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The Effects of Starvation and Alloxan-Diabetes on the Contents of Citrate and other Metabolic Intermediates in Rat Liver

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1. The content of citrate in 'freeze-clamped' livers from starved and alloxan-diabetic rats was measured by using the specific citrate assay method of Gruber & Moellering (1966). 2. The content of citrate fell progressively during a period of 48hr. starvation to reach a plateau value that is 50% of the value for livers from fed rats. Some possible explanations for the conflicting reports of changes in hepatic citrate content during starvation are discussed. 3. The hepatic contents of ATP, pyruvate, lactate, glycogen and the hexose phosphates were decreased during starvation, whereas those of acetyl-CoA and AMP were increased. 4. Acute alloxan-diabetes produced similar changes in the contents of these metabolic intermediates. 5. The effects of starvation and diabetes on the citrate and acetyl-CoA contents are discussed in relation to control of gluconeogenesis, fatty acid synthesis and the activity of citrate synthase.

Changes in the citrate content in liver during starvation and alloxan-diabetes are of interest for two reasons. First, citrate inhibits hepatic phosphofructokinase (Underwood & Newsholme, 1965), and, as this enzyme may be regulatory for glycolysis and gluconeogenesis (for full discussion see Newsholme & Gevers, 1967), an elevated citrate concentration might explain the decreased glycolysis and increased gluconeogenesis under these conditions. Evidence for such a mechanism of control has been obtained with kidney-cortex slices (Underwood & Newsholme, 1967). Secondly, a fall in the concentration of citrate might explain the decreased rate of fatty acid synthesis under these conditions, as citrate is an activator of acetyl-CoA carboxylase, a regulatory enzyme in fatty acid synthesis (Numa, Ringelman & Lynen, 1965); also, citrate is considered to be the immediate source of extramitochondrial acetyl-CoA for fatty acid synthesis via the ATP citrate lyase reaction (see Spencer & Lowenstein, 1962, 1967).

Previous reports of changes in the citrate content of rat liver during starvation are conflicting: thus D. M. Regen (unpublished work cited by Lynen, Matsuhashi, Numa & Schweizer, 1963) found that the citrate content was decreased by 50–60% of the normal value after 24hr. starvation, although no actual values were reported and the method of assay was not described; Angielski & Szutowicz (1967) reported a fall in citrate content after 24hr. and a return to normal after 48hr. starvation; Tubbs & Garland (1964) and Williamson, Herczeg, Coles & Danish (1966) have reported that the citrate content is increased by starvation, whereas Spencer & Lowenstein (1967) and Tarnowski & Seeman (1967) have shown that the liver citrate content remains unchanged by starvation. Several workers (Parmeggiani & Bowman, 1963; Dixit, De Villiers & Lazarow, 1967) have reported a rise in hepatic citrate content in alloxan-diabetes.

This inconsistency might be explained either by unsatisfactory assays for citrate, or by variations in the direction of change of citrate content during the starvation period. It was for these reasons that citrate was measured by the specific enzymic method of Gruber & Moellering (1966), and the changes were followed at intervals throughout a period of 48hr. starvation. The contents of acetyl-CoA, adenine nucleotides, hexose phosphates, lactate, pyruvate and glycogen were also measured during this period of starvation, and these values are reported in this paper. A preliminary report of the changes in the content of citrate with starvation and diabetes has been published (Start & Newsholme, 1967).

MATERIALS

Animals. Male albino Wistar rats weighing 250–280g. and fed on a laboratory small-animal diet (Spillers Ltd., Gainsborough, Lincs.) were used. By observation it was established that the rats ate during the night and stopped eating at 6 a.m. Therefore fully fed animals were used at 6 a.m. and starved animals were starved from this time (starved rats were kept in wire-bottomed cages); thus rats used at midday are referred to as '6hr. starved'. Rats were made alloxan-diabetic as described by Newsholme & Randle

(1964), and were used only if the blood glucose concentration was in excess of 5mg./ml. Diabetic rats were allowed access to food at all times.

