The Regulation of Phosphoenolpyruvate Synthesis in Pigeon Liver

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1. The intracellular location and maximal activities of enzymes involved in phosphoenolpyruvate synthesis have been investigated in pigeon liver. Enolase and pyruvate kinase were cytoplasmic, and the activities were 50–60 and 180–210 $\mu\text{moles/min./g. dry wt.}$ at 25° respectively. Phosphoenolpyruvate carboxykinase was present exclusively, and nucleoside diphosphokinase predominantly, in the mitochondria; the particles had to be disrupted to elicit maximal activities, which were 27–33 and 400–600 $\mu\text{moles/min./g. dry wt.}$ at 25° respectively. The activities of all four enzymes did not change significantly during 48 hr. of starvation. 2. Conditions for incubation of washed isolated mitochondria were established, to give high rates of synthesis of phosphoenolpyruvate, linear with time and proportional to mitochondrial concentration. Inorganic phosphate and added adenine nucleotides were stimulatory, whereas added Mg^{2+} inhibited, partly owing to activation of contaminant pyruvate kinase. Phosphoenolpyruvate formation occurred from oxaloacetate, malate, fumarate, succinate, α -oxoglutarate and citrate, in decreasing order of effectiveness. 3. The steady-state ATP/ADP ratio of mitochondrial suspensions was decreased in the presence of added 2.5 mM Mg^{2+} (owing to stimulation of adenylate kinase and possibly of an adenosine triphosphatase), 0.5 mM Ca^{2+} or 0.04 mM dinitrophenol. In each case the rate of substrate removal and oxygen uptake was increased, whereas phosphoenolpyruvate synthesis was inhibited. Citrate formation was enhanced, owing to de-inhibition of citrate synthase. These effects were not primarily related to changes in the oxaloacetate concentration. 4. Both phosphoenolpyruvate carboxykinase and nucleoside diphosphokinase were active within the atractyloside-sensitive barrier to the mitochondrial metabolism of added adenine nucleotides. There was no correlation between the rate of substrate-level phosphorylation associated with the oxidation of α -oxoglutarate, and the synthesis of phosphoenolpyruvate. 5. The results suggest that phosphoenolpyruvate formation in pigeon-liver mitochondria is regulated partly by the phosphorylation state of the adenine and guanine nucleotides, and partly by variations in the oxaloacetate concentration, all in the mitochondrial matrix. 6. Phosphoenolpyruvate is assumed to be the metabolite transported from the mitochondria to the cytoplasm during gluconeogenesis from oxaloacetate in pigeon liver.

The discovery of species differences in the intracellular distribution of hepatic PEP† carboxykinase [GTP-oxaloacetate carboxy-lyase (trans-phosphorylating), EC 4.1.1.32] by Nordlie & Lardy (1963a) suggests that the control mechanisms of PEP synthesis may also be significantly different in species such as the rat, where this enzyme is predominantly cytoplasmic, and the pigeon, where it is found in the mitochondria (Stickland, 1959). Other pointers in this direction are the kinetic differences between the mitochondrial and cyto-

plasmic PEP carboxykinases of guinea-pig liver described by Holten & Nordlie (1965), and the fact that in certain altered hormonal states, and in starvation, only the total activities of the extra-mitochondrial enzymes of rat and guinea-pig liver are affected (Nordlie, Varriecchio & Holten, 1965).

Another problem in this area of metabolism is the possibility of a special functional relationship between the substrate-level phosphorylation associated with α -oxoglutarate oxidation and the formation of PEP catalysed by PEP carboxykinase (Azzone & Ernster, 1961; Nordlie & Lardy, 1963b; see also Scholte & Tager, 1965).

To assist in the clarification of these problems,

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† Abbreviation: PEP, phosphoenolpyruvate.

the control of PEP synthesis in pigeon liver was examined, to establish the intracellular location and maximal activities of enzymes involved in this process, including nucleoside diphosphokinase (ATP-nucleoside diphosphate phosphotransferase, EC 2.7.4.6), and to study regulatory factors in isolated mitochondria.

METHODS AND MATERIALS

Materials. These were generally as described by Gevers & Krebs (1966). GDP and GTP were obtained as lithium salts from C. F. Boehringer und Soehne G.m.b.H., Mannheim, W. Germany. Potassium PEP, sodium PEP, sodium D-3-phosphoglycerate, barium D-2-phosphoglycerate, sodium pyruvate, sodium oxaloacetate, sodium fumarate, sodium succinate and sodium α -oxoglutarate were also from this source. L-Malic acid was from Koch-Light Laboratories Ltd., Colnbrook, Bucks. IDP and ITP were products of the Sigma Chemical Co., St Louis, Mo., U.S.A. 2,4-Dinitrophenol was from British Drug Houses Ltd., Poole, Dorset. Oligomycin was a product of Mann Research Laboratories Inc., New York 6, N.Y., U.S.A., and atractyl-loside was a gift of Dr A. Bruni, Padua, Italy.

Aconitase was prepared from pig heart by the method of Siebert (1963), modified to include the addition of chloroform at the step of tissue homogenization to precipitate haemoglobin (Morrison, 1954). All other enzymes were products of C. F. Boehringer und Soehne G.m.b.H.

Pigeons. Adult pigeons (males and females) were maintained on a diet of 'chicken-corn', consisting primarily of maize, barley and wheat corn. When they were to be starved for periods described in the text, food was withheld at 9 a.m., water being freely available.

Preparation of mitochondria. Since pigeons have no gall bladders and store bile in the intrahepatic system of bile ducts, and since bile may damage mitochondria (Christ & Hülsmann, 1962), it was important to remove as much bile as possible before intracellular structures were exposed to it by homogenization of the liver. Two pigeons were decapitated and exsanguinated. Livers were removed and kept for 2 min. in a polythene bag buried in crushed ice. After cutting away connective tissue, each liver was divided into five to seven pieces, which were placed on hard filter paper and cut with a razor blade at 1–2 mm. intervals in a criss-cross fashion. The pieces were washed six times by suspension and repeated inversion in 5 vol. of freshly prepared 0.25 M-sucrose, also containing 10 mM-tris and 0.2 mM-EDTA, all at pH 7.4 and 0°. After mincing in a Fischer mincer (Jouan, Paris, France), the livers were homogenized in about 4 vol. of sucrose solution in a stainless-steel homogenizer as described by Krebs, Dierks & Gascoyne (1964). Portions (10 ml.) of the homogenate were centrifuged (in 15 ml. conical tubes) for 5 min. at 450 g in the cold. The supernatants were carefully decanted into 5 in. $\times$ $\frac{3}{8}$ in. thick-walled test tubes so that none of the reddish material (erythrocytes, nuclei and debris) was included, and were centrifuged at 2500 g for 10 min. at 2°. The mitochondrial pellet was washed twice with 5–7 vol. of sucrose solution. Resuspension in each case was achieved by homogenization with a motor-driven loose-fitting Teflon pestle.

