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The Activity of Phosphoenolpyruvate Carboxykinase in Rat Tissues

ASSAY TECHNIQUES AND EFFECTS OF DIETARY AND HORMONAL CHANGES

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1. Phosphoenolpyruvate carboxykinase was assayed by three methods: (i) incorporation of $H^{14}CO_3^-$ into oxaloacetate: (ii) conversion of oxaloacetate into phosphoenolpyruvate, subsequently assayed enzymically; and (iii) transfer of ^{32}P from [γ-^{32}P]GTP to oxaloacetate. 2. Enzyme activity is increased in liver and epididymal adipose tissue in alloxan-diabetes and starvation, and in kidney in starved, acidotic and steroid-treated animals. 3. The ratios of the 'back' to the 'forward' reactions in liver, kidney and epididymal adipose tissue are different and characteristic of each tissue; they differ markedly from values reported for the purified mitochondrial enzyme. 4. The ratio of the 'back' to 'forward' reaction in any one tissue is constant in adrenalectomized, diabetic, acidotic and steroid-treated animals. 5. In starved animals, the ratio is increased in liver and kidney, but decreased in epididymal adipose tissue. 6. Administration of L-tryptophan results in an acute (1h) increase in activity measured in the 'forward' direction alone in liver and epididymal adipose tissue, but not in kidney.

There is now substantial evidence that phosphoenolpyruvate carboxykinase [GTP–oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32] plays an important role in the regulation of gluconeogenesis in mammalian systems (Record *et al.*, 1972; Ui *et al.*, 1973). Nevertheless, studies of the mechanisms in which phosphoenolpyruvate carboxykinase may be involved have raised many problems. As an example, the published K_m values for oxaloacetate (see Table 1) vary considerably from one report to another, and, in the case of the higher values, may not easily be reconciled with both the observed concentrations of oxaloacetate in tissues (Utter & Kolenbrander, 1972) and the rates of glucose synthesis.

Some, at least, of these problems may have arisen from the absence of any simple, generally applicable, continuous assay system for phosphoenolpyruvate carboxykinase. As a result of this, a number of widely differing 'stopped' procedures have been developed. Since the conditions under which the various assays are performed are so diverse, simple numerical comparison between the systems is both difficult and suspect; the situation is particularly complicated by the fact that the enzyme has been measured *in vitro* in both the forward (i.e. conversion of oxaloacetate into phosphoenolpyruvate) and backward (phosphoenolpyruvate into oxaloacetate) directions, and that these measurements may not be readily interconverted. To our knowledge, only one study of the relative rates of the reaction in both directions has been performed with purified enzyme. This study, with phosphoenolpyruvate carboxykinase from pig liver mitochondria, indicates that the relative maximum rates of the forward and backward reactions are 8.3:1, 11.3:1 and 18.5:1, at pH 6.8, 7.3 and 8.0 respectively (Chang *et al.*, 1966). A brief survey of the literature, however, reveals that these ratios may not necessarily be reflected in measurements in unpurified tissue extracts (see, e.g., Scrutton & Utter, 1968; Meyuhas *et al.*, 1971; Williamson *et al.*, 1971; Utter, 1971). In the present paper we report a systematic investigation of phosphoenolpyruvate carboxykinase activities in rat tissues as assayed by three independent methods.

These studies have been extended to conditions in which gluconeogenic flux is modified by various agents, namely starvation, diabetes and tryptophan in the liver, starvation and NH_4Cl-induced acidosis in the kidney. We have also included measurements on adipose tissue phosphoenolpyruvate carboxykinase, which may be important in glyceride glycerol synthesis (Hanson *et al.*, 1973), and/or as a necessary enzyme for the return of cytoplasmic oxaloacetate to the mitochondrion during fat synthesis from certain substrates (Martin & Denton, 1970).

The ratios of the forward and backward reactions are at variance with those derived from the earlier data with purified enzyme, are characteristic of each of the three tissues examined, and are, in general, little affected by treatments that alter total phosphoenolpyruvate carboxykinase activity.