Chemicals and enzymes. NAD, $NADH_2$, NADP, ATP and phosphoenolpyruvate were obtained from Boehringer Corp. (London) Ltd., London, W. 5. L-Malic acid, Florisil and tricarballylic acid were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks. Citric acid was obtained from British Drug Houses Ltd., Poole, Dorset. [U-^{14}C]Citric acid and [^{3}H]glycerol were obtained from The Radiochemical Centre, Amersham, Bucks. Nembutal was obtained from Abbott Laboratories, Queenborough, Kent. Alloxan was supplied by British Drug Houses Ltd. and recrystallized from water by Mr L. V. Eggleson of the Department of Biochemistry, University of Oxford. 2-(4-*tert.*-Butylphenyl)-5-(4-biphenylyl)-1-oxa-3,4-diazole was obtained from Ciba (A.R.L.) Ltd., Duxford, Cambs.

Glucose oxidase was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A., and diazyme (Takamine) from Miles Chemical Co., Clifton, N.J., U.S.A. Aconitase was prepared from pig heart by the method of Siebert (1965); chloroform was added to the initial extraction medium to remove haemoglobin (Morrison, 1954) and tricarballylic acid was used in place of citrate to stabilize the enzyme (Goldberg, Passonneau & Lowry, 1966). The final acetone precipitate was taken up in 10ml. of triethanolamine buffer containing 4mM-tricarballylic acid, pH7·5, and centrifuged at 38000*g* for 30min. The supernatant used for citrate assay was stable for 2 weeks at −15°. All other enzymes were obtained from Boehringer Corp. (London) Ltd.; citrate lyase was a generous gift from Boehringer Corp. (London) Ltd.

METHODS

Removal of liver. Each animal was anaesthetized with a mixture of ether and air, the abdomen opened up and the small ligament holding the liver to the diaphragm severed; the animal was held so that the liver was exposed and it was rapidly frozen *in situ* with aluminium tongs cooled in liquid nitrogen (for full details of the procedure see Underwood, 1965). Two groups of animals were anaesthetized by intraperitoneal injection of Nembutal (12mg./rat) and the livers frozen *in situ* as described above. Animals were used approx. 10–15min. after Nembutal injection.

Extraction. The frozen liver was powdered in a percussion mortar cooled in solid CO_2 (see Newsholme, 1962). The powder was then extracted with 2vol. of ice-cold 6% (w/v) $HClO_4$, and the protein precipitate was removed by centrifuging twice at 1000*g* for 5min. The supernatant was neutralized with 3M-$KHCO_3$ at 0° by using an external indicator (British Drug Houses Ltd. universal indicator) at room temperature. The colour change of the external indicator was standardized (in preliminary studies) with a pH electrode in the solution at 0°, so that the final pH of this solution was 6·7–7·0. Acetyl-CoA was determined immediately in the supernatants, which were then stored at −15° for assay of other intermediates.

Assay of intermediates. Glucose was assayed by using the glucose oxidase method of Huggett & Nixon (1957) as modified by Krebs, Bennett, de Gasquet, Gascoyne & Yoshida (1963). Glycogen was measured by the method of Krebs *et al.* (1963). Acetyl-CoA was assayed fluorimetrically on a recording fluorimeter (Dalziel, 1962) after $NADH_2$ formation in the presence of malate, malate dehydrogenase and citrate synthase; the correction suggested by Pearson (1965) was applied. All other intermediates except citrate were measured as described by Gevers & Krebs (1966) or Underwood & Newsholme (1967), by using a Gilford recording spectrophotometer.

Assay of citrate. (1) The aconitase–isocitrate dehydrogenase method of Siebert (1965) was used, but was found to be inadequate for the assay of citrate in liver extracts (see the Results section).

