A Study of Regulation of Gluconeogenesis and the Supply of Cytosolic Reducing Equivalents for Lactate Formation in Rat Kidney-Cortical-Tubule Fragments Incubated with Pyruvate

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1. Tubule fragments were isolated after treatment of rat kidney cortex with collagenase. The formation of glucose and lactate on incubation with 5mM-pyruvate was then measured under various conditions. 2. When tubule fragments were isolated from fed rats in the absence of Ca^{2+} and then incubated with various Ca^{2+} concentrations, an incubation period of 15–30min was necessary to establish a metabolic steady state. Under these conditions glucose formation was increased by Ca^{2+} , adrenaline or 3':5'-cyclic AMP to a greater extent than was lactate formation. Data show that appreciable lactate formation could not have resulted from glycolytic metabolism of glucose formed by gluconeogenesis during incubation. 3. When tubule fragments were isolated from fed rats in the presence of 1.27mM- Ca^{2+} and adjustments made to the Ca^{2+} concentration at the commencement of incubation, metabolic steady state was rapidly established. Under these conditions lactate formation was almost insensitive to Ca^{2+} concentration (0.16–4.5mM), whereas glucose formation varied with Ca^{2+} concentration in a sigmoidal manner. 3':5'-Cyclic AMP decreased this sigmoidicity. 4. Ca^{2+} depletion of the tissue before incubation appeared to change permanently the relationship between extracellular Ca^{2+} concentration and the measured rates of metabolic processes. 5. Under conditions of metabolic steady state, glucose formation by tubule fragments from fed rats was less sensitive than lactate formation to inhibition by 3-mercaptopycolinate or 2-n-butylmalonate. Lactate formation by tubule fragments prepared from 48h-starved rats was more sensitive to these inhibitors. 6. Estimates were made of the rate of futile cycling of C_3 species through pyruvate kinase. This was greater in the starved than in the fed state, was decreased by 3':5'-cyclic AMP in both the fed and the starved state, but was unaffected by Ca^{2+} . 7. These results suggested that formation of lactate and glucose is less tightly linked in kidney cortex than in liver. A considerable amount of the supply of reducing equivalents for lactate formation did not appear to be associated with an energy-dependent translocation from mitochondria to cytosol involving a pyruvate $\rightarrow$ oxaloacetate $\rightarrow$ phosphoenolpyruvate $\rightarrow$ pyruvate cycle.

In vitro, preparations of rat liver and kidney cortex produce considerable amounts of lactate as well as glucose when pyruvate is the sole substrate presented (Nishiitsutsuji-Uwo *et al.*, 1967; Krebs *et al.*, 1967; Rognstad & Katz, 1972; Meijer & Williamson, 1974; Mapes & Harris, 1976). It was pointed out by Krebs and co-workers (Krebs, 1966; Krebs *et al.*, 1967) that under these circumstances the necessary reducing equivalents must originate from mitochondrial oxidative processes and be transported to the cytosol across the nicotinamide nucleotide-impermeable inner mitochondrial membrane. Several studies have established the likelihood that malate may serve as the vehicle for transport from the mitochondrion of both reducing equivalents and gluconeogenic carbon when pyruvate is the sole substrate (Lardy *et al.*, 1965; Shrago & Lardy, 1966; Walter *et al.*, 1966;

Rostand *et al.*, 1969; Rognstad & Katz, 1970; Berry & Kun, 1972; Longshaw *et al.*, 1972; Zahlten *et al.*, 1974). Studies with perfused rat liver, but mainly with hepatocytes isolated from starved rats, have provided evidence that the lactate formation is associated with a metabolic cycle: cytosolic pyruvate $\rightarrow$ mitochondrial pyruvate $\rightarrow$ mitochondrial oxaloacetate $\rightarrow$ mitochondrial malate $\rightarrow$ cytosolic malate $\rightarrow$ cytosolic oxaloacetate $\rightarrow$ cytosolic phosphoenolpyruvate $\rightarrow$ cytosolic pyruvate (Friedmann *et al.*, 1971; Rognstad, 1975; Mapes & Harris, 1976). This essentially constitutes an energy-dependent translocation of reducing equivalents from mitochondria to cytosol (Berry, 1971; Meijer & Williamson, 1974) and appears to result in production of lactate and glucose in a remarkably strict 2:1 molar ratio (Mapes & Harris, 1976). Rognstad & Katz (1972), on the basis of

radioisotopic-labelling studies, proposed that a similar pyruvate $\rightarrow$ oxaloacetate $\rightarrow$ phosphoenolpyruvate $\rightarrow$ pyruvate cycle operated in kidney-cortex slices from starved rats. However, perfused rat kidney or kidney-cortex slices do not produce lactate and glucose from pyruvate in a 2:1 molar ratio (Nishiitsutsuji-Uwo *et al.*, 1967; Krebs *et al.*, 1967). Also, later studies of enzyme activities in different segments of the rat nephron (Schmidt *et al.*, 1975; Guder & Schmidt, 1976) showed that phosphoenolpyruvate carboxykinase and pyruvate kinase activities are inversely distributed along this structure. This could be inconsistent with an active cycle involving these two enzymes.

Accordingly, in the present study the relationship between formation of lactate and glucose from pyruvate has been investigated in cortical-tubule fragments under a variety of conditions. The findings show a clear difference between liver and kidney cortex and suggest that a pyruvate $\rightarrow$ oxaloacetate $\rightarrow$ phosphoenolpyruvate $\rightarrow$ pyruvate cycle is not a major process in kidney cortex from fed animals.

In addition, differences in metabolic behaviour between Ca^{2+} -depleted tubules and those prepared in physiological concentrations of free Ca^{2+} were explored, since it became obvious in the course of this study that these could be quite appreciable. Ca^{2+} -free buffers are frequently used at some stage in obtaining renal-tubule fragments and isolated hepatocytes and it was thought worthwhile to investigate perturbations caused by this treatment.

Materials and Methods

Chemicals

These were obtained and treated as described by Macdonald & Saggerson (1977). In addition, amino-oxyacetic acid (hemihydrochloride) was from Sigma (London) Chemical Co. (London S.W.6, U.K.) and 2-n-butylmalonic acid was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). 3-Mercaptopicolinic acid (SK&F 34288) was a generous gift from Smith, Kline and French Laboratories (Welwyn Garden City, Herts., U.K.).

Animals

These were male Sprague-Dawley rats bred in the animal colony at University College and maintained on diet GR3EK (E. Dixon and Sons, Ware, Herts., U.K.). Fed animals weighed 160–180 g at death. Animals were selected for starvation (48 h) from this weight range. All animals had constant access to tap water to drink.