Materials and Methods

Chemicals and enzymes

Tris, 2-mercaptoethanol, dithiothreitol, cycloheximide and Tween 80 were from Sigma (London) Chemical Co. (Norbiton, Surrey, U.K.). Imidazole and 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole were from Koch–Light Laboratories Ltd. (Colnbrook, Bucks., U.K.), and Norit GSX was from Norit Clydesdale Co. (Glasgow, U.K.). $NaH^{14}CO_3$ and [γ-^{32}P]GTP were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

ATP, GTP, oxaloacetic acid, GSH, NADH and all enzymes were purchased from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K. Phosphoenolpyruvate (monocyclohexylammonium salt) was prepared as described by Clark & Kirby (1966). ITP was made by treatment of ATP with HNO_2 as described by Kaplan (1957); this was converted into IDP with myosin, a gift from Dr. D. R. Trentham, University of Bristol. Triamcinolone (9α-fluoro-16α, 17α-isopropylidenedioxy-1-dehydrocorticosterone) acetonide was a gift from E. R. Squibb and Sons Ltd., Moreton, nr. Liverpool, Lancs., U.K. All other chemicals, of the purest grade available, were obtained from Fisons Scientific Apparatus, Loughborough, Leics., U.K., or BDH Chemicals Ltd., Poole, Dorset, U.K.

Animals

Male Wistar rats (180–250g) were used throughout. Unless otherwise stated, all animals were allowed access to diet (no. 1 maintenance diet, Cooper Nutrition Products Ltd., Witham, Essex, U.K.) and water *ad libitum*.

Adrenal glands were removed by means of a midline dorsal incision under diethyl ether anaesthesia. Adrenalectomized animals were given 1% NaCl in place of water, and were left for 5–8 days before use. Diabetes was induced by intravenous injection of alloxan (60mg/kg body wt.). Animals were used after 2 days; hyperglycaemia was confirmed enzymically (Huggett & Nixon, 1957). Rats were made acidotic by the oral administration of 0.4M-NH_4Cl (2.5ml/kg body wt.). The procedure was repeated after 12h, and animals were killed at 24h. Controls throughout received an equivalent volume of 0.4M-NaCl. Acidotic animals were given 0.18% NaCl in place of drinking water.

Appropriate dilutions of triamcinolone acetonide in 0.9% NaCl were administered intraperitoneally (5mg/kg body wt.) at zero time and 12h. These animals were killed at 24h, together with the appropriate saline-treated controls.

L-Tryptophan suspension (40mg/ml in 0.1% Tween 80, 0.9% NaCl) was injected intraperitoneally through a 16-gauge needle (dosage 0.8g/kg body wt.); controls received a similar volume of the suspending medium. Animals were killed 1h after injection. Cycloheximide (0.5mg/ml in 0.9% NaCl) was injected intraperitoneally at a dose of 1mg/kg body weight, 30min before each oral administration of NH_4Cl.

Preparation of extracts

Animals were bled after decapitation. Tissues were removed, rinsed in ice-cold 0.9% NaCl, blotted gently, weighed and extracted in either 3 (fat-pads) or 10 (liver, kidney) ml of 0.25M-sucrose–5mM-Tris–HCl–1mM-2-mercaptoethanol (pH7.4)/g of tissue, by using a motor-driven all-glass homogenizer (Kontes Glass Co., Vineland, N.J., U.S.A.). This procedure ensures complete breakage of both whole cells and mitochondria. Suitable portions were centrifuged at 12000*g* for 8min in an Eppendorf 3200 centrifuge; the resulting supernatants were stored on ice until assay (0–5h).

Assay of phosphoenolpyruvate carboxykinase

(*i*) *$H^{14}CO_3^-$ incorporation into oxaloacetate* (*Ballard & Hanson*, 1967) The medium contained 0.1M-imidazole, 2mM-$MnCl_2$, 1mM-dithiothreitol, 50mM-$KHCO_3$ (containing 2μCi of $NaH^{14}CO_3$), 1.5mM-phosphoenolpyruvate, 1.25mM-IDP, 0.5mM-NADH, malate dehydrogenase (EC 1.1.1.37, 2.5μg/ml) and extract, final pH7.0, volume 1.0ml. The reaction was started by the addition of extract, after 2min preincubation of medium. Samples, in duplicate, were incubated at 37°C for 10min; the reaction was stopped with 0.5ml of 10% (w/v) trichloroacetic acid. After CO_2 has been bubbled through each sample for at least 3min, 0.5ml portions were removed for radioactivity counting [scintillator: 0.6% (w/v) 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole, 8% (w/v) naphthalene, 40% (v/v) 2-methoxyethanol and 60% (v/v) toluene]. The time-course of the reaction was linear throughout.

