The Biochemistry of Fatty Liver and Kidney Syndrome

BIOTIN-MEDIATED RESTORATION OF HEPATIC GLUCONEOGENESIS *IN VITRO* AND ITS RELATIONSHIP TO PYRUVATE CARBOXYLASE ACTIVITY

By DONALD W. BANNISTER

*Agricultural Research Council Poultry Research Centre, King's Buildings,
West Mains Road, Edinburgh EH9 3JS, Scotland, U.K.*

(Received 14 November 1975)

Liver slices from chicks affected by the fatty liver and kidney syndrome display an extremely low extent of hepatic gluconeogenesis which is associated with decreased activities of certain rate-limiting gluconeogenic enzymes. Pyruvate carboxylase activity is particularly severely affected, being less than 4% of control values. Incubation of affected slices in a biotin-containing nutrient medium restores both gluconeogenesis and pyruvate carboxylase activity (the latter to approx. 35% of the control value). Activities of the other enzymes studied were not greatly affected by this treatment. Restoration of gluconeogenesis did not occur if biotin was excluded from the nutrient medium, nor was it prevented by protein-synthesis inhibitors. It is concluded that the syndrome involves the lack of available biotin in the liver rather than suppression of apocarboxylase synthesis.

Fatty liver and kidney syndrome is a cause of significant mortality in broiler chicks and consequently is a disorder of some economic importance in the United Kingdom and elsewhere. The syndrome, as its name suggests, is characterized by extensive lipid infiltration of liver and kidneys when observed *post mortem*, and was first recognized as a clinical entity by Marthedal & Vellinge (1958). Until recently, however, little progress in its understanding has been made.

Under industrial conditions mortality attributable to the syndrome is usually low (<1%), but occasional outbreaks occur in which up to 20% of a flock have been lost. In both industrial and experimental conditions chicks are maximally susceptible between 3 and 5 weeks old, although deaths do occur outside these limits. Before onset of the syndrome, the birds appear normal and cannot be distinguished from their flock-mates in terms of growth rate or food intake, nor do they exhibit symptoms of previous illness. The onset is sudden; the bird becomes motionless and sinks on to its sternum, in which position it remains until death supervenes, usually within 1 day and frequently within 6-10h.

The syndrome was reproduced experimentally by feeding a low-protein, low-fat, high-carbohydrate diet (Whitehead & Blair, 1974) and mortalities consistently above 20% have been obtained since its introduction (Whitehead & Blair, 1976). The interested reader is referred to the following accounts for further information on dietary and environmental factors affecting the incidence of mortality (Whitehead *et al.*, 1974, 1975; Blair *et al.*, 1975), on histopathology (Wight, 1975*a,b*; Wight & Siller,

1975; Siller & Wight, 1976) and on some aspects of lipid (Evans *et al.*, 1975) and carbohydrate metabolism (Bannister *et al.*, 1975*a,b*).

In this syndrome the most significant observations made to date are that hepatic gluconeogenesis is extremely low when measured by a tissue-slice technique, and the chicks are severely hypoglycaemic (Bannister *et al.*, 1975*a*); supplementation of syndrome-inducing regimens with biotin eliminates, or greatly decreases, death attributable to the syndrome (Payne *et al.*, 1974; Whitehead *et al.*, 1976*a*); gluconeogenesis is restored to nearly normal values both *in vivo* and *in vitro* by biotin (Bannister, 1976).

Despite the biotin-responsive nature of the syndrome, chicks do not exhibit any of the symptoms associated with experimental biotin deficiency such as poor growth rate, poor feathering, perosis and dermatitis (Jukes & Bird, 1942). Indeed, chicks fed on a number of biotin-deficient diets in which the relative proportions of protein, fat and carbohydrate were varied, showed a reciprocal relationship between the severity of such symptoms and susceptibility to fatty liver and kidney syndrome in that those diets that elicited the most severe symptoms of biotin deficiency did not cause death from the syndrome (Whitehead *et al.*, 1976*b*).

