Availability of sulphur amino acids in protein foods

2.* Assessment of available methionine by chick and microbiological assays†

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In the first paper of this series (Miller & Carpenter, 1964) we reported that the total sulphur amino acid content of meat, fish and whale-meat meals was not directly related to their net protein utilization (NPU) for the rat even when sulphur amino acids had been shown to be limiting. The results were interpreted to indicate a degree of unavailability of the sulphur amino acids, especially in those meals of low NPU. Estimates of 'digestible sulphur amino acids' were found to correlate closely with NPU.

Recently Ford (1962) has described microbiological procedures for the determination of various individual amino acids, including methionine, in protein concentrates, available to *Streptococcus zymogenes*. For a series of whale-meat meals his results for available amino acids correlated closely with those for NPU. Such correlations indicate that the protein materials are ranked in the same order by both microbiological and rat procedures, but do not provide evidence that values for amino acids available to *Strep. zymogenes* are the same as for those available to higher animals.

The purpose of the work now described was to determine the availability of methionine in animal protein concentrates by growth assays on the chick and to evaluate the microbiological procedure as a means of predicting the available methionine content for the chick.

Guttridge, Lewis & Morgan (1961) and Guttridge & Lewis (1964*a*) have described a procedure with chicks for the assay of available methionine, and in our preliminary experiment we used a basal diet modelled on theirs. In subsequent work a basal diet more deficient in methionine was obtained by replacing soya-bean meal with groundnut meal as the main source of amino acids.

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† This work formed part of a thesis submitted by one of us (E. L. M.) to the University of Cambridge in partial fulfilment of the requirements for the degree of PhD. A preliminary report on this work was read before the 6th International Congress of Nutrition (Miller, Carpenter & Morgan, 1963).

EXPERIMENTAL

Materials

Amino acids. DL-Methionine (Brown and Forth Ltd, London, NW 1) was used in preliminary chick experiments. L-Methionine (Light and Co., Colnbrook, Bucks) used in the main chick experiments was supplied as chromatographically pure and, before inclusion in the diets, was ground in a mortar to pass a BSI 60-mesh sieve. In the microbiological assays, L-methionine (Mann Assayed Grade; Mann Research Laboratories, New York 6) was used as the standard, and L-isomers of the other amino acids (Light and Co., Colnbrook, Bucks) were also used.

Oxidized casein. Casein was oxidized with performic acid in 400 g batches, to convert methionine into the sulphone derivative, by the procedure of Toennies (1942). The purification of the oxidized casein was modified to reduce the cost of the preparation. Instead of washing the precipitated oxidized protein with 12 l. methanol, it was washed by suspending it in 2 × 15 l. water and decanting. Then the precipitate was dispersed in 15 l. water and dissolved by adding NaOH to pH 7. The oxidized casein was next reprecipitated at pH 4·7 by addition of HCl, filtered on a Buchner funnel, washed with 1 l. methanol and dried in a desiccator over $CaCl_2$ and then in an oven at 70° under reduced pressure. The preparation contained 6·6% moisture and 89·5% crude protein (N × 6·25).

By assay with *Strep. zymogenes*, the preparation was found to contain 0·06% total methionine or methionine sulphoxide; thus it contained only slight traces of incompletely oxidized material, since the organism has been found to respond equally to methionine and its sulphoxide, but not to the sulphone (Carpenter, Miller & Morgan, unpublished results). The available methionine content measured after digestion in 0·36% papain was 0·02%.

Soya-bean meal. This was a commercial solvent-extracted meal. It had a low urease activity equivalent to 0·8 ml of 0·1 N-NaOH when incubated for 60 min at 60° with urea by the procedure of Croston, Smith & Cowan (1955), indicating that it had received sufficient heat treatment to inactivate any trypsin inhibitors.

Decorticated groundnut meal. This was a commercial solvent-extracted meal, which contained 50·4% crude protein and 0·42% methionine available to *Strep. zymogenes* after digestion with 0·36% crude papain. Aflatoxin was detected in the range 0·5–2·0 ppm by the procedure of Broadbent, Cornelius & Shone (1963).

Papain. Three papain preparations were used: a 'purified' preparation (Light and Co., Colnbrook, Bucks), 'crude' papain (non-crystalline grade; British Drug Houses Ltd, Poole, Dorset) and crystalline papain (British Drug Houses Ltd). The purified preparation is no longer commercially available. The proteolytic activities of purified and crude papain were 5·7 and 1·3 units/g, respectively, as determined by the method of Anson (1938–9) with dialysed haemoglobin as substrate.

Protein concentrates. These were commercial meat, fish and whale-meat meals coded MM, FM, and WM, respectively, distributed under the aegis of the Agricultural Research Council (Zuckerman, 1959; Boyne, Carpenter & Woodham, 1961) in connexion with collaborative trials on protein quality.

Management of the chicks

For most experiments 250 Maxilay day-old cockerels were purchased from a commercial hatchery. The chicks were 'de-beaked' on arrival, wing-banded and reared to 10 days of age on a commercial diet. They were weighed at 5 and 10 days of age, and chicks up to a maximum of 144 were selected on the basis of their weight and rate of growth. The randomization of chicks to dietary treatments is further described in relation to the individual experimental designs.

At 10 days of age, the selected chicks were transferred to the experimental battery previously described (Carpenter, March, Milner & Campbell, 1963) and fed *ad lib.* on the experimental diets as dry powders. The chicks were weighed individually at the end of the experimental period of 10 days. In Expts 1 and 2, two estimates were made of the amounts of each experimental diet consumed, one for the first three replicate cages and the other for the remaining three cages. In subsequent experiments the food consumption of each cage of chicks was separately recorded at the end of the experimental period.

