Biochem. J. (1973) **136**, 259–264
Printed in Great Britain

Phosphopyruvate Carboxylase Induction by L-Tryptophan

EFFECTS ON SYNTHESIS AND DEGRADATION OF THE ENZYME

By F. J. BALLARD and M. F. HOPGOOD
Commonwealth Scientific and Industrial Research Organization, Division of Nutritional Biochemistry, Adelaide, S. Austral. 5000, *Australia*

(*Received* 5 *March* 1973)

1. The administration of L-tryptophan to fed rats produces a twofold increase in hepatic phosphopyruvate carboxylase activity that represents a comparable increase in enzyme protein. With specific antibody against the enzyme we have shown that the increase in phosphopyruvate carboxylase is partially mediated via an actinomycin D-sensitive increase in enzyme synthesis. 2. In starved animals tryptophan increases the enzyme activity without any change in the relative rate of phosphopyruvate carboxylase synthesis. In this condition degradation of the enzyme is retarded by tryptophan by a mechanism that is not prevented by cycloheximide.

The injection of L-tryptophan into rats blocks gluconeogenesis at the level of phosphopyruvate carboxylase (EC 4.1.1.32), probably as a result of the action of quinolinate, an intermediate in tryptophan degradation (Ray *et al.*, 1966; Snoke *et al.*, 1971). However, the activity of phosphopyruvate carboxylase, as measured *in vitro*, is paradoxically increased in livers of tryptophan-treated rats (Foster *et al.*, 1966). This increase in activity is comprised of a short-term activation phenomenon that is not inhibited by actinomycin D or by other inhibitors of RNA or protein synthesis, as well as a gradual increase in activity that does not occur when protein synthesis is inhibited (Foster *et al.*, 1966, 1967).

In this study we have used specific immunochemical techniques to show that tryptophan increases the amount of phosphopyruvate carboxylase in liver. The increase in enzyme protein is a result of an acceleration of the synthesis and a decrease in the degradation rate of phosphopyruvate carboxylase.

Experimental

Animals

Male rats aged 7 weeks, weighing 190–210g were used. Fed animals had continual access to food, and starved rats had food withdrawn for the 20h preceding an experiment. L-Tryptophan (150mg) was injected intraperitoneally as a suspension in saline. The amounts of other compounds that were injected intraperitoneally were cycloheximide, 200μg, and actinomycin D, 200μg.

For the pulse labelling of phosphopyruvate carboxylase, 100μCi of [4,5-^{3}H]leucine (specific radioactivity 50–60Ci/mmol, from The Radiochemical Centre, Amersham, Bucks., U.K.) was injected intraperitoneally. Then 30min later the animals were either killed, or if degradation rates were to be measured, injected with a chase of 25μmol of L-leucine. After the rats were killed, livers were removed, weighed and homogenized in 3vol. of 0.25M-sucrose. Cytosol fractions prepared by centrifugation of the homogenates at 100000*g* for 30min were assayed for phosphopyruvate carboxylase activity (Chang & Lane, 1966). One unit of enzyme activity is the amount that catalyses the fixation of 1μmol of $H^{14}CO_3^-$ into oxaloacetate/min, at 37°C.

Immunochemical procedures

We have reported previously methods for the purification of phosphopyruvate carboxylase (Ballard & Hanson, 1969), for the preparation of antibodies specific to phosphopyruvate carboxylase, for the use of these antibodies to isolate the enzyme from cytosol fractions of liver and to make appropriate corrections (Philippidis *et al.*, 1972; Hopgood *et al.*, 1973).

Results

The injection of L-tryptophan into fed rats results in an increase in hepatic phosphopyruvate carboxylase activity from 2.51 units/g to 3.96 units/g after 3h and to 4.76 units/g after 6h (Table 1). An increase of similar magnitude is also found when activities are expressed as units of enzyme per total liver, a procedure adopted to take into account any changes in liver weight that might result from a treatment. For example, the liver weight decreases when animals are starved, so that a portion of the resultant increase in phosphopyruvate carboxylase activity per unit weight does not represent enzyme accumulation. In starved animals tryptophan injection also increases the activity of phosphopyruvate carboxylase in 8h from 5.85 to 7.74 units/g (Table 1).

Table 1. *Increases in hepatic phosphopyruvate carboxylase activity after tryptophan injection*

Details of treatments are given in the Experimental section. Values are expressed as the means ± S.E.M. for determinations on six animals.

