Availability of sulphur amino acids in protein foods

4.* Effect of heat treatment upon the total amino acid content of cod muscle

By E. L. MILLER
School of Agriculture, University of Cambridge
AND A. W. HARTLEY
Central Laboratory, Spillers Limited, Cambridge
AND D. C. THOMAS
Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford

(*Received* 26 *April* 1965—*Accepted* 22 *July* 1965)

With the advent of ion-exchange chromatography, and more recently with the automation of the technique, amino acid analysis of feed components has become almost routine in any work connected with the nutritive value of protein components in diets for man or non-ruminant livestock. When a wide variety of protein foodstuffs is considered, the essential amino acid composition is related to the protein quality (Block & Mitchell, 1946–7; Oser, 1951). However, attempts have been made to predict from the amino acid composition the protein quality of a restricted range of processed materials such as meat meals and fish meals (Wiechers & Laubscher, 1962; De Vuyst, Vervack, Vanbelle, Arnould & Moreels, 1964*a*, *b*) and it is the validity of this application that is in doubt.

Heat treatment of cod muscle has been shown to reduce greatly the quality of the protein for both rats and chicks (Carpenter, Ellinger, Munro & Rolfe, 1957; Carpenter, March, Milner & Campbell, 1963; Miller, Carpenter & Milner, 1965). The purpose of the study now presented was to determine whether heat treatment of cod muscle also caused considerable changes in total amino acid composition, i.e. amino acids recovered after acid hydrolysis. Our finding has been that the composition of two preparations of cod muscle, heated in different ways so as to produce large reductions in protein quality, differed from the composition of the control material only by relatively small changes in a few amino acids. Chemical scores calculated by using two different reference amino acid patterns (FAO, 1957; Bender, 1958) very much underestimated the extensive differences in protein quality between the materials.

Particular attention was directed to methionine since three different methods of analysis had previously given discrepant results (Miller *et al.* 1965), and inspection of the literature has shown a similar discrepancy between the same three methods in assessing changes in total methionine content as a result of heating protein (Lea & Hannan, 1950; Ford, 1962; Donoso, Lewis, Miller & Payne, 1962). Both Donoso *et al.*

* Paper no. 3: *Br. J. Nutr.* (1965), **19**, 547.

(1962) and Miller *et al.* (1965) found that when methionine was determined by performic acid oxidation of the protein, followed by acid hydrolysis and chromatographic separation of methionine sulphone, results obtained with heated materials were lower than with control materials. To guard against possible artifacts resulting from the method of analysis, one laboratory determined the amino acid composition of the unoxidized protein and another laboratory carried out the determination on the protein oxidized with performic acid.

EXPERIMENTAL

Materials

Three preparations of cod muscle were selected from a larger series (Miller *et al.* 1965) to represent extremes of protein quality. The materials were: C23, control freeze-dried cod muscle; C35, the control material heated for 27 h at 116° and 13·8% moisture in a sealed container under an atmosphere of N_2; C25, 90 parts dry matter of control material mixed with 10 parts by weight of D-glucose and heated for 27 h at 85° and 13·4% moisture in a sealed container under an atmosphere of N_2. Full details of the preparation of these materials were given in the preceding paper of this series (Miller *et al.* 1965).

Analytical procedures

Nitrogen content

Nitrogen was determined by the Kjeldahl method using a macrodigestion procedure (Association of Official Agricultural Chemists, 1960) followed by semi-micro distillation of the ammonia produced into 1% boric acid containing a mixed indicator and titration with 0·014N-HCl (Ma & Zuazaga, 1942).

Total amino acids

Laboratory A. A sample containing approximately 12 mg nitrogen was hydrolysed by refluxing with 6 N-HCl for 24 h in an oil-bath at 130°. The ratio of acid volume to sample weight was kept as near as possible to 200 ml 6 N-HCl/8 mg nitrogen (Dustin, Czajkowska, Moore & Bigwood, 1953). The hydrolysate was made up to 500 ml, 1 ml was measured into a 5 ml beaker, 0·1 μmole norleucine was added as an internal standard and the whole was taken to dryness in a vacuum desiccator over $CaCl_2$ and CaO. The amino acids were redissolved in 1 ml 0·1N-HCl, quantitatively transferred to a column of Chromobeads Type A resin and the eluate was analysed with a Technicon AutoAnalyser (both from Technicon Instruments Co. Ltd, Hanworth Lane, Chertsey, Surrey). The buffer solutions and procedure were those of Piez & Morris (1960), except that the pH 2·9 buffer was made with 5% (v/v) aqueous methanol to improve the resolution of threonine and serine. Ninhydrin colour factors were calculated from a chromatogram of a standard mixture of pure amino acids, with norleucine present as the reference standard. No corrections were made for possible losses, even for threonine and serine, that might have occurred during acid hydrolysis.

