The availability to the chick of pantothenic acid in foods

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1. The content of total pantothenic acid (PaA) in two foodstuffs of microbial origin was measured by standard microbiological and chick biological assays.

2. Substantially and consistently higher values were found by the chick assay than by the microbiological determination, indicating that PaA-containing compounds were present that were not utilized by the test micro-organisms but were available for chicks. Attempts to identify these compounds did not explain the source of excess PaA activity for the chick.

3. It is suggested that forms of PaA that are undetected by standard methods of microbiological assay differ from one food to another and require different treatments to make the vitamin available to the assay organism.

In natural materials vitamins frequently occur in association with proteins or other large molecules. These bound forms are not readily utilized by micro-organisms so may escape detection by microbial assays unless the vitamin is first released by chemical or enzymic treatment. The vitamins may however be available to higher animals if the bound forms are broken down by the animal's digestive processes.

Pantothenic acid (PaA) occurs naturally as coenzyme A (CoA) and as a component of the acyl carrier proteins (ACP). Plants and most micro-organisms are able to synthesize PaA and most probably contain ACP-bound pantothenate since the ACP are components of metabolically-important systems. ACP-bound pantothenate is therefore likely to be present in many foods, particularly in feeding stuffs of microbial origin. Animals do not synthesize PaA and must obtain it from their diet, hence it is important to know the extent to which bound forms of PaA contribute towards the nutrition of higher animals, especially in view of the current use of proteins of microbial origin in animal food.

In the work reported here PaA was measured in dried brewer's yeast (DBY) and in a microbially-produced protein food by biological assay with chicks and by a standard microbiological method. When appreciably higher values were found by the chick assay, attempts were made to characterize the bound form of PaA responsible for the discrepancy and to devise procedures to release it in a form measurable by the microbiological technique.

MATERIALS AND METHODS

Chicks

Rhode Island Red × Light Sussex cross chicks hatched from the eggs produced by the Institute's specified pathogen free breeding flock were used for the biological assays. They were housed from 1 d of age in three-tier brooders with wire-floored compartments. In the heated section the initial temperature of 30° was gradually reduced to 21° during the following 3 weeks. Artifical illumination was provided for 14 h/d.

Samples tested

DBY was bought in the open market. Pruteen (a protein-rich microbial cell product of bacterial origin developed by Imperial Chemical Industries Ltd, Agricultural Division, Billingham, Cleveland) and a sample of freeze-dried cells of the organisms used for production of Pruteen were kindly supplied by the manufacturer.

Table 1. *Composition of diets for pantothenic acid chick biological assay on Pruteen (g/kg diet)*

Ingredients	Standard*	Designation of diets PRa	PRb	PRc	PRd
Maize starch	602·5	561·3	575·02	584·25	590·33
Casein (low-vitamin)†	180·0	79·2	112·8	135·2	150·1
Gelatin	100	100	100	100	100
Pruteen (ICI Ltd)	—	133·3	88·9	59·3	39·5
L-cystine	3·0	3·0	3·0	3·0	3·0
L-histidine	—	0·787	0·524	0·350	0·233
L-isoleucine	—	1·079	0·719	0·479	0·320
L-leucine	—	1·602	1·068	0·712	0·474
L-lysine	—	0·923	0·615	0·410	0·273
L-methionine	—	0·321	0·214	0·143	0·095
L-phenylalanine	—	1·037	0·691	0·461	0·307
L-tyrosine	—	1·671	1·114	0·743	0·495
L-valine	—	1·255	0·836	0·558	0·372
myo-Inositol	1·0	1·0	1·0	1·0	1·0
Salt mixture‡	60·0	60·0	60·0	60·0	60·0
Choline chloride	1·5	1·5	1·5	1·5	1·5
Vitamin mixture§	2·0	2·0	2·0	2·0	2·0
Maize oil	50·0	50·0	50·0	50·0	50·0

* Calcium pantothenate was included in the standard diets at dose levels of (mg/kg): 1·78, 2·67, 4·0, 6·0.

† Casein (low-vitamin content, Calbiochem Ltd, Bishops Storford).

‡ Salt mixture provided (g/kg diet) $CaCO_3$, 17·1; $CaHPO_4.2H_2O$, 17·1; KH_2PO_4, 13·3; NaCl, 8·67; $MgSO_4.H_2O$, 2·67; $FeSO_4.7H_2O$, 0·67; $MnSO_4.4H_2O$, 0·27; $ZnSO_4.7H_2O$, 0·13; KI, 0·037; $CuSO_4.5H_2O$, 0·16.