(*ii*) *Conversion of oxaloacetate into phosphoenolpyruvate* (*Seubert & Huth* (1965). The medium contained 0.1M-Tris–HCl, 18mM-$MgCl_2$, 15mM-NaF, 6mM-ITP, 1mM-GSH, 4.5mM-oxaloacetate and extract, final pH8.1, volume 1.0ml. The reaction was started by the addition of extract after 2min preincubation of medium. Samples, in duplicate, were incubated at 37°C for 10min; reaction was stopped by the addition of 0.1ml of KBH_4 (50mg/ml) in 1mM-NaOH. After further addition of 10μl of 20% (v/v) octan-2-ol in ethanol (as antifoaming agent), samples were chilled on ice for 2–3min. Excess of KBH_4 was then destroyed with 0.2ml of 20% (w/v) $HClO_4$, and the protein precipitate removed by centrifugation. Supernatant (1ml) was carefully

neutralized with approx. 0.2ml of 0.5M-triethanolamine hydrochloride, pH7.4, containing 2M-KOH. Phosphoenolpyruvate was measured enzymically in the supernatant after further centrifugation. The time-course of the reaction was linear throughout.

(*iii*) *Conversion of [γ-^{32}P]GTP into [^{32}P]phosphoenolpyruvate; modified from Walsh & Chen* (1971). The medium contained 20mM-Tris–HCl, 4mM-NaF, 5mM-$MgCl_2$, 0.1mM-GSH, 1mM-oxaloacetate, 0.1mM-[γ-^{32}P]GTP (0.2μCi) and extract in a final volume of 0.2ml, pH8.0. The reaction was started by the addition of extract after 2min preincubation of medium. Samples were assayed in triplicate, and were incubated at 37°C for both 2 and 4min. The reaction was terminated by the addition of 1.0ml of 0.2M-$HClO_4$ containing 0.2mM-NaH_2PO_4; tubes were subsequently kept at 0°C. To each sample were added sequentially 1ml of 2% (w/v) ammonium molybdate containing 50mM-triethylamine hydrochloride, 0.2ml of Norit GSX (50mg/ml in water) and 5μl of 30mM-phosphoenolpyruvate. After 10min tubes were centrifuged at 1200*g* for 4min. Then 1.5ml portions of supernatants were added to 1.5ml of 0.25M-$HgCl_2$; after 45min, 20μl of 0.16M-triethylamine hydrochloride–10mM-NaH_2PO_4 was added, and the tubes were left for a further 30min. After centrifugation, the supernatants were carefully removed by aspiration, and the pellets washed with 3×2ml of 0.2M-$HClO_4$–0.5% (w/v) ammonium molybdate–50mM-triethylamine hydrochloride. The precipitates were finally dissolved in 10ml of scintillator and counted for radioactivity as described above. Blanks without enzyme or oxaloacetate were carried through the same procedure. Reaction rates were linear with time.

Results and Discussion

Assay systems

Three assay systems were used as a routine. The conversion of phosphoenolpyruvate into oxaloacetate ('back' reaction) was measured by the widely used method of Chang & Lane (1966), as modified by Ballard & Hanson (1967). The 'forward' reaction, in the physiological direction of phosphoenolpyruvate synthesis, was measured in two ways. The first, that of Seubert & Huth (1965), has again been widely used and involves conditions under which the K_m value for oxaloacetate is high (Holten & Nordlie, 1965), although lower values have also been reported (Ballard, 1970). The second, modified from that devised by Walsh & Chen (1971), and based on the transfer of ^{32}P from [^{32}P]GTP to oxaloacetate, is both more sensitive and gives the lowest K_m values reported for oxaloacetate (Table 1).

