# THE DETERMINATION OF PROTEIN DIGESTIBILITY AND AVAILABILITY IN VITRO

A.T.A. KAROSSI* and T.M. SUTHERLAND*

## SUMMARY

The usefulness and limitations of determination of protein digestibility in vitro are discussed. Single pronase digestions for 24h were found to be incomplete and delayed by enzyme instability and product inhibition. A rapid method for digestibility determination based on successive digestive extractions with pronase is reported.

## INTRODUCTION

The biological testing of proteins before feed formulation, although desirable in terms of economy of protein useage, is often precluded by the time and money required to mount such assays, so it is not surprising that much work has gone into developing laboratory methods (Raynor and Fox 1976; Saunders and Kohler, 1972). An important determinant of quality is digestibility and many methods have been offered for its determination in vitro (Scheffner et al. 1956; Akeson and Stahman, 1964; Kerese, 1976 and many others). These methods depend on the use of proteolytic enzymes to simulate the digestive processes of the animal.

The availability of amino acids to the animal depends on 'the physical and chemical nature of the proteins in the source, the quantity and nature of the enzymes secreted or available in the intestinal brush border, the presence of inhibitors including the products of'digestion and the rate of flow of digesta and the rate of removal of end products among many other factors,. In digestions in vitro it'should be possible to take proteolysis to a limit where further action is impossible because of restricted access, cross bridging or amino acid modification. . Digestibility in vivo is usually highly efficient and so should be related to the limit digestibility.

It is perhaps worth clarifying the concept of digestibility with regard to proteins a little further. Amino acids are not necessarily absorbed as such. Transport systems exist for di- and tri-peptides, the latter constituting an upper limit so that for intra-lumenal diqestion we can define digestibility as that part of the protein, which may 'be converted to amino acids, di- and tri-peptides by enzymes of the gastro-intestinal tract.

Tripeptides have a mean molecular weight (M.W.) of 374 (range 189-576) so operationally a digestibility could be defined as that portion converted to materials of M.W. below 500. This would exclude some higher M.W. tripeptides and include some higher oligopeptides of smaller amino acids but would allow digestibility to be defined in practical terms.

Usually because most commercial protein sources are largely insoluble, solubilisation is taken as an indicator of digestibility. This may be estimated directly or after treatment with a deproteinising agent such as trichloroacetic, tungstic or picric acids.

---

*Department of Biochemistry and Nutrition, University of New England, Armidale, N.S.W. 2351.

The closest simulation of conditions *in vivo* is by pepsin (or more strictly gastric enzyme) attack under acid conditions followed by treatment with pancreatin and mucosal enzymes under neutral conditions. The general experience with such systems is that they are slow and involve the addition of large quantities of protein to get digestions of reasonable duration. From the point of view of convenience there are obvious advantages in an *in vitro* system in which a single set of experimental conditions are used.

Pronase from S.griseus is a wide spectrum proteolytic source in which at least four neutral proteinases, three alkaline proteinases, three aminopeptidases and a carboxypeptidase have been reported (Nomoto *et al.* 1960; Nurahashi *et al.* 1968). Trypsin-like and elastolytic enzymes and peptidases similar to carboxy-peptidases A and B and aminopeptidases have been separated and characterised (Trop and Birk, 1970). Pronase preparations have been shown to hydrolyse the specific synthetic substrates of pepsin, trypsin, chymotrypsin, cathepsin C, carboxypeptidase, leucine aminopeptidase, aminotripeptidase and aminodipeptidase (Nomoto *et al.* 1960). Pronase is thus capable of providing a multi-enzyme attack similar to that given by the gastro-intestinal tract.

Pronase has been used by a number of authors for *in vitro* assessments of digestibility (Saunders and Kohler 1972; Ford and Salter 1966; Raynor and Fox 1976). We describe in the following section the development of a rapid method for *in vitro* digestibilities based on this enzyme source.

## EXPERIMENTAL AND RESULTS

Our first experiments were directed to examining the effect of pronase on a series of commonly available protein sources, soybean meal, maize gluten, blood meal, fish meal and fortified protein meal. The proteins (125 mg N in 25 ml phosphate pH 7.5) were incubated with pronase (1125 P.K.U.) for 24 h and the extent of digestion examined by:

(i) using solubilisation of N as an index of protein digestion,
(ii) estimating available lysine in the original proteins and their undigested insoluble residue.,
(iii) submitting the soluble digests to molecular exclusion chromatography on Sephadex G-25 and monitoring the eluates at 280 mm (Steinhart and Kirchgessener 1973).