(2) The citrate lyase method of Gruber & Moellering (1966) was adopted for measurement of citrate in liver extracts. The production of oxaloacetate from citrate was followed by oxidation of $NADH_2$ with malate dehydrogenase at 340mμ on a Gilford recording spectrophotometer. Any oxaloacetate that was decarboxylated to pyruvate by a decarboxylase present in the citrate lyase preparation was simultaneously measured at 340mμ by the addition of lactate dehydrogenase to the assay system. The reaction was complete in 15min. and recoveries of added standard citrate varied between 95 and 105%. Any interference by $NADH_2$ oxidases in the liver extract could be removed by pretreating the extract with 60–100 mesh-Florisil; this treatment did not affect the citrate content.

Recovery of citrate. The use of $HClO_4$ as a protein precipitant has been reported to result in only 70% recovery of citrate in certain cases (Dagley, 1965). It seemed possible that the variations in changes in hepatic citrate content with starvation (see the introduction) might be explained by variation in percentage extraction of citrate. Therefore in the present work the recovery of citrate during extraction was tested by three independent methods. (1) Citrate, at a similar concentration to that present in liver extracts, was added to 6% (w/v) $HClO_4$ that was used for extraction of frozen liver powder. The citrate measured at the end of this extraction was compared with the citrate content of the same powder extracted with $HClO_4$ without citrate addition. The recovery of the added citrate (0·35μmole/g. of liver powder) was 100%. (2) This method used the isotope-dilution principle to calculate the concentration of citrate at the stage of addition of $HClO_4$ to the frozen liver powder, and this was compared with the citrate content measured spectrophotometrically at the end of the extraction. An $HClO_4$ solution containing standard [^{14}C]citrate (0·04–0·4μmole/g. of liver powder) was used for extraction of the liver powder. At the end of the extraction the citrate content was assayed with citrate lyase and the radioactivity was measured with a liquid-scintillation counter. Thus, with a knowledge of the specific radioactivity of citrate at the end of extraction and of that of the standard citrate, the content of citrate present at the time of addition of $HClO_4$ could be calculated by the isotope-dilution principle. This was compared with that measured at the end of the extraction with citrate lyase minus the [^{14}C]citrate; in seven separate experiments with livers from fed and starved animals the values agreed within 10%. (3) If citrate is lost during the extraction procedure it is presumably due to adsorption on the protein or perchlorate precipitates. This was tested by comparing the [^{14}C]citrate/[^{3}H]glycerol ratios in each precipitate with the ratio in the corresponding supernatant; it was assumed that glycerol was not adsorbed by these precipitates. An $HClO_4$ solution containing [^{14}C]citrate (0·002μmole/ml.) and [^{3}H]glycerol (0·01μmole/ml.), both of high specific radioactivity (citrate 20mc/m-mole; glycerol 70mc/m-mole), was used for the extraction

of three livers from fed animals and three livers from starved animals (2ml. of $HClO_4$ soln./g. of liver). The protein precipitate from about 1g. of liver was taken up in 15ml. of 2N-NaOH and the corresponding perchlorate precipitate was dissolved in 10ml. of warm water. Samples of these solutions and the corresponding supernatants were counted in a Beckman liquid-scintillation counter model 1650 to determine both [^{3}H]glycerol and [^{14}C]citrate. The scintillant used consisted of 4g. of 2-(4-*tert.*-butylphenyl)-5-(4-biphenylyl)-1-oxa-3,4-diazole and 80g. of naphthalene in 600ml. of toluene and 400ml. of 2-methoxyethanol. After correction for quenching the results obtained indicated that in all six cases no detectable citrate was adsorbed on the protein precipitate and less than 10% of the total citrate was adsorbed on the perchlorate precipitate.