Preliminary experiments revealed that the dependence of enzyme activity on ionic composition was different in the 'forward' and 'back' reactions. Maximum rates were attained under standard assay conditions; the replacement of Mg^{2+} by Mn^{2+} in the Seubert & Huth (1965) 'forward' assay, and of Mn^{2+} by Mg^{2+} in the 'back' assay, resulted in substantially lower rates in both cases. The inclusion of NaF, at up to 15mM, was without effect throughout. The replacement of inosine nucleotide by guanine nucleotide in the same two assays again produced somewhat lower than maximum activities.

In the ^{32}P assay, oxaloacetate concentrations were maintained at saturating values (1mM). An increase in the concentration of GTP from 0.1 to 2.0mM led to a 30–70% increase in rate. It is difficult, however,

Table 1. *Published values for Michaelis constants for phosphoenolpyruvate carboxykinase from various sources*

Substrate	Source	$10^5 \times K_m$ (M)	Reference
Oxaloacetate	Guinea-pig liver cytosol	90	Holten & Nordlie (1965)
	Mouse liver cytosol	10	Berndt & Ulbrich (1970)
	Rat liver cytosol	14	Foster *et al.* (1967)
	Rat liver cytosol	2.2	Meyuhas *et al.* (1971)
	Rat liver cytosol	15	Marco & Sols (1970)
	Rat liver cytosol	2.5	Ballard (1970)
	Rat liver cytosol	0.15–0.5	Walsh & Chen (1971)
	Rat kidney cytosol	20	Flores & Alleyne (1971)
	Rat adipose tissue cytosol	2.2	Meyuhas *et al.* (1971)
	Sheep liver cytosol	2.2	Ballard (1970)
	Guinea-pig liver mitochondria	250	Holten & Nordlie (1965)
	Pig liver mitochondria	14	Chang *et al.* (1966)
	Sheep liver mitochondria	0.9	Ballard (1970)
	Chicken liver mitochondria	400	Felicioli *et al.* (1970)
	Chicken liver mitochondria	0.9	Ballard (1970)
GTP	Guinea-pig liver cytosol	140	Holten & Nordlie (1965)
	Pig liver mitochondria	16	Chang *et al.* (1966)
	Rat kidney cytosol	0.25	Guder & Schmidt (1974)

Table 2. *Activities of phosphoenolpyruvate carboxykinase in rat liver, kidney and adipose tissue*

Enzyme activities in extracts from fed control animals were measured as follows: (A) 'back' direction, $^{14}CO_2$ incorporation (Ballard & Hanson, 1967); (B) 'forward' direction, ^{32}P incorporation (modified from Walsh & Chen, 1971); (C) 'forward' direction, spectrophotometric assay (Seubert & Huth, 1965). Other details were as given in the Materials and Methods section. Results are means ±S.E.M.; the numbers of determinations from individual animals are shown in parentheses.

	Enzyme Activity (μmol·min^{-1} g wet wt.$^{-1}$)			Ratios of Activities		
	A	B	C	A/B	A/C	C/B
(I) Liver	1.47±0.08 (70)	0.58 ±0.08 (70)	2.39±0.18 (35)	3.7±0.24 (70)	0.60±0.03 (35)	6.6±0.41 (35)
(II) Kidney	5.33±0.15 (89)	0.74 ±0.03 (82)	5.86±0.24 (54)	7.3±0.21 (82)	0.91±0.03 (54)	8.7±0.29 (47)
(III) Epididymal fat-pad	0.038±0.004 (39)	0.0042±0.0004 (39)	—	9.5±0.8 (39)	—	—
P (I) versus (II)				<0.001	<0.001	<0.001
P (I) versus (III)				<0.001	—	—
P (II) versus (III)				<0.001	—	—

Table 3. *Effect of adrenalectomy and steroid administration on phosphoenolpyruvate carboxykinase activities in rat liver, kidney and adipose tissue*

Details of the treatment of animals and preparation of extracts are given in the Materials and Methods section. Enzyme activities are expressed as described in the legend to Table 2. Results are means ±S.E.M.; the numbers of observations are given in parentheses. *P* (versus corresponding controls): † <0.05; * <0.01; ** <0.005; *** <0.001; all other differences not significant.