Basal diets

Soya-bean basal diet. This was the diet described by Guttridge *et al.* (1961), except for the use of a different fat and different mineral and vitamin mixtures, and had the percentage composition: extracted soya-bean meal 35·0, dried whey 5·0, partially hydrogenated vegetable fat (Trex; J. Bibby and Sons Ltd, Liverpool; iodine value 77; m.p. 27°) 5·0, feeding bone-flour 2·0, limestone flour 1·0, mineral mixture 0·5, starch + vitamin mixture 1·0, glycine 0·2, choline chloride 0·2 and maize starch to 100. The mineral and vitamin premixes contributed (mg/kg diet): NaCl 4690, KI 13, $MnSO_4.4H_2O$ 300 and Rovimix A and D (Roche Products Ltd, Welwyn Garden City; containing 4×10^6 i.u. vitamin A and 1×10^6 i.u. vitamin D_3/lb) 1000, Rovimix E (Roche Products Ltd; containing 10% DL-α-tocopheryl acetate) 200, thiamine hydrochloride 10, riboflavine 10, pyridoxine hydrochloride 10, nicotinic acid 50, calcium pantothenate 30, biotin 1, pteroylglutamic acid 4, cyanocobalamin 0·013, menaphthone (vitamin K) 0·55. Supplements to this basal diet were added at the expense of maize starch.

Groundnut basal diet 1. This had the percentage composition: decorticated extracted groundnut meal 40, dried whey 5, partially hydrogenated vegetable fat 5, ground oat husks 5, choline chloride 0·3, L-lysine hydrochloride 0·3, glycine 0·2, L-cystine 0·2, mineral mixture 5, starch + vitamin mixture 1, oxytetracycline supplement (TM-5, containing 1·1% oxytetracycline hydrochloride; Pfizer Ltd, Folkestone) 0·07 and maize starch to 100. The mineral mixture was that of Hegsted, Mills, Elvehjem & Hart (1941). The starch + vitamin mixture was the same as that used in the soya-bean basal diet. This basal diet was used in preliminary Expts 2 and 4. Supplements to this diet were added at the expense of maize starch.

Groundnut basal diet 2. This was the same as basal diet 1 except for the mineral composition. The amounts of $CaCO_3$, K_2HPO_4 and $CaHPO_4.2H_2O$ contributed by the mineral mixture of Hegsted *et al.* (1941) were halved and steamed bone flour

containing 79·5% ash was included at 3·9% of the diet. This basal diet was used in Expt 3 and assay Expts 5–10. Test-protein supplements were added at the expense of maize starch and steamed bone flour, the latter being replaced on the basis of an equivalent contribution of ash from the test meat, fish or whale-meat meals. When test materials were calculated to contribute less metabolizable energy than starch, additional fat was added to correct for the difference. (No signs of zinc deficiency have been observed in this work, but, in the light of recent work showing reduced zinc availability in the presence of high calcium and phytate levels (O'Dell, Yohe & Savage, 1964), it is proposed in future assays to increase the level of zinc by adding 130 mg basic zinc carbonate/kg diet, as recommended by Fox & Briggs (1960).)

Experimental design and diets

Expt 1. With groups of three chicks, eight treatments were replicated six times. By weight, 144 selected chicks were arranged into six blocks, each of twenty-four chicks. Within each block three chicks were allotted to each of eight cages so that the initial weight of each group of birds was approximately the same. The eight experimental diets were randomized to the eight cages in each block. The experimental diets were designed to study the response obtained when supplements of L-cystine or DL-methionine (with or without 0·4% cystine) were added to the soya-bean basal diet. The levels of supplements used are detailed in Table 1.

Expt 2. The design and randomization procedures were similar to those of Expt 1, except that four dietary treatments were replicated six times. The four diets were the groundnut basal diet 1, alone or supplemented with one of three levels of DL-methionine as detailed in Table 1.

Expt 3. This was designed to ensure that the groundnut basal diet contained adequate cystine. Basal diet 2 alone and supplemented, as detailed in Table 1, with one of two levels of L-cystine in addition to that already present in the basal diet, were each given to four replicate groups of chicks.

Expt 4. The design and randomization were as detailed for Expt 1. The experiment was meant to investigate in greater detail the response obtained when the groundnut basal diet 1 was supplemented with graded levels of L-methionine and also with oxidized casein, a protein devoid of methionine, at two levels of methionine supplementation. Cystine, tyrosine and tryptophan are also transformed in the oxidation of casein. Therefore tyrosine and tryptophan were included with supplements of oxidized casein at the level present in casein. Extra cystine was not added as the basal diet already contained an excess of cystine. The levels of supplementary L-methionine and crude protein contributed by oxidized casein in the diets are detailed in Table 2.

Expts 5 *and* 6. In Expt 5, six animal protein concentrates were assayed for their available methionine content. Each protein concentrate was assayed at two levels of addition to groundnut basal diet 2. The lower level was chosen to contribute an expected 0·025–0·03% available methionine. The higher level of supplementary protein was 1·75 times the lower. The standard response was constructed from the results with basal diet 2 alone or supplemented with 0·0200, 0·0350 or 0·0613% L-

methionine (i.e. with logarithmic increments). The stratification of the selected chicks and randomization of diets were similar to those of Expt 1 except that each block consisted of sixteen cages, one for each of the sixteen diets, and there were three replicate blocks. The experiment was repeated (Expt 6), with fresh preparation of all diets to give a total over both experiments of six replicates for each dietary treatment.

Expts 7–10. In Expts 7 and 8, a further six materials were assayed, as described for Expts 5 and 6. However, for reasons given later, the results were rejected, and the materials were assayed again in Expts 9 and 10. These were carried out as described before, except that standard L-methionine was added at levels of 0, 0·020, 0·040 and 0·060% and the test proteins were added to contribute approximately 0·025 and 0·050% available methionine, the higher level of addition being thus twice the lower. This arrangement of dose levels with arithmetic increments facilitates statistical analysis. Of the six test materials, three were cod muscle preparations, used as part of an investigation into the effects of heat treatment; the results for these materials are not presented here.

Statistical analysis

The results of the preliminary Expts 1–3 were subjected to normal analysis of variance for randomized block designs. Those of Expt 4 were subjected to more detailed analysis so as to examine the suitability of three sets of dose and response metameters. These were: (1) g weight gain and percentage added methionine in diet; (2) food conversion efficiency (FCE; g weight gain/g food eaten) and percentage added methionine in diet; (3) g weight gain and g available methionine eaten.

For the third method of calculation a value was assumed for the available methionine content of the basal diet: on the basis of the microbiologically determined available methionine of the groundnut meal, it was taken to be 0·18% of the diet.