RESULTS

Aconitase assay of citrate. This method involved converting citrate into α-oxoglutarate by aconitase and isocitrate dehydrogenase and following the reduction of NADP at 340mμ. Although standard solutions of citrate could be assayed quantitatively, the presence of liver extract resulted in inconsistent recoveries of standard (30–80%). It was found that the aconitase preparation contained lactate dehydrogenase activity, which was in part responsible for the poor recoveries of standard citrate. Thus addition of pyruvate to the cuvette resulted in oxidation of $NADPH_2$, and this was inhibited by oxalate, an inhibitor of lactate dehydrogenase. The presence of oxalate in the assay medium for citrate increased the value for citrate content of liver from 0·02 to 0·10μmole/g. of fresh tissue; but the citrate lyase method gave a value of 0·26μmole/g. of fresh tissue, showing that, even in the presence of a lactate dehydrogenase inhibitor, the aconitase method was still unsatisfactory. The presence of lactate dehydrogenase in the aconitase preparation may explain why some workers have observed that starvation increases the citrate content of liver, in contrast with the findings of the present paper. Because the content of pyruvate in the liver would be decreased markedly in starvation and diabetes (see Table 2), oxidation of $NADPH_2$ due to pyruvate would be less in livers from starved than from fed animals; this would result in a higher $NADPH_2$ response for such an extract and therefore apparently in more citrate.

Effect of anaesthesia. Underwood (1965) investigated the effect of ether–air and Nembutal anaesthesia, and of stunning the animal by a blow on the head, on the adenine-nucleotide and ketone-body contents of the 'freeze-clamped' liver. Since ether resulted in the highest ATP/ADP ratio and the lowest ketone-body concentration, he concluded that the use of this anaesthetic produced the least metabolic stress. Therefore in the present studies the rats were anaesthetized with an ether–air mixture. In one experiment Nembutal was used

Table 1. *Effects of starvation, acute alloxan-diabetes and Nembutal anaesthesia on the contents of citrate, acetyl-CoA and adenine nucleotides in rat liver*

Except for the experiment involving Nembutal administration, rats were anaesthetized by ether–air as described in the Methods section. Livers were removed, extracts prepared and intermediates assayed as described in the Methods section. Results are expressed as means ± S.E.M. The numbers of animals are given in parentheses.

Treatment of rat	Hepatic content of acetyl-CoA (mμmoles/g. fresh wt.)	Hepatic content (μmoles/g. fresh wt.)					
		Citrate	ATP	ADP	AMP	ATP/ADP	ATP/AMP
Fed control	29·3 ± 3·8 (8)	0·262 ± 0·020 (8)	2·45 ± 0·06 (8)	0·760 ± 0·031 (4)	0·130 ± 0·020 (4)	3·2	18·8
6hr. starved	34·2 ± 2·7 (7)	0·226 ± 0·006 (7)	2·40 ± 0·07 (7)	0·796 ± 0·036 (4)	0·159 ± 0·033 (4)	2·9	15·6
12hr. starved	51·1 ± 5·3 (4)†	0·154 ± 0·010 (4)†	1·94 ± 0·09 (4)†	0·695 ± 0·030 (4)	0·184 ± 0·036 (4)*	2·8	11·0
27hr. starved	82·7 ± 9·2 (5)†	0·151 ± 0·024 (5)†	1·78 ± 0·14 (5)†	0·778 ± 0·031 (5)	0·155 ± 0·012 (5)	2·2	11·7
35hr. starved	81·4 ± 2·0 (4)†	0·127 ± 0·002 (4)†	1·82 ± 0·14 (4)†	0·767 ± 0·020 (4)	0·171 ± 0·013 (4)	2·9	10·8
48hr. starved	82·4 ± 8·0 (4)†	0·136 ± 0·008 (4)†	1·86 ± 0·14 (4)†	0·825 ± 0·028 (4)	0·184 ± 0·020 (4)*	2·3	10·4
Nembutal anaesthesia							
6hr. starved	41·3 ± 4·4 (5)	0·200 ± 0·018 (5)	2·40 ± 0·06 (5)	0·813 ± 0·039 (5)	0·194 ± 0·021 (5)	3·0	12·9
48hr. starved	102 ± 2·1 (3)‡	0·161 ± 0·013 (3)	2·06 ± 0·09 (3)	0·846 ± 0·041 (3)	0·189 ± 0·044 (3)	2·6	12·1
Alloxan-diabetic	79·8 ± 7·3 (4)	0·178 ± 0·011 (8)	2·33 ± 0·14 (8)	0·889 ± 0·006 (8)	0·230 ± 0·016 (8)	2·6	10·1