The present paper reports that the severe impairment of gluconeogenesis is associated with decreased activities of four rate-limiting enzymes, namely glucose 6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9), fructose 1,6-bisphosphatase (D-fructose 1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11), phosphopyruvate carboxylase [GTP-

oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32] and pyruvate carboxylase [pyruvate-CO₂ ligase (ADP-forming), EC 6.4.1.1]. Pyruvate carboxylase, a biotin-containing enzyme, is the most grossly affected. Further, it is demonstrated that the activity of pyruvate carboxylase and gluconeogenesis (from lactate) are restored quickly in the presence of biotin, but the other three enzymes are unaffected. The restoration of gluconeogenesis is not dependent on protein synthesis, indicating that the syndrome involves a lack of available biotin in the liver rather than suppression of apocarboxylase synthesis.

Experimental

Birds

All birds were 1-day-old Ross I broiler chicks obtained either from the Centre's outstation or from a commercial hatchery. They were housed in a heated four-tiered brooder and allowed unrestricted access to food and water, except that they were starved overnight (17–18 h) before being killed. Chicks were used between 3.5 and 4.5 weeks old. Overnight starvation was used because it precipitated the syndrome in a proportion of the chicks (Whitehead *et al.*, 1975) and provided starved but healthy chicks that served as controls. In a number of cases, however, birds were used in which the syndrome had arisen spontaneously.

Diet

Chicks were fed on a diet containing 18% (w/w) crude protein, 1.9% (w/w) fat (measured by ether extraction) and having a calculated metabolizable energy of 12.55 MJ/kg, known to cause a high spontaneous death rate from the syndrome. Full details of this diet and its relationship to normal commercial practice were described by Whitehead & Blair (1974).

Additionally, some chicks were fed on a normal commercial-type ration (Bolton & Blair, 1974) that does not cause mortality from the syndrome. Overnight starvation of this group provided normal chicks, so called to distinguish them from the controls referred to above.

Diagnosis of fatty liver and kidney syndrome

Affected chicks were selected initially on the basis of their clinical appearance (sitting motionless on the brooder floor and not responding to intrusion with normal escape behaviour) and the pale and swollen appearance of both liver and kidney when examined *post mortem*. Confirmation of diagnosis (or, in the case of controls, absence of the syndrome) was established by histological examination of liver, kidney and heart muscle for the presence of fatty infiltration (Wight & Siller, 1975), and by measurement of plasma glucose concentration (Morley *et al.*, 1968)

to establish the presence of hypoglycaemia (values usually between 150 and 50 mg/100 ml as compared with a mean of 267 mg/100 ml for healthy chicks as reported by Bannister *et al.*, 1975a).

Biotin-mediated restoration of gluconeogenesis

Liver slices (0.5 mm thick) were prepared with a Mickle (Gomshall, Surrey, U.K.) tissue slicer and washed in Krebs–Ringer solution (Krebs & Henseleit, 1932) at 23–25°C. Approx. 1.2–1.4 g of sliced tissue was transferred to 50 ml conical flasks containing 25 ml of Eagle's minimum essential medium (Wellcome Reagents Ltd., Beckenham, Kent, U.K.) supplemented with D-glucose to a final concentration of 2.5 mg/ml and treated with silicone antifoam. To appropriate flasks was added 1.25 µmol of D-biotin in 1.25 ml of water, and to reference flasks an equivalent volume of water. They were then incubated at 38°C in a shaking water bath while being gassed continuously with O₂+CO₂ (95:5). Tissue was removed at 1.5 h intervals between 0.5 and 0.6 h from the beginning of the experiment, washed in Krebs–Ringer solution and assayed for gluconeogenic activity for 2 h with lactate (final concn. 10 mM) as precursor and under the conditions described previously (Bannister, 1976; Bannister *et al.*, 1975a). In all experiments glucose was assayed in the medium and that formed in the absence of added precursor was subtracted from the total produced when precursor was present. Results are therefore presented as net glucose released into the medium, as µmol/h per g dry weight. Formation of glycogen was ignored because the capacity for hepatic glycogen storage is low in birds of this age (Pearce & Brown, 1971).