Laboratory B. A quantity of material containing 5–7 mg nitrogen was oxidized and hydrolysed according to the procedure of Bidmead & Ley (1958). After reducing the hydrolysate to near dryness by rotary evaporation norleucine was added as an internal standard so that when the hydrolysate was made up to 50 ml the concentration of

norleucine was 0·4 μmole/ml. The amino acids in 0·5 ml of the hydrolysate were chromatographed on a column of Chromobeads Type A resin and the eluate was analysed with a Technicon AutoAnalyser. The compositions of the buffer solutions which differed from those described by Piez & Morris (1960) were as follows.

(1) Stock buffer solution: 73·55 g $Na_3C_6H_5O_7.2H_2O$, 125 ml 2N-NaOH, 50 ml of a solution of 100 g BRIJ 35 detergent (Honeywill and Stein Ltd, London, W1) dissolved in 200 ml of water, and 25 ml thiodiglycol; the solids were dissolved in water and the whole was made up to 4500 ml.

(2) Buffer solutions pH 2·5, pH 3·0, pH 3·8: 900 ml stock buffer were titrated with 6 N-HCl to the required pH, diluted to 1 l., and the pH was readjusted.

(3) Buffer solutions pH 2·5, pH 3·0 containing methanol: prepared as in (2) above but 55 ml methanol were added before diluting to volume.

(4) Buffer solution pH 5·0: 36·78 g $Na_3C_6H_5O_7.2H_2O$, 62·5 ml 2 N-NaOH, 87·68 g NaCl and 25 ml of the BRIJ 35 solution were dissolved in 2250 ml water, titrated to pH 5·0 with 6 N-HCl, made up to 2500 ml with water, and the pH was re-adjusted to 5·0.

The volumes of the buffers used in the preparation of the gradient-elution device are given in Table 1.

Table 1. *Volumes of buffers* used in the preparation of the gradient-elution device (ml)*

Chamber	pH 2·5	pH 2·5 with methanol	pH 3·0	pH 3·0 with methanol	pH 3·8	pH 5·0
1	75	—	—	—	—	—
2	—	75·0	—	—	—	—
3	—	—	—	75·0	—	—
4	—	—	—	75·0	—	—
5	—	—	—	—	70	5
6	—	—	6	—	9	60
7	—	—	—	—	—	75
8	—	—	—	—	—	75
9	—	—	—	—	—	75

* See above.

Ninhydrin colour factors were calculated from a chromatogram of a standard amino acid mixture which had been subjected to the same process of oxidation, hydrolysis and ion-exchange chromatography.

Laboratory C. Cystine and methionine were determined by performic acid oxidation, hydrolysis and chromatographic separation of cysteic acid and methionine sulphone on a manually operated 40 cm × 1·46 cm column of Zeo-Karb 225 ion-exchange resin (the Permutit Co. Ltd, London, W4), the amino acids being eluted with pH 2·8, 0·5 M citrate buffer as previously described (Miller & Carpenter, 1964). Recoveries of cystine and methionine added to the control cod muscle preparation and carried through all stages of the procedure were 99% and 93% respectively. The methionine values were therefore multiplied by the factor 1·075. Lysine was determined in hydrolysates of unoxidized protein by manual operation of a 15 cm resin

column using the conditions previously described. Results for each amino acid were calculated by reference to standard curves of ninhydrin colour developed with graded levels of the appropriate amino acid. Tryptophan was determined in $Ba(OH)_2$ hydrolysates (Greene & Black, 1944) by modification (Miller, 1965, unpublished procedure) of the method of Horn & Jones (1945) based on the colour reaction with *p*-dimethylaminobenzaldehyde. A mean recovery of 94% was obtained for tryptophan added to samples of cod muscle and carried through all stages of the procedure, and apparent values were corrected accordingly.