* $0·01 < P < 0·05$, and † $P < 0·01$, for differences from fed controls. ‡ $0·01 < P < 0·05$ for difference from 48hr. starved, ether–air anaesthetized.

Table 2. *Effects of starvation and acute alloxan-diabetes on the contents of glycogen, glucose 6-phosphate, fructose 6-phosphate, lactate and pyruvate in rat liver*

Rats were anaesthetized, livers removed and extracted and intermediates assayed as described in the Methods section. Results are expressed as means ± S.E.M. Numbers of animals are given in parentheses.

Treatment of rat	Hepatic content (μmoles/g. fresh wt.)				
	Glycogen	Glucose 6-phosphate	Fructose 6-phosphate	Lactate	Pyruvate
Fed control	218 ± 25 (4)	0·283 ± 0·018 (4)	0·060 ± 0·010 (4)	1·53 ± 0·12 (4)	0·212 ± 0·025 (4)
6hr. starved	231 ± 20 (4)	0·271 ± 0·029 (4)	0·069 ± 0·011 (4)	1·01 ± 0·16 (4)	0·192 ± 0·030 (3)
12hr. starved	55·9 ± 18 (4)*	0·254 ± 0·018 (4)	0·070 ± 0·011 (4)	1·03 ± 0·12 (4)	—
27hr. starved	9·1 ± 3·8 (4)*	0·180 ± 0·047 (5)	0·070 ± 0·017 (5)	0·534 ± 0·159 (5)*	—
35hr. starved	1·95 ± 0·23 (4)*	0·073 ± 0·011 (4)*	0·031 ± 0·004 (4)*	0·427 ± 0·088 (4)*	0·086 ± 0·018 (4)*
48hr. starved	4·25 ± 1·10 (4)*	0·088 ± 0·017 (4)*	0·036 ± 0·004 (4)*	0·657 ± 0·069 (4)*	0·073 ± 0·018 (3)*
Alloxan-diabetic	12·6 ± 3·0 (4)	0·118 ± 0·010 (4)	0·019 ± 0·002 (4)	0·260 ± 0·036 (4)	0·013 ± 0·002 (4)

* $P < 0·01$ for differences from fed controls.

instead of ether to compare the effect of these anaesthetics on the change of citrate content with starvation. No difference was observed when Nembutal was used as the anaesthetic in the citrate content of either fed or starved rats (Table 1).

Effect of starvation and alloxan-diabetes on content of intermediates. A large number of male rats were simultaneously starved from 6a.m., and groups of five rats were used at intervals over a period of 48hr. Starvation caused a gradual fall in the hepatic citrate content to 50% of the value of the control fed animal (Table 1); there were also decreases in the contents of ATP, lactate and pyruvate, and very large decreases in the contents of glycogen and glucose 6-phosphate (Tables 1 and 2). The latter results confirmed the findings of Steiner & Williams (1959) and Potter & Ono (1961). Starvation increased the hepatic content of acetyl-CoA and AMP. Thus during starvation there was a progressive fall in the ATP/ADP and ATP/AMP ratios. Similar changes after 6hr. and 48hr. starvation were obtained for citrate, acetyl-CoA and the adenine nucleotides when the animals were anaesthetized by injection of Nembutal (Table 1).