In one series of experiments the effect of including the following protein-synthesis inhibitors in the Eagle's minimum essential medium was investigated: puromycin (final concn. 0.1 mM), chloramphenicol (0.2 mM) and cycloheximide (2.0 mM).

Activity of gluconeogenic enzymes

Enzyme activity was assayed in liver slices after incubation in Eagle's minimum essential medium as described above. Tissue for assay of glucose 6-phosphatase, fructose 1,6-bisphosphatase and phosphopyruvate carboxylase was frozen in liquid N₂ and stored frozen until required. Pyruvate carboxylase activity was assayed in fresh tissue. All assays were performed at 38°C.

Glucose 6-phosphatase was assayed in a 10% (w/v) homogenate prepared with 0.1 M-citrate buffer adjusted to pH 6.5 with 5 M-NaOH by using a Pye model 291 pH-meter and without subsequent centrifugation, as described by Harper (1963). The glucose released was determined enzymically.

Fructose 1,6-bisphosphatase was assayed by using the conditions for avian liver described by Underwood

& Newsholme (1965) and Wallace & Newsholme (1967) in an extract prepared by homogenizing liver in 39 vol. of 20 mM-Tris/5 mM-MgSO₄/0.1 mM-EDTA (adjusted to pH 8.0 with HCl at 23–25°C). The homogenate was centrifuged at 600g for 10 min at 2°C and subsequently at 100 000g for 1 h at 4°C.

Phosphopyruvate carboxylase was measured as described by Seubert & Huth (1965).

Pyruvate carboxylase was assayed as follows. Tissue was disrupted in a Potter–Elvehjem homogenizer (in ice) in a medium comprising 0.3 M-sucrose/50 mM-Tris (adjusted to pH 7.8 with HCl at 25°C)/5 mM - ATP/5 mM - MgSO₄/2 μ M - phenylmethanesulphonyl fluoride. Sodium deoxycholate was added to give a final concentration of 1% (w/v) and further medium was added to give a tissue concentration of approx. 10% (wet wt./v). A portion of the preparation (0.1 ml) was assayed immediately in a medium (final vol. 1 ml) containing (in μ mol): Tris, 100 (adjusted to pH 7.8 at 38°C with HCl); ATP, 5; MgSO₄, 5; NaH¹⁴CO₃ (0.5 μ Ci), 30; acetyl-CoA, 0.43. Incubation was terminated after 3 min by addition of 0.5 ml of 10% (w/v) trichloroacetic acid and precipitated protein was removed by centrifuging at approx. 2500g for 10 min. A portion (0.5 ml) of the supernatant was dried *in vacuo* and the residue dissolved in 0.5 ml of Hyamine hydroxide (10%, w/v) methanol, and 4 ml of 0.4% 2,5-diphenyloxazole in toluene/Triton X-100 (4:1, v/v) and counted for radioactivity in a Phillips scintillation analyser PW 4510/00. Counting efficiency, determined by using *n*-[1-¹⁴C]hexadecane as internal standard, was 78.2%. The incorporation of ¹⁴CO₂ was corrected by subtracting a control from which acetyl-CoA was omitted.

Chemicals

All chemicals were of analytical or reagent grade, and were used as supplied, except for the Tris used in the pyruvate carboxylase assay, which was recrystallized as described by Sutherland & Waselait (1956). NaH¹⁴CO₃ (0.1 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Biotin-mediated restoration of gluconeogenesis

Typical results for net glucose formation from lactate for untreated liver slices from normal, control and syndrome-affected chicks were (as μ mol of glucose/h per g dry wt. $\pm$ s.e.m.) 66.5 $\pm$ 6.3 (5), 95.1 $\pm$ 15.9 (13) and 8.3 $\pm$ 2.4 (11) respectively (where the number in parenthesis represents the number of observations), showing that affected liver is almost incapable of gluconeogenesis from this precursor.