§ Vitamin mixture in glucose, provided (mg/kg diet) pteroylmonoglutamic acid 1·5, thiamin hydrochloride 3·0, pyridoxin hydrochloride 4·0, riboflavin 6·0, nicotinic acid 40·0, biotin 0·2, cyanocobalamin 0·02, Rovimix A-500 (Roche Products, Dunstable, Beds.; providing 5·1 retinol) 34. The following (mg/kg diet) were dissolved in the maize oil: menaphthone 5·0, DL-α-tocopheryl acetate 10·0, cholecalciferol 0·04.

Diets

Food and water were available *ad lib*. The standard diet (Table 1) contained casein as the source of protein. Since the protein content and amino acid composition of Pruteen were different from those of casein the test diets were supplemented with appropriate amounts of casein and individual amino acids to made them comparable with the equivalent standard diets. The content of protein and of essential amino acids in granular Pruteen (according to Imperial Chemical Industries Ltd) was as follows (g/kg): crude protein (nitrogen × 6·25) 720, arginine 35, cystine 5, glycine 40, histidine 13, isoleucine 35, leucine 53, lysine 46, methionine 18, phenylalanine 27, threonine 35, tryptophan 10, tyrosine 23, valine 68. The compositions of diets for the assay on Pruteen are described in Table 1. The protein and amino acid contents of DBY and casein were calculated according to the (US) National Academy of Sciences' (1969) food composition tables. No adjustments were made when DBY was tested as the amounts incorporated in the diet were smaller and its protein and essential amino acid contents were near to those of the standard diet. The DBY replaced part of the standard mix in amounts (g/kg) of 26·7, 40·0, 60·0 and 90·0. In the standard diets of all chick assays four dose levels of calcium pantothenate (CaPa) (mg/kg) 1·78, 2·67, 4·0 and 6·0 were included.

The ready-mixed diets were moistened with water, granulated by pushing through a sieve of 5 mm mesh and dried on stainless steel trays in a current of air at 40°.

Biological assay for PaA

The chick biological assays were carried out using the method described by Coates *et al.* (1950). The assay depends on the fact that a curvilinear relationship exists between the growth of the chicks and the logarithm of the dose of PaA, but over a limited range of doses the response is linear and equal proportional increases in the dose produce approximately equal increases in body-weight.

One-day-old chicks from within a narrow weight range (36–41 g in Expt 1; 34–37 g in Expt 2) were distributed into groups of ten, five males and five females, so that within an experiment the mean weight of each group was similar. They received the experimental diets from the first day of life. They were weighed at weekly intervals and examined regularly for signs of PaA deficiency. These became very obvious during the fourth week of life. Birds receiving the low supplements of PaA were severely affected and several died. The weights at 21 d were therefore used to calculate the potency of the test materials.

The standard groups of chicks were maintained on diets containing sub-optimal amounts of PaA. The DBY or Pruteen were incorporated into the test diets in amounts estimated from published figures of microbiological assays to be roughly similar in PaA content to the standard diets. Doses were chosen in geometrical progression in the ratio of 3:2.

In Expt 1 Pruteen and DBY were tested against a common standard, using two groups of ten chicks at each of four doses. In the second trial, of Pruteen alone, two groups of ten birds were put on test but a mechanical failure in the water supply interfered with the growth of half the birds so that only one group of ten could be legitimately used in the calculation.

Numerical interpretation of results of the biological assay

Calculations of potency and limits of error were based on the methods recommended by the British Standards Institution for the biological assay of vitamin D_3 by the chick method (British Standards Institution, 1940). The mean body-weights of the groups were plotted *v.* the logarithms of the doses of test and standard materials. In Expt 1 results of duplicate groups were added together and treated as one group of twenty individuals. A common regression line was calculated for the responses to the two test materials and the standard in Expt 1, and to Pruteen and standard in Expt 2. The responses to test and standard were checked for linearity and parallelism to each other. On one occasion the response failed to pass the test for linearity because the dose range chosen was too close to the minimal requirement, so the group receiving the lowest dose was discarded and only those falling on the linear part of the response curve were used for calculation of potency. The relative potency of the test material was estimated from the difference betwen the logarithmic doses of test and standard that produced the same response. The standard error was calculated as recommended by the British Standards Institution (1940).

Analytical procedures

To obtain a common homogenate available for all analyses the test material was first suspended in a solution of 0·1 M-disodium hydrogen phosphate at pH 7·0 and sonicated for 15 min under ice to rupture the microbial cells.