		Enzyme activity (μmol·min^{-1}·g wet wt.$^{-1}$)			Ratio of activities		
		A	B	C	A/B	A/C	C/B
Expt. (I)							
Liver							
Control	(5)	1.28 ±0.22	0.34 ±0.06	2.24±0.37	3.8±0.1	0.57±0.04	6.7±0.5
Adrenalectomized	(7)	1.48 ±0.17	0.35 ±0.04	2.28±0.47	4.4±0.6	0.67±0.04	6.5±0.4
Kidney							
Control	(5)	6.80 ±0.42	1.09 ±0.10	7.87±0.49	6.3±0.3	0.87±0.01	7.3±0.3
Adrenalectomized	(7)	4.16 ±0.43**	0.69 ±0.10†	4.75±0.49**	6.2±0.4	0.85±0.01	7.3±0.6
Epididymal fat-pad							
Control	(5)	0.056±0.006	0.0042±0.0005	—	13.5±0.7	—	—
Adrenalectomized	(7)	0.062±0.004	0.0062±0.0006	—	10.1±0.7*	—	—
Expt. (II)							
Liver							
Control	(8)	1.53 ±0.13	0.39 ±0.04	2.88±0.41	4.0±0.3	0.61±0.09	7.7±1.3
Plus triamcinolone	(8)	1.38 ±0.15	0.21 ±0.02**	2.46±0.15	6.9±0.8**	0.58±0.08	12.6±1.2†
Kidney							
Control	(8)	5.72 ±0.22	0.87 ±0.09	7.15±0.54	7.0±0.7	0.83±0.06	8.4±0.4
Plus triamcinolone	(8)	15.8 ±0.3***	2.40 ±0.38***	21.9 ±0.7***	7.8±1.1	0.72±0.02	10.5±1.5
Epididymal fat-pad							
Control	(8)	0.034±0.005	0.0029±0.0004	—	12.6±2.0	—	—
Plus triamcinolone	(8)	0.018±0.002†	0.0029±0.003	—	7.2±1.3†	—	—

to obtain accurate measurements at the higher concentrations (above 1mM), since the corresponding blank values are very greatly increased. For this reason, all subsequent assays contained GTP at 0.1mM, and units of enzyme activity refer to this condition.

Phosphoenolpyruvate carboxykinase activities in normal rat tissues

Values for enzyme activity in liver, kidney and epididymal adipose tissue, measured simultaneously with the three assay systems, are given in Table 2. The activities for any one tissue under one set of assay conditions agree with the available values in the literature. At the same time, the data diverge markedly from expected values with respect to the relative rates of the 'back' and 'forward' reactions. For example, the 'back'/'forward' ratio in liver (A/C, see Table 2) is 0.60±0.03; this compares with an expected value of approx. 0.09 (Chang *et al.*, 1966). Despite the near-saturating substrate concentrations in the ^{32}P assay method, the rates recorded are as a routine much

lower than those in the Seubert & Huth (1965) system.

The ratios obtained from any two of the three assay methods are reproducible in each tissue examined, but vary between tissues. Thus the ratio of the rates in the $^{14}CO_2$-fixation and ^{32}P-transfer systems (A/B) is twice as high in the kidney as in the liver, and that in adipose tissue is consistently higher again (Table 2). Similarly, significant, although smaller, differences are seen between liver and kidney when the 'forward' reaction is measured by the Seubert & Huth (1965) method. The relatively lower sensitivity of this latter method unfortunately precludes its application for similar measurements in adipose-tissue extracts.

Phosphoenolpyruvate carboxykinase activity in various dietary and hormonal states

The effects of steroid imbalance on phosphoenolpyruvate carboxykinase activities are demonstrated in Table 3. Adrenalectomy has no effect on enzymic activity in the liver, as previously noted by Reshef *et al.* (1969). Corticosteroids have been reported variously both to increase (Foster *et al.*, 1966*a*, 1967; Longshaw *et al.*, 1972; Suda *et al.*, 1973) and to decrease (Reshef *et al.*, 1969) hepatic phosphoenolpyruvate carboxykinase. In the present study, we have observed that little significant change occurs with two assay systems, but that there is a fall in activity in the liver when the ^{32}P assay method is used. It is probable that the variability of response after steroid administration is related to a number of extraneous factors, such as the source, age and weight of animals as well as the composition of diet and nutritional status.