The results are given in Tables 1 and 2.

TABLE 1 Digestion *in vitro* of protein meals by pronase

| Protein Source | N% | N Digestibility (% solubilised) | Available Lysine Digestibility % |
|---|---|---|---|
| Soybean meal | 7.97 | 80.8 (86)[a] | 78.5 |
| Maize gluten | 12.23 | 76.3 (87-99)[b] | 73.9 |
| Blood meal | 14.79 | 83.2 (60-83) | 76.3 |
| Fish meal | 9.90 | 74.0 | 62.4 |
| Fortified protein meal | 8.98 | 83.1 | 63.6 |

Figures in parentheses are apparent digestibilities *in vivo* from (a) Fitzpatrick and Bayley (1971) (b) De Muelenaere *et al.* (1967)

TABLE 2 Percentage distribution of molecular weights from pronase treatment (24 h) estimated by Sephadex G-25 exclusion chromatography

| | Molecular weight range | | | |
|---|---|---|---|---|
| | Insoluble | Soluble 1000 | 500 - 1000 | <500 |
| Soybean meal | 19.2 | 12.8 | 47.2 | 20.8 |
| Maize gluten | 23.7 | 11.1 | 49.6 | 15.6 |
| Blood meal | 16.8 | 13.0 | 48.4 | 21.8 |
| Fish meal | 26.0 | 16.8 | 42.8 | 14.4 |
| Fortified protein meal | 16.9 | 31.7 | 50.6 | 0.8 |

The following points emerged from these experiments:

(i) the digestibilities as measured by solubility are of the order of digestibilities observed in vivo.

(ii) available lysine digestibilities can differ appreciably from N digestibilities.

(iii) in no case has the pronase digestion converted the protein in quantity to the M.W. range for absorption although a large part has been converted to the range below 1000 daltons.

(iv) deproteinisatiop with picric acid although in general tending to remove higher peptides showed no clear cut off and left in solution oligopeptides in the range 1000 daltons.

(v) the insoluble residues from these experiments were tested separately with pronase, trypsin, chymotrypsin, collagenase and pepsin and in each case showed susceptibility to further digestion especially with pepsin.

More extensive kinetic studies were set up in which the protein source and the pronase were enclosed in a dialysis sac in 2.5 volumes of buffer and digestion monitored by 280 nm light absorption in the diffusate. The results are given in Fig. 1.

It was noted in this experiment:

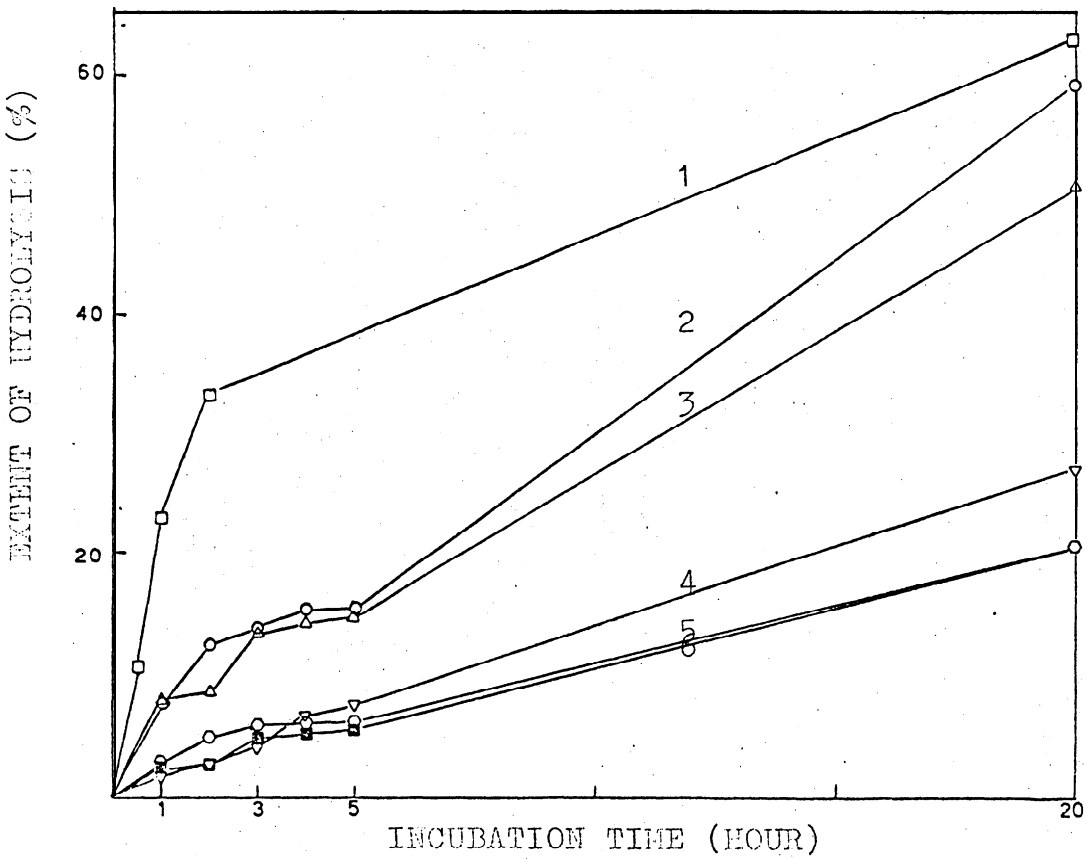
(i) that digestion measured in this way at 20 h was much lower than would occur in vivo,