The assay Expts 5–10 were analysed by the slope–ratio procedure (Finney, 1952) with the dose and response metameters 1 and 2 (see above). The slope–ratio procedure was used since these responses were found to be linearly related to arithmetic increments of methionine or test protein, and the error variability did not increase with increases in the levels either of methionine or of the test proteins.

In calculating the fiducial limits, Fieller's equation was used with the modification recommended by Finney (1952) that when the asymmetry of the upper and lower limits was small it was replaced, as in Table 3, by approximate symmetrical limits indicated by a single estimate of the standard error.

These standard errors cannot, however, be used for comparison of the ratios one with another, as the direct comparison of, say, WM 9 with MM 10 does not involve the use of the standards, and the fiducial limits of any such ratios would have to be calculated separately.

Microbiological determination of total and available methionine

The procedures of Ford (1962) with *Strep. zymogenes* NCDO 592 were followed. In the method for estimation of total methionine, the protein concentrate (containing 100 mg N) is hydrolysed with dilute hydrochloric acid, neutralized and suitably diluted. Measured portions of the hydrolysate are incubated for 48 h at 37° with the

organism in the presence of a synthetic medium containing all the necessary growth factors except methionine, which has to be contributed by the test hydrolysate. The growth of the organism is determined turbidimetrically. The amount of methionine in the hydrolysate is estimated by comparison with a standard growth response obtained by adding different amounts of methionine to the medium in place of test hydrolysate. For estimation of available methionine, Ford (1962) recommends that the same amount of test protein be predigested for 2 h at 56° after addition of 1 ml of 1% papain solution and 20 ml of buffer, i.e. at a papain concentration of 0·05%. Portions of the diluted digests are then incubated with the medium and organism, and its methionine content is estimated as before.

In our experiments the concentration of K_2HPO_4 in the basal medium recommended by Ford (1962) was increased by 50% in order to achieve a more nearly rectilinear response with standard methionine at the high concentrations (J. E. Ford, personal communication). Growth of the organism was measured by determining optical density with a Lumetron Model 401 A colorimeter (Photovolt Corporation, New York) and a 580 nm filter. As detailed below, conditions of papain predigestion were modified, but all other conditions used were those recommended by Ford (1962).

In preliminary experiments we investigated the effect of using different papain preparations and of varying their concentration. Different volumes of a 1% solution of crude or purified papain in buffer were used to provide 0–8 ml, and buffer was added to give a total volume of 22 ml for each 100 mg N from the test material. The concentration of papain in the digest thus ranged from 0 to 0·36%. In a few experiments crystalline papain was used at concentrations up to 0·023%. During the standard 2 h incubation the tubes were shaken every 10 min. Methionine contributed by papain itself was estimated with an enzyme blank at ten times the concentration used to prepare the test protein digest, and a correction was made when calculating the amount of methionine in the protein concentrates.

Finally, single digests of each of the commercial protein concentrates studied in experiments with chicks were prepared with either 0·09 or 0·36% crude papain and assayed for available methionine in one experiment. Single (MM and FM) or duplicate (WM) acid hydrolysates were prepared and assayed in a number of experiments for total methionine.

No attempt was made in the work now reported to study the variability of the microbiological assays, since this aspect is being investigated under the aegis of the Agricultural Research Council. However, from an earlier series of results subjected to statistical analysis (Carpenter, Miller & Morgan, unpublished) it would seem that a difference of approximately 10% could be considered significant.

RESULTS

Experiments with chicks

Preliminary Expts 1–4. Expt 1 was conducted to test the procedure of Guttridge *et al.* (1961) under our conditions. The results obtained are shown in Table 1. Supplementation of the soya-bean basal diet with cystine did not produce any change in rate

of live-weight gain, but did improve FCE. This response was small and only statistically significant ($P < 0.05$) when 0·3% cystine or more was added. Maximum response to cystine was obtained at the 0·3% level of addition. Supplementation with methionine in the presence and absence of 0·4% cystine brought about increased weight gain and FCE. In the presence of excess cystine most of the response to methionine was obtained with the lowest level used. The higher levels of methionine supplement appeared to bring about slight increases in both response metameters but the differences were not statistically significant ($P < 0.05$). We concluded that under our conditions it was not possible to use this procedure for the assay of cystine plus methionine. The use of groundnut in place of soya-bean meal to construct a basal diet

Table 1. *Effect of supplementing three basal diets with cystine and methionine upon the weight gain and food conversion efficiency of chicks from the 10th to the 20th day of age*

Expt no. and basal diet	Supplement (% of diet)		Daily weight gain (g/chick)	Food conversion efficiency
	L-cystine	DL-methionine		
1, soya-bean basal	None	None	5·21	0·319
	0·20	None	4·95	0·346
	0·30	None	5·06	0·365
	0·40	None	5·18	0·365
	0·40	0·06	8·88	0·493
	0·40	0·08	9·36	0·496
	0·40	0·10	9·70	0·524
	None	0·25	9·36	0·504
SE (df)	—	—	±0·30 (35)	±0·013 (7)
2, groundnut basal 1	None	None	2·77	0·301
	None	0·10	8·48	0·509
	None	0·20	9·03	0·520
	None	0·30	9·13	0·537
SE (df)	—	—	±0·20 (15)	±0·012 (3)
3, groundnut basal 2	None	None	3·31	0·297
	0·100	None	3·21	0·309
	0·175	None	3·21	0·309
SE (df)	—	—	±0·20 (6)	±0·023 (6)

Table 2. *Effect of supplementing groundnut basal diet 1 with methionine and oxidized casein upon the weight gain and food conversion efficiency of chicks from the 10th to the 20th day of age*

Diet code		L-methionine supplement (% of diet)	Daily weight gain (g/chick)		Food conversion efficiency	
(i)	(ii)		(i)	(ii)	(i)	(ii)
A	—	None	3·54	—	0·344	—
B	—	0·0200	4·59	—	0·388	—
C	G	0·0350	6·12	4·63	0·448	0·406
D	H	0·0613	8·27	6·55	0·512	0·484
E	F	0·40	9·61	10·13	0·548	0·616
SE (35 df)			±0·19		±0·010	

(i) Without supplement of oxidized casein.
(ii) With supplement of 5·4% crude protein from oxidized casein, 0·08% L-tryptophan and 0·35% L-tyrosine.

more deficient in methionine and thereby to increase the potential range of an assay for methionine only was investigated in Expts 2–4.