Isolation of tubule fragments from renal cortex

This was an adaptation of the techniques developed by Nagata & Rasmussen (1970) and Guder *et al.*

(1971) with the modifications described by Macdonald & Saggerson (1977). All preparations were in Krebs-Ringer bicarbonate medium (Krebs & Henseleit, 1932) containing fatty acid-poor albumin (10 mg/ml). In some experiments Ca^{2+} was omitted from these media. In other cases the preparation media contained 1.27 mM- Ca^{2+} .

Incubation techniques

These were described by Macdonald & Saggerson (1977).

Analytical methods

Glucose, lactate, oxaloacetate and aspartate were assayed enzymically by the methods of Slein (1965), Hohorst (1965), Hohorst & Reim (1965) and Pfeleiderer (1965) respectively, in extracts prepared by deproteinization of flask contents (Macdonald & Saggerson, 1977). The small amount of glucose and lactate initially present in tubule suspensions before incubation was also determined and subtracted from experimental values. DNA was measured by the method of Burton (1956) in duplicate portions of stock tubule suspensions.

Statistical methods

Analysis of data was performed on a paired basis and statistical significance determined by Student's *t* test.

Results and Discussion

Supply of cytosolic reducing equivalents in Ca^{2+} -depleted tubule fragments from fed rats

Though the effect of Ca^{2+} on renal gluconeogenesis from pyruvate has been extensively studied (Krebs *et al.*, 1963; Nagata & Rasmussen, 1970; Kurokawa & Rasmussen, 1973; Alleyne *et al.*, 1973), actions of this cation on lactate formation do not appear to have been investigated. In initial experiments tubule fragments were depleted of Ca^{2+} during preparation in Ca^{2+} -free media (approx. 55 min). EGTA (0.1 mM) was added to incubations to establish a low- Ca^{2+} basal condition, and Ca^{2+} was then added, as well as the EGTA. Similar conditions were used by Nagata & Rasmussen (1970). Table 1 shows that, although lactate formation was increased by Ca^{2+} , the dependence of this process on Ca^{2+} was different from that of gluconeogenesis, as shown by the progressive decrease in the ratio $[\text{lactate}]/[\text{glucose}]$ with increasing Ca^{2+} . With zero addition of Ca^{2+} $81 \pm 1\%$ of the cytosolic reducing equivalents was used in lactate formation. This decreased to $35 \pm 2\%$ with 6 mM- Ca^{2+} .

Table 1. *Effect of Ca²⁺ on formation of glucose and lactate by Ca²⁺-depleted tubule fragments from fed rats*
Tubule fragments were prepared in Ca²⁺-free media and incubated for 1 h in Krebs–Ringer bicarbonate buffer containing fatty acid-poor albumin (10mg/ml), 100 μM-disodium EGTA and 5 mM-sodium pyruvate. The results are means ± S.E.M. of four experiments and are expressed as μmol/h per mg of DNA. The mean tubule DNA per ml of flask contents was 49 μg. Net cytosolic reducing-equivalent provision was calculated as 2 mol of NADH/mol of glucose + 1 mol of NADH/mol of lactate.

Ca ²⁺ addition (mM)	Glucose production	Lactate production	Net cytosolic reducing-equivalent provision	Ratio [lactate]/[glucose]
0	0.23 ± 0.02	1.91 ± 0.07	2.37 ± 0.12	8.6 ± 0.6
0.5	0.92 ± 0.09	3.50 ± 0.16	5.34 ± 0.34	3.9 ± 0.2
1.0	1.58 ± 0.06	3.90 ± 0.23	7.06 ± 0.36	2.5 ± 0.1
2.5	2.65 ± 0.26	4.05 ± 0.22	9.34 ± 0.74	1.6 ± 0.1
4.0	3.83 ± 0.36	4.54 ± 0.15	12.20 ± 0.86	1.2 ± 0.1
6.0	4.67 ± 0.49	5.12 ± 0.18	14.36 ± 1.16	1.1 ± 0.1

Table 2. *Effect of Ca²⁺, adrenaline and 3':5'-cyclic AMP on formation of glucose and lactate by Ca²⁺-depleted tubule fragments from fed rats*

Tubule fragments were prepared in Ca²⁺-free media and incubated for 1 h in Krebs–Ringer bicarbonate buffer containing fatty acid-poor albumin (10mg/ml), 100 μM-disodium EGTA and 5 mM-sodium pyruvate. The results are means ± S.E.M. of five and four independent measurements for Expts. 1 and 2 respectively and are expressed as μmol/h per mg of DNA. The mean tubule DNA per ml of flask contents was 47 μg (Expt. 1) and 44 μg (Expt. 2). *, **, *** indicate *P* < 0.05, <0.01, <0.001 respectively versus controls.

Expt. no.	Ca ²⁺ addition (mM)	Other additions	Glucose production	Lactate production	Ratio [lactate]/[glucose]
1	0.2	—	0.61 ± 0.15	3.53 ± 0.44	6.5 ± 0.9
	0.2	Adrenaline (1 μM)	0.82 ± 0.16**	3.66 ± 0.41	4.7 ± 0.3*
	2	—	3.60 ± 0.57	5.83 ± 0.54	1.7 ± 0.1
	2	Adrenaline (1 μM)	4.79 ± 0.77**	5.93 ± 0.75	1.3 ± 0.1**
2	0.2	—	0.78 ± 0.17	3.16 ± 0.43	4.5 ± 0.7
	0.2	3':5'-Cyclic AMP (0.1 mM)	1.90 ± 0.48*	3.94 ± 0.32**	2.5 ± 0.6***
	2	—	4.75 ± 0.96	5.71 ± 0.51	1.4 ± 0.3
	2	3':5'-Cyclic AMP (0.1 mM)	7.46 ± 1.63*	6.89 ± 0.65	1.1 ± 0.2*

Scheme 1. *Relationship between cytosolic formation of oxaloacetate and lactate*

Renal gluconeogenesis may also be increased by 3':5'-cyclic AMP (Pagliara & Goodman, 1969; Rasmussen & Nagata, 1970; Guder *et al.*, 1971;