The total PaA (free and bound) was determined using a conventional method similar to that of Barton-Wright (1963), in which the vitamin was microbiologically determined after previous digestion of the sample with alkaline phosphatase (*EC* 3.1.3.1) and chick liver enzymes. The freed PaA was determined by microbiological assay using *Lactobacillus plantarum* strain DCDO82, ATAA 8014 by a procedure similar to that described by Bird (1963). The bacterial growth was measured in terms of acid production by measuring the

Table 2. *Results of pantothenic acid (mg/kg) determination by chick biological assay, standard microbiological assay and alkali digestions followed by the microbiological determination*

Material tested	Test no.	Chick biological assay	Limits of error $P = 0{\cdot}95$	Microbiological assay: Standard method	Microbiological assay: After incubation at pH 12 at 70° for 1 h	Microbiological assay: After incubation with 3 M-sodium hydroxide at 102° for 2 h
Dried brewer's yeast	1	59·4	55·0–67·1	42·2	21·3	13·0
Pruteen (batch no. 1)	1	40·5	36·9–45·2	25·9	39·4	42·2
	2	35·2	30·5–39·2	20·0	32·7	41·1
Combined results for Pruteen		38·8	36·2–41·7	23·0	36·1	41·7

Table 3. *Measurement of acyl-carrier-protein (ACP)-bound pantothenic acid (PaA) in Pruteen determined by a malonyl-CoA-CO_2 exchange reaction*

(The molecular weight of ACP was assumed to be 9750)

Pruteen sample	ACP (mg/kg)	ACP bound PaA (mg/kg)
Batch no. 1 heat-dried	4·819	0·1084
Batch no. 2 heat-dried	1·892	0·0425
Freeze-dried unheated	16·479	0·3697

amount of 0·1 M-sodium hydroxide needed to bring the pH of standards and samples to 7·5. The values of required amounts of NaOH for the standards were plotted *v.* concentration of PaA per tube and from the graph obtained the PaA concentration per tube of a test sample was read. This standard method does not liberate PaA bound in ACP which is therefore not included in the measurement accomplished entirely by this assay (Majerus *et al.* 1965).

The design of the method is such that no simple statistical procedure is applicable, but in the experience of this laboratory the between-assay variance is not more than 10%.

Liberation of PaA from ACP by incubation with 3 M-NaOH

A portion of the common homogenate was freeze dried and then incubated in 3 M-NaOH in sealed ampoules at 102° for 2 h. During this procedure the ACP-bound pantothenate is released as 4′-phosphopantothenate as described by Majerus *et al.* (1965), after which treatment the pH of the sample was adjusted to 8·3. The sample was then treated with enzymes, extracted and microbiologically analysed as described previously.

Release of PaA from ACP by incubation at pH 12

The freeze-dried sample was incubated in 0·01 M-Tris HCl at pH 12 and 70° for 1 h as described by Larrabee *et al.* (1965). The pH of the sample was adjusted to 8·3, treated with enzymes and assayed according to the procedure described for the standard microbiological assay.

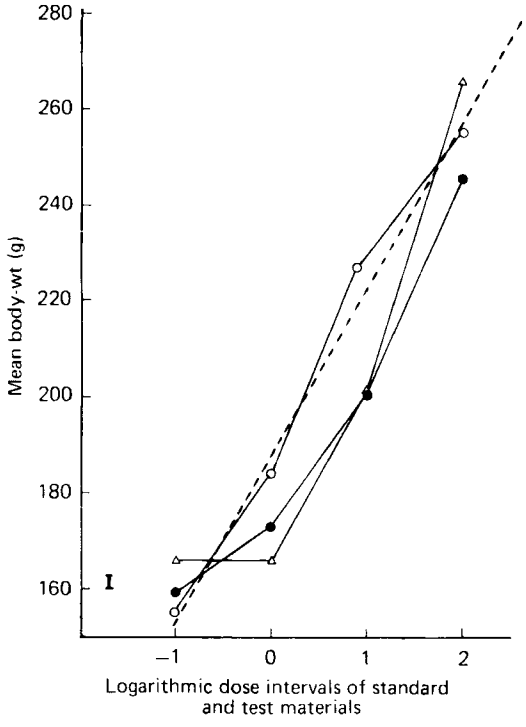

Fig. 1. Regression of body-weight on logarithmic dose of calcium pantothenate (○—○); dried brewer's yeast (●—●); Pruteen (△—△) in Expt 1. Points represent mean values for twenty chicks and the vertical bar represents the standard deviation. Response to the lowest dose of Pruteen was omitted from the calculation. Common regression line (b = 35·1; - - -) calculated for responses to standard and test materials.

Enzymic determination of ACP of fatty acid synthetase (FAS)

The activity of ACP of FAS was determined in two samples of Pruteen and the freeze-dried cells using a combination of the methods of Alberts *et al.* (1963), Majerus *et al.* (1969) and Simoni & Stumpf (1969) based on the malonyl-CoA-CO_2 exchange reaction. No sample of pure ACP of FAS was available as a standard, so the results were assessed on the assumption of Majerus *et al.* (1969) that 1 μmol pure ACP of FAS from *Escherichia coli* catalyses the fixation of 360 μmol $^{14}CO_2$ into malonyl-CoA in 15 min at 30°.