(ii) that initial rates of hydrolysis would be a poor guide to digestibility,

(iii) the rate of digestion of casein was falling off in the late stages' and that complete digestion would be very protracted.

We concluded from these experiments that the possibility of converting proteins to the M.W. range for. absorption by pronase digestion in a single incubation within a reasonable time period was remote and that an empirical approach would have to be adopted. Two possibilities seemed open:

FIGURE 1 Hydrolysis of protein meals by pronase with dialysis, (I) casein; (2) blood meal; (3) maize gluten; (4) soybean meal; (5) fortified protein meal; (6) fish meal




(i) to examine' further the usefulness of initial rates of digestion as a digestibility indicator,

(ii) to use solubilisation as an indicator and to concentrate on the differences between the initial protein and the digestion-resistant residue.

The first approach has been advocated by Hsu et al. (1977) using tryptic digestion. Results of our experiment testing this possibility are given in Table 3 where tryptic digestion rates for the proteins are given as percentages of the rate for casein which is assumed to be 100% digestible.

TABLE 3 Hydrolysis of protein meals. with trypsin

| Test material | Rate of hydrolysis ($\Delta OD_{280}$ $min^{-1}$).$10^3$ | Relative rate (%) |
|---|---|---|
| Soybean meal | 22.83 | 45 |
| Maize gluten | 5.67 | 11 |
| Blood meal | 14.00 | 28 |
| Fish meal | 8.50 | 17 |
| Fortified proteinmeal | 4.50 | 9 |
| Casein | 50.33 | 100 |

The relative rates bear no resemblance to the expected digestibilities and this method, which has a weak theoretical foundation was not pursued further.

For obtaining an insoluble resistant residue rapidly, it occurred to us that repeated short term incubations with fresh portions of a soluble pronase preparation might be more effective than a single incubation for the same overall time. This was tested experimentally and found to be true (Table 4).

TABLE 4 *In vitro* nitrogen digestibility of protein meals (Mean ± SEM)% after pronase digestion

| Test material | Successive digestion | | | Single step digest. | |
|---|---|---|---|---|---|
| | 1st-2-hr | 2nd-2-hr | 3rd-2-hr | 4 hours | 6 hours |
| Soybean meal | 51.3±0.8 | 93.9±1.6 | 97.2±0.3 | 77.2±2.1 | 77.2±1.3 |
| Maize gluten | 48.3±1.6 | 74.5±0.7 | 90.7±2.1 | 63.1±0.9 | 66.3±0.3 |
| Blood meal | 60.1±2.4 | 71.4±1.9 | 87.1±1.3 | 67.0±3.7 | 67.4±1.1 |
| Fish meal | 48.9±2.8 | 70.4±0.3 | 82.7±1.3 | 51.3±1.1 | 51.7±1.2 |
| Fortified protein meal | 57.2±0.4 | 74.5±0 | 83.7±0.4 | 66.7±0.2 | 78.6±1.0 |

Samples of protein source (50mg N) were incubated with soluble pronase (25 mg, 1700 P.K.U.) at 370C for the periods indicated in Phosphate buffer pH 7.5 before centrifuging at 9,000 g for 10 min.