Roobol & Alleyne, 1973) and by adrenergic stimulation of an α-receptor (Guder & Rupprecht, 1975; Macdonald & Saggerson, 1977). Table 2 shows that stimulation of gluconeogenesis by these agents at two Ca²⁺ concentrations was accompanied by smaller increases in lactate formation. Again this is clearly seen in the changed [lactate]/[glucose] ratio. Hepatocytes isolated from starved rats and incubated with pyruvate under a variety of conditions produced lactate and glucose in a remarkably constant molar ratio of 2:1 (Meijer & Williamson, 1974; Rognstad, 1975; Mapes & Harris, 1976) and it has been shown that the rate of lactate formation is similar to the net pyruvate kinase flux from phosphoenolpyruvate to pyruvate (Rognstad, 1975). This is not the case in renal cortex (Tables 1 and 2), where the processes of formation of glucose and lactate obviously have a greater degree of independence. If the lactate formation shown in Tables 1 and 2 represented a reasonable measurement of pyruvate kinase flux as

in liver (Rognstad, 1975), there would be an inconsistency with the conclusions of Nagata & Rasmussen (1970), Rasmussen & Nagata (1970) and Kurokawa & Rasmussen (1973), i.e. that Ca^{2+} or 3':5'-cyclic AMP may decrease pyruvate kinase flux in gluconeogenic cells in renal cortex. However, these conclusions arose from studies of metabolite crossovers, which may be open to criticism (see below).

Formation of lactate through a dismutation between cytosolic lactate dehydrogenase and NAD-malate dehydrogenase (Scheme 1) leaves stoichiometric amounts of cytosolic oxaloacetate. It is most unlikely that cytosolic oxaloacetate can be removed by transamination to aspartate followed by return of aspartate to the mitochondria, since energized mitochondria are reported to be virtually impermeable to cytosolic aspartate (La Noue & Williamson, 1971; La Noue *et al.*, 1974). In support of this argument, amino-oxycetate did not inhibit lactate formation in tubule fragments (see below). Furthermore, accumulation of neither oxaloacetate nor aspartate was detectable in the experiments summarized in Table 1 (results not shown). Krebs *et al.* (1967) proposed that cytosolic oxaloacetate is returned to the mitochondria as part of a 'malate-oxaloacetate cycle'. However, the question whether oxaloacetate may or may not be readily transported between mitochondria and cytosol is still not fully answered. Chappell (1961) reported impermeability of rat kidney mitochondria to oxaloacetate, and Lardy *et al.* (1965) observed little oxaloacetate efflux from rat liver mitochondria. However, other studies have suggested that mitochondria from rat heart, liver or kidney have some permeability to oxaloacetate (Robinson & Chappell, 1967; Haslam & Griffiths, 1968; Haslam & Krebs, 1968; Wojtczak, 1969; Bohnensack & Kunz, 1971; Gimpel *et al.*, 1973), with those from kidney showing higher rates of penetration of oxaloacetate than do those from liver in the presence of a respiratory energy source (Haslam & Krebs, 1968). In liver mitochondria at least, it is generally concluded that the relatively high K_m value for oxaloacetate transport on the dicarboxylate translocator (Gimpel *et al.*, 1973) renders this process relatively unimportant [see Meijer & Williamson (1974) and Williamson (1976)].

Meijer & Williamson (1974), in a study of the effects of quinolinate on formation of glucose and lactate from pyruvate by hepatocytes isolated from starved rats, concluded that the activity of the oxaloacetate-malate cycle was negligible under their experimental conditions, and that cytosolic oxaloacetate was removed via the sequential actions of phosphoenolpyruvate carboxykinase and pyruvate kinase. The hypoglycaemic agent 3-mercaptopicolinate, which inhibits gluconeogenesis in rat renal-cortex slices (Di Tullio *et al.*, 1974; Bennett & Alleyne, 1976) through non-competitive inhibition of

Fig. 1. Effect of 3-mercaptopicolinate on formation of glucose and lactate by Ca^{2+} -depleted tubule fragments from fed rats

Tubule fragments were prepared in Ca^{2+} -free media and then incubated for 1 h in Krebs-Ringer bicarbonate buffer containing fatty acid-poor albumin (10 mg/ml), 5 mM-sodium pyruvate, 100 μM -disodium EGTA, the indicated concentrations of 3-mercaptopicolinate and either 0.2 mM- Ca^{2+} (○, ●), 2 mM- Ca^{2+} (□, ■) or 6 mM- Ca^{2+} (△, ▲). The values are means $\pm$ S.E.M. of three experiments. Where not shown, error bars lie within the symbols. At 0.2 mM-, 2 mM- and 6 mM- Ca^{2+} control values for glucose formation were 0.48 ± 0.03 , 1.99 ± 0.02 and $3.92 \pm 0.24 \mu\text{mol/h}$ per mg of DNA respectively and for lactate formation were 3.11 ± 0.05 , 4.06 ± 0.11 and $4.93 \pm 0.20 \mu\text{mol/h}$ per mg of DNA respectively. The mean tubule DNA per ml of flask contents was 47 μg . Open symbols: glucose formation; closed symbols: lactate formation.

phosphoenolpyruvate carboxykinase (Kostos *et al.*, 1975; Jomain-Baum *et al.*, 1976), is reported to be a more potent and selective agent than quinolinate. The effect of this agent was tested on formation of glucose and lactate from pyruvate in Ca^{2+} -depleted tubules from fed rats (Fig. 1). At three Ca^{2+} concentrations glucose formation was considerably decreased by 3-mercaptopicolinate. Lactate formation, however, was decreased by a much smaller extent. These data indicated that a large proportion of lactate formation was unlikely to be associated with a pyruvate $\rightarrow$ oxaloacetate $\rightarrow$ phosphoenolpyruvate $\rightarrow$ pyruvate cycle (see below for quantitative estimates). It is concluded therefore that either the oxaloacetate-malate cycle or some other unknown process must supply appreciable amounts of cytosolic reducing equivalents in kidney cortex from fed rats.

Table 3. *Formation of glucose and lactate by Ca^{2+} -depleted tubule fragments from fed rats*

Tubule fragments were prepared in Ca^{2+} -free media and incubated in Krebs-Ringer bicarbonate buffer containing fatty acid-poor albumin (10mg/ml), 100 μM -disodium EGTA, 2mM- CaCl_2 and the indicated substrate additions. The results are means of two independent measurements and are expressed as $\mu\text{mol}/\text{mg}$ of DNA. The mean tubule DNA per ml of flask contents was 68 μg .