For incubations, the washed mitochondria were finally suspended in ice-cold 0.154 M-KCl, containing 10 mM-tris,

pH 7.4. They were incubated in manometer vessels (gas phase oxygen or air) in a water bath at 30°, with shaking; the centre wells contained 0.2 ml. of 2 N-NaOH. The final fluid volume was 4 ml. and 18–32 mg. dry wt. of trichloroacetic acid-precipitable material was added to each cup. Other additions are described in the text. Incubations were terminated by rapid freezing and acidification, and neutral extracts were prepared, all as described by Gevers & Krebs (1966). To obtain a reference standard (tissue dry wt.), known volumes of mitochondrial suspensions were treated in previously weighed tubes with 4% and then 2% trichloroacetic acid and finally water (with centrifugation at 3000 rev./min. between each mixing), and were dried to constant weight at 110°.

For assays of PEP carboxykinase, nucleoside diphosphokinase, pyruvate kinase and enolase, sucrose homogenates were prepared as described above, except that 10 mM-2-mercaptoethanol was present throughout. The dry wt./wet wt. ratio of tissue in the homogenates varied from 1:2.9 to 1:3.6, depending on the nutritional state. To study the intracellular distribution of enzymes, a fresh homogenate was centrifuged at 30000 g for 20 min. at 2°, or a pure mitochondrial fraction was prepared. The first two of the above enzymes were assayed before and after treatment of the extracts for two separate periods of 60 sec. each in an MSE ultrasonic disintegrator (60 W, $\frac{3}{8}$ in. probe) (Measuring and Scientific Equipment Ltd., London, S.W. 1).

Analytical procedures. D-2- and D-3-Phosphoglycerate, PEP and pyruvate were determined as described by Czok & Eckert (1963). L-Malate, α -oxoglutarate, L-glutamate, L-aspartate, ADP and AMP were determined as described by Gevers & Krebs (1966). GTP was determined as the difference between the value for total nucleoside triphosphates given by the method of Adam (1963) and that by the method of Lamprecht & Trautschold (1963) for ATP + ITP. Citrate was determined as described by Siebert (1963).

Enzyme assays. Pyruvate kinase (ATP-pyruvate phosphotransferase, EC 2.7.1.40) was assayed by the method of Krebs & Eggleston (1965), and enolase (D-2-phosphoglycerate hydro-lyase, EC 4.2.1.1) by the method of Delbrück, Schimassek, Bartsch & Bücher (1959).

PEP carboxykinase was assayed generally by the method of Holten & Nordlie (1965), except that 2-mercaptoethanol was present during preparation and assay of the extracts. Also, ultrasonic disruption of the whole homogenates or mitochondria was employed to solubilize the enzyme, rather than repeated freezing and thawing (which inhibited after four or five 'cycles' and which never yielded more than two-thirds of the activity released by ultrasonic disruption). Sodium cyanide (0.2 mM) was added to inhibit the NADH-oxidase activity activated by ultrasonic disruption. Blank pyruvate-kinase activity was decreased by excluding K⁺ and NH₄⁺ ions (both activators of pyruvate kinase; Kachmar & Boyer, 1953), by using sodium salts of reactants and by dialysing the malate dehydrogenase suspension for 24 hr. against ten changes of ice-cold distilled water, in previously boiled and rinsed dialysis tubing. IDP was used in preference to GDP since it is only half as reactive as the latter with pyruvate kinase (W. Gevers, unpublished work).

Nucleoside-diphosphokinase activity was measured by the method of Mourad & Parks (1966), except that 10 μ g.

(1400 units) each of hexokinase and glucose 6-phosphate dehydrogenase were added to permit the accurate determination of maximum initial velocities. The extracts used were identical with those in which PEP carboxykinase was assayed. The blank activity due to adenylate kinase was subtracted.

RESULTS

Enzyme activity and distribution

Pyruvate-kinase and enolase activities in livers from fed pigeons were 50–60 and 180–210 μ moles/min./g. dry wt. at 25° respectively (Table 1). No significant changes occurred during starvation for 48 hr. and re-feeding with standard diet, in contrast with the marked fall in pyruvate kinase during starvation observed in rat livers by Krebs & Eggleston (1965) and Tanaka, Harano, Morimura & Mori (1965). The bulk of both enzymes was found in the soluble fraction (Table 2), although twice-washed mitochondrial preparations were always significantly contaminated with pyruvate kinase.

Maximal PEP-carboxykinase activities could

not be elicited without disruption of the particles and practically all of the enzyme resided in the particulate fraction from which more than 80% could be solubilized by ultrasonic disruption (Table 3). In another experiment, 66% of the original activity of a homogenate was recovered in the pure washed mitochondrial fraction, 20% in the pellet containing intact cells, nuclei and debris, and 10% in the two supernatants of the washing procedures. It is evident that there is little of the enzyme in the cytoplasm of pigeon liver, contrasting strongly with rat liver (Nordlie & Lardy, 1963a). The intracellular distribution was the same in fed and starved birds. There was no significant variation in PEP-carboxykinase activity (27–33 μ moles/min./g. dry wt. at 25°) during a cycle of starvation and re-feeding (Table 4). Also, the activity in washed mitochondria was 70–85 μ moles/min./g. dry wt. at 25°, irrespective of the nutritional state of the pigeons.

Nucleoside diphosphokinase was present mainly in the particles; 12% was found in the cytoplasm. This appears also to be the case in rat liver (Klingenberg & Pfaff, 1966), although Heldt &

Table 1. *Activities of pyruvate kinase and enolase in pigeon liver during starvation (48 hr.) and re-feeding (with standard diet)*

The assays were carried out on whole homogenates as described in the Methods and Materials section. Mean activities are expressed as μ moles/min./g. dry wt. at 25°, $\pm$ s.d.

Nutritional state	No. of birds	Pyruvate-kinase activity	Enolase activity
Well-fed	4	56 $\pm$ 4	202 $\pm$ 17
Starved for 24 hr.	3	69 $\pm$ 5	175 $\pm$ 29
Starved for 48 hr.	4	58 $\pm$ 9	162 $\pm$ 12
Starved for 48 hr., then:			
Re-fed for 6 hr.	3	60 $\pm$ 4	174 $\pm$ 26
Re-fed for 18 hr.	3	51 $\pm$ 3	149 $\pm$ 22
Re-fed for 40 hr.	3	49 $\pm$ 7	192 $\pm$ 31

Table 2. *Intracellular distribution of pyruvate kinase and enolase in pigeon liver*

A fresh sucrose homogenate was prepared (1:4, w/v; see the Methods and Materials section) with the liver of a pigeon starved for 48 hr. Two sets of particles were prepared; the first was the unwashed sediment of a 20 min. centrifugation at 30000g at 0°, and the other consisted of twice-washed mitochondria. Both pellets were resuspended in homogenizing medium to the original volume of homogenate from which they were prepared; percentage yields could then be derived for each fraction.