RESULTS

The individual amino acid analyses obtained in laboratories A and B together with the values of the heated materials expressed relative to those of the control materials are given in Table 2. Where duplicate results from one laboratory are reported, these were obtained by replication of all stages of sample preparation and hydrolysis. Cystine and methionine were not satisfactorily determined in hydrolysates of unoxidized protein. Cystine was detected in only two of the hydrolysates prepared by laboratory A. Methionine was calculated from the sum of methionine and methionine sulphoxide. Twice when the methionine sulphoxide peak was incompletely separated from the aspartic acid peak no value could be calculated. Tyrosine is destroyed in the procedure used by laboratory B and proline was not determined. The values obtained in laboratory C have been previously reported (Miller *et al.* 1965) but are included in Table 2 for comparison.

Heating cod muscle for 27 h at 116° caused a loss of cystine of approximately 60%. A small decrease in lysine ranging from 3 to 10% was noted in each laboratory. No other amino acid showed a consistent decrease in all the laboratories although individual results sometimes suggested destruction. Thus the 17% loss of serine found by laboratory B was not confirmed by laboratory A.

In contrast to the results obtained on heating cod muscle alone at 116°, heating for 27 h at 85° in the presence of D-glucose resulted in less destruction of cystine and greater loss of lysine, approximately one-third of each amino acid being lost. In addition the arginine content decreased by approximately 20 %. The total contents of other amino acids were not affected.

The decrease in methionine content upon heating observed in laboratory C was not confirmed in the other laboratories. In a further investigation of this discrepancy, samples of C23 and C25 were oxidized with performic acid and hydrolysed in laboratory C and then analysed in laboratory B. The results, which are not presented in detail here, showed no change in methionine content with heating.

DISCUSSION

In reviewing the literature on amino acid content of flesh of fish, Connell & Howgate (1959) commented on the scarcity of data and the variability of reported values even for the same species. The amino acid contents determined by these authors were generally slightly higher than literature values, and especially so for threonine,

Table 2. *Total amino acid content (g/16 g N) of a control and two heated preparations of cod muscle in replicate analyses 1 and 2 in laboratories A, B and C*

	Cod 23, unheated control					Cod 35, 27 h, 116°, 14% water				Cod 25, 27 h, 85°, 14% water (+10% glucose)			$\frac{\text{Cod 35}}{\text{Cod 23}} \times 100$			$\frac{\text{Cod 25}}{\text{Cod 23}} \times 100$		
	A		B		C	A		B	C	A	B	C	A	B	C	A	B	C
Amino acid	1	2	1	2		1	2											
Cystine	1·1	—	—	—	—	0·45	—	—	—	—	—	—	40	—	—	—	—	—
Cystine (measured as cysteic acid)	—	—	1·1	1·1	1·2	—	—	0·50	0·4	—	0·73	0·8	—	45	33	—	66	67
Methionine + sulphoxide	3·5	3·4	—	—	—	3·55	—	—	—	—	—	—	105	—	—	—	—	—
Methionine (measured as the sulphone)	—	—	3·5	3·3	3·5	—	—	3·3	2·6	—	3·1	2·6	—	97	74	—	91	74
Aspartic acid	9·2	9·25	9·8	9·7	—	10·1	9·75	8·9	—	9·2	10·5	—	107	91	—	100	108	—
Threonine	4·0	3·95	6·0	4·3	—	4·3	4·65	5·6	—	4·05	6·2	—	112	109	—	101	120	—
Serine	3·8	3·85	3·8	4·2	—	3·7	4·0	3·3	—	3·75	4·4	—	101	83	—	99	110	—
Glutamic acid	13·55	12·2	13·9	12·6	—	15·05	14·5	13·5	—	12·6	14·0	—	114	102	—	98	106	—
Proline	3·35	3·4	—	—	—	3·7	3·75	—	—	3·4	—	—	109	—	—	100	—	—
Glycine	4·05	4·3	3·9	3·9	—	4·4	4·15	4·1	—	4·15	4·3	—	102	105	—	99	110	—
Alanine	5·75	5·35	5·4	7·4	—	6·2	6·2	5·8	—	5·4	5·8	—	112	91	—	97	91	—
Valine	4·9	4·95	4·6	4·4	—	5·15	4·95	4·8	—	4·75	4·8	—	103	107	—	97	107	—
Isoleucine	4·5	4·35	4·5	3·9	—	4·9	4·65	4·8	—	4·35	4·5	—	109	114	—	99	107	—
Leucine	7·65	7·7	7·2	7·1	—	8·2	7·65	8·0	—	7·8	8·3	—	103	112	—	101	116	—
Tyrosine	3·5	3·6	—	—	—	3·75	3·35	—	—	3·7	—	—	100	—	—	104	—	—
Phenylalanine	3·9	4·0	6·8	6·6	—	4·3	3·85	7·2	—	3·95	7·5	—	104	107	—	100	112	—
Lysine	8·35	9·05	9·3	8·9	9·3	8·6	8·15	8·5	8·4	5·6	6·9	6·1	97	94	90	64	76	66
Histidine	2·05	2·25	2·3	2·1	—	2·35	2·15	2·1	—	2·1	2·3	—	105	96	—	98	104	—
Arginine	5·65	5·55	6·1	—	—	5·8	6·0	6·6	—	4·35	5·2	—	105	108	—	78	85	—
Tryptophan	—	—	—	—	1·24	—	—	—	1·28	—	—	1·34	—	—	103	—	—	108
Hydroxylysine	0·0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Taurine	0·55	0·6	—	—	—	0·45	0·5	—	—	0·3	—	—	—	—	—	—	—	—
Ammonia	0·7	0·55	0·8	0·8	—	1·3	1·1	0·7	—	0·6	1·0	—	—	—	—	—	—	—
Recovery of nitrogen (%)*	80·3	79·7	85·6	84·3	—	87·8	85·0	84·6	—	72·7	85·6	—	—	—	—	—	—	—