The effect of incubating tissue in Eagle's minimum essential medium, with and without biotin, is shown

in Fig. 1. Biotin had little effect on liver slices from control birds, though the mean values tended to be higher throughout the experiment when the vitamin was included. Fig. 1(a) shows a similar experiment in which slices from normal chicks were used (results for reference experiments, in which biotin was omitted, are not given since they were little different). Apart from the 30 min value, the results were very similar (i.e. between 60 and 80 μ mol of glucose/h per g dry wt.) to those for the control slices. The difference between normal and control values at 30 min, although substantial, was not statistically significant because of the large standard errors.

Slices from syndrome-affected birds behaved differently; initial values were very low and remained as such if biotin was omitted. However, in the presence of biotin they rose substantially within a few hours to reach 50.4 μ mol/h per g dry wt. by 6 h (Fig. 1b). This value was lower than that for control tissue similarly treated, and the difference bordered

Fig. 1. Effect of biotin on gluconeogenesis by liver slices

Liver slices were incubated in Eagle's minimum essential medium in the presence (final concn. 47.6 μ M) or absence of biotin, washed in Krebs–Ringer solution (Krebs & Henseleit, 1932) and assayed for gluconeogenic activity with lactate (final concn. 10 mM) as precursor. Full details are given in the text. Results are expressed as means (vertical bars indicate the s.e.m.) for the number of observations shown on the right (in parentheses). (a) ●, Normal birds, biotin present ('normal' in this context refers to birds fed on a diet that does not cause fatty liver and kidney syndrome). (b) ■, Controls, biotin present; □, controls, biotin absent; ▲, fatty liver and kidney syndrome, biotin absent.

Table 1. *Effect of protein-synthesis inhibitors on biotin-mediated restoration of gluconeogenesis in liver slices from syndrome-affected birds*

Liver slices were incubated in Eagle's minimum essential medium in the presence of biotin (final concn. 47.6 μ M), washed in Krebs-Ringer solution (Krebs & Henseleit, 1932) and assayed for gluconeogenic activity with lactate (final concn. 10 mM) as precursor. Full details are given in the text. Results are expressed as the means $\pm$ S.E.M. for the numbers of observations in parentheses.

Duration of incubation (h) ...	Glucose (μ mol/h per g dry wt.)				
	0.5	1.5	3.0	4.5	6.0
Puromycin (final concn. 0.1 mM)					
Controls (4)	76.0 $\pm$ 11.6	94.2 $\pm$ 13.0	83.3 $\pm$ 11.5	87.6 $\pm$ 16.2	62.2 $\pm$ 13.5
Syndrome-affected (3)	22.5 $\pm$ 6.9	48.2 $\pm$ 4.9	58.8 $\pm$ 9.6	59.6 $\pm$ 11.2	62.9 $\pm$ 8.6
Chloramphenicol (final concn. 0.2 mM)					
Controls (4)	109.2 $\pm$ 22.9	83.6 $\pm$ 20.3	82.7 $\pm$ 9.1	66.5 $\pm$ 18.6	68.9 $\pm$ 12.2
Syndrome-affected (3)	41.9 $\pm$ 13.1	64.4 $\pm$ 14.8	61.2 $\pm$ 12.6	63.9 $\pm$ 7.8	64.3 $\pm$ 8.2
Cycloheximide (final concn. 2.0 mM)					
Controls (4)	83.2 $\pm$ 7.4	92.2 $\pm$ 6.1	92.9 $\pm$ 16.6	70.4 $\pm$ 5.7	64.6 $\pm$ 8.2
Syndrome-affected (4)	29.9 $\pm$ 12.1	53.3 $\pm$ 16.0	59.6 $\pm$ 11.8	65.8 $\pm$ 17.8	57.7 $\pm$ 12.4

Table 2. *Activities of gluconeogenic enzymes in control and syndrome-affected chick liver*

Tissue was removed from birds that had been starved overnight, and was frozen in liquid N₂. Assays on the frozen material were performed for glucose 6-phosphatase (Harper, 1963), fructose 1,6-bisphosphatase (Wallace & Newsholme, 1967) and phosphopyruvate carboxylase (Seubert & Huth, 1965). Pyruvate carboxylase was assayed in fresh liver by the method described in the text. Results are expressed as means $\pm$ S.E.M. for the numbers of observations given in parentheses. N.S., not significant.