In the single stage experiments, except for the fortified protein meal there was little change from 4 to 6 h. This apparent limit was obviously an artefact of the conditions as much higher digestibilities were obtained with. successive treatments.

In a'second experiment the incubation periods were reduced to 1 h. and a correspondingly greater enzyme concentrationused (Table 5).

TABLE' 5 *In vitro* nitrogen digestibility of protein meals after digestion with pronase employing the l-hour step procedure

| Test material | Nitrogen digestibility (Mean ± SEM)% | | |
|---|---|---|---|
| | 1st-1-hr | 2nd-1-hr | 3rd-1-hr |
| Soybean meal | 92.3 ± 0.5 | 96.8 ± 0.4 | 97.3 ± 0.4 |
| Maize gluten | 53.5 ± 0.7 | 91.7 ± 0.5 | 97.3 ± 0.4 |
| Blood meal | 64.8 ± 1.3 | 88.6 ± 0.6 | 96.4 ± 0 |
| Fish meal | 67.1 ± 3.4 | 86.3 ± 0.2 | 90.6 ± 0.5 |
| Meat-Bone meal | 64.0 ± 0.6 | 77.6 ± 2.4 | 81.2 ± 1.9 |

With two successive 1h treatments apparent diaestibilities were in the region of expected results *in vivo*. Results from three successive treatments suggested that further treatment-would lead to complete solubilisation of soybean, maize gluten and blood meals.

The superiority of repeated treatmentswith fresh enzyme over a single prolonged treatment could be due to:

(i) instability of the pronase activity in solution or

(ii) inhibition of the pronase activity by materials in the protein source or produced by enzyme action.

Incubation of the pronase solution for periods of 0, 2 or 4 h before adding to the protein sources allowed us to examine these possibilities. (Table 6).