Fraction	Activity (μ moles/min./fraction at 25°)		Percentage of original activity	
	Pyruvate kinase	Enolase	Pyruvate kinase	Enolase
Whole homogenate	379	640	100	100
30000g supernatant	285	562	75	88
30000g sediment	104	86	27	13
Washed mitochondria	8	3	2	1

Table 3. *Intracellular distribution of phosphoenolpyruvate carboxykinase in pigeon liver*

A pigeon starved for 48 hr. was used. A standard sucrose homogenate (see the Methods and Materials section) was centrifuged for 20 min. at 30000g at 0°. Samples were treated with ultrasound for two periods of 60 sec. each, and the ultrasonic extracts were centrifuged as before. Assays were carried out in the direction of oxaloacetate formation (see the Methods and Materials section).

No.	Fraction	Activity (μ moles/fraction/ min. at 25°)
1	Whole homogenate	10.2
2	Supernatant	3.18
3	Resuspended particles	8.85
4	Ultrasonic whole homogenate	75
5	Ultrasonic supernatant	4.10
6	Ultrasonic resuspended particles	75
7	Supernatant of (6) after centrifugation	58
8	Resuspended sediment of (6) after centrifugation	11.6

Table 4. *Changes in phosphoenolpyruvate-carboxykinase activity in pigeon liver during starvation and re-feeding (with standard diet)*

Whole homogenates were prepared and assayed as in the Methods and Materials section. The mean values are given $\pm$ S.D.

Nutritional state	No. of birds	Activity (μ moles/min./g. dry wt. at 25°)
Well-fed	5	31 $\pm$ 5
Starved for 24 hr.	3	27 $\pm$ 3
Starved for 48 hr.	6	33 $\pm$ 5
Starved for 48 hr., then:		
Re-fed for 6 hr.	3	27 $\pm$ 2
Re-fed for 18 hr.	3	32 $\pm$ 3
Re-fed for 40 hr.	3	29 $\pm$ 4

Schwalbach (1966) have reported contrary data. Ultrasonic disruption solubilized about two-thirds of the particulate activity, and was required for the demonstration of 'latency' (see Chappell & Greville, 1963). The tissue activity was very high (400–600 μ moles/min./g. dry wt. at 25°) in comparison with PEP carboxykinase.

Synthesis of phosphoenolpyruvate in pigeon liver mitochondria

General requirements and properties. Suitable conditions for the study of PEP synthesis in mitochondria were established as follows. It was initially decided to use two media, the first corresponding to that of Bartley (1954) and the second to that of Stanbury & Mudge (1954), with phosphate and tris as the principal buffer respectively. When particles were incubated with malate and with added ATP, the yield of PEP was greater at higher P_i concentrations (Table 5), and the rates of substrate removal and the ATP/ADP ratio were also increased. There was no inhibition by tris (cups 5 and 6). The rate of substrate removal and the ATP/ADP ratio were higher as the P_i concentration was increased.

When Mg^{2+} was omitted, net accumulation of PEP occurred (with a small amount of 2- and 3-phosphoglycerate) and was linear over 40 min. (Fig. 1). In this experiment, 10 μ moles of PEP were added at the start; in the presence of 2.5 mM- Mg^{2+} there was a progressive loss of PEP, whereas there was no change in the presence of 2.5 mM- Ca^{2+} . Added adenine nucleotides (ADP or ATP) were required for maximal accumulation of PEP from all substrates tested. The standard incubation medium for synthesis of PEP from oxaloacetate, malate, fumarate, succinate, α -oxoglutarate, glutamate and citrate contained 15 mM- P_i , 1–2 mM-ADP, 10–15 mM-substrate and potassium chloride, all at pH 7.4; with all substrates except malate and

Table 5. *Effect of inorganic phosphate concentration on phosphoenolpyruvate formation from malate in pigeon liver mitochondria*

Fresh washed mitochondria (21 mg. dry wt./cup) were prepared and incubated (as described in the Methods and Materials section) for 30 min. at 30°. Each cup, except no. 6, contained 40 mM-tris buffer, pH 7.4, and 1 mM-ATP. P_i was added as potassium phosphate solution, pH 7.4.

Cup. no.	1	2	3	4	5	6
Additions (mm):						
Malate	15	15	15	15	15	15
P_i	0	2.5	5	10	20	20
Metabolic changes (μ moles/cup):						
Malate	-13.6	-21.8	-27.2	-29.3	-31.7	-30.1
Metabolites formed (μ moles/cup):						
PEP + phosphoglycerates	1.81	8.16	8.38	9.44	11.2	11.4
ATP	1.30	2.70	3.40	3.34	3.41	3.29
ADP	2.15	0.85	0.33	0.37	0.34	0.40

Fig. 1. Effect of Mg²⁺ and Ca²⁺ addition on PEP accumulation from malate in pigeon liver mitochondria. Mitochondria were prepared as described in the Methods and Materials section. They were incubated (20mg. dry wt./cup) at 30° in a medium containing 15mm-P_i, pH 7.4, 2mm-ADP, 2.5mm-PEP and iso-osmotic KCl, with additions as indicated. At the times indicated, incubations were terminated and the phosphorylated compounds measured in the neutral extracts. ■, No added bivalent ions; ▲, +2.5mm-Ca²⁺; ○, +2.5mm-Mg²⁺.

oxaloacetate, 0.625mm-Mg²⁺ was included to increase the rate of oxidation, which was otherwise very slow. K⁺ was required for maximal activity; Na⁺ was not essential but was usually present. The formation of PEP from malate was proportional to the concentration of mitochondria in the range 10–40mg./cup. This is in contrast with the findings of Mendicino & Utter (1962), who included 5mm-Mg²⁺ in their incubation medium for chicken liver mitochondria.

Results with mitochondria from livers of fed and starved pigeons were qualitatively and quantitatively similar.

Table 6. Formation of phosphoenolpyruvate from various substrates in pigeon liver mitochondria under optimum conditions

Mitochondria were prepared as in the Methods and Materials section and incubated for 30min. at 30° in a medium containing 10mm-substrate as indicated, 15mm-P_i, pH7.4, 1mm-ADP and (for all substrates except malate and oxaloacetate) 0.625mm-Mg²⁺. Mitochondrial dry wt. was 26mg./cup.

Cup no.	Substrate	PEP formed (μmoles/cup)
1	Oxaloacetate	20
2	Malate	20
3	Fumarate	16
4	Succinate	7.7
5	α-Oxoglutarate	6.9
6	Citrate	5.7

Table 7. Effects of added ATP, ADP and Mg²⁺ on the formation of phosphoenolpyruvate from malate in pigeon liver mitochondria

Mitochondria were prepared as described in the Methods and Materials section, and were incubated under air at 30° for 30min. The medium contained 15mm-P_i, pH7.4, and additions as indicated. Mitochondrial dry wt. was 22mg./cup.