Enzyme ...	Glucose 6-phosphatase	Fructose 1,6-bisphosphatase	Phosphopyruvate carboxylase	Pyruvate carboxylase
Activity (μ mol of product/h per g wet wt. at 38°C)				
Control	1126 $\pm$ 102 (5)	868 $\pm$ 48 (5)	146 $\pm$ 7.8 (5)	1210 $\pm$ 32 (5)
Syndrome-affected	746 $\pm$ 90 (4)	736 $\pm$ 26 (5)	63 $\pm$ 16 (5)	41 $\pm$ 3.4 (4)
Percentage of control value	66.3	84.8	43.3	3.3
Significance of difference (<i>P</i>)	<0.05	N.S.	<0.001	<0.001

on statistical significance ($0.1 > P > 0.05$); however, observations made in the presence of protein-synthesis inhibitors (see below) did not support the inference that a significant difference exists.

Effect of protein-synthesis inhibitors on biotin-mediated restoration of gluconeogenesis

The rapid recovery of activity in the presence of biotin suggested that protein synthesis was not required, and therefore the effect of three such inhibitors was studied. Biotin was included in all these experiments. The results, given in Table 1, show that none of the inhibitors decreased gluconeogenesis by control slices or prevented the biotin-mediated recovery in affected tissue. Indeed, by 6 h there was practically no difference between control and affected liver in the presence of any inhibitor.

Activities of gluconeogenic enzymes

The results of this series of experiments are given in Table 2. Apart from fructose 1,6-bisphosphatase, activities of the enzymes were decreased significantly, the most notable decrease being that of pyruvate

carboxylase, for which activity was under 4% of the control value.

The effect of biotin on enzyme activity was studied up to 4.5 h and the results are given in Fig. 2.

The activity of glucose 6-phosphatase in affected liver slices fell gradually, but not significantly, during the course of incubation. By contrast, activity in control tissue did fall significantly in comparison with the initial value ($P < 0.02$ and $P < 0.01$ for absence and presence of biotin respectively). Nevertheless, the mean activity of control slices (results obtained in the presence and absence of biotin were pooled) remained 30.3% higher than slices from syndrome-affected birds at 4.5 h ($P < 0.01$).

In syndrome-affected liver, the activity of fructose 1,6-bisphosphatase fell progressively, and by 4.5 h was significantly decreased ($P < 0.05$) in the presence of biotin. However, in the absence of the vitamin, there was an unexplained reversal of the downward trend, so that the 4.5 h value was little different from the initial activity. It is doubtful whether any physiological significance attaches to this observation. Similarly, there were significant decreases of activity

Fig. 2. Effect of incubating syndrome-affected liver slices in the presence of biotin on the activities of four gluconeogenic enzymes

Liver slices were incubated in Eagle's minimum essential medium in the presence (final concn. $47.6 \mu\text{M}$) or absence of biotin. Samples of tissue were frozen in liquid N_2 at the times shown (zero-time values are taken from Table 2) and subsequently assayed for: (a) glucose 6-phosphatase (Harper, 1963); (b) fructose 1,6-bisphosphatase (Wallace & Newsholme, 1967); (c) phosphopyruvate carboxylase (Seubert & Huth, 1965). Pyruvate carboxylase (d) was assayed in fresh tissue as described in the text. The right-hand portion of the Figure shows the activities of the enzymes in control tissue treated in the same manner. The results are given as the mean with a vertical bar representing s.e.m. where this is larger than the symbol used. There were five observations in each case. ○, Biotin absent; ●, biotin present.

in control liver slices ($P < 0.01$ for comparisons in both the presence and the absence of biotin) and by the end of incubation there was little difference between control and syndrome-affected tissue.

Phosphopyruvate carboxylase activity, initially considerably lower in syndrome-affected liver than in the control tissue, fell slightly but not significantly during incubation. The control slices also main-

tained their activity throughout the experiment and consequently there was still a significant difference ($P < 0.001$) between the two sources of material after 4.5 h.