TABLE 6 Stability of pronase solution at 37°C; pH 7.5

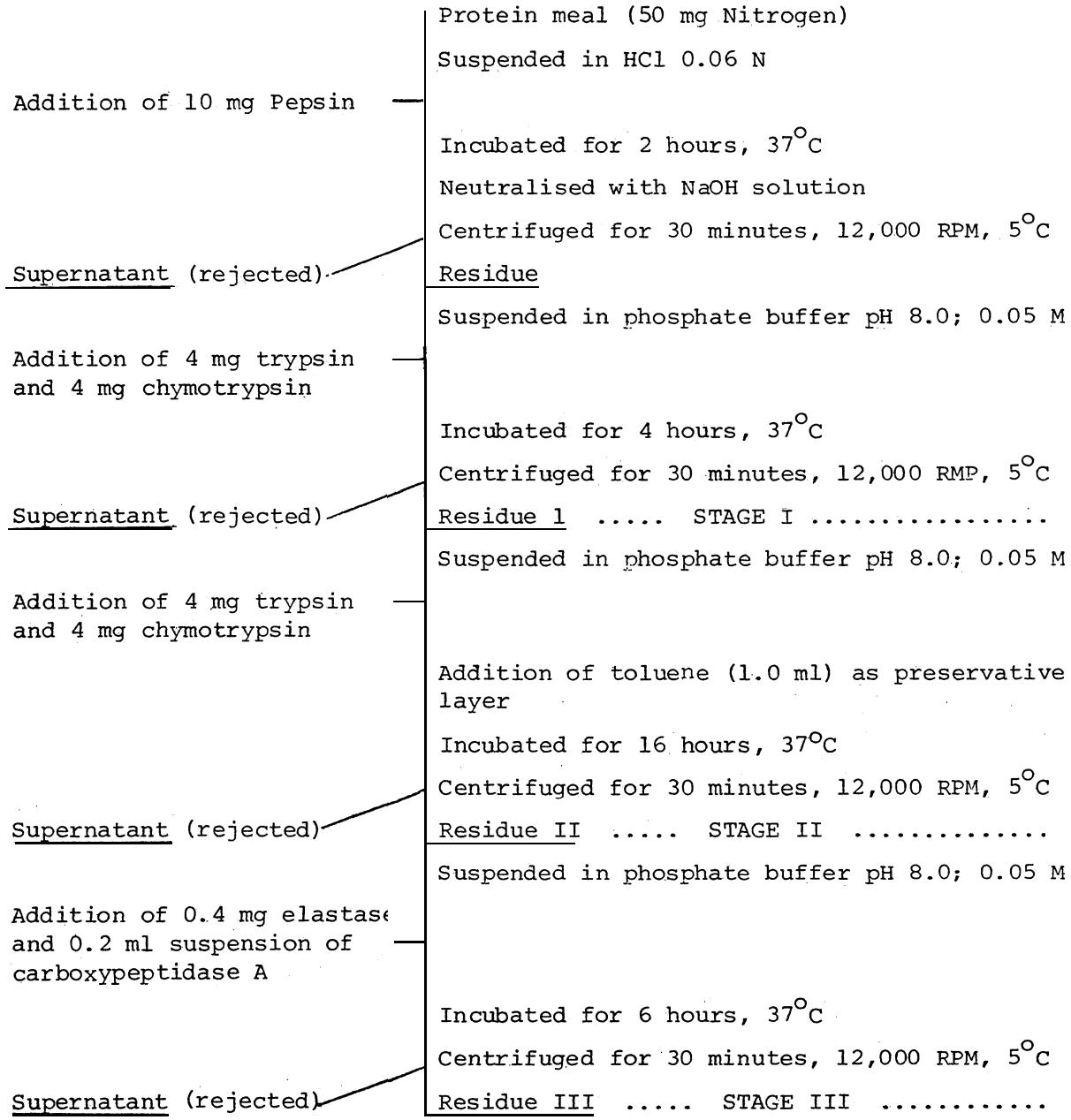
| Test material used as substrate | Remaining pronase activity (%) after the respective incubation time | | |
|---|---|---|---|
| | Control | 2 hr | 4 hr |
| Soybean meal | 100 (51.3)* | 90 | 66 |
| Maize gluten | 100 (48.3) | 87 | 58 |
| Blood meal | 100 (60.1) | 74 | 49 |
| Fish meal | 100 (48.9) | 85 | 43 |
| Fortified protein meal | 100 (57.2) | 82 | 55 |
| Average | | 84 | 54 |

*Values in parenthesis are the percentage of the solubilised nitrogen after 1st-2-hour procedure

'The pronase was clearly losing activity under the incubation conditions but the degree of loss of activity, probably exaggerated in the absence of substrate, did not cover all the observations. There was evidently inhibitor present in the soybean preparation, which was overcome in the 2 h experiments (Table 4) after the first extraction and in the 1 h experiments (Table 5) by excess enzyme. If half the original enzyme activity is still present after 4 h under conditions maximising self-destruction, one must assume that the apparent plateau seen between 4 and 6 h in the one step incubations (Table.4) is due to another cause, presumably product inhibition.

It was apparent that we now had a very rapid if empirical technique for carrying out digestions in vitro. The next points to examine were how closely did the process we were observing simulate digestion with mammalian enzymes and how well do the results obtained in vitro correlate with observations in vivo. An in vitro system similar to that of Kerese (1976) was set up using the procedure described in Figure 2 to see how our protein sources responded to mammalian enzymes.

FIGURE. 2 System for digestion with mammalian enzymes




The results shown in Table 7 show a mean digestibilitv at stage III of 87.5% compared with a mean of 88%. for stage II of successive 1 h incubations with pronase but the blood meal was 8.2% more digested by the mammalian enzyme system and fish meal 12.2% less digested (see table 5).

We were fortunate in having access to a number of grain samples which had been examined by Dr. M. Taverner (1979) in. ileally fistulated pigs for true digestibility and in vitro by his own method (Taverner, '1979). A number of these grains were examined by successive 1 h pronase treatment. The results'are compared with those of Taverner in Table 8.