RESULTS

The dose/response curves for the chick assays are shown in Figs 1 and 2 and the results given in Table 2. Substantially higher values were found by the chick biological assay than by the standard microbiological assay. After the alkali-digestion the amount of PaA measured microbiologically in Pruteen increased, but in DBY the alkali treatment resulted in a marked decrease.

The malonyl-CoA-CO_2 exchange reaction detected minute amounts of ACP in both samples of Pruteen (Table 3). The freeze-dried cells contained more, but not enough to account for the difference in results obtained by the chick and microbiological assays.

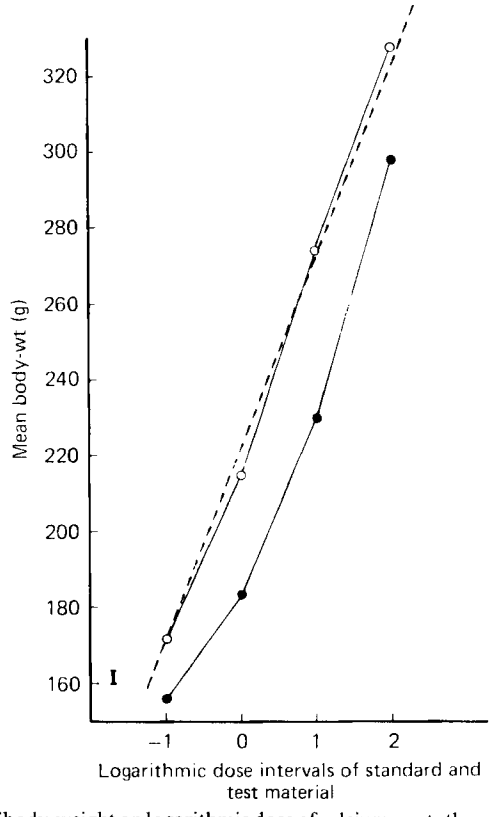

Fig. 2. Regression of body-weight on logarithmic dose of calcium pantothenate (○—○); Pruteen (●—●) in Expt 2. Points represent mean values for ten chicks and the vertical bar represents the standard deviation. Common regression line ($b = 50{\cdot}6$; – – –) calculated for responses to standard and test material.

DISCUSSION

Comparison of the results showed that in both materials the standard microbioligical assay measured a substantially and consistently smaller quantity of PaA than was found by the chick biological assay. This suggests that in these materials a considerable part of the vitamin was present in compounds which were not utilized by the test micro-organism, but from which the PaA was available to the chick. The bound forms of the vitamin recognized at present include the metabolic intermediates of CoA formation, CoA itself, and its intermediate the ACP of FAS (Alberts & Vagelos, 1961), as well as other forms of ACP such as those of citrate-(pro-3*S*)-lyase (*EC* 4.1.3.6) (Dimroth *et al.* 1973) and citramalate lyase (*EC* 4.1.3.22) (Dimroth *et al.* 1977).

The results of attempts to identify the bound forms of PaA by the malonyl-CoA-CO_2 exchange reaction, an enzymic assay in which ACP of FAS (Vagelos, 1976) and the deacetylated ACP of citrate lyase (Dimroth *et al.* 1973) are active, did not explain the source of excess PaA activity for the chick. However, Pruteen is heat-dried during the course of its production, a procedure that would be likely to inactivate a large proportion of the ACP and so render it undetectable by the malonyl-CoA-CO_2 exchange reaction. For this reason a freeze-dried sample of the live organisms from which Pruteen is prepared was assayed by the enzymic method. Although its content of ACP was substantially higher than that of Pruteen it was still not sufficient to account for the difference between results by the chick

and microbiological assays. In the absence of a sample of pure ACP the quantitative recommendation of Majerus *et al.* (1969) concerning the molecular weight of ACP was assumed to apply. Whether or not this value was applicable in the circumstances cannot be verified until a satisfactory standard for ACP becomes available.

The attempts to devise a microbiological assay by which all bound forms of PaA would be measurable gave promising results for Pruteen but not for DBY. Alkali digestion released a considerable amount of activity from Pruteen and the value found by subsequent microbiological assay was very similar to that obtained by the chick assay, but similar treatment of DBY led to even lower microbiological results. It is clear that some of the PaA in foods occurs in forms that remain undetected by the standard microbiological procedures. They apparently differ from one food to another and require different treatments to render them measurable by the routine method of microbiological assay.

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