Additions	Incubation time (min)	Net glucose production	Net lactate production
5mM-Glutamate	30	0.34	-0.03
5mM-Glutamate + 0.1mM-glucose	30	—	0.02
5mM-Glutamate	60	0.75	-0.02
5mM-Glutamate + 0.1mM-glucose	60	—	0.03
5mM-Glutamate + 0.5mM-glucose	60	—	0.39
5mM-Succinate	30	0.46	0.08
5mM-Succinate + 0.1mM-glucose	30	—	0.15
5mM-Succinate	60	0.74	0.09
5mM-Succinate + 0.1mM-glucose	60	—	0.15
5mM-Succinate + 0.5mM-glucose	60	—	0.59

Fig. 2. *Effect of Ca^{2+} concentration on time courses of formation of glucose and lactate by Ca^{2+} -depleted tubule fragments from fed rats*

Tubule fragments were prepared in Ca^{2+} -free media and then incubated in Krebs-Ringer bicarbonate buffer containing fatty acid-poor albumin (10mg/ml), 5mM-sodium pyruvate and 100 μM -disodium EGTA. Ca^{2+} additions were zero (○), 0.2mM (●), 1mM (□), 2mM (■), 6mM (▲). The values are means $\pm$ S.E.M. of five experiments. The mean tubule DNA/ml of flask contents was 51 μg . (a) Glucose formation; (b) lactate formation.

To support this argument it was thought necessary to establish that lactate was formed through cytosolic pyruvate acting as an electron sink for reducing equivalents that are presumably derived from mitochondrial processes rather than from glycolysis in non-gluconeogenic cells in the tubule preparation. Accordingly, tubule fragments from fed rats were incubated in media containing 2mM- Ca^{2+} with glutamate or succinate to provide the source for gluconeogenic flux (Table 3). Little lactate is formed by kidney with these substrates (Nishiitsutsuji-Uwo *et al.*, 1967). Glucose formation from glutamate or succinate was similar to that observed with pyruvate in the presence of 0.2mM- Ca^{2+} (compare Tables 2 and 3), yet lactate formation was negligible with the former substrates. Appreciable lactate formation was only seen when 0.5mM-glucose was also added

(Table 3). It may be calculated from Table 1 that, even after incubation for 1h with pyruvate and 6mM- Ca^{2+} , glucose accumulation was only approx. 0.23mM, whereas lactate formation was 10-fold that found with succinate + 0.5mM-glucose (Table 3). The high rates of lactate formation observed in the presence of pyruvate are therefore most unlikely to have resulted from recycling back out of the pool of newly synthesized glucose. This is supported by the time courses of formation of glucose and lactate from pyruvate shown in Fig. 2: lactate formation does not require any prior glucose accumulation.

Metabolic effects of Ca^{2+} depletion

The experiment shown in Fig. 2 led to other noteworthy findings. As in all previous experiments

described above, tubule fragments were depleted of Ca^{2+} during preparation (this takes approx. 15min in ice-cold media, then 40min thereafter at 37°C). No time course at any Ca^{2+} concentration was linear over the entire 75min of incubation. With $0.2\text{mM-}\text{Ca}^{2+}$, glucose formation was higher between 0 and 15min than at 15–30min. After 30min the rate increased and then remained steady. With 1mM- , 2mM- or $6\text{mM-}\text{Ca}^{2+}$ linear rates ensued after a lag period. This was particularly apparent with $2\text{mM-}\text{Ca}^{2+}$, where a constant rate of product formation was not seen until after 30min. Profiles of lactate formation were essentially similar to those of gluconeogenesis. Since these unusual time courses were not seen when tubules were prepared in $1.27\text{mM-}\text{Ca}^{2+}$ (see below, Fig. 4), it is likely that they were a consequence of prior Ca^{2+} depletion. Any Ca^{2+} reaccumulation into cellular components must affect the energy metabolism of the tissue. If kidney mitochondria are similar in this respect to liver, i.e. Ca^{2+} uptake takes primacy over oxidative phosphorylation in utilizing respiratory energy (Rossi & Lehninger, 1964), gluconeogenesis will be curtailed while Ca^{2+} reaccumulation proceeds, presumably to an extent and at a rate determined by the concentration of external Ca^{2+} added. The apparent relationships between production of glucose or lactate and Ca^{2+} concentration therefore must differ with the sampling time. Fig. 3 shows these relationships when increments in product formation between 30 and 60min are considered. This time period was chosen since in this interval the time courses were linear with

Fig. 3. Effect of Ca^{2+} concentration on formation of glucose and lactate by Ca^{2+} -depleted tubule fragments from fed rats

The data are derived from Fig. 2 and represent the increments in glucose and lactate between 30 and 60min of incubation. The bars represent S.E.M. $\circ$, Glucose; $\bullet$, lactate.

respect to either the preceding or succeeding interval. The exception to this was glucose production at zero Ca^{2+} , which was very low and progressively declined with time. Both curves in Fig. 3 appeared to contain more than one component. Fig. 3 is at variance with Fig. 6 of Kurokawa *et al.* (1973), but this may be explained by the use of only 20min incubation periods by these authors. Further, interpretation of metabolite-crossover data obtained by Nagata & Rasmussen (1970), Rasmussen & Nagata (1970) and by Kurokawa & Rasmussen (1973) is complicated by the likelihood that measurements were not made under steady-state conditions in their studies. These findings led to the realization that a different experimental approach was necessary to undertake satisfactory investigations of the different metabolic steady states resulting from alteration of Ca^{2+} concentration, or from the addition of agonists and inhibitors. Therefore in experiments described below, tubule fragments were prepared in media containing $1.27\text{mM-}\text{Ca}^{2+}$ and the Ca^{2+} concentration was only changed at the commencement of incubation. Fig. 4 shows that linear time courses of formation of glucose and lactate were rapidly established at various Ca^{2+} concentrations if prior Ca^{2+} depletion was so avoided. At short incubation times, however, glucose production was an appreciable overestimate of the steady-state rate finally obtained at low Ca^{2+} concentrations (Fig. 4a). This error can be eliminated by measuring the increment in product formation between 15 and 45min when steady state pertains (Fig. 4).