The results of Expts 2 and 3 are shown in Table 1. Supplementation of groundnut basal diet 1 with 0·1% DL-methionine brought about large increases in live-weight gain and FCE. Further supplementation with up to 0·3% methionine elicited slight additional responses, but the differences were not statistically significant ($P > 0·05$). Cystine supplementation of groundnut basal diet 2, which already contained free cystine, was without effect.

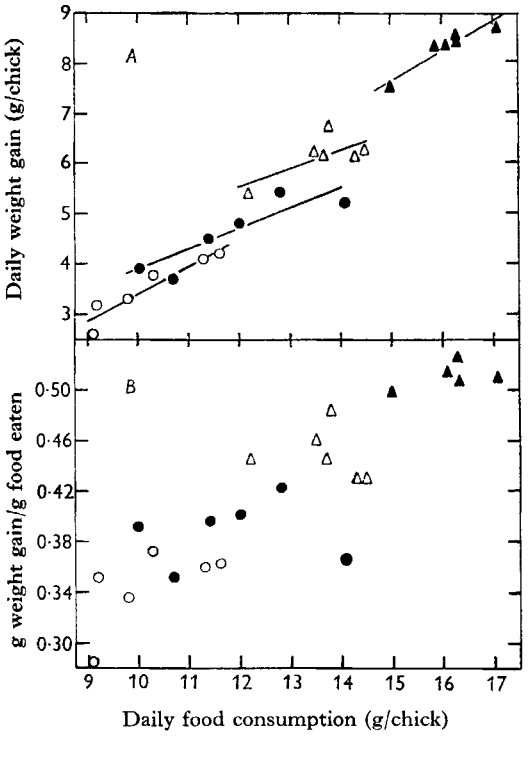

Fig. 1

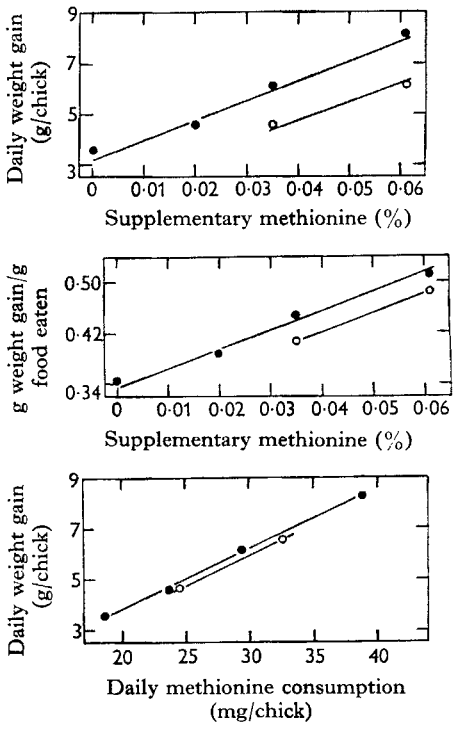

Fig. 2

Fig. 1. Expt 4. Relationship between (*A*) weight gain, or (*B*) weight gain/g food eaten, and food consumption of replicate groups of chicks. ○, basal diet; ●, basal diet+0·020% methionine; △, basal diet+0·035% methionine; ▲, basal diet+0·0613% methionine.

Fig. 2. Expt 4. Response of chicks to methionine supplementation of the groundnut basal diet in the presence (○–○) or absence (●–●) of 6% oxidized casein contributing 5·4% additional crude protein.

In Expt 4 the addition to groundnut basal diet 1 of L-methionine supplements up to 0·0613% of the diet (Table 2, diets B, C and D) brought about a marked improvement in live-weight gain and FCE. The addition of oxidized casein to the diets with 0·035 and 0·0613% methionine supplements caused a significant ($P < 0·05$) depression in weight gain and FCE. In contrast, addition of oxidized casein to the diet supplemented with an excess of methionine (0·4%) brought about further improvement in

rate of weight gain and FCE. The depression observed on addition of oxidized casein, therefore, is related to the deficiency of methionine and not to a generalized toxicity of the oxidized preparation.

In a detailed statistical analysis of the results, the weight gains of replicate groups of chicks on any one diet were found to be closely correlated with food consumption (Fig. 1*A*). In contrast, FCE for any one diet was independent of the variations in food consumption (Fig. 1*B*). The addition of 5·4% crude protein from oxidized casein produced a similar decrease in response at both limiting levels of supplementary methionine. This is shown diagrammatically in Fig. 2 for each of the three metameters used to measure response. The slope of the line joining responses obtained in the presence of oxidized casein was not significantly different from that of the standard response line. Therefore parallel lines have been drawn to fit the figures as shown in Fig. 2, and the effect of additional oxidized casein is given in terms of methionine by the horizontal distance between these lines. The amount of additional methionine (with its standard error) that would be required to increase the response in the presence of oxidized casein to that of the standard response was: for method 1, 0·0196 ± 0·0021%; for method 2, 0·0114 ± 0·0028%; and for method 3, 1·17 ± 0·33 mg/chick daily. The mean daily food intake on diets containing oxidized casein was 12·5 g/chick, so that the value of 1·17 mg methionine approximates to an increase in the methionine content of the diet of 0·0093%. The distorting effect of oxidized casein appears therefore to be greater when the first method of calculation is used than when food consumption is taken into account in either the response or the dose metameters, as in the second and third methods of calculation, respectively.

Assays of protein concentrates. Expts 5–10. All the chick experiments were carried out without any deaths or mishaps. The diet means for Expts 5 and 6 are given in Table 3, and typical patterns of response (method 2) to standard methionine and test-protein supplements are shown in Fig. 3. With one meal, FM 4, the only shell-fish meal, the response obtained failed significantly to intersect with the others, and no estimate of available methionine content was made. The remaining results were analysed statistically to estimate the available methionine content of the test proteins by methods 1 and 2, and the estimates are also given in Table 3.