The effects of alloxan-diabetes on hepatic contents of citrate and other intermediates were generally similar to the effects of starvation (Tables 1 and 2).

DISCUSSION

In this investigation it has been shown that the content of citrate shows a steady decline during starvation to a value that is 50% of the control value in fed rats. Because of this steady decline during starvation, previous reports of increases, or no changes, in hepatic citrate content cannot be explained by variations in the direction of change during a period of starvation. It is suggested that these inconsistencies in changes in citrate content may be due to non-specific assays for citrate, or possibly to contamination of aconitase with lactate dehydrogenase, as discussed in the Results section. Another problem of starvation is that the time at which food is removed from the animals may not necessarily be the time at which the animal stops eating. In the present investigation it was observed that the animals stopped eating at 6 a.m. (when the lights in the animal house were automatically switched on). At midday and 6 p.m. on the same day the mean citrate content was decreased by 14% and 41% respectively, in comparison with the 6 a.m. value. As the decrease after 48hr. was only 48%, failure to use animals immediately after cessation of feeding might lower the control value in fed rats to such a level that starvation for 48hr. would appear to have had little effect on the citrate content.

The decrease in citrate content during starvation and diabetes cannot explain the increased dephosphorylation of fructose diphosphate under these conditions (i.e. by citrate inhibition of phosphofructokinase). However, the decrease is consistent with citrate playing some role in the inhibition of fatty acid synthesis; Numa *et al.* (1965) have shown that acetyl-CoA carboxylase is inhibited by long-chain acyl-CoA and that this effect is competitive with the activation by citrate. Thus the increase in long-chain acyl-CoA (Tubbs & Garland, 1964) and decrease in citrate would ensure inhibition of fatty acid synthesis in the liver during starvation. An additional effect could be the decrease in ATP citrate lyase activity due to the decrease in citrate concentration.

The control of the early stages of the tricarboxylic acid cycle is not understood because the difficulties of 'compartmentation' of the intermediates (e.g. citrate, acetyl-CoA, oxaloacelate, CoA) preclude identification of equilibrium and non-equilibrium reactions—a necessary prerequisite in the formulation of theories of metabolic regulation (see Bücher

& Rüssmann, 1964; Rolleston & Newsholme, 1967; Newsholme & Gevers, 1967). The low activity of citrate synthase in liver in comparison with other tissues (Srere & Kosicki, 1961) suggests that this enzyme may catalyse a non-equilibrium reaction; the increase in the content of substrate (acetyl-CoA) for the reaction when the flux rate through the cycle decreases (e.g. during starvation or diabetes) suggests that citrate synthase is a regulatory enzyme for the cycle. The fact that starvation or diabetes decreases the citrate content and increases the acetyl-CoA content, despite increased conversion of acetyl-CoA into ketone bodies and decreased citrate conversion into fatty acids, supports this suggestion. Moreover, starvation or alloxan-diabetes or both increases the citrate content in rat heart (Parmeggiani & Bowman, 1963; Garland & Randle, 1964), in rat adipose tissue (Denton, Yorke & Randle, 1966) and in rat kidney cortex (Underwood & Newsholme, 1967); this suggests that in these tissues regulation at isocitrate dehydrogenase may be more important than that at citrate synthase, in contrast with the situation in liver. A number of possible mechanisms of regulation of hepatic citrate synthase have been proposed by various workers (see review by Newsholme & Gevers, 1967). Of the possible mechanisms, the one proposed by Shepherd & Garland (1966), that ATP increases under conditions of starvation and inhibits citrate synthase, is not supported by the present results, as the hepatic ATP content decreased during starvation. Obviously this and the other arguments do not take into account 'compartmentation' of adenine nucleotides or other intermediates, but, in the absence of results about such 'compartmentation', theories of metabolic control should be consistent with known changes in total intracellular contents of metabolic regulators.

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REFERENCES

Angielski, S. & Szutowicz, A. (1967). *Nature, Lond.*, **213**, 1252.

