The effect of methods of sterilization on the nutritive value of protein in a commercial rat diet

By D. J. FORD

Medical Research Council Laboratory Animals Centre,
Woodmansterne Road, Carshalton, Surrey SM5 4EF

1. The effect on protein quality of treating a commercial rat diet by autoclaving at various temperatures for different periods of time, or by irradiation with 2·5 or 10 Mrd, was studied. True digestibility (TD) and biological value (BV) were measured and the available and total amino acids in the diets were estimated using microbiological and chemical methods.

2. Autoclaving at 121° for 60 min reduced BV, TD and net protein utilization (NPU) more than autoclaving at 134° for 3 min. Availability of amino acids was reduced by both treatments but to a greater extent by autoclaving at 121° for 60 min. Total amino acids were essentially unaffected. Irradiation had no effect on BV, TD, NPU or total amino acids, and the availability of amino acids was also unaffected, with the exception of lysine which was slightly reduced.

3. When the diet was autoclaved at 115 or 121° for 15, 30 or 60 min, or at 134° for 3 min the availability of the amino acids was reduced with increasing time and temperature of treatment. Treatment at 134° for 3 min had an effect on available amino acids similar to treatment at 121° for 15 or 30 min.

4. Ethylene oxide fumigation of the diet caused reduced availability of histidine, methionine and tryptophan but had negligible effect on arginine, leucine and lysine.

5. It is concluded that from a practical point of view irradiation causes least damage to proteins in rodent diets. If such diets are to be autoclaved they should be supplemented with complete protein to counteract amino acid destruction.

As the numbers of germ-free and specified-pathogen-free animals used in research have grown in recent years, consideration of the technique for sterilizing diets has become an increasingly important factor in laboratory animal husbandry. Autoclaving, gamma-irradiation and ethylene oxide sterilization are all used but the most suitable method for a particular situation depends upon a number of factors, including the effect on the nutritive value of the diet.

Heating of protein sources such as fish and oilseed meals reduces their nutritional value (Bender, 1972). Sterilization of laboratory animal diets by autoclaving has also been found to reduce protein quality (Eggum, 1969; Schoen & Hiller, 1971). These workers and Udes, Hiller & Juhr (1971) also reported a reduction in total amino acid values in autoclaved diets, but could find little effect of gamma-irradiation on protein quality. A reduction in availability of some amino acids after treatment of casein by exposure to ethylene oxide has been reported (Windmueller, Ackerman & Engel, 1956) but Porter & Lane-Petter (1965) found no decrease in methionine, histidine or available lysine after treatment of a rat diet.

This paper reports the effect of various sterilization procedures on the protein quality and availability of amino acids of a commercial rat diet, and the results of varying the extent of treatment administered.

Table 1. *Sterilization treatments given to commercial rat diet* PRD* *used in Expts* 1 *and* 2

Method of sterilization	Treatment	
	Expt 1	Expt 2
Control	Untreated	Untreated
Autoclaving	121°, 60 min	121°, 60 min
		121°, 30 min
		121°, 15 min
		115°, 60 min
		115°, 30 min
		115°, 15 min
	134°, 3 min	134°, 3 min
Irradiation	2·5 Mrd	2·5 Mrd
	10·0 Mrd	

* C. Hill & Co. Ltd, Poole, Dorset.

In the first experiment, samples of diet were autoclaved and irradiated using a regimen similar to, and one considerably more rigorous than, those commonly used in laboratories. In this way an exaggeration of effects by excess treatment could be measured in addition to those caused by the more usual conditions. A further test was then done to study the influence on nutritive value of varying the severity of autoclaving treatment by altering the time and temperature used.

EXPERIMENTAL

Diet

A commercial rat diet PRD (C. Hill & Co. Ltd, Poole, Dorset) was used in Expts 1 and 2.

In Expt 1, samples were treated as indicated in Table 1. Samples to be autoclaved were spread on metal trays to a depth of 0·01 m and then treated in a high-vacuum autoclave which was evacuated several times before the steam was injected and the load held at the required temperature for the necessary period of time. Samples for irradiation (4 kg) were packed in two heat-sealed polyethylene bags before exposure to a ^{60}Co source (Irradiation Products Ltd, Swindon, Wilts.).