Treatment	Phosphopyruvate carboxylase activity (Units/g of liver)	(Units/total liver)
Fed; saline injection; 3h	2.51 ± 0.09	26.3 ± 2.0
Fed; tryptophan injection; 3h	3.96 ± 0.33	39.1 ± 2.8
Fed; saline injection; 6h	2.78 ± 0.07	26.7 ± 1.9
Fed; tryptophan injection; 6h	4.76 ± 0.45	46.0 ± 5.5
Starved; saline injection; 8h	5.85 ± 0.32	41.2 ± 3.3
Starved; tryptophan injection; 8h	7.74 ± 0.27	64.3 ± 0.9

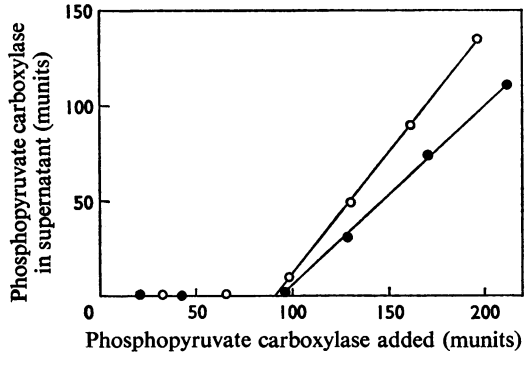

Fig. 1. *Equivalence-point determination for phosphopyruvate carboxylase*

Increasing amounts of cytosol fractions from normally fed rats (●) or fed rats injected 4h previously with tryptophan (○) were mixed with a fixed amount of antibody, incubated for 15min at 37°C and overnight at 0°C, centrifuged, and the phosphopyruvate carboxylase activity was measured in the supernatant.

Table 2. *Inactivation of phosphopyruvate carboxylase by antibody*

Cytosol fractions from control and tryptophan-treated animals were assayed and diluted to approximately the same enzyme activity. Two equal samples were taken from each cytosol, then a fixed volume of antibody was added to one, and the tubes were incubated, centrifuged, and the activity of phosphopyruvate carboxylase was measured (Philippidis *et al.*, 1972). Each value represents the mean ± S.E.M. for determinations on cytosol fractions from six animals.

	Phosphopyruvate carboxylase activity (munits)
Control	
−Antibody	267 ± 7
+Antibody	168 ± 3
Difference	99 ± 4
Tryptophan-treated	
−Antibody	273 ± 3
+Antibody	170 ± 4
Difference	103 ± 5

We have used specific antibodies to phosphopyruvate carboxylase to establish that the increase in enzyme activity after tryptophan injection represents new antigenic material, rather than an activation of existing enzyme. Thus the addition of increasing amounts of liver cytosol from either control or tryptophan-treated animals to a fixed amount of antibody, gives an equivalence point beyond which any extra phosphopyruvate carboxylase is not inhibited by the antibody. From Fig. 1 it is apparent that only one equivalence point for phosphopyruvate carboxylase is found regardless of whether the enzyme is derived from control or from tryptophan-treated animals. We conclude, therefore, that the increase in enzyme activity produced by tryptophan is not an activation phenomenon, because activation is likely to result in more enzyme activity being inhibited than if the enzyme was from control rats.

A second method used to relate enzyme activity to enzyme content is to measure the amount of enzyme inactivated by submaximal amounts of antibody. Liver cytosol fractions from tryptophan-treated animals were diluted to the same enzyme activity as found in control rats, after which sufficient antibody was added to inactivate about half of the enzyme present (Table 2). With this technique it is easier to control variability than with the equivalence-point method, where an extremely accurate measure of starting enzyme activity is needed. The data in Table 2 again show no difference between the two enzyme preparations, with the antibody inactivating 99 munits of enzyme from control rats and 103 munits from

tryptophan-treated animals. Since the animals used in this experiment showed an average increase in enzyme activity after tryptophan injection of from 2.78 to 5.07 units/g of liver, an increase in activity but not antigenicity would have required about 180 munits to be inactivated in cytosol fractions from tryptophan-injected animals.

