The determination of the availability to chicks of biotin in feed ingredients by a bioassay based on the response of blood pyruvate carboxylase (*EC* 6.4.1.1) activity

BY C. C. WHITEHEAD, J. A. ARMSTRONG AND D. WADDINGTON

Agricultural Research Council's Poultry Research Centre, Roslin, Midlothian EH25 9PS, Scotland

(*Received 9 October 1981 – Accepted 26 January 1982*)

1. Experiments were carried out to establish the conditions required for the measurement of the availability to chicks of biotin in feed ingredients by a bioassay based on the response of blood pyruvate carboxylase (*EC* 6.4.1.1; PC) activity.

2. Reference diets were formulated which gave a linear response in blood PC activity at 3 weeks of age over a wide range of supplemental dietary biotin concentrations.

3. Dietary protein concentration was found to affect blood PC activity. Hence purified ingredients in the reference diets were wholly or partially replaced by the test ingredients so that all diets in each assay contained the same amounts of protein, fat and metabolizable energy. Comparison of the blood PC activities of birds given the test diets with those given biotin-supplemented reference diets provided a measure of the available biotin content of the test ingredient.

4. Bioavailabilities of biotin were found to vary widely in the cereals and vegetable-protein sources tested.

The biotin contents of poultry feeds and feed ingredients are usually determined microbiologically. However, the values determined by this method may not represent the amounts actually available to the bird, since bioassays with chicks have indicated that biotin in some feed ingredients is not totally available.

The usual bioassay has been based on chick growth response to addition of test ingredient to a deficient diet. This method has several disadvantages. The biotin content of some feed ingredients is so low that very high inclusion levels of the ingredient are required to elicit a growth response. Moreover, growth response is non-specific and may be influenced by changes in other aspects of the diet such as nutrient concentrations or palatability. These factors probably account for the considerable variation in the availabilities of biotin in some feed ingredients reported by different authors. Thus the available biotin content of wheat has been suggested to be 90 μg/kg by Anderson & Warnick (1970), 43 μg/kg by Anderson *et al.* (1978) and virtually zero by Frigg (1976). However, the last author was able to support his conclusion with extensive statistical analyses of his results by the parallel line technique (Finney, 1978). An alternative means of assessing biotin bioavailability, based on a specific criterion of biotin status, is thus highly desirable.

The activity of pyruvate carboxylase (pyruvate–carbon dioxide ligase (ADP-forming) *EC* 6.4.1.1; PC) in the blood of young chicks has been shown to be closely related to the biotin status of birds and to the biotin content of their diet (Whitehead & Bannister, 1978, 1980). The over-all relationship between enzyme activity and dietary biotin content is sigmoid in nature, but is linear over a wide range of dietary biotin concentrations. Moreover the relative increase in enzyme activity is much higher than that in growth rate over the same range of intakes. A bioassay based on blood PC activity is thus potentially more specific and sensitive than one based on growth. Accordingly, a series of experiments were carried out to establish the conditions and techniques required to measure biotin availability by means of a bioassay based on blood PC activity.

EXPERIMENTAL

Birds and husbandry

The birds used were female broilers, obtained as 1-d-old chicks from a commercial hatchery (D. B. Marshall Ltd, Newbridge, Scotland). They were housed in groups of up to ten birds in compartments (0·36 m^2) of electrically-heated tier brooders and had continuous access to feed and water. Individual live weights and group feed consumptions were recorded weekly.

Analytical methods

Blood PC. Samples of blood (1 ml) were taken from the wing veins during the third week of age into heparinized containers. Blood PC activity was assayed by the method of Bannister & Whitehead (1976). To simplify the assay, measurements of blood protein concentration were not made; hence activities were expressed as nmol $^{14}CO_2$ incorporated/ml blood per h at 38°. For convenience, assays were conducted on batches of eight samples at a time and a maximum of four batches could be processed in 1 d. Assays for each experiment were therefore usually spread over a 3 d period.

Biotin. This was determined on hydrolysates from test feed ingredients by a microbiological method (Wright & Skeggs, 1944).

Procedure

Expt 1. Establishment of conditions. This preliminary experiment was conducted to investigate the influence of dietary protein concentration on blood pyruvate carboxylase activity. Diet 1 (Table 1) was formulated to contain 150 g starch/kg and to provide sufficient biotin to result in intermediate activities of blood PC. Other diets were obtained by adding 50, 100 or 150 g low-vitamin casein at the expense of the starch. Each diet was fed to ten chicks up to 3 weeks of age when blood PC activities were measured.