In Expt 2, samples of the diet were also treated as described in Table 1. Samples for autoclaving were packed in 4 kg quantities in paper bags and samples for irradiation were treated as described for Expt 1.

Samples of another batch of diet were also treated by exposure for 8 h to ethylene oxide (1500 ml/m³) at a temperature of 25°.

In Expt 3, available lysine was measured in a further sample of PRD and samples of diet SAD6 (Spratts Patent Ltd, Cambridge Road, Barking, Essex) and CDDR (E. Dixon & Sons (Ware) Ltd, Crane Mead Mills, Ware, Herts.) irradiated at 2·5 Mrd.

Determination of biological value (BV) and true digestibility (TD)

The method used was basically that of Mitchell (1924). Samples of treated and untreated diet were ground and diluted by addition of the nitrogen-free basal diet, so

Table 2. *Composition (g/kg) of the diets used in determinations of net protein utilization for rats*

Ingredient	Nitrogen-free basal diet	Low-N egg diet
Rice starch	640	592·5
Potato starch	100	100
Maize oil	100	100
Ground cane sugar	120	120
Salts USP XIV*	40	40
Diethyl-ether-extracted whole egg	—	47·5

To each diet was also added (mg/kg): retinol 1·0, cholecalciferol 0·025, α-tocopherol 50·0, menaphthone 2·2, thiamin 3·0, riboflavin 6·0, calcium pantothenate 15·0, nicotinic acid 40·0, pyridoxine hydrochloride 4·0, folic acid 0·75, biotin 0·2, cyanocobalamin 0·02, *myo*-inositol 110·0, choline chloride 1900.

* US Pharmacopeia XIV (1950).

that the mixture contained 80 g crude protein $(N \times 6·25)$/kg. Each rat was given every diet in turn on a randomized basis for an 8 d period. The animals were allowed 4 d in which to become accustomed to the diet, and the total collections of urine and faeces were made during the next 4 d. At the beginning and end of the experiment all rats were given the low-N egg diet (Table 2) so that body (or endogenous) N excretion could be measured.

Total N estimations for the urine and faeces were done by the Kjeldahl method (see below). After correction of these values for body N in urine and faeces, TD and BV were calculated as follows:

$$TD = \frac{\text{absorbed N}}{\text{N intake}},$$

where absorbed N = N intake − N in faeces (corrected for endogenous N);

$$BV = \frac{\text{retained N}}{\text{absorbed N}},$$

where retained N = absorbed N − N in urine (corrected for endogenous urinary N). Net protein utilization (NPU) was then calculated as follows:

$$NPU = BV \times TD.$$

Analytical methods

Total N was determined by Kjeldahl digestion using a copper–selenium catalyst and estimating the amount of ammonium sulphate formed, using an AutoAnalyzer (Technicon Instruments Co. Ltd, Basingstoke, Hants).

The fluorodinitrobenzene method of Carpenter (1960) as modified by Booth (1971) was used to determine available lysine and a correction factor of 1·2 was applied as suggested for a mixed diet.

Available and total values for other amino acids were assayed microbiologically

Table 3. *Expt 1. Effect of sterilization by autoclaving and irradiation on protein quality for rats and crude protein (nitrogen × 6·25) content of commercial rat diet PRD**

(Mean values for no. of determinations given in parentheses)

Method of sterilization ...	Control Untreated	Autoclaved		Irradiated	
Treatment		134°, 3 min	121°, 60 min	2·5 Mrd	10 Mrd
Crude protein (g/kg) (3)	189^a	189^a	188^a	188^a	188^a
Biological value (10)	0·77^a	0·64^{bc}	0·54^b	0·72^{ac}	0·72^{ac}
True digestibility (10)	0·87^a	0·83^a	0·64^b	0·87^a	0·87^a
Net protein utilization (10)	0·67^a	0·54^b	0·35^c	0·63^a	0·63^a
Relative nutritive value† (4)	1·0	0·94	0·72	1·0	1·0

Values for individual treatments with the same superscript letter were not significantly different ($P < 0.05$).

* C. Hill & Co. Ltd, Poole, Dorset.

† Microbiological measurement of over-all protein quality; for details, see below.

using *Streptococcus zymogenes*, as outlined by Ford (1962) and Ford & Salter (1966). Samples for analysis of total amino acids were hydrolysed by reflux-boiling for 16 h with 3 M-hydrochloric acid and those for analysis of available amino acids were enzymically digested with papain before being assayed.