We have measured the rate of synthesis of hepatic phosphopyruvate carboxylase by injecting [^{3}H]-leucine into animals and then isolating the enzyme as an antibody–antigen complex. In addition, we have determined incorporation into total cytosol protein, so that a relative rate of enzyme synthesis can be obtained. When synthesis is expressed as a relative rate, any changes in the pool size of the amino acid precursor will not influence the results provided that total proteins and phosphopyruvate carboxylase are synthesized from the same pool of amino acids. Tryptophan increases the relative rate of phosphopyruvate carboxylase synthesis in fed animals from 1.24 to 2.86% of cytosol protein synthesis, and there is no effect on the relative rate of synthesis in starved rats (Table 3). However, tryptophan increases the total incorporation of leucine into both cytosol proteins and phosphopyruvate carboxylase in the starved animals.

The degradation rate of phosphopyruvate carboxylase is measured as the rate of isotope loss after the enzyme pool has been labelled with [^{3}H]leucine. Data are expressed both as the total radioactivity in the enzyme pool as well as the relative amount present compared with radioactivity in total cytosol protein. For degradation studies the latter method has the advantage that it is not necessary to assume an equal total isotope incorporation during the earlier pulse. A disadvantage is that the rate of degradation of phosphopyruvate carboxylase will be underestimated because of the turnover of all proteins. However, provided that the turnover of the enzyme is rapid compared with that of the average for protein, the error will not be large.

From Tables 4 and 5 it can be seen that the relative radioactivity in phosphopyruvate carboxylase is decreased after 8h of decay from 0.88 to 0.45% of the radioactivity in total cytosol proteins of fed animals and from 1.98 to 0.89% in starved animals.

Table 3. *Effect of tryptophan on the rate of phosphopyruvate carboxylase synthesis*

At 2h after injection of tryptophan or 0.15M-NaCl into rats, [^{3}H]leucine was injected; 30min later the rats were killed, liver cytosol fractions prepared and the radioactivity in cytosol proteins and phosphopyruvate carboxylase was measured. Values are means±S.E.M. for determinations on six animals in each group.

Treatment	Radioactivity incorporated (d.p.m./mg of liver)		Incorporation into phosphopyruvate carboxylase (%)
	Cytosol protein	Phosphopyruvate carboxylase	
Fed; saline injection	625±113	7.92±2.15	1.24±0.21
Fed; tryptophan injection	786±78	22.1 ±2.0	2.86±0.13
Starved; saline injection	738±89	17.0 ±2.5	2.35±0.13
Starved; tryptophan injection	1091±59	26.4 ±2.1	2.44±0.17

Table 4. *Inhibition by tryptophan of phosphopyruvate carboxylase degradation in livers of fed rats*

At 30min after injection of 100μCi of [^{3}H]leucine animals were either killed (pulse) or injected with 25μmol of leucine and killed 8h later. Tryptophan was injected with the chase in some animals, and again after 4h. Measurements of enzyme activity and radioactivity are described in the text. Values are the means±S.E.M. for six animals in each group.

Treatment	Phosphopyruvate carboxylase (units/liver)	Radioactivity incorporated (d.p.m./mg of liver)		Radioactivity in phosphopyruvate carboxylase (%)
		Cytosol protein	Phosphopyruvate carboxylase	
Pulse	19.2±0.7	597±52	4.80±0.56	0.88±0.05
8h decay	24.9±2.5	521±63	2.37±0.64	0.45±0.09
8h decay+tryptophan	40.6±3.7	536±22	3.90±0.37	0.74±0.08

Table 5. *Effects of tryptophan and cycloheximide on phosphopyruvate carboxylase degradation in livers of starved rats*

At 30 min after injection of [^{3}H]leucine, animals were either killed (pulse) or injected with 25 μmol of leucine and killed 8 h later. Cycloheximide or tryptophan were injected with the leucine chase and again 4 h later. Measurement of enzyme activity, radioactivity in phosphopyruvate carboxylase and cytosol protein are described in the text. Values are the means ± S.E.M. for data on seven animals in each group.

Treatment		Phosphopyruvate carboxylase (units/liver)	Radioactivity incorporated (d.p.m./mg of liver)		Radioactivity in phosphopyruvate carboxylase (%)
			Cytosol protein	Phosphopyruvate carboxylase	
Pulse	—	39.0 ± 1.3	822 ± 28	16.7 ± 1.8	1.98 ± 0.20
8 h decay	—	43.9 ± 2.4	940 ± 56	9.24 ± 0.70	0.89 ± 0.07
8 h decay	Cycloheximide	25.3 ± 0.5	700 ± 44	9.35 ± 0.91	1.34 ± 0.13
8 h decay	Tryptophan	61.8 ± 3.1	692 ± 28	11.1 ± 1.1	1.62 ± 0.23
8 h decay	Cycloheximide + tryptophan	25.5 ± 1.2	760 ± 61	11.9 ± 1.6	1.61 ± 0.14

Table 6. *Actinomycin D prevents induction of phosphopyruvate carboxylase by tryptophan*

Starved rats were re-fed for 1 h before and throughout the experiment. Injections of tryptophan or actinomycin D were given at the times indicated. All animals were injected with [^{3}H]leucine 0.5 h before killing. The relative radioactivity in phosphopyruvate carboxylase was then measured. Each value represents the mean ± S.E.M. for determinations on three animals.