* When no value was reported for an amino acid, a mean value from the data of the other laboratories was inserted in order to calculate an approximate value for the recovery of nitrogen.

leucine, phenylalanine, lysine and histidine. Their analyses were performed on the alcohol-precipitated protein of the flesh of cod and other fish, and so their materials may have differed from those analysed in the work described now. Results for the control materials from laboratories A and B are generally lower than values reported by Connell & Howgate (1959), but similar to those of Braekkan & Boge (1962) and Ellinger & Boyne (1965). Approximately 85% of the total nitrogen has been accounted for by our analyses. Again, this is less than the recovery achieved by Connell & Howgate (1959) and may be a result either of the presence in our material of nitrogenous compounds not estimated with ninhydrin or of underestimation of amino acid content.

The decreases in total contents of essential and semi-essential amino acids brought about by heating cod muscle alone or in the presence of added carbohydrate are given in Table 3 along with results of studies on other proteins. Here apparent increases of amino acid content as the result of heat treatment are recorded as zero loss. Such increases can arise as a result of the known loss of ammonia on heating. In every instance given in Table 3, heating protein materials containing little carbohydrate resulted in large losses of cystine. Losses of lysine consistently occurred, but in the studies reported by Clandinin (1949) and Donoso *et al.* (1962) the loss of lysine was approximately double that reported in the other five studies. The 16% loss of methionine reported by Donoso *et al.* (1962) is not supported either by the data of Beuk, Chornock & Rice (1948), who also heated pork, or by the studies on other proteins. The method used for the determination of methionine by Donoso *et al.* (1962) was essentially that which gave the low values for heated cod muscle in laboratory C. Some workers reported small losses of other amino acids, but such losses were not found in all of the studies detailed in Table 3. In each of these studies, the heated protein was found by biological trials to be considerably poorer than the control material, and in no instance could the decreased value be predicted from the total amino acid composition. Also Bunyan & Woodham (1964) have reported amino acid analyses for two Peruvian anchovy meals, which were of very different quality in chick, rat and pig trials (Barber, Braude, Chamberlain, Hosking & Mitchell, 1964), presumably as a result of spontaneous overheating, but differed in total methionine, lysine and tryptophan content by only 10, 11 and 22% respectively.

In each instance given in Table 3 when a protein–carbohydrate mixture was heated, a large loss of lysine, arginine and cystine was reported. The conflicting results of damage to tryptophan may be due to the unsatisfactory methods available for tryptophan analysis. Also, the great difference reported by Ford (1962) in the stability on heating of arginine in white-fish meal and in dried skim milk may be attributed to the presence of carbohydrate in dried milk. In a study of the effects of processing temperature on sunflower-seed meal (Renner, Clandinin, Morrison & Robblee, 1953), increasing severity of heating again resulted in progressive destruction of lysine, arginine and tryptophan (cystine was not determined), but no change in any other essential amino acid.