In Expts 7 and 8 the response to standard methionine deviated significantly from rectilinearity, although response patterns to test proteins were normal. All these results were rejected, and the experiments were repeated (9 and 10) with the results given in Table 3. The standard response in the repeated experiment was rectilinear, but weight gain and FCE of chicks on the unsupplemented basal diet were considerably better than those obtained in any previous experiment. Statistical analysis of the weight gains (method 1, p. 253) showed a significant interaction between responses obtained in Expts 9 and 10, mainly because in Expt 9 the response lines for two cod preparations failed to intersect the others. Therefore only the results of Expt 10 were used in calculating available methionine by method 1. When FCE was used as the response, there was no significant interaction between experiments, and the combined values were used to calculate available methionine by method 2 (p. 253), with the results given in Table 3.

Table 3. *Mean weight gain and food conversion efficiency (FCE) of chicks in Expts* 5, 6, 9 *and* 10, *together with the estimated available methionine (g/16 g N) of the protein concentrates calculated in three ways*

Expt no.	Supplement	% crude protein from supplement (i)	% crude protein from supplement (ii)	Daily weight gain (g/chick)* (i)	Daily weight gain (g/chick)* (ii)	FCE* (i)	FCE* (ii)	Methionine available to the chick calculated by: Method 1†	Method 2†	Method 3†
5 and 6	—	—		3·70		0·333		—	—	—
	0·02 % L-methionine	—		5·03		0·395		—	—	—
	0·035 % L-methionine	—		6·60		0·437		—	—	—
	0·0613 % L-methionine	—		8·89		0·496		—	—	—
	MM 10	2·38	4·17	4·77	6·07	0·386	0·444	0·7 (±0·07)	0·9 (±0·08)	0·9
	MM 16	2·59	4·54	4·77	5·93	0·393	0·436	0·6 (±0·06)	0·8 (±0·07)	0·8
	FM 4	1·78	3·12	5·98	6·47	0·446	0·479	—	—	—
	FM 6	1·25	2·18	6·16	8·33	0·443	0·513	2·5 (±0·15)	3·1 (±0·14)	3·3
	WM 7	2·14	3·75	5·31	5·94	0·409	0·450	0·8 (±0·08)	1·2 (±0·09)	1·2
	WM 9	1·15	2·00	5·12	7·08	0·403	0·476	2·0 (±0·15)	2·5 (±0·16)	2·5
9 and 10	None	—		5·58		0·384		—	—	—
	0·02 % L-methionine	—		7·38		0·430		—	—	—
	0·04 % L-methionine	—		8·54		0·467		—	—	—
	0·06 % L-methionine	—		9·53		0·500		—	—	—
	WM 1	1·57	3·13	6·93	8·48	0·423	0·480	1·5 (±0·31)‡	1·5 (±0·24)	1·4
	WM 3	1·14	2·28	7·03	9·23	0·434	0·485	2·0 (±0·42)‡	2·2 (±0·35)	2·2
	WM 13	1·02	2·03	7·29	8·48	0·440	0·479	2·0 (±0·45)‡	2·4 (±0·37)	2·3

(i) and (ii) first and second levels of addition of test protein respectively.

* The residual variability was combinable for all four experiments in both daily weight gain and FCE. The standard error of each figure was ±0·24 g/chick daily or ±0·0077 g gain/g food respectively. Each figure is the mean response over 10 days for six units, each of three chicks.

† Method 1, weight gain *v.* % added methionine; method 2, FCE *v.* % added methionine; method 3, weight gain *v.* g available methionine eaten. The values given in parentheses are the standard errors of the estimates of available methionine and cannot be used for assessing differences between meals.

‡ Result from Expt 10 only.

For both sets of Expts, 5–6 and 9–10, a third estimate of available methionine was made from the relationship between weight gain and methionine consumption (method 3). This was not done statistically, but by 'reading off' from a graph of the standard response. This graphical method of calculation gives evidence of neither validity nor fiducial limits to the estimates of available methionine. The results were in close agreement with those calculated statistically with FCE as the measured response, and both these methods gave results about 24% greater than those obtained by the first method of calculation, i.e. weight gain *v.* percentage of supplementary methionine (Table 3).

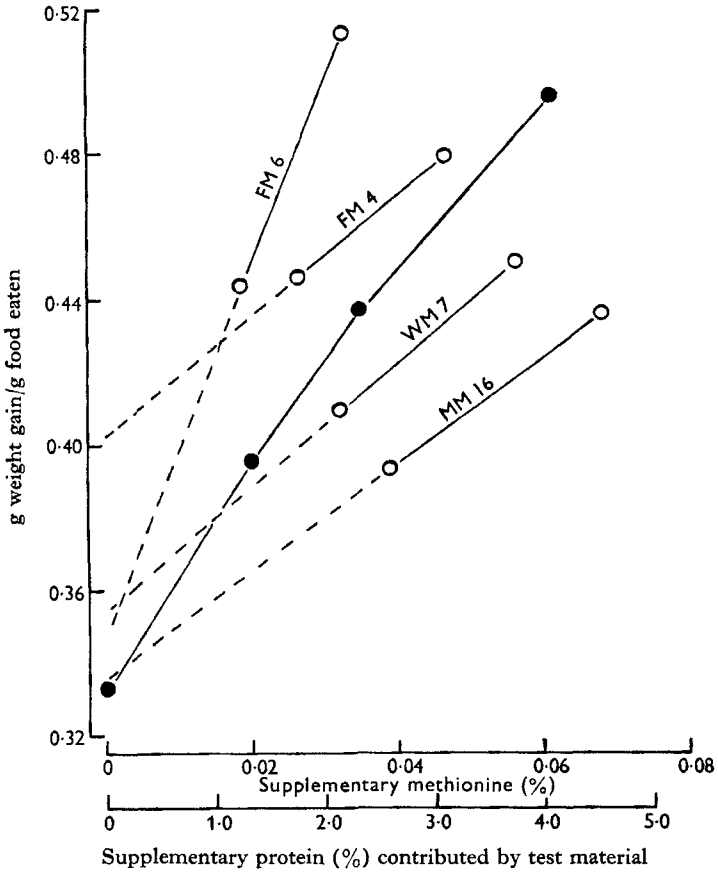

Fig. 3. Expts 5 and 6. Typical responses of chicks to supplementation of the groundnut basal diet with either test materials (see p. 250) (○–○) or methionine (●–●). The lines joining the two levels of test protein are extrapolated to zero addition to indicate the failure of the response to FM4 to intersect with the responses to the standard and the other test proteins.