Effects of Ca^{2+} and 3':5'-cyclic AMP on the steady-state metabolism of tubule fragments prepared in $1.27\text{mM-}\text{Ca}^{2+}$

By the experimental approach described above, the Ca^{2+} -dependence of formation of glucose and lactate by tubule fragments from fed rats was re-investigated (Fig. 5). Glucose formation still bore a sigmoidal relationship to Ca^{2+} concentration: 3':5'-cyclic AMP decreased this sigmoidicity and moved the curve of glucose formation versus Ca^{2+} concentration to the left. The rate of lactate formation was now almost independent of Ca^{2+} concentration and was unaffected by 3':5'-cyclic AMP. Clearly, if malate is both the vehicle for transfer of reducing equivalents from mitochondria to cytosol and the precursor of glucose, this finding, together with the results shown in Tables 1 and 2, must indicate that the major site(s) of stimulation of gluconeogenesis by Ca^{2+} and 3':5'-cyclic AMP occur after malate efflux from the mitochondria. In preliminary experiments it was established that the increased rates of glucose formation in the presence of 3':5'-cyclic AMP were constant after 10min of incubation. Fig. 5 shows that use of tubule fragments that were not

Fig. 4. Effect of Ca^{2+} concentration on time courses of production of glucose and lactate by tubule fragments from fed rats prepared in 1.27 mM-Ca^{2+}

Tubule fragments were prepared in media containing 1.27 mM-Ca^{2+} and then incubated in Krebs-Ringer bicarbonate buffer containing fatty acid-poor albumin (10 mg/ml) and 5 mM -sodium pyruvate. At the commencement of incubation Ca^{2+} concentrations were changed from 1.27 mM to 0.32 mM ($\circ$), 1.27 mM ($\bullet$) or 4.5 mM (Δ). The values are means $\pm$ s.e.m. of four experiments. The mean tubule DNA per ml of flask contents was $42 \mu\text{g}$. (a) Glucose formation; (b) lactate formation.

Fig. 5. Effects of Ca^{2+} and $3':5'$ -cyclic AMP on formation of glucose and lactate by tubule fragments from fed rats prepared in 1.27 mM-Ca^{2+}

Tubule fragments were prepared in media containing 1.27 mM-Ca^{2+} and then incubated in Krebs-Ringer bicarbonate buffer containing fatty acid-poor albumin (10 mg/ml) and 5 mM -sodium pyruvate. At the commencement of incubation Ca^{2+} concentrations were changed from 1.27 mM to those indicated. The values are means $\pm$ s.e.m. of four experiments and are increments in glucose or lactate between 15 and 45 min of steady-state incubation. The mean tubule DNA per ml of flask contents was $42 \mu\text{g}$. $\bullet$, No cyclic AMP; Δ , with cyclic AMP (0.1 mM). (a) Glucose formation; (b) lactate formation.

Ca^{2+} -depleted led to profiles of product formation versus Ca^{2+} concentration that were more compressed towards the ordinate than those obtained after prior Ca^{2+} depletion (Fig. 3), even though both sets of profiles refer to steady metabolic rates. Although Ca^{2+} -depletion during preparation may

possibly reveal aspects of Ca^{2+} -dependency that might not otherwise be seen (compare Figs. 3 and 5), it is clearly disadvantageous, since it appears (at least over the time scale of these experiments) to change permanently the relationship between extracellular Ca^{2+} concentration and metabolic rates.

Effects of 3-mercaptopycolinate, amino-oxyacetate and 2-n-butylnalonate on the steady-state metabolism of tubule fragments prepared in 1.27 mM-Ca²⁺

The effect of 3-mercaptopycolinate concentration on the metabolism of tubule fragments from fed or starved rats was investigated at 1.27 mM-Ca²⁺ under steady-state conditions (Fig. 6). Glucose production by tubules from starved rats was less sensitive to 3-mercaptopycolinate, i.e. 50% inhibition of this process was obtained with approx. 40 μ M- and 160 μ M-drug in tubules from fed and starved rats

respectively. Maximum inhibition of lactate formation was obtained with 0.1 mM-3-mercaptopycolinate in both preparations; this represented a 27% decrease in the fed state and a decrease of 48% in tubules from starved rats. Attempts were made to measure pyruvate kinase flux under conditions of steady-state incubation. This should be the same as the amount of lactate formation that is inhibited by a maximally effective dose of 3-mercaptopycolinate (0.1 mM). In this treatment it is assumed that effects of 3-mercaptopycolinate other than on phospho-

Fig. 6. Effect of 3-mercaptopycolinate on formation of glucose and lactate by tubule fragments from fed or starved (48 h) rats prepared in 1.27 mM-Ca²⁺

Tubule fragments were prepared in media containing 1.27 mM-Ca²⁺ and then incubated in Krebs-Ringer bicarbonate buffer containing 1.27 mM-Ca²⁺, fatty acid-poor albumin (10 mg/ml), 5 mM-sodium pyruvate and the indicated concentrations of 3-mercaptopycolinate. The values are means of two separate experiments and are increments in glucose or lactate between 15 and 45 min of steady-state incubation. The mean tubule DNA per ml of flask contents was: fed, 47 μ g; starved, 59 μ g. (a) Glucose production: ○, fed; ●, starved. (b) Lactate production: □, fed; ■, starved.

Table 4. Effect of 3-mercaptopycolinate and Ca²⁺ on formation of glucose and lactate by tubule fragments from fed rats prepared in 1.27 mM-Ca²⁺

Tubule fragments were prepared in media containing 1.27 mM-Ca²⁺ and then incubated in Krebs-Ringer bicarbonate buffer containing fatty acid-poor albumin (10 mg/ml) and 5 mM-pyruvate. Ca²⁺ concentrations were changed, where appropriate, at the start of the incubation. The results, which are means $\pm$ S.E.M. of four independent determinations, represent increments between 15 and 45 min of steady-state incubation and are expressed as μ mol/30 min per mg of DNA. The values in parentheses indicate the percentages of glucose or lactate formation remaining in the presence of 3-mercaptopycolinate. The mean tubule DNA per ml of flask contents was 47 μ g. **, *** indicate $P < 0.01$, < 0.001 respectively for effects of 3-mercaptopycolinate.

Additions to incubation	Glucose formation	Lactate formation	Ratio [lactate]/[glucose]	3-Mercaptopycolinate-sensitive lactate production
0.32 mM-Ca ²⁺	1.81 $\pm$ 0.13	3.20 $\pm$ 0.25	1.77 $\pm$ 0.02	0.73 $\pm$ 0.05
0.32 mM-Ca ²⁺ + 3-mercaptopycolinate (0.1 mM)	0.53 $\pm$ 0.03*** (29.7 $\pm$ 0.3%)	2.47 $\pm$ 0.21*** (77.0 $\pm$ 1.2%)	4.62 $\pm$ 0.16	
1.27 mM-Ca ²⁺	3.17 $\pm$ 0.15	3.22 $\pm$ 0.26	1.02 $\pm$ 0.06	0.75 $\pm$ 0.12
1.27 mM-Ca ²⁺ + 3-mercaptopycolinate (0.1 mM)	0.90 $\pm$ 0.01*** (28.7 $\pm$ 1.1%)	2.46 $\pm$ 0.16** (77.0 $\pm$ 2.2%)	2.73 $\pm$ 0.16	
4.5 mM-Ca ²⁺	3.73 $\pm$ 0.11	3.11 $\pm$ 0.14	0.83 $\pm$ 0.01	0.72 $\pm$ 0.07
4.5 mM-Ca ²⁺ + 3-mercaptopycolinate (0.1 mM)	1.16 $\pm$ 0.07*** (31.1 $\pm$ 1.5%)	2.39 $\pm$ 0.20** (76.5 $\pm$ 3.2%)	2.08 $\pm$ 0.19	