Treatment		Phosphopyruvate carboxylase activity (units/liver)	Radioactivity in phosphopyruvate carboxylase (%)
Injection	Killed (h)		
Control	0.5	41.6 ± 1.4	1.01 ± 0.30
Actinomycin D, 0 h	3	32.9 ± 4.8	1.46 ± 0.07
Tryptophan, 1 h	3	40.5 ± 4.7	2.50 ± 0.21
Actinomycin D, 0 h; tryptophan, 1 h	3	26.4 ± 9.7	1.02 ± 0.19
Actinomycin D, 0 h, 3 h	6	20.5 ± 3.4	1.14 ± 0.27
Tryptophan, 1 h, 4 h	6	49.5 ± 2.3	3.43 ± 0.48
Actinomycin D, 0 h, 3 h; tryptophan, 1 h, 4 h	6	23.1 ± 6.3	1.06 ± 0.19

Tryptophan markedly inhibits the rate of isotope loss from the phosphopyruvate carboxylase pools in livers from both fed and starved rats. However, the effect of tryptophan would not be directly on enzyme degradation if the rate of isotope re-incorporation was accelerated by the amino acid. Such a possibility is decreased by our procedure of injecting a leucine chase, and can be eliminated entirely by stopping protein synthesis with cycloheximide. At an initial dose of 200 μg of cycloheximide immediately after the pulse period, followed by similar doses at 3 h intervals, total protein and phosphopyruvate carboxylase synthesis are inhibited by 90% or more in both fed and starved animals. In the presence of cycloheximide, tryptophan still inhibits enzyme degradation in starved rats, although cycloheximide alone decreases the degradation rate (Table 5). The inhibition by cycloheximide of phosphopyruvate carboxylase degradation in starved rats has also been shown in diabetic and insulin-treated diabetic rats (S. M. Tilghman, R. W. Hanson, L. Reshef, M. F. Hopgood & F. J. Ballard, unpublished work), an effect that precludes the use of cycloheximide for measurement of enzyme turnover.

Tryptophan might act to increase phosphopyruvate carboxylase synthesis either by increasing the amount of specific mRNA or by accelerating the translation of existing mRNA. To distinguish between these possibilities we have stopped the synthesis of mRNA by the injection of actinomycin D before the tryptophan stimulus. This actinomycin D treatment completely prevents the increase in enzyme synthesis after trypto-

phan injection (Table 6), suggesting that tryptophan acts to increase the formation of phosphopyruvate carboxylase messenger rather than to facilitate translation of the template.

Discussion

The effects of tryptophan on phosphopyruvate carboxylase represent two distinct events. One, an activation of the enzyme, occurs rapidly *in vivo*, and is not prevented by actinomycin D, puromycin or acetoxycycloheximide (Foster *et al.*, 1966, 1967). This activation possibly represents the dissociation of inhibitory quinolinate from a tertiary complex with the enzyme and Fe^{2+} (Snoke *et al.*, 1971), but is not found when phosphopyruvate carboxylase activity is measured by the carboxylation assay used in the present study. Inhibition of protein and RNA synthesis blocks the slow effect of tryptophan (Foster *et al.*, 1966, 1967), which we have shown to be due to an increase in the amount of enzyme protein resulting in turn from both an increase in the rate of phosphopyruvate carboxylase synthesis and a decrease in the degradation rate of the enzyme.