Microbiological assays

Preliminary experiments. Effect of conditions of papain predigestion on estimation of available methionine. The results of a number of experiments in which different concentrations of crude papain were used are summarized in Fig. 4. Increasing

concentrations of papain up to 0·36% of the digest gave increasing estimates of available methionine for a number of protein concentrates. Changes were greatest over the lower range of papain concentration and appeared to be approaching maximum values at the highest concentration used.

The results of an experiment in which purified and crude papain were compared are also given in Table 4. Purified papain gave higher results than an equal concentration (0·045%) of crude papain. When the two papain preparations were used at equal-activity concentrations, similar estimates of available methionine were obtained.

In another experiment, predigestion in a 0·023% solution of crystalline papain gave results equivalent to the use of 0·36% crude papain.

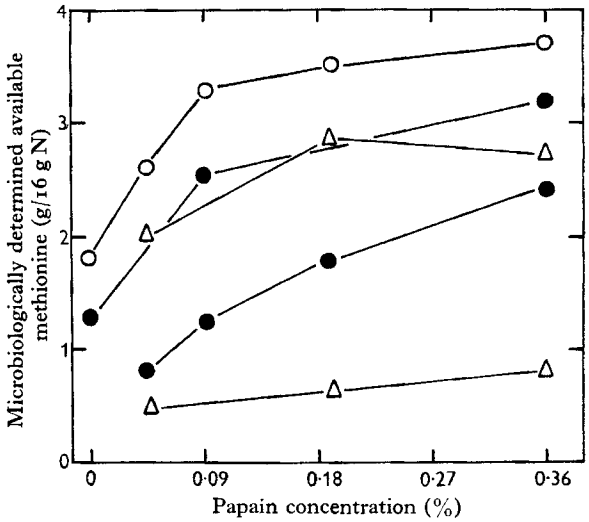

Fig. 4. Effect of concentration of crude papain in the preliminary digestion period upon the values obtained with *Strep. zymogenes* for available methionine. O–O, freeze-dried cod fillets; ●–●, commercial fish meals; △–△, commercial whale-meat meals.

Table 4. *Effect of papain activity on the determination of available methionine in papain digests of protein concentrates by the* Streptococcus zymogenes *assay*

Papain concentration ... (g/100 ml digest)	Purified*	Crude*		
	0·045	0·045	0·195	0·360
Papain activity ... ... (units/100 ml digest)	0·057	0·013	0·056	0·104
Protein concentrate	Available methionine (g/16 g N)			
MM 16	0·47	0·36	0·49	0·53
WM 7	0·63	0·47	0·62	0·74
MM 10†	0·67	0·60	0·61	0·84
WM 13	2·72	1·98	2·87	2·68
Cod muscle	3·37	2·57	3·51	3·76

* The purified papain was estimated to have 4·4 times the proteolytic activity of the crude papain (see p. 250). The units are those defined by Anson (1938–9).

† Mean values for duplicate papain digests assayed in separate experiments. The values for the other materials were obtained in a single experiment.

Microbiological assays of protein concentrates. The results for total, and for available methionine after digestion in either 0·09% or 0·36% crude papain solution, are given in Table 5. As in the preliminary experiments, the use of the higher concentration of papain resulted in a higher available methionine value. Even these higher figures for available methionine gave a range from 35 to 86% of the corresponding values for total methionine in the commercial meals.

Table 5. *Methionine available to the chick, together with microbiological estimates of total and available methionine and FDNB-available lysine of commercial protein concentrates and laboratory preparations of cod muscle*

Protein concentrate	Methionine available to the chick (g/16 g N)	Total methionine, *Strep. zymogenes* (g/16 g N)	Methionine available to *Strep. zymogenes* after pretreatment with 0·09% crude papain (g/16 g N)	Methionine available to *Strep. zymogenes* after pretreatment with 0·36% crude papain (g/16 g N)	FDNB-available lysine (g/16 g N)
Commercial protein concentrate					
FM 6	3·1	3·1	2·2	2·7	6·6
WM 9	2·5	2·7	1·8	2·1	6·7
WM 13	2·4	3·5	2·3	2·8	6·8
WM 3	2·2	3·2	1·5	1·8	5·9
WM 1	1·5	2·5	1·0	1·1	4·9
WM 7	1·2	2·8	0·5	1·0	3·5
MM 10	0·9	2·0	0·8	0·9	3·6
MM 16	0·8	1·6	0·5	0·5	2·7
Cod muscle preparation					
Cod 23	3·4	3·6	3·4	3·6	8·8
Cod 24	3·2	3·7	2·5	3·3	9·0
Cod 27	3·2	3·5	1·9	2·6	9·0
Cod 26	2·9	3·6	2·0	2·9	8·2
Cod 28	2·8	3·6	1·3	1·9	8·4
Cod 35	2·2	3·6	1·6	2·0	5·9

FDNB, fluorodinitrobenzene procedure.

DISCUSSION

Chick assays

Four procedures have been reported in the literature for the direct estimation of sulphur amino acids available to either the rat or chick. Of these, only that of Guttridge *et al.* (1961) gave results suitable for statistical analysis. In the other procedures, the test proteins were usually assayed at one level only. The danger of doing this is exemplified by the anomalous response obtained with FM 4 in our work, and also by the data of Schweigert & Guthneck (1954), which showed that, when four proteins were tested at a higher level of supplementation than routinely used by these workers, the apparent availability of methionine was always greater. The conventional tests of validity can be made by statistical methods, but it is more difficult to detect errors resulting from a consistent bias. Such an effect may exist in our experiments, since the test-protein supplement contributes amino acids other than that being assayed. The procedures of Grau & Almquist (1945) and of Ousterhout, Grau & Lundholm (1959) were designed to keep all diets isonitrogenous. However, even this does not

necessarily prevent modification of growth response due to antagonisms brought about by varying proportions of amino acids. Guttridge *et al.* (1961) could show no effect upon growth response when an amino acid mixture devoid of sulphur amino acids was tested as a supplement to their diet, and therefore concluded that simple supplementation of the basal diet with the test protein, without maintaining nitrogen or amino acid balance constant, gave reliable results.