In kidney, adrenalectomy leads to a significant decrease in phosphoenolpyruvate carboxykinase activity; the degree of 'repression' is similar in all three assay systems, so that ratios between these remain constant. Triamcinolone produces a substantial increase, again independent of the assay system used; this agrees with the data of Longshaw *et al.* (1972). Conversely, adrenalectomy increases phosphoenolpyruvate carboxykinase activity in adipose tissue; this correlates with the fall that occurs after administration of triamcinolone.

Although total enzyme activity is altered by a number of hormonal changes (Tables 3 and 4), the ratios of the 'back'/'forward' reactions remain essentially constant. Thus acute alloxan-diabetes increases phosphoenolpyruvate carboxykinase activity in both liver and adipose tissue, but the corresponding changes in the ratios are not significant.

The activity in kidney is likewise increased, but to a lesser extent. Experimental 'chronic' diabetes is reported to cause somewhat greater increases (Anderson & Stowring, 1973; Murphy & Anderson, 1974); this has been attributed to the prevailing ketoacidosis rather than to insulin deficiency itself (Kamm *et al.*, 1974). In other experiments (not shown) we have found that acute alloxan-diabetes (48h) is associated variously with insignificant or barely significant increases in kidney enzyme activity.

Overnight starvation increases the 'back' reaction disproportionately to the 'forward' reaction in both liver and kidney, but produces an opposite effect in epididymal adipose tissue (Table 4). The mechanism and significance of these changes are unknown but it seems from the other data that changes in the concentrations of circulating insulin and corticosteroids are not involved.

L-Tryptophan increases total hepatic phosphoenolpyruvate carboxykinase activity and simultaneously and paradoxically, inhibits gluconeogenic flux at this reaction (Foster *et al.*, 1966*b*; Ray *et al*, 1966). The long-term increase in enzyme activity corresponds to an inhibition of specific degradative processes by tryphophan and a concomitant rise in enzyme protein (Ballard & Hopgood, 1973). The increase in assayable phosphoenolpyruvate carboxykinase after 1h is, however, not blocked by either transcription or translation inhibitors and has been attributed to activation of pre-existing enzyme (Foster *et al.*, 1966*a*, 1967). This short-term increase may only be observed in the 'forward' direction (Williamson *et al.*, 1971; Ballard & Hopgood, 1973); the actinomycin D-sensitive rise over a longer time-period is, however, measurable in both directions. Expt. (III) in Table 4 confirms these earlier findings, although a small increase in the 'back' reaction is detectable. The ratios of the 'back' to the 'forward' reactions are markedly decreased; the percentage increases measured by the 'forward' assay methods are similar, the ratio of the two being unchanged. Phosphoenolpyruvate carboxykinase in kidney is markedly less sensitive to tryptophan administration; this correlates with the absence of tryptophan 2,3-dioxygenase (EC 1.13.11.11), obligatory for the conversion of tryptophan into quinolinate, in this tissue (Rose, 1972). Adipose tissue, however, resembles liver in sensitivity towards tryptophan.

The phosphoenolpyruvate carboxykinase activity in kidney, but not liver, is increased by NH_4Cl-induced acidosis (Alleyne & Scullard, 1969; Longshaw *et al.*, 1972). Table 5 shows that the activity is increased similarly, but not identically, in both 'back' and 'forward' directions. The effective inhibition by cycloheximide suggests that protein synthesis *de novo* is required for the increase in enzyme activity.

It is clear that the rise in total phosphoenolpyruvate carboxykinase activities seen in livers and kidneys from animals treated variously as above correlates well with increases in gluconeogenic flux, and provides further circumstantial evidence for the role

Table 4. *Effect of alloxan-diabetes, starvation and L-tryptophan on phosphoenolpyruvate carboxykinase activities in rat liver, kidney and adipose tissue*

Details of the treatment of animals and preparation of extracts are given in the Materials and Methods section. Enzyme activities are expressed as described in the legend to Table 2. In the eight diabetic animals, the plasma glucose concentration was 34.3±2.2mM. Results are means ±S.E.M.; the numbers of observations are given in parentheses. *P* (versus corresponding controls): † 0.05; * <0.01; ** <0.005; *** <0.001; all other differences not significant.