Chemical scores, calculated by comparison of the values given in Table 2 both with the FAO (1957) reference pattern of amino acids, which is based on the requirements of man, and with the stated amino acid requirements of the rat (Bender, 1958), are

Table 3. *Loss (%) of essential and semi-essential amino acids on heating protein materials either in the absence or in the presence of appreciable quantities of carbohydrate*

Material	Heat treatment	Lysine	Cystine	Methionine	Arginine	Histidine	Threonine	Valine	Isoleucine	Leucine	Phenylalanine	Tyrosine	Tryptophan	Reference
						A, containing little carbohydrate								
Cod	27 h, 116°	6	61	0	0	0	0	0	0	0	0	0	0	Present paper
Cod	36 h, 105°	11	64	0	0	12	0	0	0	0	0	6	—	Ellinger & Boyne (1965)
Herring meal	Flame dried 105° compared with vacuum dried	25	—	0	9	5	11	4	1	2	8	—	0	Clandinin (1949)
Fish meal	20 h, 120°	6	68	3	5	14	13	7	5	3	4	0	11	Mason & Weidner (1964)
Fish meal	12 h, 121°	12	—	0	2	7	8	2	0	0	0	0	—	Smith & Scott (1965)
Pork	24 h, 112°	5	44	0	4	0	0	0	0	0	0	—	0	Beuk, Chornock & Rice (1948)
Pork	24 h, 110°	20	44	16	2	8	13	11	3	20	10	11	5	Donoso, Lewis, Miller & Payne (1962)
						B, containing carbohydrate								
Cod + glucose	27 h, 85°	31	33	9	18	0	0	0	0	0	0	0	0	Present paper
Casein + glucose	24 h, 96·5°	26	—	0	31	7	0	0	6	3	—	—	39*	Patton, Hill & Foreman (1948)
Soya-bean protein + sucrose	4 h, 121°	47	22	2	42	10	1	0	0	0	0	—	—	Evans & Butts (1949)
Soya-bean meal	4 h, 121°	52	—	0	41	0	0	5	0	0	0	—	19	Riesen, Clandinin, Elvehjem & Cravens (1947)

* Enzymic hydrolysis.

given in Table 4. In our experience the standards of Bender for calculating chemical scores give values in accord with biological findings when applied to materials that have not been heat-damaged. Thus the cystine + methionine combination has been shown to be the limiting factor of fish protein for rats (Miller, 1956; Chalupa & Fisher, 1963), and the chemical score of 93 for the control material (C 23) agrees with the determined biological value of 95. The poor agreement obtained by Wiechers & Laubscher (1962) using the 'target' figures of Bender was partly due to a misprint of these figures (Bender, 1960) and partly to the failure by Wiechers & Laubscher to allow for the fact that often DL amino acids had been used in the experiments of Bender. On the other hand, chemical scores do not predict the value of heat-damaged materials for the rat. Decreases in net protein ratio (Table 4) brought about by heat treatment are clearly much greater than the corresponding decreases in chemical score.

Table 4. *Net protein ratio and calculated chemical scores of cod-muscle protein subjected to various heat treatments*

Material	Net protein ratio*	Chemical score calculated from standards of	
		FAO (1957)	Bender (1958)
C23, unheated control	4·6	90†	92‡
C35, 27 h, 116°, 14% moisture	2·9	90†	79‡
C25, 27 h, 85°, 14% moisture (+10% glucose)	0·69	90†	79‡

* Data from Miller, Carpenter & Milner (1965).
† Tryptophan calculated as limiting.
‡ Cystine + methionine calculated as limiting.

Similarly the methionine available to the chick decreased from 3·4 g/16 g N for the control to 0·4 g/16 g N in the most severely damaged material, whereas the total methionine content remained unchanged. We conclude from both the present results and the literature reviewed that the failure of total amino acid analyses to indicate changes in protein quality brought about by heat treatment, as judged by several different criteria with rats and chicks, clearly indicates the limitations of such analyses for the nutritional evaluation of heat-processed foods. Our study thus confirms earlier conclusions based on work using microbiological methods for the assay of total amino acids.

SUMMARY

1. The amino acid contents of freeze-dried cod muscle, cod muscle heated for 27 h at 116° and 13·8% moisture under N_2, and cod muscle mixed with 10 parts by weight of D-glucose and heated for 27 h at 85° and 13·4% moisture under N_2 were determined in three laboratories by ion-exchange chromatography.

2. Heating cod muscle alone resulted in a large loss of cystine and a small loss of lysine. Heating cod muscle mixed with glucose resulted in losses of cystine, lysine and arginine.

3. The decrease in protein quality of the heated preparations was much greater than the decrease in total amino acid content.

One of us (E.L.M.) is indebted to the United States Department of Agriculture for a research grant (FG-UK-127).