Pyruvate carboxylase underwent an approximately tenfold increase in activity during the incubation period when biotin was included (Fig. 2). Even so, the activity reached only 34.5% of that in similarly

treated control tissue and this difference was significant ($P < 0.001$). In the absence of the vitamin, pyruvate carboxylase activity remained minimal.

Discussion

The results show that in syndrome-affected liver slices, gluconeogenesis from lactate is restored rapidly *in vitro*, confirming observations by Bannister (1976). Further, restoration is not dependent on protein synthesis. The findings also imply that the severe hypoglycaemia observed as an invariable accompaniment of the syndrome (Bannister *et al.*, 1975a) stems from the extremely low pyruvate carboxylase activity in affected liver.

These observations have a number of interesting implications. First, there is some similarity between fatty liver and kidney syndrome and experimentally induced classical biotin deficiency in that lower pyruvate carboxylase activity is a consequence of the lack of biotin. The apocarboxylase is present in normal amounts in biotin-deficient animals (Achuta Murthy & Mistry, 1972), though in fatty liver and kidney syndrome the activity in recovering slices suggest that the concentrations of apoenzyme could be less than in control tissue. Second, the failure of gluconeogenesis via the pyruvate carboxylase pathway is apparently sudden and severe and the consequences to the bird are drastic. Two possible explanations may be suggested. Under normal circumstances only precursors dependent on the pyruvate carboxylase pathway are of physiological importance to the chick. Some support for such a hypothesis comes from the observation that, whereas glucose formation from malate by normal and syndrome-affected liver slices was minimal, that in slices from moderately biotin-deficient chicks was appreciable (Bannister, 1976), suggesting adaptation to the gradual impairment of pyruvate carboxylase activity. However, the activity of this enzyme, even in severe biotin deficiency (Arinze & Mistry, 1970), does not fall as low as reported here. The second suggestion is that more than one block in the gluconeogenic pathway occurs, presumably arising as a result of the failure of pyruvate carboxylase. Evidence for a secondary impairment was demonstrated by Deodhar & Mistry (1969) in biotin-deficient rats, which they attributed to a lower $[NADH]/[NAD^+]$ ratio. Further, a virtual absence of gluconeogenesis from any potential precursor other than fructose, glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-bisphosphate was found in the syndrome (D. W. Bannister, unpublished work). Detailed knowledge of gluconeogenesis in the domestic fowl is poor compared with laboratory animals such as the rat, and further information is required before these or other suggestions can be assessed.

The rate of gluconeogenesis achieved by the slice

technique is not as high as that attained by the perfused organ (Hems *et al.*, 1966; Ross *et al.*, 1967) and in this context it is noteworthy that, whereas the net rate of glucose formation by recovering syndrome-affected slices was 68.7% of that by biotin-treated control tissue (at 4.5h), pyruvate carboxylase activity was only 34.5% of the corresponding control value. Indeed, the activities of two of the other three enzymes studied were also significantly below control values, but it is not yet possible to state whether this occurs as a result of the onset of the syndrome or as an integral part of a pre-clinical development process that renders an individual bird susceptible to the syndrome.

In earlier work, Bannister *et al.* (1975a) did not detect any significant decrease in the activity of glucose 6-phosphatase in affected liver. In those experiments, however, semi-industrial husbandry conditions were used (it was not then known how to reproduce the syndrome on a laboratory scale) and non-starved chicks on a high-protein diet were used as controls. These factors, and the differing experimental approach they necessitated, probably account for the difference.

I thank Dr. W. G. Siller and Dr. P. A. L. Wight for conducting the diagnoses of fatty liver and kidney syndrome and for making known the results of their work before publication. Similarly, I thank Dr. C. C. Whitehead and Dr. R. Blair for allowing me access to their work before publication. I also thank Mr. J. Bray and Miss A. Campbell for their technical assistance.