Formulation of reference and test diets. The reference diets were formulated to meet the following requirements: (1) an adequacy of all nutrients except biotin, (2) a high content of essentially biotin-free ingredients, i.e. starch, low-vitamin casein, maize oil and cellulose, which could be wholly or partially replaced by test ingredients, (3) a sufficient content of available biotin to permit a blood PC activity at the lower end of the linear part of the relationship between dietary biotin content and blood PC activity. Reference diet R1 (Table 1) contained a very high proportion (700 g/kg) of replaceable ingredients to permit high inclusion levels of test ingredients of very low biotin content. Diet R2, with 350 g replaceable ingredients/kg, was meant for use with test ingredients that contained large amounts of biotin and therefore did not require high rates of inclusion. The difference in non-replaceable constituents between the reference diets was made up with wheat, with the result that diet R2 contained slightly more available biotin.

The test diets were obtained by adding an appropriate amount of the test ingredient at the expense of the replaceable ingredients so as to permit all the diets in each bioassay to contain the same amounts of metabolizable energy (ME), crude protein (nitrogen × 6·25; CP) and fat. For this purpose the ME contents of the replaceable ingredients were assumed to be 36·0, 16·7, 15·3 and 0 MJ/kg for maize oil, low-vitamin casein, starch and cellulose respectively. The compositions of typical test diets for use in conjunction with both reference diets are given in Table 1.

Expt 2. Test of linearity of response with diet R1. This experiment investigated whether reference diet R1 contained sufficient available biotin to permit a linear response of blood PC activity to the addition of low levels of supplemented biotin and also the range of higher supplemental levels over which linearity of response was maintained. Accordingly diet R1 and seven other diets in which diet R1 was supplemented with 5, 10, 15, 25, 50, 75 or 100 μg

Table 1. *Compositions (g/kg) of diets*

Standard ingredients	1	Reference diets R1	Reference diets R2	Test diets for use with diet R1 T1	Test diets for use with diet R1 T2	Test diets for use with diet R2 T3	Test diets for use with diet R2 T4
Maize	200						
Wheat	340	53	400	53	53	400	400
Herring meal	140	90	90	90	90	90	90
Meat-and-bone meal		70	70	70	70	70	70
Soya-bean meal	110	40	40	40	40	40	40
Isolated soya-bean protein*	20	30	30	30	30	30	30
DL-Methionine	1	2	2	3	3	2	2
Limestone flour	16	5	5	5	5	5	5
Dicalcium phospate	15	4	4	4	4	4	4
Salt	3	2	2	2	2	2	2
Vitamin and mineral supplement†	5	5	5	5	5	5	5
Replaceable ingredients							
Maize starch	150	400	194	37	43	194	190
Low-vitamin casein		82	43	14	31	6	
Maize oil		20	13	10		11	12
Cellulose		197	102	87	124	61	70
Test ingredients							
Wheat				550			
Maize					500		
Sunflower-seed meal						80	
Soya-bean meal							80
Analytical composition							
Calculated							
Crude protein (nitrogen × 6·25)	221	222	224	222	222	224	224
Diethyl ether extract	28	38	38	38	38	38	38
Metabolizable energy (MJ/kg)	12·7	11·6	11·9	11·6	11·6	11·9	11·9
Available biotin (μg/kg)	70	38	40				

* FDP 950; Food Production Developments Ltd, 320 Kilburn Road, London.

† Supplied (/kg diet): retinol 2000 μg, cholecalciferol 20 μg, α-tocopherol 17 mg, menadione 1·3 mg, thiamin 10 mg, riboflavin 4 mg, nicotinic acid 28 mg, pantothenic acid 10 mg, pyridoxine 4 mg, folic acid 3 mg, choline 500 mg, copper 3·5 mg, iodide 0·4 mg, iron 80 mg, magnesium 300 mg, manganese 100 mg, zinc 50 mg.

biotin/kg were each fed to groups of ten chicks and blood PC activities were measured at 3 weeks of age.

Expt 3. Test of linearity of response with diet R2. This experiment investigated the response of blood PC activity to the addition of supplemental biotin to reference diet 2 in the amounts likely to be used in the bioassay. Diet R2 and two others, containing 30 or 60 μg supplemental biotin/kg were each fed to groups of twenty chicks up to 3 weeks of age when blood PC activities were measured.