Table 5. *Effect of metabolic inhibitors on formation of glucose and lactate by tubule fragments from fed or starved rats prepared in 1.27 mM-Ca²⁺*

Tubule fragments were prepared in media containing 1.27 mM-Ca²⁺ and then incubated in Krebs-Ringer bicarbonate buffer containing fatty acid-poor albumin (10 mg/ml) and 5 mM-pyruvate. n-Butylmalonate was added as the neutral sodium salt. The values, which are means $\pm$ S.E.M. of four independent determinations in each case, represent increments between 15 and 45 min of steady-state incubation and are expressed as μ mol/30 min per mg of DNA. The values in parentheses indicate the percentages of glucose or lactate formation remaining in the presence of the inhibitor. The mean tubule DNA per ml of flask contents was 50 μ g and 64 μ g for fed and starved rats respectively. *, **, *** indicate $P < 0.05$, < 0.01 , < 0.001 respectively for effects of inhibitors.

Dietary status	Additions to incubation	Glucose formation	Lactate formation	Ratio [lactate]/[glucose]	3-Mercaptopicolinate-sensitive lactate production
Fed	None	2.94 $\pm$ 0.09	2.65 $\pm$ 0.03	0.90 $\pm$ 0.03	
	Amino-oxyacetate (0.1 mM)	3.08 $\pm$ 0.16 (104.8 $\pm$ 4.6%)	2.59 $\pm$ 0.09 (98.1 $\pm$ 4.0%)	0.85 $\pm$ 0.04	
	n-Butylmalonate (5 mM)	1.90 $\pm$ 0.09*** (64.7 $\pm$ 1.6%)	2.15 $\pm$ 0.13* (81.1 $\pm$ 4.6%)	1.13 $\pm$ 0.01	
	None	3.77 $\pm$ 0.14	3.26 $\pm$ 0.08	0.87 $\pm$ 0.04	1.43 $\pm$ 0.20
Starved (48 h)	3-Mercaptopicolinate (0.1 mM)	1.63 $\pm$ 0.08*** (43.5 $\pm$ 2.8%)	1.83 $\pm$ 0.16** (56.4 $\pm$ 5.4%)	1.12 $\pm$ 0.05	
	Amino-oxyacetate (0.1 mM)	4.03 $\pm$ 0.16** (106.8% $\pm$ 0.5%)	3.90 $\pm$ 0.13*** (119.3 $\pm$ 1.4%)	0.98 $\pm$ 0.06	
	n-Butylmalonate (5 mM)	2.38 $\pm$ 0.10** (63.5 $\pm$ 3.5%)	1.95 $\pm$ 0.13** (59.9 $\pm$ 4.3%)	0.82 $\pm$ 0.03	
	None				

enolpyruvate carboxykinase are small. There is no published information on other effects of this drug, although Kostos *et al.* (1975) have implied that some inhibition of mitochondrial transport of dicarboxylate anions may occur. On this basis, lactate formation associated with a pyruvate $\rightarrow$ oxaloacetate $\rightarrow$ phosphoenolpyruvate $\rightarrow$ pyruvate cycle, calculated as (total lactate formation) - (lactate formation in the presence of 0.1 mM-3-mercaptopicolinate) and referred to as '3-mercaptopicolinate-sensitive lactate formation', may be an overestimate, but is unlikely to be an underestimate, of pyruvate kinase flux. Table 4 shows that 3-mercaptopicolinate-sensitive lactate production was totally unaffected by Ca²⁺, suggesting that alteration of pyruvate kinase flux was unlikely to have been involved in the promotion of gluconeogenesis seen with increasing Ca²⁺ concentration. This is at variance with the conclusions of Nagata & Rasmussen (1970) and Kurokawa & Rasmussen (1973), which were based on studies of metabolite crossovers. In addition, the results of Table 4 suggest that recycling of C₃ units through pyruvate kinase in tubules from fed rats is rather small compared with the flux of C₃ units to glucose, i.e. at 0.32 mM-Ca²⁺ 3-mercaptopicolinate-sensitive lactate production was only 20% of the C₃-unit flux to glucose. At 1.27 mM- and 4.5 mM-Ca²⁺ the corresponding percentages were only 12 and 10% respectively. As suggested by Fig. 6(b) and seen by comparison of Tables 4 and 5, 3-mercaptopicolinate-sensitive lactate production was greater in tubules

from starved rats. In media containing 1.27 mM-Ca²⁺ the rate of this process was 22% of the rate of C₃-unit flux to glucose, which is very much less than the rate of recycling via pyruvate kinase estimated from radioactive isotopic data in renal-cortex slices from starved rats by Rognstad & Katz (1972). Table 6 shows that 3-mercaptopicolinate-sensitive lactate production was decreased in tubules from fed and starved rats by 3':5'-cyclic AMP. These experiments were performed in 0.32 mM-Ca²⁺ rather than with 1.27 mM-Ca²⁺ to increase the percentage effect of 3':5'-cyclic AMP on glucose production (see Fig. 5a). In tubules from starved rats total lactate production also was significantly decreased by 3':5'-cyclic AMP. This is similar to the findings of Rognstad (1975) with hepatocytes. However, in contrast with findings with hepatocytes from starved rats (Rognstad, 1975; Mapes & Harris, 1976) 3':5'-cyclic AMP did not decrease glucose formation from pyruvate in tubules from starved rats (Table 6). This is further evidence that in kidney cortex the linkage between lactate formation, glucose formation and the operation of a pyruvate $\rightarrow$ oxaloacetate $\rightarrow$ phosphoenolpyruvate $\rightarrow$ pyruvate cycle is looser. The 'K'-type pyruvate kinase isoenzyme is the predominant form in rat kidney cortex (Ibsen & Krueger, 1973) and has kinetic properties more similar to those of the 'L'- than of the 'M'-type isoenzyme (Ibsen & Trippet, 1973). At present it is unknown whether the K-type, like the L-type, can be phosphorylated by a 3':5'-cyclic AMP-dependent

Table 6. *Effect of 3-mercaptopycolinate and 3':5'-cyclic AMP on formation of glucose and lactate by tubule fragments from fed or starved rats prepared in 1.27 mM-Ca²⁺*

Tubule fragments were prepared in media containing 1.27 mM-Ca²⁺ and then incubated in Krebs-Ringer bicarbonate buffer containing fatty acid-poor albumin (10mg/ml) and 5mM-pyruvate. Ca²⁺ concentration was changed to 0.32 mM at the commencement of incubation. The values, which are means $\pm$ S.E.M. of five independent determinations in each case, represent increments between 15 and 45 min of steady-state incubation and are expressed as μ mol/30 min per mg of DNA. The values in parentheses indicate the percentages of glucose or lactate formation remaining in the presence of 3-mercaptopycolinate. The mean tubule DNA per ml of flask contents was 43 μ g and 53 μ g for fed and starved rats respectively. **, *** indicate $P < 0.01$, < 0.001 respectively for effects of 3-mercaptopycolinate. †, ††, ††† indicate $P < 0.05$, < 0.01 , < 0.001 respectively for effects of 3':5'-cyclic AMP.