Relative nutritive value (RNV) using *Strep. zymogenes* was determined as described by Ford (1960). The results for the treated diet samples were expressed as a proportion of those for the corresponding untreated samples.

Testing for sterility in samples of treated food

In Expt 2, 5 g samples of the treated diets were taken from the bags using aseptic techniques and cultured in media suitable for growth of anaerobic and aerobic organisms and yeasts.

Statistical treatment

The results for TD, BV and NPU in Expt 1 were analysed using analysis of variance, and Tukey's range test (Snedecor, 1957) was used to determine the significance of differences between individual means.

In Expt 2 the available amino acid values were ranked and analysed using Friedmann's Rank Test. Kendall's Concordance Coefficient (Bradley, 1968) was calculated in order to decide whether the relationship between treatments was similar for each amino acid.

RESULTS

Expt 1

Crude protein values were not significantly altered by any treatment (Table 3).

The effects of the treatments on protein quality and amino acids are shown in Tables 3 and 4 respectively. Significant reduction in TD and BV was caused by autoclaving at 121° for 60 min, but at 134° for 3 min, although BV was reduced, TD was unaltered. The combined result of these effects was indicated by the value for NPU,

Table 4. *Expt 1. Effect of sterilization by autoclaving and irradiation on the total and available levels of some amino acids (g/kg) in commercial rat diet PRD**

(Mean values for three determinations/treatment)

Method of sterilization ...		Control Untreated	Autoclaved		Irradiated	
Treatment			134°, 3 min	121°, 60 min	2·5 Mrd	10 Mrd
Arginine:	Available	8·75	6·89	4·50	9·93	9·30
	Total	10·59	10·11	8·98	10·46	10·92
Histidine:	Available	2·70	2·47	1·81	2·57	2·68
	Total	3·77	3·44	4·13	3·86	3·84
Leucine:	Available	12·03	10·44	9·33	12·83	12·63
	Total	12·60	12·76	12·77	13·05	12·92
Methionine:	Available	3·16	2·70	2·21	3·31	3·24
	Total	3·57	3·75	3·86	3·48	3·50
Valine:	Available	9·03	8·35	6·71	8·33	9·18
	Total	9·06	8·98	8·99	8·91	9·29
Lysine:	Available	7·86	5·50	3·54	6·74	6·89
Tryptophan:	Available	1·58	1·25	0·93	1·28	1·31

* C. Hill & Co. Ltd, Poole, Dorset.

which was reduced by 50 % during treatment at 121° and 25 % at 134°. TD, BV and NPU values were not affected by either of the irradiation treatments. RNV (microbiological measurement of over-all protein quality) followed a trend similar to that of the rat test results.

Availability of all the amino acids measured was reduced by autoclaving, the effects again being greater in the diet autoclaved at 121° for 60 min. Available tryptophan and lysine were slightly reduced by irradiation but in each instance the higher dose had no additional effect compared to the lower dose. Of the total amino acids estimated, arginine was reduced by autoclaving at 121° for 60 min.

Expt 2

As in the previous experiment, no alteration of crude protein levels was found (Table 5).

The RNV and available amino acid values (Table 5) were dependent on time and temperature of autoclaving and an interrelationship was apparent between these two factors. When results were ranked in order of extent of reduction in availability of each amino acid and RNV, a close correlation between the relative severity of each treatment on each amino acid was found (Kendall's Concordance Coefficient 0·92). When RNV was the criterion, treatment at 134° for 3 min caused no significant reduction but its effect on most of the available amino acids was rather more severe, the extent of destruction being similar to that of autoclaving at 121° for 15 or 30 min or at 115° for 30 or 60 min.

As found in Expt 1, irradiation had no significant effect on RNV or any of the amino acids studied.