Effects of tryptophan on enzyme synthesis

The omission of tryptophan from an otherwise complete diet causes a general decrease in protein synthesis and in the dissociation of hepatic polyribosomes (Drysdale & Munro, 1967). These changes can be reversed either by tryptophan administration to intact animals (Wunner *et al.*, 1966), or by the addition of the amino acid to a protein-synthesizing medium *in vitro* (Baliga *et al.*, 1968). Since the effect of tryptophan is apparent *in vitro* and is not sensitive to actinomycin D (Fleck *et al.*, 1965), it is improbable that formation of new messenger RNA is involved. Rather, it has been proposed that the decrease of protein synthesis and dissociation of polyribosomes is caused by a relative deficiency of tryptophyl transfer RNA (Allen *et al.*, 1969; Munro, 1970). Tryptophan administration would restore high amounts of tryptophanyl transfer RNA and thereby promote aggregation of polyribosomes for an increased rate of protein synthesis. However, it is unlikely that this explanation of tryptophan effects on protein synthesis would account for the increase in phosphopyruvate carboxylase synthesis, because not only are larger amounts of tryptophan required than would be needed to satisfy a tryptophan deficiency, but actinomycin D prevents the increase in enzyme activity. In addition to phosphopyruvate carboxylase, tryptophan produces large increases in the activities of hepatic threonine dehydratase, ornithine aminotransferase (Peraino *et al.*, 1965), tyrosine aminotransferase (Kenney & Flora, 1961), serine dehydratase (Jost *et al.*, 1968) and tryptophan pyrrolase (Schimke *et al.*, 1965). With the exception of tryptophan pyrrolase, all these effects could be prevented by the prior administration of actinomycin D, possibly because the stimulus is at the level of transcription. If tryptophan was either stabilizing the specific messengers or accelerating the translation of existing templates, we would not expect actinomycin D to prevent the appearance of new enzyme or to prevent the increased rate of enzyme synthesis unless actinomycin D was also inhibiting the processing or translation of mRNA. Alternatively, the effect of tryptophan can be interpreted as a translational stimulus that cannot occur in the presence of actinomycin D because the enzyme template is so labile that inhibition of mRNA formation by actinomycin D decreases the concentration of specific messenger to values so low that an increased translation rate would not result in a detectable increase in the rate of enzyme synthesis. The effect of actinomycin D on some enzyme activities in cultured cells is quite different from that noted with phosphopyruvate carboxylase. Thus Tomkins argues for an indirect stabilization of messenger RNA by actinomycin D as an explanation for the elevated tyrosine aminotransferase activities (Tomkins *et al.*, 1969), though other explanations of this super-induction effect can also account for the results (Reel *et al.*, 1970; Rhoads *et al.*, 1973). With phosphopyruvate carboxylase we can detect no super-induction (Table 6), but rather a gradual fall in the rate of enzyme synthesis that is adequately explained as an inhibition of mRNA formation with no major effect of actinomycin D on translation of existing template (M. F. Hopgood, F. J. Ballard, S. M. Tilghman, R. W. Hanson & L. Reshef, unpublished work).

Kenney (1970) has suggested that tryptophan and dietary effectors that increase the rates of synthesis of tyrosine aminotransferase, phosphopyruvate carboxylase and other enzymes, function indirectly, via glucagon or cyclic AMP. Certainly, cyclic AMP will induce these enzymes, but it has been reported that the cyclic AMP induction of phosphopyruvate carboxylase in Reuber H35 cells is not prevented by actinomycin D (Wicks & McKibbin, 1972). In foetal and adult rats, however, we find that glucagon or cyclic AMP increase hepatic phosphopyruvate carboxylase activity, and that this increase is prevented by actinomycin D (R. W. Hanson, L. Reshef & F. J. Ballard, unpublished work). Whether the tryptophan effects can be explained by changes in cyclic AMP remains an open question.

Effects of tryptophan on enzyme degradation

Although tryptophan has been shown to increase the activities of several liver enzymes by stimulating their respective synthesis rates, effects on degradation comparable with those shown in Table 4 have not previously been reported. Tryptophan pyrrolase,