Standard bioassay procedure. Four diets were fed in each bioassay, two test diets and the corresponding reference diet on its own and supplemented with an amount of biotin in the range of 30–60 μg/kg. The amounts of the test ingredients and supplemental biotin were chosen so that the blood PC activities with the test diets would be approximately intermediate to the activities obtained with basal and supplemented reference diets. However this was not achievable at even the highest inclusion rates for some of the cereals tested, such as wheat and barley. With these ingredients, the biotin supplement for the reference diet was 30 μg/kg.

All the diets were made up freshly for each bioassay to ensure that the contribution of

Table 2. *Expt 1. Effect of dietary protein content on blood pyruvate carboxylase (pyruvate: CO_2 ligase (ADP-forming), EC 6.4.1.1; PC) activity of broilers at 3 weeks of age*

(Mean values with their standard errors for measurements on ten birds per group)

Dietary protein content (g/kg)	Blood PC activity (nmol $^{14}CO_2$ incorporated/ml per h at 38 °)	
	Mean	SE
220	478	34
266	376	37
312	356	35
358	325	21

biotin from common ingredients was the same. CP and diethyl ether extract (EE) of test ingredients were determined before mixing to ensure that appropriate adjustments were made to the amounts of purified ingredients included in the test diets. The similarities of the protein and fat contents of test and reference diets in each bioassay were confirmed by analyses.

Each diet was fed to twenty chicks and enzyme activities were measured in each bird during the 3rd week of life. Each batch of blood samples assayed contained samples from two birds on each diet. This procedure minimized any effects of changes in assay conditions or age of birds over the assay period.

Of the seven feed ingredients tested, the wheat, barley, maize and soya-bean meal (extracted; 445 g CP/kg), were of unknown origin, having been purchased for the Poultry Research Centre feed mill through normal commercial channels. The sunflower oil cake meal (380 g CP/kg, 17 g EE/kg) came from South Africa, the safflower meal (245 g CP/kg, 10 g EE/kg) came from Mexico and the rapeseed meal (352 g CP/kg, 12 g EE/kg) came from Switzerland. All the samples tested were from the same batch of each ingredient except for maize and soya-bean meal which came from two batches.

RESULTS AND DISCUSSION

Establishment of conditions

Results from earlier studies (Whitehead & Bannister, 1978) indicated that although blood PC activity changes with age, a good relationship with dietary biotin intake is established by 2 weeks of age. However, variability is high between young birds, so the age for measuring enzyme activities was set at 3 weeks to represent a compromise between decreasing variation and increasing consumption of feed containing expensive ingredients. Female chicks were used since they have shown larger responses in blood PC activities than males.

To establish nutritional guidelines for the assay, information was required on the effects of nutrients other than biotin on blood PC activity. Previous results (Whitehead & Bannister, 1978) suggested that dietary fat content could affect activity but no information was available on the effect of protein. However, the results of Expt 1 (given in Table 2) showed that increasing the dietary protein content markedly depressed activity. It was therefore decided that the diets used in each bioassay should contain the same amounts of CP, fat and ME.

Reference diets R1 and R2 were formulated according to the criteria described and the response of enzyme activity to biotin supplementation of each was investigated. The results

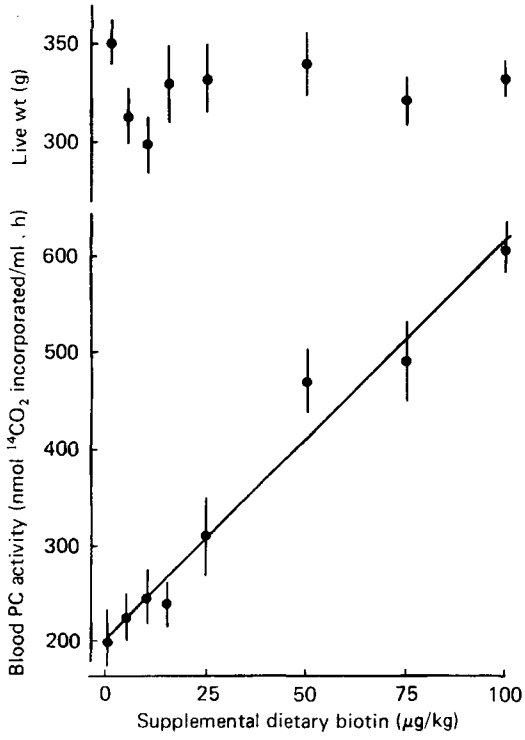

Fig. 1. Expt 2. Response of live weight (g) and blood pyruvate carboxylase (pyruvate–CO_2 ligase (ADP-forming) *EC* 6.4.1.1; PC; nmol $^{14}CO_2$ incorporated/ml per h) at 3 weeks of age in chicks given reference diet R1 (for details, see Table 1) supplemented with graded levels of biotin. Each point indicates the mean value for ten birds with their standard errors represented by vertical bars. Regression equation for blood PC activity is: Mean (PC) = 201·3+4·174 biotin.

with R1 are shown in Fig. 1. Deficiency lesions or growth depression did not occur at the lowest dietary biotin concentrations. This is consistent with previous observations that biotin requirements of broilers for growth are comparatively low when husbandry conditions do not allow achievement of full genetic potential for growth (Whitehead & Bannister, 1980). However enzyme activity showed a response over the whole range of supplemental biotin levels. Tests for quadratic, cubic and linear responses suggested that the response was linear throughout the range.