Additions (mm):	Cup no.	1	2	3	4	5	6
Malate		15	15	15	15	15	15
ATP		0	1.25	0	0	1.25	0
ADP		0	0	1.25	0	0	1.25
Mg ²⁺		0	0	0	2.5	2.5	2.5
Metabolic changes (μmoles/cup):							
O ₂		- 8.4	-13.0	-10.4	- 8.6	-22.0	-18.1
Malate		-20.6	-25.2	-22.6	-21.0	-30.1	-29.4
Compounds found (μmoles/cup):							
PEP+ phosphoglycerates		11.6	17.7	14.8	11.8	9.2	8.2
Oxaloacetate		0.13	0.54	0.39	0.09	0.44	0.47
α-Oxoglutarate		1.12	1.38	1.68	0.99	3.53	3.50
Citrate		1.36	1.51	1.59	1.19	3.69	4.04
ATP		0.15	4.27	3.78	0.06	1.99	1.53
ADP		0.12	0.77	0.65	0.10	1.51	1.67
AMP		<0.01	0.04	0.03	<0.01	0.67	0.64

Phosphoenolpyruvate synthesis from malate and oxaloacetate. Under optimum conditions, these two substrates gave the highest rates of PEP accumulation of all substrates tested (Table 6). In the absence of added Mg^{2+} but presence of added ADP or ATP, between 60 and 80% of the substrate removed was converted into PEP (Table 7). This was about a quarter of the maximum PEP-carboxykinase activity when assayed in the same particles after ultrasonic disruption. The oxygen uptake declined progressively during the incubation, but with malate it was at all times sufficient for the conversion of malate into oxaloacetate before phosphorylation. The ATP/ADP ratio was about 6. Small amounts of citrate and α -oxoglutarate were formed. Omission of added adenine nucleotides decreased PEP formation by 20–35%. Addition of Mg^{2+} (2.5 mm) decreased the accumulation of PEP from malate in the presence of added adenine nucleotides by 80–90%, and the oxygen uptake was increased, as was the removal of malate and the formation of citrate and α -oxoglutarate. The ATP/ADP ratio was about 1, and significant quantities of AMP were formed. Addition of Mg^{2+} had little effect in the absence of added adenine nucleotides. The oxaloacetate concentration was increased three- to four-fold by the addition of ADP or ATP, independent of the presence of Mg^{2+} .

The data suggest that contaminant pyruvate kinase was activated by the presence of added Mg^{2+} and by ADP, added as such or formed from ATP. The measured activity of pyruvate kinase in the particles was sufficient to account for the differences observed. However, the failure of the mitochondria to maintain ATP in the presence of Mg^{2+} , ADP and malate (in agreement with Bartley, 1955) cannot be explained on this basis, since no net loss of 'energy-rich' phosphates occurs in the sequence of reactions: oxaloacetate $\rightarrow$ PEP $\rightarrow$ pyruvate. Freshly prepared liver mitochondria are thought to contain little Mg^{2+} -activated adenosine triphosphatase (Kielley & Kielley, 1953), but the simplest explanation of the data is that the fall in ATP was due to the action of Mg^{2+} -activated adenylate kinase, and possibly of an adenosine triphosphatase, both outside the inner mitochondrial membrane (Klingenberg & Pfaff, 1966).

The results with oxaloacetate as substrate were similar to those with malate. In both cases the oxygen uptake was low, partly because of the absence of precursors of acetyl-CoA for citrate formation. Pyruvate was thus added with these substrates to saturate the tricarboxylic acid cycle (Table 8). There was a marked effect of Mg^{2+} in decreasing both the ATP/ADP ratio (especially at high added ADP concentrations) and the accumulation of PEP. More citrate and α -oxoglutarate

Table 8. *Effects of ADP concentration on phosphoenolpyruvate formation from malate (or oxaloacetate) + pyruvate in the presence and absence of Mg^{2+}*
Mitochondria were prepared and incubated at 30° for 25 min. under air, as described in the Methods and Materials section. The medium contained 15 mm P_i , pH 7.4, and 4 mm HCO_3^- . Mitochondrial dry wt. was 20 mg./cup.

	Cup no.											
	1	2	3	4	5	6	7	8	9	10	11	12
Additions (mm):												
ADP	0	1	2.5	5	0	1	2.5	5	0	2.5	0	2.5
Malate	10	10	10	10	10	10	10	10	0	0	0	0
Oxaloacetate	0	0	0	0	0	0	0	0	10	10	10	10
Pyruvate	10	10	10	10	10	10	10	10	10	10	10	10
Mg^{2+}	0	0	0	0	2.5	2.5	2.5	2.5	0	2.5	2.5	2.5
Compounds found (μmoles/cup):												
PEP + phosphoglycerates	9.95	27.3	25.2	23.8	10.1	12.7	6.34	5.10	18.2	26.6	16.6	2.94
Oxaloacetate	0.03	0.07	0.20	0.35	0.06	0.29	0.34	0.30	1.65	3.40	6.00	9.72
Citrate	2.36	2.40	2.17	4.48	2.10	5.90	5.66	5.61	3.90	3.30	3.96	7.45
α -Oxoglutarate	1.70	3.20	4.92	11.7	1.72	12.0	11.5	10.4	5.00	8.65	0.04	1.11
ATP	0.08	3.76	9.08	18.0	0.05	2.57	4.34	5.00	0.06	0.68	0.06	2.78
ADP	0.04	0.25	0.76	0.68	0.08	1.33	3.20	5.55	0.04	0.68	0.02	4.22
AMP	<0.01	<0.01	<0.01	<0.01	0.02	0.08	0.74	6.00	<0.01	0.17	0.02	
Metabolic changes (μmoles/cup):												
Malate	-12.0	-20.4	-23.2	-25.2	-12.4	-25.3	-25.0	-20.0				
Pyruvate	-9.72	-14.9	-19.3	-31.8	-9.5	-31.6	-29.7	-29.6				

Table 9. *Effects of Ca²⁺ and Mg²⁺ on the formation of phosphoenolpyruvate from pyruvate + malate in pigeon liver mitochondria*

Conditions were exactly as described for Table 8. Mitochondrial dry wt. was 24.6 mg./cup.

Cup no.	1	2	3	4	5	6
Additions (mm):						
Pyruvate	10	10	10	10	10	10
Malate	10	10	10	10	10	10
ADP	0	2	0	2	0	2
Mg ²⁺	0	0	2.5	2.5	0	0
Ca ²⁺	0	0	0	0	0.5	0.5
Compounds found (μmoles/cup):						
PEP + phosphoglycerates	11.8	24.7	9.1	7.4	10.4	11.4
Oxaloacetate	0.04	0.18	0.12	0.31	0.10	0.24
Citrate	1.28	1.08	1.42	8.00	1.21	5.82
α-Oxoglutarate	1.33	1.24	1.12	8.59	1.00	4.39
ATP	0.04	7.06	0.08	5.22	0.06	4.95
ADP	0.08	0.74	0.07	1.84	0.06	1.82
AMP	<0.01	0.03	0.03	0.48	<0.01	0.58
Metabolic changes (μmoles/cup):						
Pyruvate	-4.2	-11.5	-5.7	-35.4	-4.6	-20.1
Malate	-16.1	-22.9	-16.0	-21.0	-14.8	-19.5
O ₂	-6.1	-9.65	-5.95	-28.0	-6.3	-20.0

Table 10. *Effects of 2,4-dinitrophenol and oligomycin on phosphoenolpyruvate formation from malate and oxaloacetate in pigeon liver mitochondria*

Mitochondria were prepared and incubated for 30 min. exactly as described for Table 8. Mitochondrial dry wt. was 18 mg./cup.