Dietary status	Additions to incubation	Glucose formation	Lactate formation	Ratio [lactate]/[glucose]	3-Mercaptopycolinate-sensitive lactate production
Fed	None	1.90 $\pm$ 0.17	2.92 $\pm$ 0.31	1.63 $\pm$ 0.12	0.83 $\pm$ 0.10
	3-Mercaptopycolinate (0.1 mM)	0.57 $\pm$ 0.04*** (30.1 $\pm$ 1.2%)	2.09 $\pm$ 0.27*** (71.4 $\pm$ 3.1%)	3.65 $\pm$ 0.38	
	3':5'-Cyclic AMP (0.1 mM)	3.55 $\pm$ 0.18†††	2.93 $\pm$ 0.25	0.83 $\pm$ 0.07	0.61 $\pm$ 0.05 (76.2 $\pm$ 9.2% compared with control)
	3':5'-Cyclic AMP (0.1 mM)	0.92 $\pm$ 0.05***††	2.33 $\pm$ 0.21***	2.55 $\pm$ 0.25	
	+ 3-mercaptopycolinate (0.1 mM)	(26.0 $\pm$ 0.3%)	(79.4 $\pm$ 1.0%)		
Starved (48 h)	None	2.64 $\pm$ 0.24	3.07 $\pm$ 0.29	1.19 $\pm$ 0.15	1.23 $\pm$ 0.03
	3-Mercaptopycolinate (0.1 mM)	1.27 $\pm$ 0.24*** (46.7 $\pm$ 5.4%)	1.83 $\pm$ 0.32** (57.8 $\pm$ 5.2%)	1.63 $\pm$ 0.46	
	3':5'-Cyclic AMP (0.1 mM)	3.26 $\pm$ 0.40†	2.69 $\pm$ 0.24†	0.86 $\pm$ 0.13	0.83 $\pm$ 0.13† (67.0 $\pm$ 9.8% compared with control)
	3':5'-Cyclic AMP (0.1 mM)	1.61 $\pm$ 0.28***††	1.86 $\pm$ 0.30**	1.32 $\pm$ 0.36	
	+ 3-mercaptopycolinate (0.1 mM)	(49.2 $\pm$ 7.6%)	(67.8 $\pm$ 6.7%)		

protein kinase (Ljungström *et al.*, 1974, 1976; Titanji *et al.*, 1976). Evidence has been provided that this may be an indirect means of achieving hormonal stimulation of hepatic gluconeogenesis (Blair *et al.*, 1976; Feliu *et al.*, 1976; van Berkel *et al.*, 1976), although this has been disputed (Clark, 1976). In the present study (Table 6) the changes in 3-mercaptopycolinate-sensitive lactate formation caused by 3':5'-cyclic AMP were quite small compared with the associated increases in flux of C₃ units to glucose.

Table 5 shows that lactate formation in either the fed or starved state was not inhibited by amino-oxyacetate. In fact this agent caused a small, but significant, increase in this process in tubules from starved rats. It is therefore most unlikely that lactate formation is linked with any process in which it is necessary to dispose of cytosolic oxaloacetate by aspartate aminotransferase.

Table 5 also shows that, although gluconeogenesis in the fed or starved state was equally sensitive to inhibition by 2-n-butylmalonate, lactate formation in tubules from starved rats had a considerably

greater sensitivity to this agent. Lactate formation in association with a pyruvate $\rightarrow$ oxaloacetate $\rightarrow$ phosphoenolpyruvate $\rightarrow$ pyruvate cycle, which, on the basis of 3-mercaptopycolinate sensitivity, is judged to be greater in the starved state, should involve a malate/phosphate exchange on the dicarboxylate translocator (Mapes & Harris, 1976), which is sensitive to 2-n-butylmalonate inhibition (Meijer & Tager, 1969; Robinson & Williams, 1970). Whether lactate formation in association with a malate-oxaloacetate cycle involving a malate/oxaloacetate exchange on the dicarboxylate translocator should be less sensitive to 2-n-butylmalonate is unclear. Dicarboxylate/phosphate exchange in rat liver mitochondria has been reported to be far more sensitive to 2-n-butylmalonate than are various dicarboxylate/dicarboxylate exchanges (Meijer & Tager, 1969; Robinson & Williams, 1970). However, Gimpel *et al.* (1973) found extensive inhibition of oxaloacetate penetration of rat liver mitochondria by 2-n-butylmalonate. The metabolic processes involved in 3-mercaptopycolinate-insensitive lactate formation therefore cannot be ascertained at present.