Thus the procedure of Guttridge *et al.* (1961) appeared to be the most satisfactory and was tried in Expt 1 of our work. The lack of response to cystine supplementation of the soya-bean basal diet was in contrast with the results of Guttridge, but the response to methionine in the presence of excess cystine was similar in the two investigations, being linearly related to the logarithm of the concentration of supplementary methionine. Guttridge (1962) used an underheated soya-bean meal, whereas the meal used in our work was adequately heat-treated as judged by the urease test. Evans & McGinnis (1946) have noted that the growth of chicks fed on raw soya-beans supplemented with methionine was not as good as that obtained with optimally heated soya-beans plus methionine. The use of raw or underheated soya-beans containing factors inhibitory to chick growth was considered to be undesirable.

Replacement of soya-bean meal by groundnut extended the useful range of response to methionine and also permitted a higher protein level in the diet, thereby reducing the proportional changes in protein level upon supplementation with test materials. Growth rates approaching the maximum obtainable with this relatively slow-growing type of chick were achieved when the basal diet was adequately supplemented with methionine. The slight increase in growth rate noted with oxidized casein plus excess methionine indicated the possibility that another amino acid became limiting when the deficiency of methionine was corrected. In subsequent work, a supplement of threonine induced a further slight response in chicks fed on a diet containing 20% groundnut protein and adequately supplemented with lysine and methionine (Milner & Carpenter, unpublished). Slight inadequacy of threonine for maximum growth is not expected to influence the validity of methionine assays, since these are conducted within the range of linear response to methionine. The response to arithmetical increments of methionine up to 0·08% of the groundnut basal diet was linear and permitted statistical analysis by the slope–ratio technique. The same batch of groundnut meal was used for all the experiments reported here. However, in some subsequent assays a second batch (X. 210) has been used and has given essentially the same pattern of response. In both batches a similar level of aflatoxin was present, as determined fluorimetrically. This level did not appear to have any detrimental effect, since meal X. 210, properly supplemented with amino acids and supplied as sole source of protein to chicks, was found to support the same rapid and efficient growth that resulted from a practical diet containing a variety of protein sources (Milner & Carpenter, unpublished).

In a similar assay for available lysine with chicks (Carpenter *et al.* 1963), zein in the basal diet was replaced by test proteins on an equal-nitrogen basis. A similar procedure could not be used in the methionine assays, since there is no suitable food protein devoid of methionine. The use of amino acids or oxidized protein as a source of

replaceable nitrogen was not considered possible in routine assays, on the grounds of expense. Therefore, as in the procedure of Guttridge *et al.* (1961), the test proteins were incorporated in the diets without maintaining constant nitrogen or amino acid balance. Such a procedure must be considered a compromise between the ideal conditions and those to be achieved practically, and the results obtained may be biased as a result of not maintaining constant all factors other than methionine. Certainly it has been demonstrated that an additional 5·4% protein from oxidized casein modified the response. The methionine sulphone present in the oxidized casein does not appear to show anti-methionine activity for either the rat (Bennett, 1941; Njaa, 1962) or the chick (Miller & Carpenter, unpublished). The depression of growth may have resulted from the increased protein level, which exaggerated the methionine deficiency. A similar imbalance has been demonstrated on supplementing a lysine-deficient diet with a mixture of amino acids (Fisher, Griminger, Leveille & Shapiro, 1960). As shown in Table 4, the levels of supplementary protein supplied by test materials in assays were much less than those supplied by oxidized casein. Therefore, any depressant effect of protein itself in the assays is expected to be correspondingly less and the error introduced to be less than other experimental errors inherent in biological assays. Of the three methods of calculating available methionine, that using FCE as the response is preferred, since this appears to overcome partially the depressant effects of additional protein and the results can be statistically analysed by a routine procedure. Experiments are in progress to compare results obtained by this procedure with those obtained under conditions more closely approaching constant amino acid balance.

Recently, Combs (1964) has briefly reported the application of a methionine assay with chicks on a soya-bean basal diet, and involving a method of calculation that takes food consumption into account, to a series of menhaden and soya-bean meals.

Microbiological assays

The degree of enzymic hydrolysis to which the test protein concentrate is subjected in the papain predigestion is extremely important in determining the available methionine values, as has been found both in this and other studies (Carpenter, Lea & Parr, 1963; Ford, 1964). Thus, to obtain consistent results of the correct order, the conditions of papain predigestion must be closely defined. For example, in experiments in which different papain preparations were compared, similar results were obtained only when the preparations were used at concentrations providing equivalent proteolytic activity.

The most appropriate concentration of papain activity will be that giving results most closely predicting the available amino acid content for higher animals. As will be seen by comparison with results obtained with the chick, this concentration, for the materials used by us, was found to be one providing 0·10 units enzyme activity/100 ml digest, equivalent to 0·36% of our crude papain. At the same time it should be stressed that, if a completely different type of protein-containing material were under investigation by the microbiological method, it would then be necessary to confirm

that the correlation between availability to *Strep. zymogenes* and to higher animals is still valid. Other problems, such as interference from turbidity contributed by large amounts of starch, may be encountered with certain foods.

Correlation of chick estimates of available methionine with laboratory analyses

In Table 5 estimates of total and available methionine by *Strep. zymogenes* and of FDNB-available lysine are presented along with estimates of methionine available to the chick as calculated by the preferred procedure (method 2). The materials listed include preparations of cod muscle (but excluding samples containing added carbohydrates) that were being used in a study on the effects of heating. Detailed results of this investigation are being submitted for publication.