Cup no.	1	2	3	4	5	6
Additions (mm):						
Malate	15	15	0	0	15	0
Oxaloacetate	0	0	15	15	0	15
2,4-Dinitrophenol	0	0.04	0	0.04	0	0
Oligomycin (μg.)	0	0	0	0	5	5
ADP	1.25	1.25	1.25	1.25	1.25	1.25
Compounds formed (μmoles/cup):						
PEP + phosphoglycerates	14.8	4.51	19.0	3.72	1.85	1.90
Oxaloacetate	0.60	0.37			0.39	
Citrate	1.12	3.36	2.00	4.16	0.92	1.40
α-Oxoglutarate	1.68	4.32	1.15	5.25	1.12	0.96
ATP	3.78	1.40	3.97	1.25	0.04	0.08
ADP	0.65	2.65	0.54	3.04	0.54	0.74
AMP	0.03	0.38	0.06	0.50	3.36	3.20
Metabolic changes (μmoles/cup):						
Malate	-24.6	-28.7	+5.1	+3.9	-13.9	+5.4
O ₂	-9.4	-16.5	-5.7	-10.8	-4.6	-3.1

were formed in the presence of a decreased ATP/ADP ratio; the exception (cup 4) may have been due to the occurrence of high ADP concentrations, during part of the incubation, before the 20 μmoles of ADP could be phosphorylated. These findings support the postulate of Hathaway & Atkinson (1965) and Shepherd & Garland (1966) that high ATP/ADP ratios may inhibit the activity of citrate synthase, since there were no differences in the overall oxaloacetate concentrations.

Low concentrations of Ca²⁺ (0.5 mm), like Mg²⁺,

stimulated oxygen uptake, increased the removal of added substrates, inhibited PEP accumulation and lowered the concentration of ATP (Table 9). Although this concentration of Ca²⁺ does not inhibit pyruvate kinase to a significant degree (Kachmar & Boyer, 1953), the absence of added Mg²⁺ is markedly inhibitory; assay of pyruvate kinase in the particle suspensions under these conditions showed negligible activity. It may be concluded that the fall in the ATP/ADP ratio was responsible for the inhibition of PEP formation

and for the increased synthesis of citrate and α -oxoglutarate. This was confirmed by the effects of agents inhibiting oxidative phosphorylation. At $40\mu\text{M}$, 2,4-dinitrophenol inhibited PEP formation from malate and oxaloacetate in the absence of added Mg^{2+} (Table 10, cups 1-4). This was accompanied by a fall in ATP and an increase in the amount of citrate found. Oligomycin ($0.3\mu\text{g./mg.}$ mitochondrial dry wt.) was also inhibitory (cups 5-6) but in this case the oxygen uptake and substrate removal were diminished.

Atractyloside is an inhibitor of mitochondrial reactions involving added or 'unbound' adenine nucleotides, and its action may be that of an inhibitor, competitive with adenine nucleotides, of a specific 'translocase' catalysing the exchange of 'bound' mitochondrial and added adenine nucleotides (Chappell & Crofts, 1965; Pfaff, Klingenberg & Heldt, 1965). Guanine nucleotides do not exchange in this manner in intact mitochondria (Klingenberg & Pfaff, 1966). Experiments were carried out with atractyloside ($100\mu\text{M}$ in the presence of 15-17 mg. dry wt. of particles) to define the location of PEP carboxykinase and nucleotide diphosphokinase in relation to the atractyloside barrier (Table 11). Oxaloacetate was used as substrate to saturate PEP carboxykinase with this substrate. Atractyloside eliminated the stimulation of PEP formation by added ATP (cups 2 and 4), but did not affect the ATP-independent phosphorylation (cups 1 and 3). PEP synthesis was totally

prevented when 4mm-potassium cyanide was added; atractyloside inhibited the PEP formation that occurred when ATP was added in the presence of potassium cyanide (cups 5, 6 and 7). Similar results were obtained when ATP synthesis and hydrolysis were inhibited by high concentrations of oligomycin (cups 8, 9 and 10). Added GTP (or ITP) did not affect the rate of PEP formation, nor was there an effect of atractyloside (cups 11 and 12). PEP carboxykinase and nucleoside diphosphokinase both appear to be located functionally within the atractyloside-sensitive 'barrier'; this is confirmed by the fact that no added metal ions were required for PEP synthesis *in situ*, whereas the solubilized PEP carboxykinase has an absolute requirement for Mg^{2+} or Mn^{2+} . Endogenous 'bound' metal ions are presumably involved (Klingenberg & Pfaff, 1966).

The results indicate that the ultimate phosphate donor (via nucleoside diphosphokinase) for GTP-linked PEP synthesis from malate and oxaloacetate was ATP, derived either from oxidative phosphorylation of added or endogenous ADP, or from added ATP. The rate of substrate-level phosphorylation during incubations with these substrates could not have been sufficient to supply the GTP required. Bartley (1955) reported that the kinetics of ^{32}P incorporation into PEP and ATP during mitochondrial synthesis of PEP from malate were such as to suggest that ATP was not a precursor of PEP. It is possible that these results were affected by 'compartmentation' of adenine nucleotide pools inside and outside the mitochondrial membranes (Utter, 1960), although this would not explain why the incorporation pattern was different when α -oxoglutarate rather than malate was the substrate (Bartley, 1954). Differences in the net rates of PEP synthesis from the two substrates may account for the discrepancy.

α -Oxoglutarate as precursor of phosphoenolpyruvate. The oxidation rate of α -oxoglutarate ($5\text{--}10\text{mM}$) was doubled by the addition of $50\mu\text{M}$ 2,4-dinitrophenol (Table 12), with a fall in ATP and an increase in the concentrations of both malate and oxaloacetate. PEP formation was decreased. 'Energy-rich' phosphate synthesized during the enhanced oxidation of α -oxoglutarate in the presence of 2,4-dinitrophenol was thus available to the 2,4-dinitrophenol-stimulated adenosine triphosphatase via the nucleoside diphosphokinase reaction. When malate was incubated with α -oxoglutarate and ADP, in the presence of increasing concentrations of 2,4-dinitrophenol such that α -oxoglutarate oxidation was increased, there was only a slight stimulation of PEP synthesis over that in the presence of malate alone (Table 13). These results suggest that there is no functional relationship between the substrate-level phosphorylation and

Table 11. *Effects of atractyloside on phosphoenolpyruvate synthesis from oxaloacetate in pigeon liver mitochondria*

Mitochondria were prepared and incubated for 10 min. under air, at 20.5° , as described in the Methods and Materials section. The medium contained 15mM-P_i , $\text{pH } 7.4$, and 10mM -oxaloacetate. The concentrations of additions were as follows: ATP, 1.5mM ; GTP, 0.4mM ; KCN, 5mM ; oligomycin, $10\mu\text{g./cup}$; atractyloside, $100\mu\text{M}$. Mitochondrial dry wt. was 17mg./cup .

