 239 (1964) 1694.
8 S. G. VAN DEN BERGH, *Biochim. Biophys. Acta*, 98 (1965) 442.
9 S. W. STANBURY AND G. H. MUDGE, *J. Biol. Chem.*, 210 (1954) 949.
10 G. H. MUDGE, H. W. LEUBERG AND S. W. STANBURY, *J. Biol. Chem.*, 210 (1954) 965.
11 K. A. SCHELLENBERG AND E. C. WEINBACH, *Biochim. Biophys. Acta*, 45 (1960) 593.
12 H. R. SCHOLTE AND J. M. TAGER, *Biochim. Biophys. Acta*, 110 (1965) 252.
13 R. C. NORDLIE AND H. A. LARDY, *Biochem. Z.*, 338 (1963) 356.
14 G. F. AZZONE AND L. ERNSTER, *J. Biol. Chem.*, 236 (1961) 1501.
15 R. CHARLES, J. M. TAGER AND E. C. SLATER, *Biochim. Biophys. Acta*, 74 (1963) 33.
16 J. B. CHAPPELL AND G. D. GREVILLE, *Nature*, 190 (1961) 502.
17 W. GEVERS, *Biochem. J.*, 103 (1967) 141.
18 W. C. SCHNEIDER AND G. H. HOGEBOOM, *J. Biol. Chem.*, 183 (1950) 123.
19 R. CZOK AND E. ECKERT, in H. U. BERGMEYER, *Methods of Enzymatic Analysis*, Academic Press, New York, 1963, p. 228.
20 Y. SUGINO AND Y. MIYOSHI, *J. Biol. Chem.*, 239 (1964) 2360.
21 J. A. MUNTZ AND J. HURWITZ, *Arch. Biochem. Biophys.*, 32 (1951) 137.
22 G. R. BARTLETT, *J. Biol. Chem.*, 234 (1959) 459.
23 N. MOURAD AND R. E. PARKS, Jr., *J. Biol. Chem.*, 241 (1966) 271.
24 E. C. WEINBACH AND J. GARBUS, *J. Biol. Chem.*, 240 (1965) 1811.
25 J. B. CHAPPELL AND A. R. CROFTS, *Biochem. J.*, 95 (1965) 707.
26 E. PAFF, M. KLINGENBERG AND H. W. HELDT, *Biochim. Biophys. Acta*, 104 (1965) 312.
27 M. KLINGENBERG AND E. PAFF, in J. M. TAGER, S. PAPA, E. QUAGLIARIELLO AND E. C. SLATER, *Regulation of Metabolic Processes in Mitochondria* (B.B.A. Library, Vol. 7), Elsevier, Amsterdam, 1966, p. 180.
28 H. W. HELDT, in J. M. TAGER, S. PAPA, E. QUAGLIARIELLO AND E. C. SLATER, *Regulation of Metabolic Processes in Mitochondria* (B.B.A. Library, Vol. 7), Elsevier, Amsterdam, 1966, p. 51.