TABLE 7 *In vitro* nitrogen digestibility of protein meals after digestion with the mammalian digestive enzyme system

| Test material | Nitrogen digestibility (% Mean ± SEM) Stage I | Stage II | Stage III |
|---|---|---|---|
| Soybean meal | 82.8 ± 0.90 | 90.8 ± 1.12 | 92.7 ± 0.25 |
| Maize gluten | 66.9 ± 1.42 | 85.2 ± 1.29 | 92.5 ± 0.32 |
| Blood meal | 87.4 ± 1.81 | 93.2 ± 0.30 | 96.8 ± 0.35 |
| Fish meal | 51.1 ± 1.24 | 61.8 ± 1.25 | 74.1 ± 3.03 |
| Fortified protein meal | 62.7 ± 0.86 | 77.5 ± 0.94 | 81.5 ± 0.11 |

TABLE 8 *In vitro* nitrogen digestibility of cereal grains after 2nd 1-hour method as compared to true digestibilities with pigs

| Grain | *In vitro* digestibility (% ± SEM) | True N digestibility (%)* |
|---|---|---|
| Wheat 1 | 89.3 ± 2.63 (91.4)* | 92.3 |
| Wheat 2 | 83.6 ± 0.88 (87.0)* | 90.9 |
| Wheat 3 | 85.3 ± 2.52 (81.1 * | 87.0 |
| Wheat 4 | 88.6 ± 0.33 (79.9)* | 83.0 |
| Wheat 5 | 91.5 ± 0.14 (87.8)* | 91.6 |
| Triticale | 93.4 ± 1.05 (95.8)* | 91.4 |
| Barley | 84.1 ± 0.04 (82.1)* | 84.4 |
| Sorghum | 58.8 ± 0.78 (44.5)*(75.6 ± 0.43)** | 88.5 |
| Corn | 65.0 ± 1.65 (51.1)*(80.0 ± 0.23)** | 86.4 |

*Quoted from Taverner, M.R., 1979, Ph.D. thesis, University of New England

**With 3rd 1-hour procedure

With the wheat, triticale and barley samples there is a reasonably good agreement between the *in vitro* and the *in vivo* estimates (means 88.0 and 88.5 respectively) but for sorghum and maize the *in vitro* methods seriously underestimate. Even a third pronase treatment fails 'to bring the sorghum and maize digestibilities up to the value observed *in vivo*.

## SUMMARY AND CONCLUSIONS

Pronase preparations in digestions *in vitro* failed to convert common protein sources to tripeptides and beyondin quantities comparable to the expected digestibilities. They can however be used in rapid digestion extraction systems to give approximations to digestibilities *in vivo* by equating solubilisation to digestion. The effectiveness of pronase digestion measured in this way on different materials does not truly parallel the course of digestion *in vivo* and separate calibration would be necessary for different protein sources. The rapidity of the digestion extraction technique is its major advantage. By analysing the source and the resistant residue by hydrolysis and amino acid estimation it is possible to complete an estimate of aminoacid availability in two working days. The possibilities of combining the rapid pronase technique with Tetrahymena assays are being explored.

ACKNOWLEDGEMENTS

We thank Associate Professor Farrell and Dr, M.R. Taverner for making their grain samples available.

REFERENCES

AKESON, W.R. and STAHMAN, M.A. (1964). J. Nutr., 83: 257.

De MUELANAERE, H.J.H., CHEN, M.L. and HARPER, A.E. (1967). J. Agr. Food Chem., 15(2): 310.

FITZPATRICK, D.W. and BAYLEY, H.S. (1967). Can. J. Anim. Sci., 57: 745.

FORD, J.E. and SALTER, D.N. (1966). Brit. J. Nutr., 20: 843.

HSU, H.W., VAVAK, D.L., SATTERLEE, I.D. and MILLER, G.A. (1977). J. Food Sci., 42(5): 1269.

KERESE, I. (1976). Acta Agr. Acad. Sci. Hung., 25(3,4): 473.

NOMOTO.M., NARAHASHI, Y. and MURAKAMI, M. (1960). J. Biochem. (Tokyo), 48(6): 906.

RAYNER, C.J. and FOX, M. (1976). J. Sci. Fd. Agric., 27: 643.

SAUNDERS, R.M. and KOHLER, G.O. (1972). Cereal Chem., 49(1): 98.

SCHEFFNER, A.L., ECKFELDT, G.A. and SPECTOR, H. (1956). J. Nutr., 60: 105.

STEINHART; H. and KIRCHGESSENER, M. (1973). Landwirtsch. Forsch., 26: 200.

TAVERNER, M.R. (1979). Ph.D. Thesis, University of New England.

TROP, M. and BIRK, Y. (1970). Biochem. J., 116: 19.