Cup no.	Additions	PEP + phosphoglycerates formed ($\mu\text{moles/cup}$)
1	None	2.52
2	ATP	3.35
3	Atractyloside	2.42
4	ATP + atractyloside	2.47
5	KCN	0.11
6	KCN + ATP	0.92
7	KCN + ATP + atractyloside	0.12
8	Oligomycin	0.50
9	Oligomycin + ATP	1.38
10	Oligomycin + ATP + atractyloside	0.52
11	GTP	2.36
12	GTP + atractyloside	2.41

Table 12. *Effects of 2,4-dinitrophenol on the formation of phosphoenolpyruvate from α -oxoglutarate in pigeon liver mitochondria*

Mitochondria were prepared and incubated for 30 min. at 30° under air, as described in the Methods and Materials section. The medium contained 15 mM- P_i , pH 7.4, 0.625 mM- Mg^{2+} and 10 mM- α -oxoglutarate. Mitochondrial dry wt. was 20 mg./cup.

Cup no.	1	2	3	4
Additions (mm):				
ADP	1.25	1.25	1.25	1.25
2,4-Dinitrophenol	0	0.02	0.05	0.1
Compounds found (μ moles/cup):				
PEP + phosphoglycerates	3.39	4.37	1.93	0.88
Oxaloacetate	0.06	0.14	0.24	0.20
Malate	0.23	0.47	2.42	1.40
ATP	4.09	4.03	1.33	0.10
ADP	0.85	0.95	2.82	1.10
Metabolic changes (μ moles/cup):				
α -Oxoglutarate	-5.02	-6.42	-10.9	-10.9
O ₂	-7.05	-9.4	-15.8	-16.1

Table 13. *Effect of α -oxoglutarate and 2,4-dinitrophenol addition on the formation of phosphoenolpyruvate from malate in pigeon liver mitochondria*

Mitochondria were prepared and incubated at 30° for 30 min. as described in the Methods and Materials section. The gas phase was O₂. The medium contained 15 mM- P_i , pH 7.4, 0.625 mM- Mg^{2+} , 4 mM- HCO_3^- and 1 mM-ATP. The dry wt. was 31 mg./cup.

Cup no.	1	2	3	4	5	6	7	8	9
Additions (mm):									
Malate	10	10	10	0	0	0	10	10	10
α -Oxoglutarate	0	0	0	5	5	5	5	5	5
2,4-Dinitrophenol	0	0.05	0.1	0	0.05	0.1	0	0.05	0.1
Metabolic changes (μ moles/cup):									
PEP + phosphoglycerates + 23.1		+6.71	+3.32	+8.00	+2.25	+1.7	+27.2	+8.04	+4.77
O ₂	-17.4	-32.6	-30.4	-19.7	-34.7	-29.1	-21.6	-28.2	-34.9

PEP formation in intact pigeon liver mitochondria, and that the nucleotides are able freely to interact in the mitochondrial matrix.

It is difficult to reconcile this view of the control of PEP synthesis by the ratios of nucleotides with the results of Nordlie & Lardy (1963b), where 2,4-dinitrophenol or a glucose-hexokinase trap (both agents likely to depress the ATP/ADP ratio) increased the formation of PEP from α -oxoglutarate in guinea-pig liver mitochondria in the absence of added adenine nucleotides. This implies either that 'compartmentation' of nucleotides occurred or that there was dis-equilibrium in the nucleoside diphosphokinase system under the conditions used. Species differences may account for these discrepancies.

Role of the oxaloacetate concentration in the regulation of phosphoenolpyruvate synthesis. Assuming that oxaloacetate was evenly distributed between medium and mitochondria, the malate/oxaloacetate ratios varied between 20:1 and 2:1 (when these compounds were not added as substrates). The

oxaloacetate concentrations were highest with malate as the substrate (Table 6) and were lower in the presence of pyruvate + malate (Table 7) or α -oxoglutarate (Table 12). The formation of oxaloacetate from α -oxoglutarate was enhanced by addition of 2,4-dinitrophenol, but the unfavourable nucleotide ratios prevented the synthesis of PEP (Table 12). This applies also to the effects of adenine nucleotide addition, which resulted in an increase in the oxaloacetate concentration independent of the presence of Mg^{2+} (Tables 6 and 7) but which was associated with increased PEP synthesis only when the ATP/ADP ratios were high. An explanation for the effects of AMP addition on PEP formation from lactate in pigeon liver homogenates (Gevers & Krebs, 1966) is also afforded in that the inhibition observed was perhaps due to the high concentration of ADP.

Succinate (10 mM) was added to mitochondria to test whether, under conditions where high ATP/ADP ratios might be expected, the synthesis of PEP would be affected by a fall in the oxaloacetate

Table 14. *Effect of succinate addition on phosphoenolpyruvate synthesis from malate and α -oxoglutarate in pigeon liver mitochondria*

Mitochondria were prepared and incubated at 30° for 30 min. under air, as described in the Methods and Materials section. The medium contained 15 mM- P_i , pH 7.4, 1 mM-ADP, 4 mM- HCO_3^- and 0.625 mM- Mg^{2+} . Mitochondrial dry wt. was 19 mg./cup.

Cup no.	1	2	3	4	5
Additions (mm):					
Malate	10	10	0	0	0
α -Oxoglutarate	0	0	10	10	0
Succinate	0	10	0	10	10
Compounds found (μ moles/cup):					
PEP + phosphoglycerates	14.4	10.4	6.22	4.55	8.91
Oxaloacetate	0.18	0.08	0.22	0.11	0.09
Malate	21.3	42.4	1.37	25.5	24.6

concentration. This was based on the known effects of succinate oxidation on intramitochondrial NADH (Krebs, Eggleston & D'Alessandro, 1961). Succinate addition decreased the oxaloacetate concentration, as well as the formation of PEP, with malate and α -oxoglutarate as substrates (Table 14). This supports the conclusion that the oxaloacetate concentration is an important regulatory factor for PEP synthesis *in situ*, even if no estimate can be made of the effective concentration of the substrate at the active site of the enzyme.

DISCUSSION

The control of the net rate of PEP synthesis depends on the regulation both of the reaction forming this metabolite in the mitochondrial matrix, and of the reactions removing it in the cytoplasm.

Control of phosphoenolpyruvate formation by nucleotides. The conversion of oxaloacetate into PEP is controlled in part by the [ATP]/[ADP][P_i] ratio (the 'phosphorylation potential'; Klingenberg, 1964). It is most likely that this effect is exerted indirectly via the equilibrium reaction catalysed by nucleoside diphosphokinase, through an effect on the GTP/GDP ratio. The nucleotides react freely with each other in the mitochondrial matrix, and there appears to be no special relationship of PEP carboxykinase to the substrate-level phosphorylation. Scholz & Bücher (1966) have shown that the overall tissue GTP/GDP ratio follows the ATP/ADP ratio in perfused rat liver very closely during an aerobic-anaerobic transition. The GTP/GDP ratio probably influences the activity of PEP carboxykinase by variations in the degree of substrate saturation (GTP) and competitive inhibition (GDP) (W. Gevers, unpublished work; see also Chang, Maruyama, Miller & Lane, 1966).