		Enzyme activity (μmol·min⁻¹·g wet wt.⁻¹)			Ratio of activities		
		A	B	C	A/B	A/C	C/B
Expt. (I)							
Liver							
Control	(8)	1.54 ±0.19	0.44 ±0.05	2.92±0.47	3.8±0.4	0.56±0.04	6.7±0.6
Diabetic	(8)	7.22 ±0.84***	1.70 ±0.19***	12.8 ±1.5***	4.5±0.1	0.57±0.02	8.0±0.3
Kidney							
Control	(8)	3.30 ±0.20	0.44 ±0.02	4.83±0.37	7.5±0.3	0.69±0.02	10.8±0.6
Diabetic	(8)	5.37 ±0.67†	0.71 ±0.08*	8.25±1.2†	7.6±0.6	0.65±0.01	11.6±0.6
Epididymal fat-pad							
Control	(8)	0.057±0.007	0.0062±0.0011		10.4±1.4		
Diabetic	(8)	0.138±0.021**	0.0210±0.0033***		7.1±1.2		
Expt. (II)							
Liver							
Control	(4)	1.15 ±0.09	0.73 ±0.18		1.7±0.3		
Starved 18h	(8)	7.1 ±0.2***	1.21 ±0.09†		6.1±0.5***		
Kidney							
Control	(8)	4.2 ±0.1	0.58 ±0.04		7.6±0.6		
Starved 18h	(8)	12.3 ±0.8***	1.02 ±0.11**		12.6±1.0***		
Epididymal fat-pad							
Control	(7)	0.021±0.002	0.0032±0.0006		7.6±0.7		
Starved 18h	(8)	0.062±0.007***	0.0210±0.003***		3.0±0.3***		
Expt. (III)							
Liver							
Control	(6)	0.89 ±0.11	0.18 ±0.02	1.34±0.23	4.9±0.2	0.71±0.07	7.2±0.7
Tryptophan	(6)	1.22 ±0.06†	0.45 ±0.03***	3.22±0.33***	3.1±0.2***	0.39±0.02***	8.0±0.5
Kidney							
Control	(6)	7.02 ±0.23	0.76 ±0.08	7.55±0.50	9.6±0.8	0.95±0.06	10.1±0.3
Tryptophan	(6)	6.50 ±0.31	0.78 ±0.06	8.92±0.25†	8.5±0.7	0.73±0.02*	11.7±1.0
Epididymal fat-pad							
Control	(6)	0.067±0.008	0.0016±0.0003		39.3±5.9		
Tryptophan	(6)	0.051±0.002	0.0048±0.0008**		12.7±3.3		

Table 5. *Effect of NH_4Cl-induced acidosis and cycloheximide on phosphoenolpyruvate carboxykinase activities in rat kidney*

Details of the treatment of animals and preparation of extracts are given in the Materials and Methods section. Enzyme activities are expressed as described in the legend to Table 2. Results are means ±S.E.M.; the numbers of observations are given in parentheses. N.S., Not significant.

	Enzyme activity (μmol·min⁻¹·g wet wt.⁻¹)		Ratio of activities
	A	C	A/C
(I) Control	(7) 4.32±0.52	5.93±0.88	0.77±0.05
(II) Control, plus cycloheximide	(6) 4.02±0.51	5.70±0.81	0.72±0.02
(III) Acidotic	(7) 7.66±0.37	12.2±0.66	0.63±0.02
(IV) Control, plus cycloheximide	(6) 4.09±0.85	6.26±1.23	0.65±0.04
P (I) versus (II)	N.S.	N.S.	N.S.
P (I) versus (III)	<0.001	<0.001	<0.025
P (II) versus (IV)	N.S.	N.S.	N.S.
P (III) versus (IV)	<0.005	<0.005	N.S.

of the enzyme as a regulator of glucose synthesis. Thus acute alloxan-diabetes substantially increases both the hepatic enzyme concentration and glucose synthesis, but has negligible effects on the same processes in kidney; the opposite is true where the stimulus is a change in acid–base status (Kamm & Cahill, 1969; Exton 1972). Glucocorticoid, which facilitates gluconeogenesis in both tissues (Exton *et al.*, 1970; Stumpf *et al.*, 1972), increases phosphoenolpyruvate carboxykinase concentrations in both tissues.