however, an enzyme whose rate of synthesis is not altered by tryptophan, is increased in activity by a mechanism that is not sensitive to actinomycin D (Yatvin & Pitot, 1970). This action on tryptophan pyrrolase occurs via a stabilization process that can be measured *in vitro* (Schimke *et al.*, 1965), so that the addition of tryptophan to liver homogenates prevents inactivation of tryptophan pyrrolase by heat, urea, ethanol or by proteolytic degradation by trypsin, chymotrypsin or *Streptomyces* proteinase (Schimke *et al.*, 1965). Differences in the kinetics of catalysis and stabilization with respect to tryptophan lead Schimke *et al.* (1965) to propose that the amino acid may bind to the enzyme at two different sites. A similar protective effect could possibly explain the stabilization of phosphopyruvate carboxylase by tryptophan in intact animals. Although tryptophan does not alter the phosphopyruvate carboxylase activity when added to or preincubated with the assay mixture, a metabolic product of tryptophan metabolism, quinolinate, is inhibitory (Snoke *et al.*, 1971). To explain the findings that tryptophan inhibits gluconeogenesis at the same time as phosphopyruvate carboxylase activity is stimulated, Snoke *et al.* (1971) propose that quinolinate combines with the enzyme and a divalent cation to form a complex of low catalytic activity. It is conceivable that such a complex would stabilize phosphopyruvate carboxylase towards degradation *in vivo*, but would dissociate in homogenates and thus satisfy our findings that the immunochemical reactivity of the enzyme from tryptophan-treated animals was not altered. Should this explanation be valid, we might expect that tryptophan could stabilize phosphopyruvate carboxylase *in vivo* after protein synthesis is inhibited by cycloheximide. Although Treadow & Khairallah (1972) have reported this effect, we are unable to confirm it (see Table 5). We do find that cycloheximide decreases the degradation rate of the enzyme, but that substantial loss of activity still occurs in the presence of tryptophan.

References

Allen, R. E., Raines, P. L. & Regen, D. M. (1969) *Biochim. Biophys. Acta* **190**, 323–336

Baliga, B. S., Pranczuk, A. W. & Munro H. N. (1968) *J. Mol. Biol.* **34**, 199–218

Ballard, F. J. & Hanson, R. W. (1969) *J. Biol. Chem.* **244**, 5625–5630

Chang, M. E. & Lane, M. D. (1966) *J. Biol. Chem.* **241**, 2413–2420

Drysdale, J. W. & Munro, H. N. (1967) *Biochim. Biophys. Acta* **138**, 616–618

Fleck, A., Shepherd, J. & Munro, H. N. (1965) *Science* **150**, 628–629

Foster, D. O., Ray, P. D. & Lardy, H. A. (1966) *Biochemistry* **5**, 563–569

Foster, D. O., Lardy, H. A., Ray, P. D. & Johnston, J. B. (1967) *Biochemistry* **6**, 2120–2128

Hopgood, M. F., Ballard, F. J., Reshef, L. & Hanson, R. W. (1973) *Biochem. J.* **134**, 445–453

Jost, J.-P., Khairallah, E. A. & Pitot, H. C. (1968) *J. Biol. Chem.* **243**, 3057–3066

Kenney, F. T. (1970) in *Mammalian Protein Metabolism* (Munro, H. N., ed.), vol. 4, pp. 131–176, Academic Press, New York and London

Kenney, F. T. & Flora, R. M. (1961) *J. Biol. Chem.* **236**, 2699–2702

Munro, H. N. (1970) in *Mammalian Protein Metabolism* (Munro, H. N., ed.), vol. 4, p. 333, Academic Press, New York and London

Peraino, C., Blake, R. L. & Pitot, H. C. (1965) *J. Biol. Chem.* **240**, 3039–3043

Philippidis, H., Hanson, R. W., Reshef, L., Hopgood, M. F. & Ballard, F. J. (1972) *Biochem. J.* **216**, 1127–1134

Ray, P. D., Foster, D. O. & Lardy, H. A. (1966) *J. Biol. Chem.* **241**, 3094–3908

Reel, J. R., Lee, K. L. & Kenney, F. T. (1970) *J. Biol. Chem.* **245**, 5806–5812

Rhoads, R. E., McKnight, G. S. & Schimke, R. T. (1973) *J. Biol. Chem.* **248**, 2031–2039

Schimke, R. T., Sweeney, E. W. & Berlin, C. M. (1965) *J. Biol. Chem.* **240**, 4609–4620

Snoke, R. E., Johnston, J. B. & Lardy, H. A. (1971) *Eur. J. Biochem.* **24**, 342–346

Tomkins, G. M., Gelehrter, T. D., Granner, D. K., Martin, D. W., Jr., Samuels, H. H. & Thompson, E. B. (1969) *Science* **166**, 1474–1480

Treadow, B. R. & Khairallah, E. A. (1972) *Nature (London) New Biol.* **239**, 131–133

Wicks, W. D. & McKibbin, J. B. (1972) *Biochem. Biophys. Res. Commun.* **48**, 205–211

Wunner, W. H., Bell, J. & Munro, H. N. (1966) *Biochem. J.* **101**, 417–428

Yatvin, M. B. & Pitot, H. C. (1970) *J. Biol. Chem.* **245**, 4673–4676