A second possibility is that nucleoside diphosphokinase may be inhibited by ADP when the con-

centration of the latter rises relative to that of the other substrates, owing to the formation of an 'abortive complex' (Mourad & Parks, 1966). Because of the high activity of the enzyme in pigeon liver mitochondria and the results obtained with α -oxoglutarate as substrate, this explanation is not favoured. Inhibition of PEP carboxykinase by AMP (Holten & Nordlie, 1965) or ADP (Utter & Kurahashi, 1954) requires higher concentrations of these nucleotides than may reasonably be expected to have been present in the mitochondria in these experiments.

Another possibility is that energy is required for the maintenance of functional mitochondrial compartments, such that the movements of oxaloacetate are controlled (see Tager, 1963, 1966; Schollmeyer & Klingenberg, 1961). Thus energy may be required to translocate oxaloacetate to the active site of PEP carboxykinase, and to increase the rate of malate oxidation, since oxaloacetate is a potent inhibitor of mitochondrial malate dehydrogenase (Siegel & England, 1960).

Control of phosphoenolpyruvate synthesis by the oxaloacetate concentration. The oxaloacetate concentration is directly related to the rate of PEP synthesis, except when the ATP/ADP ratio is unfavourable. Control by the nucleotides is thus of a 'permissive' nature, in that it permits operation of other control mechanisms only when energy is available. This is analogous to the control of the interconversion of fructose 1,6-diphosphate and fructose 6-phosphate in pigeon liver homogenates (Gevers & Krebs, 1966). Seubert & Huth (1965) have proposed that variations in the cytoplasmic oxaloacetate concentration could regulate the activity of PEP carboxykinase in rat liver; subsequently this concept was withdrawn in favour of emphasis on the amount of PEP carboxykinase in the cytoplasm (Henning, Stumpf, Ohly & Seubert, 1966), which permits 'accelerated gluconeogenesis even at lowered oxaloacetate levels' such as occur

in diabetic-rat liver (Wieland & Löffler, 1963). The role of nucleotides in the control of PEP synthesis in rat liver has not yet been studied.

Path of carbon from mitochondria to the cytoplasm. The absence of enolase from the mitochondria suggests that PEP must be the metabolite transported from the mitochondrial matrix to the cytoplasm during gluconeogenesis from oxaloacetate. The effect of P_i on PEP formation in isolated mitochondria may be due to changes in the 'phosphorylation potential' (see Table 5), or it could be related to the facilitation of a 'carrier' mechanism of PEP transport into and out of mitochondria, as suggested for various carboxylic acids by Chappell (1966). This point requires further investigation.

Control of phosphoenolpyruvate removal in the cytoplasm. The activity of pyruvate kinase is an important determinant of the net rate of PEP synthesis in isolated mitochondria and presumably also in the intact cell. Since activity of the pathway: $\text{pyruvate} + \text{ATP} \rightarrow \text{oxaloacetate} + \text{GTP} \rightarrow \text{PEP} + \text{ADP} \rightarrow \text{pyruvate} + \text{ATP}$, would constitute an energy-consuming 'futile' cycle, mechanisms are required by which the activity of pyruvate kinase can be controlled when gluconeogenesis from oxaloacetate is occurring. In pigeon liver this is not achieved by variations in the overall activity of pyruvate kinase, as it is, for example, in yeast (Ruiz-Amil, de Torrentegui, Palacián, Catalina & Losada, 1965) and rat liver (Krebs & Eggleston, 1965), although even in this case the assayed maximal activity of pyruvate kinase after prolonged starvation exceeds that of PEP carboxykinase. It is possible that the effects of ATP and Ca^{2+} (inhibitors) and of ADP, K^+ and Mg^{2+} (activators) are of importance in the control of the enzyme activity in the cell (see Reynard, Hass, Jacobsen & Boyer, 1961; Siebert, Kesselring & Fischer, 1965).

The role of enolase in the metabolism of PEP may lie in its catalysis (with phosphoglycerate mutase) of the equilibrium reactions between PEP and 2- and 3-phosphoglycerate. In this way the effects of adenine nucleotides, P_i and the NAD^+/NADH ratio on the enzyme system converting 3-phosphoglycerate into triose phosphates might indirectly affect the disposal of PEP.

The ATP/ADP ratio, both in the mitochondrial and cytoplasmic compartments, is thus an important determinant of the rate of carbohydrate synthesis in the liver, although part of the effect is exerted by AMP, related to the other adenosine phosphates by the action of adenylate kinase. The control expresses itself in a number of ways: (a) inhibition of citrate synthase, closing off a major 'leak' from the mitochondrial pool of oxaloacetate (Shepherd & Garland, 1966); (b) 'de-inhibition' of

pyruvate carboxylase (Berry, 1965; Walter, Paetkau & Lardy, 1966); (c) enhancement of PEP synthesis as described above; (d) inhibition of pyruvate kinase, closing off a 'leak' at the level of PEP; (e) enhancement of the net conversion of fructose 1,6-diphosphate into fructose 6-phosphate; (f) promotion of glycogen synthesis by an effect on the UTP/UDP ratio (Kornfeld, 1965; Steiner, Younger & King, 1965).

The mechanism of the 'switch-over' of principal storage product from glycogen to triglyceride, which occurs at a certain point during carbohydrate feeding, is not yet clearly understood. An important regulatory function of nucleotides in gluconeogenesis would imply that the ATP/ADP ratio must be depressed during the 'switch-over' to permit reversal of the reactions previously favoured by the high ATP/ADP ratios and the low AMP concentrations. No such changes have yet been observed in studies of the adenine nucleotide content of rat liver under various conditions (Underwood, 1965; Söling, Kettermann, Schmidt & Kneer, 1966). Gluconeogenesis from lactate in the perfused rat liver is accompanied by a fall in the ATP/ADP ratio of tissue samples (Hems, Ross, Berry & Krebs, 1966), and Hohorst, Arese, Bartels, Stratmann & Talke (1964) found that the overall ATP/ADP ratios were depressed in alloxan-diabetic-rat livers. It is feasible that a large part of the ATP measured in whole tissues by the standard 'freeze-clamping' and perchloric acid-extraction techniques may be inactive in the sense that it is not available to certain metabolic reactions, since the values reflect an average of the amounts of the compound bound at many sites to proteins and complex-forming agents, and of the concentrations in spatially separate compartments. Scholz & Bücher (1966) have shown that the overall CTP/ADP ratio changes from 30:1 to 3:1 in a few seconds after anoxia in the perfused rat liver, whereas that of ATP/ADP only falls from 5:1 to 2:1.

These considerations indicate that the mechanisms of regulation of metabolic processes revealed by studies with disrupted systems may have importance in intact cells despite the contradictory results so far obtained with the technique of metabolite assay in frozen-arrested tissues.