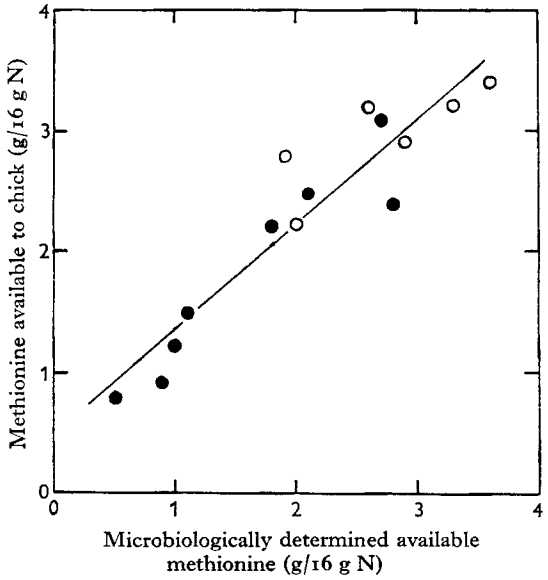

Fig. 5. Relationship between values obtained biologically (chick) and those obtained microbiologically (*Streptococcus zymogenes*) for available methionine in protein concentrates. The response metameter used for calculating the results of the chick assays was food conversion efficiency. The microbiological values were obtained by assay with *Strep. zymogenes* after predigestion with 0·36% crude papain. ○, laboratory preparations of cod muscle; ●, commercial meat, fish and whale-meat meals. The least squares regression line, 'chick value = 0·49+0·87 (microbiological value)', is also shown.

Comparison of the chick estimates with total methionine content by microbiological assay indicates that availability ranged from 42 to 98% in the materials selected. This demonstrates the inadequacy of total methionine as a guide to the quantity of methionine available to animals in foods or feeding-stuffs of the type that we have been investigating. Similarly, Guttridge & Lewis (1964*b*) have reported available methionine values for the chick of three meat meals, one of which (MM 16) was included in our assays. For each meal their available values are approximately half the corresponding total methionine content. Our values for MM 16 calculated by both methods 1 and 2 are higher than the corresponding values of Guttridge & Lewis (1964*b*), but the

conclusion from each study is that a considerable proportion of the methionine present is unavailable to the chick.

The total values now reported are in some instances different from values obtained by iodometric and chromatographic techniques on the same samples (Miller & Carpenter, 1964). Neither the cause of this discrepancy nor the correct value is known. Only the microbiological value has been reported here but none of the other series of total values gave any closer agreement with chick estimates of available methionine.

Estimation of available methionine by treatment with 0·09% crude papain followed by *Strep. zymogenes* assay significantly underestimated ($P < 0{\cdot}001$) the chick value by an average 28%. The microbiological values obtained after treatment with 0·36% crude papain were on average 9% lower than the chick estimate but this did not constitute a significant bias. Further the least squares equation predicting the chick value from the *Strep. zymogenes* assay of the 0·36% crude papain digest,

$$\text{chick value} = 0{\cdot}49 \pm 0{\cdot}87 \text{ (microbiological value) (Fig. 5; } r = 0{\cdot}93; P < 0{\cdot}001),$$

had a residual standard deviation of $\pm 0{\cdot}33$. This is not appreciably better than the residual standard deviation of $\pm 0{\cdot}41$ obtained when the chick value is equated directly with the 0·36% crude papain–*Strep. zymogenes* value. From these results, demonstrated graphically in Fig. 5, it is clear that the assay of available methionine with *Strep. zymogenes* under these conditions of predigestion provides a useful method of predicting the availability of this amino acid to the chick in materials of the types that we have used.

The chick methionine values also correlated closely ($r = 0{\cdot}96$; $P < 0{\cdot}001$) with the FDNB-available lysine contents of the commercial meals (Boyne *et al.* 1961) and the cod samples, treated as a single series. This correlation is in agreement with that emerging from the values presented by Boyne *et al.* (1961), which show the FDNB-available lysine values of commercial samples of fish, meat and whale-meat meals treated as a single series to correlate with NPU for the rat, although under the conditions of this animal test the sulphur amino acids would usually be limiting. Similar correlations between microbiologically available levels of different amino acids in whale-meat meals have been reported (Ford, 1962). In part such correlations may result from the nature of the raw materials used in the manufacture of animal protein concentrates; for example, muscle tissue is rich in both lysine and methionine, whereas connective tissue is a poor source of both. Further, excessive heat treatment of fish meals or pork muscle has been shown to reduce the microbiological availability of several amino acids (Ford, 1962), to reduce the FDNB-available lysine (Carpenter, Morgan, Lea & Parr, 1962; Donoso, Lewis, Miller & Payne, 1962) and to decrease the nitrogen and sulphur digestibility (Donoso *et al.* 1962). These correlations between available levels of different amino acids suggest the possibility that either the chemical estimation of available lysine or the microbiological procedure with *Strep. zymogenes* will not only assess the particular amino acid assayed, but also give some indication of the overall value of an animal protein concentrate.

SUMMARY

1. Chicks, fed from 10 to 20 days of age on a basal diet containing 40% groundnut meal, dried whey, fat, oat husks, glycine, cystine, lysine, oxytetracycline, vitamins, minerals and maize starch, showed a rectilinear relationship of response (live-weight gain or food conversion efficiency) to dietary supplements of up to approximately 0·08% L-methionine.

2. The addition of 5·4% crude protein from oxidized casein depressed live-weight gain and to a lesser extent FCE of chicks receiving limiting levels of methionine.

3. In the selected assay procedure, test materials were added, mainly at the expense of maize starch, at two levels chosen to contribute estimated levels of 0·025 and 0·05% available methionine to the diet, and such that the supplementary protein levels were well below the 5·4% level tested with oxidized casein. The responses were compared with those to pure L-methionine by the slope–ratio method.

4. Potencies of commercial meat, fish and whale-meat meals in terms of methionine were greater when differences in food consumption were taken into account, and these higher values may also have been less affected by systematic errors due to substances other than methionine contributed by the test materials. With FCE as the response, methionine potency ranged from 0·8 to 3·1 g/16 g N in eight samples of commercial meals.

5. Laboratory estimates of available methionine by the *Streptococcus zymogenes* method were found to be influenced by the concentration and the activity of the papain used in the predigestion stage.

6. Chick estimates of available methionine were closely correlated ($r = 0{\cdot}93$; $P < 0{\cdot}001$) with available methionine measured by *Strep. zymogenes* after predigestion of the meals in 0·36% crude papain (British Drug Houses Ltd) in buffer solution. When the microbiological values were taken to be equal to the chick values, the standard deviation was $\pm 0{\cdot}41$.

7. Chick estimates of available methionine were also closely correlated ($r = 0{\cdot}96$; $P < 0{\cdot}001$) with FDNB-available lysine.

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