Hanson *et al.* (1973) have proposed that the increased concentration of phosphoenolpyruvate carboxykinase in the adipose tissue of starved rats may correlate with an increased demand for glyceride glycerol synthesis from citric acid-cycle precursors. Since, however, there is no firm evidence that this pathway is significant *in vivo*, one clearly cannot at present attempt to draw conclusions from the changes observed here.

Two problems arise from these observations, namely: (i) why do the ratios of the 'back' and 'forward' reactions differ between crude tissue extracts and the pure enzyme? (ii) why does this ratio vary from one tissue to another? It is clear that the results obtained in the present study agree reasonably with earlier tissue-extract data where these are available. Moreover, it is known that the phosphoenolpyruvate carboxykinase activities in rat liver, kidney and adipose tissue are immunologically indistinguishable (Ballard & Hanson, 1969; Longshaw & Pogson, 1972), and presumably reflect the presence in all three tissues of a single protein species. The observed ratios therefore must be a reflexion rather of specific tissue factors than of the enzyme itself.

Although modification of enzymic activity can never alter the ultimate equilibrium position, it is theoretically permissible for an inhibitor or other modifying agent to affect the reaction velocity in one direction alone. This may for example, involve changes in substrate-binding constants or more complex events leading to altered reaction mechanisms. An example of this is already known in the case of phosphoenolpyruvate carboxykinase, in that quinolinate is an inhibitor in the physiological ('forward') direction alone (Ballard & Hopgood, 1973; Williamson *et al.*, 1971). Further, the paradoxical increase in enzyme activity in liver extracts from quinolinate- or tryptophan-treated animals is also a unidirectional effect. It is difficult to fit a simple explanation to these observations. Snoke *et al.* (1971) have, however, drawn attention to the capacity of quinolinate to chelate Fe^{2+}, an ion which stimulates phosphoenolpyruvate carboxykinase activity *in vitro*, and have proposed that changes in the ionic environment may be involved in the regulation of the enzyme *in vivo*.

Since tryptophan administration is associated with a fall in the ratio of the 'back' and the 'forward' reaction in liver and adipose tissue, it is possible that the higher ratio normally found in kidney and adipose tissue is due to similar binding of some as yet unknown effector. The tissue-dependent variable binding of such a ligand therefore offers one plausible explanation of the observations in this paper.

The increase in gluconeogenesis in livers perfused with glucagon may originate at the phosphoenolpyruvate carboxykinase reaction (Ui *et al.*, 1973). The possibility of a direct connection between raised cyclic AMP concentrations and phosphoenolpyruvate carboxykinase activity has been investigated, but no positive evidence has been found to support a phosphorylation–dephosphorylation mechanism analogous to that known for glycogen phosphorylase (W. D. Wicks & D. A. Walsh quoted in Wicks *et al.*, 1972). Further, extensive investigations with the purified enzyme designed to discover potential physiological effectors have been markedly unsuccessful (Foster *et al.*, 1967; Utter & Kolenbrander, 1972).

Although phosphoenolpyruvate carboxykinases from lower eukaryotes are apparently tetrameric (Cannata, 1970; Diesterhaft *et al.*, 1972), the mammalian forms so far purified to homogeneity are monomers of approx. 70000 molecular weight (Chang & Lane, 1966; Ballard & Hanson, 1969). Antibody to purified rat phosphoenolpyruvate carboxykinase titrates similarly against crude extracts and pure antigen; this is consistent with the presence of the monomeric form in normal tissue homogenates (Ballard & Hanson, 1969). There are, however, indications that higher-molecular-weight species may also occur *in vivo* (Longshaw & Pogson, 1972); if present, these species must possess structures such that monomers are readily produced during the homogenization process, presumably either by dissociation alone or by removal of covalently bound groups followed by dissociation.

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