Bücher, T. & Rüssmann, W. (1964). *Angew. Chem. (Engl. int. Ed.)*, **3**, 426.

Dagley, S. (1965). In *Methods in Enzymatic Analysis*, p. 313. Ed. by Bergmeyer, H. U. New York and London: Academic Press Inc.

Dalziel, K. (1962). *Biochem. J.* **84**, 244.

Denton, R. M., Yorke, R. E. & Randle, P. J. (1966). *Biochem. J.* **100**, 407.

Dixit, P. K., De Villiers, D. C., jun. & Lazarow, A. (1967). *Metabolism*, **16**, 285.

Garland, P. B. & Randle, P. J. (1964). *Biochem. J.* **93**, 678.

Gevers, W. & Krebs, H. A. (1966). *Biochem. J.* **98**, 720.

Goldberg, N. D., Passonneau, J. V. & Lowry, O. H. (1966). *J. biol. Chem.* **241**, 3997.

Gruber, W. & Moellering, H. (1966). *Biochem. Z.* **346**, 85.

Huggett, A. St G. & Nixon, D. A. (1957). *Biochem. J.* **66**, 12P.

Krebs, H. A., Bennett, D. A. H., de Gasquet, P., Gascoyne, T. & Yoshida, T. (1963). *Biochem. J.* **86**, 22.

Lynen, F., Matsuhashi, M., Numa, S. & Schweizer, E. (1963). *Symp. biochem. Soc.* **24**, 43.

Morrison, J. F. (1954). *Biochem. J.* **56**, 99.

Newsholme, E. A. (1962). Ph.D. Thesis: University of Cambridge.

Newsholme, E. A. & Gevers, W. (1967). *Vitam. & Horm.* **25**, 1.

Newsholme, E. A. & Randle, P. J. (1964). *Biochem. J.* **93**, 641.

Numa, S., Ringelman, E. & Lynen, F. (1965). *Biochem. Z.* **343**, 243.

Parmeggiani, A. & Bowman, R. H. (1963). *Biochem. biophys. Res. Commun.* **12**, 268.

Pearson, D. J. (1965). *Biochem. J.* **95**, 23C.

Potter, V. R. & Ono, T. (1961). *Cold Spr. Harb. Symp. quant. Biol.* **26**, 355.

Rolleston, F. S. & Newsholme, E. A. (1967). *Biochem. J.* **104**, 524.

Shepherd, D. & Garland, P. B. (1966). *Biochem. biophys. Res. Commun.* **22**, 89.

Siebert, G. (1965). In *Methods in Enzymatic Analysis*, p. 318. Ed. by Bergmeyer, H. U. New York and London: Academic Press Inc.

Spencer, A. F. & Lowenstein, J. M. (1962). *J. biol. Chem.* **237**, 3640.

Spencer, A. F. & Lowenstein, J. M. (1967). *Biochem. J.* **103**, 342.

Srere, P. A. & Kosicki, G. W. (1961). *J. biol. Chem.* **236**, 2557.

Start, C. & Newsholme, E. A. (1967). *Biochem. J.* **104**, 46P.

Steiner, D. F. & Williams, R. H. (1959). *J. biol. Chem.* **234**, 1342.

Tarnowski, W. & Seeman, M. (1967). *Hoppe-Seyl. Z.* **348**, 829.

Tubbs, P. K. & Garland, P. B. (1964). *Biochem. J.* **93**, 550.

Underwood, A. H. (1965). D.Phil. Thesis: University of Oxford.

Underwood, A. H. & Newsholme, E. A. (1965). *Biochem. J.* **95**, 868.

Underwood, A. H. & Newsholme, E. A. (1967). *Biochem. J.* **104**, 300.

Williamson, J. R., Herczeg, B., Coles, H. & Danish, R. (1966). *Biochem. biophys. Res. Commun.* **24**, 437.