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REFERENCES

- Adam, H. (1963). In *Methods of Enzymatic Analysis*, p. 539. Ed. by Bergmeyer, H.-U. New York: Academic Press Inc.

- Azzone, G. F. & Ernster, L. (1961). *J. biol. Chem.* **236**, 1501.
- Bartley, W. (1954). *Biochem. J.* **56**, 387.
- Bartley, W. (1955). *Biochem. J.* **61**, 555.
- Berry, M. N. (1965). *Biochem. J.* **95**, 587.
- Chang, H.-C., Maruyama, H., Miller, R. S. & Lane, M. D. (1966). *J. biol. Chem.* **241**, 2421.
- Chappell, J. B. (1966). *Biochem. J.* **100**, 43r.
- Chappell, J. B. & Crofts, A. R. (1965). *Biochem. J.* **95**, 707.
- Chappell, J. B. & Greville, G. D. (1963). *Symp. biochem. Soc.* **23**, 39.
- Christ, E. J. & Hülsmann, W. C. (1962). *Biochim. biophys. Acta*, **60**, 72.
- Czok, R. & Eckert, L. (1963). In *Methods of Enzymatic Analysis*, p. 224. Ed. by Bergmeyer, H.-U. New York: Academic Press Inc.
- Delbrück, A., Schimassek, H., Bartsch, K. & Bücher, T. (1959). *Biochem. Z.* **331**, 297.
- Gevers, W. & Krebs, H. A. (1966). *Biochem. J.* **98**, 720.
- Hathaway, J. A. & Atkinson, D. E. (1965). *Biochem. biophys. Res. Commun.* **20**, 661.
- Heldt, H. W. & Schwalbach, K. (1966). *Abstr. 3rd Meet. Fed. Europ. biochem. Soc., Warsaw*, p. 134. New York: Academic Press Inc.
- Hems, R., Ross, B. D., Berry, M. N. & Krebs, H. A. (1966). *Biochem. J.* **101**, 284.
- Henning, H.-V., Stumpf, B., Ohly, B. & Seubert, W. (1966). *Biochem. Z.* **344**, 274.
- Hohorst, H.-J., Arese, P., Bartels, H., Stratmann, D. & Talke, H. (1964). *Ann. N.Y. Acad. Sci.* **119**, 974.
- Holten, D. D. & Nordlie, R. C. (1965). *Biochemistry*, **4**, 723.
- Kachmar, J. F. & Boyer, P. D. (1953). *J. biol. Chem.* **200**, 669.
- Kielley, W. W. & Kielley, R. K. (1953). *J. biol. Chem.* **200**, 213.
- Klingenberg, M. (1964). *Angew. Chem. (int. Ed.)*, **3**, 54.
- Klingenberg, M. & Pfaff, E. (1966). In *Regulation of Metabolic Processes in Mitochondria*, p. 180. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.
- Kornfeld, S. (1965). *Fed. Proc.* **24**, 536.
- Krebs, H. A., Dierks, C. & Gascoyne, T. (1964). *Biochem. J.* **93**, 112.
- Krebs, H. A. & Eggleston, L. V. (1965). *Biochem. J.* **94**, 3c.
- Krebs, H. A., Eggleston, L. V. & D'Alessandro, A. (1961). *Biochem. J.* **79**, 537.
- Lamprecht, W. & Trautschold, I. (1963). In *Methods of Enzymatic Analysis*, p. 543. Ed. by Bergmeyer, H.-U. New York: Academic Press Inc.
- Mendicino, J. & Utter, M. F. (1962). *J. biol. Chem.* **237**, 1716.
- Morrison, J. F. (1954). *Biochem. J.* **56**, 99.
- Mourad, N. & Parks, R. E., jun. (1966). *J. biol. Chem.* **241**, 271.
- Nordlie, R. C. & Lardy, H. A. (1963a). *J. biol. Chem.* **238**, 2259.
- Nordlie, R. C. & Lardy, H. A. (1963b). *Biochem. Z.* **338**, 356.
- Nordlie, R. C., Varricchio, F. E. & Holten, D. D. (1965). *Biochim. biophys. Acta*, **97**, 214.
- Pfaff, E., Klingenberg, M. & Heldt, H. W. (1965). *Biochim. biophys. Acta*, **104**, 312.
- Reynard, A. M., Hass, L. F., Jacobsen, D. D. & Boyer, P. D. (1961). *J. biol. Chem.* **236**, 2277.
- Ruiz-Amil, M., de Torrentegui, G., Palacián, E., Catalina, L. & Losada, M. (1965). *J. biol. Chem.* **240**, 3485.
- Schollmeyer, P. & Klingenberg, M. (1961). *Biochem. biophys. Res. Commun.* **4**, 43.
- Scholte, H. R. & Tager, J. M. (1965). *Biochim. biophys. Acta*, **110**, 252.
- Scholz, R. & Bücher, T. (1966). In *Control of Energy Metabolism*, p. 393. Ed. by Chance, B., Estabrook, R. W. & Williamson, J. R. New York: Academic Press Inc.
- Seubert, W. & Huth, W. (1965). *Biochem. Z.* **343**, 176.
- Shepherd, D. & Garland, P. B. (1966). *Biochem. biophys. Res. Commun.* **22**, 89.
- Siebert, G. (1963). In *Methods of Enzymatic Analysis*, p. 318. Ed. by Bergmeyer, H.-U. New York: Academic Press Inc.
- Siebert, G., Kesselring, K. & Fischer, F. (1965). *Hoppe-Seyl. Z.* **341**, 44.
- Siegel, L. & Englard, S. (1960). *Biochem. biophys. Res. Commun.* **3**, 253.
- Söling, H.-D., Kettermann, R., Schmidt, H. & Kneer, P. (1966). *Biochim. biophys. Acta*, **115**, 1.
- Stanbury, S. W. & Mudge, G. H. (1954). *J. biol. Chem.* **210**, 949.
- Steiner, D. F., Younger, L. & King, J. (1965). *Biochemistry*, **4**, 740.
- Stickland, R. G. (1959). *Biochem. J.* **73**, 660.
- Tager, J. M. (1963). *Biochim. biophys. Acta*, **77**, 258.
- Tager, J. M. (1966). In *Regulation of Metabolic Processes in Mitochondria*, p. 202. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.
- Tanaka, T., Harano, Y., Morimura, H. & Mori, R. (1965). *Biochem. biophys. Res. Commun.* **21**, 55.
- Underwood, A. H. (1965). D.Phil. Thesis: University of Oxford.
- Utter, M. F. (1960). In *The Enzymes*, vol. 2, p. 75. Ed. by Boyer, P. D., Lardy, H. A. & Myrback, K. New York: Academic Press Inc.
- Utter, M. F. & Kurahashi, K. (1954). *J. biol. Chem.* **207**, 821.
- Walter, P., Paetkau, V. & Lardy, H. A. (1966). *J. biol. Chem.* **241**, 2523.
- Wieland, O. & Löffler, G. (1963). *Biochem. Z.* **339**, 204.