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References

- Alleyne, G. A. O., Flores, H. & Roobol, A. (1973) *Biochem. J.* **136**, 445–453
- Bennett, F. I. & Alleyne, G. A. O. (1976) *FEBS Lett.* **65**, 125–129
- Berry, M. N. (1971) *Biochim. Biophys. Res. Commun.* **44**, 1449–1456
- Berry, M. N. & Kun, E. (1972) *Eur. J. Biochem.* **27**, 395–400
- Blair, J. B., Cimbala, M. A., Foster, J. L. & Morgan, R. A. (1976) *J. Biol. Chem.* **251**, 3756–3762
- Bohnensack, B. & Kunz, W. (1971) *Biochim. Biophys. Acta* **226**, 33–41
- Burton, K. (1956) *Biochem. J.* **62**, 315–323
- Chappell, J. B. (1961) in *Biological Structure and Function*, vol. 2 (Goodwin, T. W. & Lindberg, O., eds.), pp. 72–83, Academic Press, London and New York
- Clark, M. G. (1976) *Biochem. Biophys. Res. Commun.* **68**, 120–126
- Di Tullio, N. W., Bekoff, C. E., Blank, B., Kostos, V., Stack, E. J. & Saunders, H. L. (1974) *Biochem. J.* **138**, 387–394
- Feliu, J. E., Hue, L. & Hers, H. G. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2762–2766
- Friedmann, B., Goodman, E. H., Saunders, H. L., Kostos, V. & Weinhouse, S. (1971) *Arch. Biochem. Biophys.* **143**, 506–578
- Gimpel, J. A., De Haan, E. J. & Tager, J. M. (1973) *Biochim. Biophys. Acta* **292**, 582–591
- Guder, W. G. & Rupprecht, A. (1975) *Eur. J. Biochem.* **52**, 283–289
- Guder, W. G. & Schmidt, U. (1976) *Kidney Int.* **10**, S-32–S-38
- Guder, W. G., Wiesner, W., Stukowski, B. & Wieland, O. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 1319–1328
- Haslam, J. M. & Griffiths, D. E. (1968) *Biochem. J.* **109**, 921–928
- Haslam, J. M. & Krebs, H. A. (1968) *Biochem. J.* **107**, 659–667
- Hohorst, H.-J. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 266–270, Academic Press, New York and London
- Hohorst, H.-J. & Reim, M. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 335–339, Academic Press, New York and London
- Ibsen, K. H. & Krueger, E. (1973) *Arch. Biochem. Biophys.* **157**, 509–513
- Ibsen, K. H. & Trippet, P. (1973) *Arch. Biochem. Biophys.* **156**, 730–744
- Jomain-Baum, M., Schramm, V. L. & Hanson, R. W. (1976) *J. Biol. Chem.* **251**, 37–44
- Kostos, V., Di Tullio, N. W., Rush, J., Cieslinski, L. & Saunders, H. L. (1975) *Arch. Biochem. Biophys.* **171**, 459–465
- Krebs, H. A. (1966) in *Biochemistry of Mitochondria* (Slater, E. C., Kaninga, Z. & Wojtczak, L., eds.), pp. 105–113, Academic Press, London and New York
- Krebs, H. A. & Henseleit, H. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Krebs, H. A., Bennett, D. A. H., de Gasquet, P., Gascoyne, T. & Yoshida, T. (1963) *Biochem. J.* **86**, 22–27
- Krebs, H. A., Gascoyne, T. & Notton, B. M. (1967) *Biochem. J.* **102**, 275–282
- Kurokawa, K. & Rasmussen, H. (1973) *Biochim. Biophys. Acta* **313**, 17–31
- Kurokawa, K., Ohno, T. & Rasmussen, H. (1973) *Biochim. Biophys. Acta* **313**, 32–41
- La Noue, K. F. & Williamson, J. R. (1971) *Metabolism* **20**, 119–140
- La Noue, K. F., Meijer, A. J. & Brouwer, A. (1974) *Arch. Biochem. Biophys.* **161**, 544–550
- Lardy, H. A., Paetkau, V. & Walter, P. (1965) *Proc. Natl. Acad. Sci. U.S.A.* **53**, 1410–1415
- Ljungström, O., Hjelmquist, G. & Engström, L. (1974) *Biochim. Biophys. Acta* **358**, 289–298
- Ljungström, O., Berglund, L. & Engström, L. (1976) *Eur. J. Biochem.* **68**, 497–506
- Longshaw, I. D., Bowen, N. L. & Pogson, C. I. (1972) *Eur. J. Biochem.* **25**, 366–371
- Macdonald, D. W. R. & Saggerson, E. D. (1977) *Biochem. J.* **168**, 33–42
- Mapes, J. P. & Harris, R. A. (1976) *J. Biol. Chem.* **251**, 6189–6196
- Meijer, A. J. & Tager, J. M. (1969) *Biochim. Biophys. Acta* **189**, 136–139
- Meijer, A. J. & Williamson, S. R. (1974) *Biochim. Biophys. Acta* **331**, 1–11
- Nagata, N. & Rasmussen, H. (1970) *Biochim. Biophys. Acta* **215**, 1–16
- Nishiitsutsuji-Uwo, J. M., Ross, B. D. & Krebs, H. A. (1967) *Biochem. J.* **103**, 852–862
- Pagliara, A. S. & Goodman, A. D. (1969) *J. Clin. Invest.* **48**, 1408–1412
- Pfleiderer, G. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 381–383, Academic Press, New York and London
- Rasmussen, H. & Nagata, N. (1970) *Biochim. Biophys. Acta* **215**, 17–28
- Robinson, B. H. & Chappell, J. B. (1967) *Biochem. J.* **105**, 18 P
- Robinson, B. H. & Williams, G. R. (1970) *Biochim. Biophys. Acta* **216**, 63–70
- Rognstad, R. (1975) *Biochim. Biophys. Res. Commun.* **63**, 900–905
- Rognstad, R. & Katz, J. (1970) *Biochem. J.* **116**, 483–491
- Rognstad, R. & Katz, J. (1972) *J. Biol. Chem.* **247**, 6047–6054
- Roobol, A. & Alleyne, G. A. O. (1973) *Biochem. J.* **134**, 157–165
- Rossi, C. S. & Lehninger, A. L. (1964) *J. Biol. Chem.* **239**, 3971–3980
- Rostand, S., Peterson, M. & Williamson, J. R. (1969) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **28**, 335
- Schmidt, U., Dubach, U. C., Guder, W. G., Funk, B. & Paris, K. (1975) *Curr. Problems Chem. Biochem.* **4**, 22–32
- Shrago, E. & Lardy, H. A. (1966) *J. Biol. Chem.* **241**, 663–668
- Slein, M. W. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 117–123, Academic Press, New York and London

- Titanji, V. P. K., Zetterqvist, O. & Engström, L. (1976) *Biochim. Biophys. Acta* **422**, 98–108
- van Berkel, T. J. C., Kruijt, J. K., Koster, J. F. & Hülsmann, W. C. (1976) *Biochem. Biophys. Res. Commun.* **72**, 917–925
- Walter, P., Paetkau, V. & Lardy, H. A. (1966) *J. Biol. Chem.* **241**, 2523–2532
- Williamson, J. R. (1976) in *Gluconeogenesis: Its Regulation in Mammalian Species* (Hanson, R. W. & Mehlman, M. A., eds.), pp. 165–220, John Wiley and Sons, New York
- Wojtczak, A. B. (1969) *Biochim. Biophys. Acta* **172**, 52–65
- Zahlten, R. N., Kneer, N. M., Stratman, F. W. & Lardy, H. A. (1974) *Arch. Biochem. Biophys.* **161**, 528–535