The test with reference diet R2 (Expt 3) showed that the response of enzyme activity to added biotin was also linear with this diet. Mean (±SE) blood PC activities were 112±11, 224±25 and 338±23 nmol $^{14}CO_2$ incorporated/ml per h at 38° for diet R2 alone or supplemented with 30 or 60 μg biotin/kg respectively. The results of Expts 2 and 3 thus confirmed that there was a linear response of enzyme activity with both basal reference diets to added biotin concentrations of up to and beyond 60 μg/kg which was the largest supplement used in the subsequent bioassays.

Bioassay procedure

The bioassay procedure adopted as standard contained four treatments so that each batch of enzyme assays could be balanced. The treatments comprised two test diets to maximize throughput, together with a basal and a biotin-supplemented reference diet. The accuracy

of the determination was dependent on the number of birds in each group. Twenty birds were used as an acceptable compromise between accuracy and effort. Assays of pooled samples were tried but were discontinued in favour of individual assays for methodological and statistical reasons.

Examples of the compositions of some of the test diets are given in Table 1. The inclusion rate for the cereals was maximized since their available biotin contents were low. With the richer biotin sources, variable levels of inclusion were used. The assumption that these test ingredients were included in diets at the expense of biotin-free ingredients was not entirely valid. Cellulose and maize oil and starch do not contain biotin but the low-vitamin casein was found to contain 20 μg/kg on microbiological analysis. On the assumption that this was fully available, the appropriate small correction was made in the calculation of results.

Enzyme activities with the basal reference diets varied appreciably between bioassays. This was attributable not only to differences in enzyme assay conditions and reagents but also to changes in the natural biotin contents of the standard practical ingredients in the diets. One batch of herring meal in particular was suspected to contain an abnormally-large amount of biotin. These fluctuations were not thought to affect the accuracy of the results since the enzyme activities with the basal diets in the two linearity tests were amongst the lowest observed and the activities in the bioassays normally extended over only the lower half of the linear part of the relationship between dietary biotin content and enzyme activity.

Birds in bioassays based on reference diet R2 grew better to 3 weeks of age than those with R1 (400 g *v.* 330 g), presumably because the higher proportion of practical ingredients made the former diet more palatable. Within individual bioassays there were occasionally significant differences in live weight between groups. However this was not thought to affect the validity or accuracy of results since there was no within-group relationship between bird weight and enzyme activity and, between bioassays involving the same diets, there were no relationships between activities and group mean live weights.

Assessment of results

When values for the blood PC activities with the reference diets were combined they showed a marked increase in variability as the mean level of activity rose. Since this relationship was largely linear, variances were considered to be directly proportional to the observed mean activity. Weighting the regressions by the inverse of the variance thus determined had a sufficiently small effect on the resultant estimates such that any further refinements of the variance-mean relationship were considered unnecessary.

The reference diets were used to calibrate the response line in each assay. Amounts of biotin corresponding to the mean activity of PC were then read off for each feed ingredient. Fieller's theorem was used to find 95% confidence intervals for these estimates. Variation about the line was high, leading to an imprecise estimate of the slope and rather wide confidence intervals for any individual assay.

Results of assays with the same test ingredient were combined using the method of Armitage, Bennett and Finney (Finney, 1978). A useful by-product of the combination procedure is a test that the assays, being combined, all measure a common concentration of biotin for any particular ingredient. This has been the situation for all values combined so far, despite the possible inhomogeneity of the slopes (χ^2 of 32·4 on 13 df). Bartlett's test suggested that the error variance estimates were homogeneous and these were pooled to give an over-all estimate in the previously-mentioned method. Examples of the individual and combined values from one test ingredient (maize) are given in Table 3.