References

- Achuta Murthy, P. N. & Mistry, S. P. (1972) *J. Sci. Ind. Res.* **31**, 554–563
- Arinze, J. C. & Mistry, S. P. (1970) *Proc. Soc. Exp. Biol.* **135**, 553–556
- Bannister, D. W. (1976) *Comp. Biochem. Physiol.* **53**, 573–579
- Bannister, D. W., Evans, A. J. & Whitehead, C. C. (1975a) *Res. Vet. Sci.* **18**, 149–156
- Bannister, D. W., Evans, A. J. & Whitehead, C. C. (1975b) *Res. Vet. Sci.* **19**, 90–92
- Blair, R., Whitehead, C. C. & Teague, P. W. (1975) *Res. Vet. Sci.* **18**, 76–81
- Bolton, W. & Blair, R. (1974) *Poultry Nutrition*, Bulletin 174, Ministry of Agriculture, Fisheries and Food, H.M.S.O., London
- Deodhar, A. D. & Mistry, S. P. (1969) *Arch. Biochem. Biophys.* **129**, 321–328
- Evans, A. J., Bannister, D. W. & Whitehead, C. C. (1975) *Res. Vet. Sci.* **18**, 26–31
- Harper, A. E. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 788–792, Verlag Chemie, Academic Press, London and New York
- Hems, R., Ross, B. D., Berry, M. N. & Krebs, H. A. (1966) *Biochem. J.* **101**, 284–292
- Jukes, T. H. & Bird, F. H. (1942) *Proc. Soc. Exp. Biol.* **49**, 231–232

- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–43
- Marthedal, H. E. & Vellinge, G. (1958) *Nord. Veterinar-motet. Helsinki Proc.* **8**, 250–255
- Morley, G., Dawson, A. & Marks, V. (1968) *Proc. Ass. Clin. Biochem.* **5**, 42–45
- Payne, C. G., Gilchrist, P., Pearson, J. A. & Hemsley, L. A. (1974) *Br. Poult. Sci.* **15**, 489–498
- Pearce, J. & Brown, W. O. (1971) in *Physiology and Biochemistry of the Domestic Fowl* (Bell, D. J. & Freeman, B. M., eds.), vol. 1, pp. 295–319, Academic Press, London and New York
- Ross, B. D., Hems, R. & Krebs, H. A. (1967) *Biochem. J.* **102**, 942–951
- Seubert, W. & Huth, W. (1965) *Biochem. Z.* **343**, 176–191
- Siller, W. G. & Wight, P. A. L. (1976) *Res. Vet. Sci.* in the press
- Sutherland, E. W. & Waselait, W. D. (1956) *J. Biol. Chem.* **218**, 459–468
- Underwood, A. H. & Newsholme, E. A. (1965) *Biochem. J.* **95**, 767–774
- Wallace, J. C. & Newsholme, E. A. (1967) *Biochem. J.* **104**, 378–384
- Whitehead, C. C. & Blair, R. (1974) *Res. Vet. Sci.* **17**, 86–90
- Whitehead, C. C. & Blair, R. (1976) *Res. Vet. Sci.* in the press
- Whitehead, C. C., Bannister, D. W., Blair, R. & Evans, A. J. (1974) *Res. Vet. Sci.* **17**, 222–225
- Whitehead, C. C., Blair, R., Bannister, D. W. & Evans, A. J. (1975) *Res. Vet. Sci.* **18**, 100–104
- Whitehead, C. C., Blair, R., Bannister, D. W., Evans, A. J. & Morley Jones, R. (1976a) *Res. Vet. Sci.* in the press
- Whitehead, C. C., Bannister, D. W., Evans, A. J., Siller, W. G. & Wight, P. A. L. (1976b) *Br. J. Nutr.* **35**, 115–125
- Wight, P. A. L. (1975a) *Neuropath. Appl. Neurobiol.* **1**, 231–245
- Wight, P. A. L. (1975b) *J. Comp. Pathol.* **85**, 539–547
- Wight, P. A. L. & Siller, W. G. (1975) *Res. Vet. Sci.* **19**, 173–184