REFERENCES

Association of Official Agricultural Chemists (1960). *Official Methods of Analysis*, 9th ed., para. 2.034. Washington, DC: Association of Official Agricultural Chemists.
Barber, R. S., Braude, R., Chamberlain, A. G., Hosking, Z. D. & Mitchell, K. G. (1964). *Br. J. Nutr.* **18**, 545.
Bender, A. E. (1958). *Proc. Nutr. Soc.* **17**, xxxix.
Bender, A. E. (1960). *Clinica chim. Acta*, **5**, 1.
Beuk, J. F., Chornock, F. W. & Rice, E. E. (1948). *J. biol. Chem.* **175**, 291.
Bidmead, D. & Ley, F. J. (1958). *Biochim. biophys. Acta*, **29**, 562.
Block, R. J. & Mitchell, H. H. (1946–7). *Nutr. Abstr. Rev.* **16**, 249.
Braekkan, O. R. & Boge, G. (1962). *FiskDir. Skr. Serie Teknologiske undersøkelser*, **4**, no. 3.
Bunyan, J. & Woodham, A. A. (1964). *Br. J. Nutr.* **18**, 537.
Carpenter, K. J., Ellinger, G. M., Munro, M. I. & Rolfe, E. J. (1957). *Br. J. Nutr.* **11**, 162.
Carpenter, K. J., March, B. E., Milner, C. K. & Campbell, R. C. (1963). *Br. J. Nutr.* **17**, 309.
Chalupa, W. & Fisher, H. (1963). *J. Nutr.* **81**, 139.
Clandinin, D. R. (1949). *Poult. Sci.* **28**, 128.
Connell, J. J. & Howgate, P. F. (1959). *J. Sci. Fd Agric.* **10**, 241.
De Vuyst, A., Vervack, W., Vanbelle, M., Arnould, R. & Moreels, A. (1964*a*). *Agricultura, Louvain*, **12**, 141.
De Vuyst, A., Vervack, W., Vanbelle, M., Arnould, R. & Moreels, A. (1964*b*). *Agricultura, Louvain*, **12**, 153.
Donoso, G., Lewis, O. A. M., Miller, D. S. & Payne, P. R. (1962). *J. Sci. Fd Agric.* **13**, 192.
Dustin, J. P., Czajkowska, C., Moore, S. & Bigwood, E. J. (1953). *Analytica chim. Acta*, **9**, 256.
Ellinger, G. M. & Boyne, E. B. (1965). *Br. J. Nutr.* **19**, 587.
Evans, R. J. & Butts, H. A. (1949). *Science, N.Y.*, **109**, 569.
FAO (1957). *F.A.O. nutr. Stud.* no. 16.
Ford, J. E. (1962). *Br. J. Nutr.* **16**, 409.
Greene, R. D. & Black, A. (1944). *J. biol. Chem.* **155**, 1.
Horn, M. J. & Jones, D. B. (1945). *J. biol. Chem.* **157**, 153.
Lea, C. H. & Hannan, R. S. (1950). *Biochim. biophys. Acta*, **5**, 433.
Ma, T. S. & Zuazaga, G. (1942). *Ind. Engng Chem. analyt. Edn*, **14**, 280.
Mason, V. C. & Weidner, K. (1964). *Acta Agric. scand.* **14**, 87.
Miller, D. S. (1956). *J. Sci. Fd Agric.* **7**, 337.
Miller, E. L. & Carpenter, K. J. (1964). *J. Sci. Fd Agric.* **15**, 810.
Miller, E. L., Carpenter, K. J. & Milner, C. K. (1965). *Br. J. Nutr.* **19**, 547.
Oser, B. L. (1951). *J. Am. diet. Ass.* **27**, 396.
Patton, A. R., Hill, E. G. & Foreman, E. M. (1948). *Science, N.Y.*, **107**, 623.
Piez, K. A. & Morris, L. (1960). *Analyt. Biochem.* **1**, 187.
Renner, R., Clandinin, D. R., Morrison, A. B. & Robblee, A. R. (1953). *J. Nutr.* **50**, 487.
Riesen, W. H., Clandinin, D. R., Elvehjem, C. A. & Cravens, W. W. (1947). *J. biol. Chem.* **167**, 143.
Smith, R. E. & Scott, H. M. (1965). *Poult. Sci.* **44**, 401.
Wiechers, S. G. & Laubscher, H. (1962). *Hand. Voedveren. Suid. Afr.* **3**, 169.

Printed in Great Britain