A summary of the combined results of the bioassays is given in Table 4. The available biotin values are estimated from repeated determinations on samples from single batches of all test ingredients except maize and soya-bean meal. However values from different

Table 3. *Slope of the relationship between blood pyruvate carboxylase (pyruvate:CO_2 ligase (ADP-forming) EC 6.4.1.1) activity and dietary biotin content, residual variances and estimates of the means and confidence intervals in individual biosassays for the available biotin content of maize*

(Estimates are for analyses weighted (by the inverse of the variance) (A) and unweighted (B) (see p. 86). The test statistic for the uniformity of the estimates of available biotin content is distributed as $F_{4,161}$ and has a value of 0·17, suggesting that the estimates are similar)

Bioassay	No. of reference birds	No. of test birds	Slope	Standard error of slope	Residual variance	Estimated available biotin (μg/kg)	95% confidence intervals (μg/kg)
BA12	33	18	3·97	A 0·90	0·730	58·2	35·1, 101·2
				B 0·89	5840		37·3, 95·2
BA18	35	19	4·97	A 1·28	1·238	68·5	38·7, 131·8
				B 1·33	13980		40·4, 128·4
BA24	40	19	6·42	A 0·82	1·032	58·7	12·9, 102·4
				B 0·78	15240		9·4, 102·1
BA27	33	18	4·07	A 1·24	0·988	87·0	−14·4, 200·4
				B 1·25	20440		−20·9, 194·6
BA30	30	32	7·84	A 0·90	0·680	63·4	38·3, 89·6
				B 0·94	10560		33·8, 90·8
Combined						64·6	48·8, 82·5

Table 4. *Summary of results of measurements of the available biotin contents of feed ingredients by a chick bioassay based on the activity of blood pyruvate carboxylase (pyruvate:CO_2 ligase (ADP-forming), EC 6.4.1.1) activity*

Test ingredient	Levels of inclusion in test diets (g/kg)	Reference diet	No. of assays	Estimated available biotin (μg/kg)	95% confidence limits (μg/kg)	Total biotin (μg/kg)*	Estimated bioavailability
Barley	600	R1	2	12	−2, 25	109	0·11
Maize	500	R1	5	65	49, 82	50	1·33
Wheat	550	R1	5	4	−5, 12	84	0·05
Rapeseed meal	50,100	R2	2	574	414, 825	930	0·62
Safflower meal	50,100	R2	2	385	283, 502	1211	0·32
Sunflower meal	40,80	R2	5	415	274, 580	1190	0·35
Soya-bean meal	80	R2	3	278	207, 363	258	1·08

* Determined microbiologically.

batches of these ingredients were combined when microbiological and PC assays suggested that the total and available biotin contents of the different batches were very similar.

The results show that the bioavailability of biotin varies widely from one feed ingredient to another. Bioavailabilities in the wheat and barley samples were very low and were nearer the values obtained by Frigg (1976) than those of Anderson *et al.* (1978). The negative confidence limits obtained were included in the Table, rather than being replaced by zero values, because the possible presence of biotin-binding proteins in the cereals cannot be excluded.

In contrast, the available content of maize was repeatedly found to be higher than the

amount determined microbiologically. Indeed, over all the assays the latter value was at the extremity of the 95% confidence limits of the combined estimate. If this disparity is real, there are two possible explanations. The hydrolytic procedures employed during the preparation of samples for the microbiological assay may destroy biotin or not liberate it fully (Scheiner & De Ritter, 1975). Alternatively, other compounds may occur in maize which have biotin-like activity for chicks but not for micro-organisms.

Biotin availability also varied widely in the oil seed meals tested. Biotin was fully available in soya-bean meal: the amount determined by PC assay was slightly higher than the microbiological value but the latter was well within the confidence limits for the enzymic method. Bioavailability in sunflower- and safflower-seed meals was comparatively low but, because of the high total contents, these meals were still good sources of biotin. Rapeseed meal was the richest source of available biotin among the ingredients tested.

The results of these studies show that a bioassay based on the response of PC can be used to determine the available biotin content of feed ingredients. Measurements of the enzyme in the blood of chicks gives a specific indication of the relative biotin contents of their diets, provided the dietary contents of protein and fat are similar. The method should also work using hepatic PC since the activity of this enzyme is also related to dietary content (Atwal *et al.* 1971). However, hepatic PC activity is also influenced by dietary fat and protein (Whitehead *et al.* 1978) and, since in their work these were not equalized, it is not surprising that Anderson *et al.* (1978) did not obtain satisfactory results with a method based on this enzyme.

The authors thank Mrs C. E. McNeill for expert technical assistance and F. Hoffmann-La Roche & Co., Basle, for microbiological assays for biotin.

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Printed in Great Britain