Exposure of the diet to ethylene oxide (Table 6) reduced the availability of all the

Table 5. *Expt. 2. Effect of sterilization by various autoclaving treatments and by irradiation at 2·5 Mrd on crude protein (nitrogen × 6·25) content, relative nutritive value (RNV), and available levels of some amino acid in commercial rat diet PRD**

(Mean values for three determinations/treatment)

Method of sterilization ...	Untreated	Autoclaved							Irradiated (2·5 Mrd)
Temperature (°)	—	121	121	121	115	115	115	134	—
Time (min)	—	60	30	15	60	30	15	3	—
Crude protein (g/kg diet)	193	193	195	192	194	195	192	193	193
RNV†	1·0^c	0·76^a	0·85^{ab}	0·90^{abc}	0·76^a	0·86^{abc}	1·02^c	0·96^{abc}	1·07^c
Available (g/kg diet):									
Arginine	9·77de	6·49^a	8·02^b	8·35^{bc}	6·49^a	9·01^{cd}	10·13^e	7·77^b	10·19^e
Histidine	3·73^c	2·32^a	2·66^{ab}	3·20^{abc}	2·61^a	3·08^{abc}	3·74^c	2·68^{ab}	3·63^{bc}
Leucine	11·60^{cd}	8·65^a	9·32^{ab}	10·43^{bc}	9·41^{ab}	11·35^{cd}	11·66^{cd}	9·57^{ab}	12·25^d
Lysine	8·28^e	4·78^a	6·23^{bc}	7·06^{cd}	5·28^a	6·24^{bc}	7·82de	6·15^b	7·85de
Methionine	3·37^b	2·10^a	2·27^{ab}	2·61^a	2·16^a	2·59^{ab}	2·97^{ab}	2·68^{ab}	3·44^b
Tryptophan	2·31^{bc}	1·61^a	1·96^{ab}	2·01^{ab}	1·71^{ab}	2·27^{bc}	2·60^c	2·01^{ab}	2·49^{bc}

Mean values for individual treatments with the same superscript letter were not significantly different (P < 0·05).

* C. Hill & Co. Ltd, Poole, Dorset.

† Microbiological measurement of over-all protein quality, see p. 270.

Table 6. *Expt 2. Effect of sterilization by treatment with ethylene oxide (Etox) on available levels of some amino acids (g/kg diet) in commercial rat diet PRD**

(Mean values for two determinations/treatment)

	Untreated	Etox-treated
Arginine	9·14	8·67
Histidine	3·17	2·09
Leucine	13·26	12·84
Lysine	8·83	8·68
Methionine	3·72	2·99
Tryptophan	2·17	1·85

* C. Hill & Co. Ltd, Poole, Dorset.

Table 7. *Expt 3. Effect of sterilization by irradiation at 2·5 Mrd on levels of available lysine (g/kg diet) in three commercial rat diets*

(Mean values for three determinations/treatment)

Diet	Expt§ no.	Untreated	Irradiated
PRD*	1	7·86	6·74
	2	8·28	7·85
	3	8·32	7·31
SAD6†	3	8·49	8·89
CDDR‡	3	12·12	11·86

* C. Hill & Co. Ltd, Poole, Dorset.
† Spratts Patent Ltd, Cambridge Road, Barking, Essex.
‡ E. Dixon & Sons (Ware) Ltd, Crane Mead Mills, Ware, Herts.
§ For details, see p. 268.

amino acids studied but this was only significant for histidine, methionine and tryptophan.

Viable organisms were isolated only from samples of the untreated diet and of that treated at 115° for 15 min.

Expt 3

Samples of the other two commercial rat diets irradiated at 2·5 Mrd did not show the reduction in available lysine found with three batches of PRD (Table 7).

DISCUSSION

The results obtained in this study emphasize the destructive action of autoclaving and negligible effect of irradiation on protein quality.

Bender (1972) describes three possible ways in which damage to proteins can occur and so affect their nutritive value. First, certain of the linkages between amino acids may become modified, delaying their release during digestion. Secondly, linkages may form between amino acids and other substances which prevent digestion of the proteins. Thirdly, amino acids may be destroyed by oxidation.

Total N was unaltered by any of the treatments in either experiment, and because in Expt 1 total amino acids also remained unaffected, except for some loss of arginine, they were not measured in Expt 2.

Other workers have also failed to show any decrease in crude protein content after

autoclaving a rat diet for 10 min at 134° (Schoen & Hiller, 1971), or at 105, 118 and 133° (Eggum, 1969), although Udes *et al.* (1971) reported an 8% loss of crude protein after autoclaving at 134° for 20 min.

Both Udes *et al.* (1971) and Eggum (1969) reported a loss of some amino acids during treatment and since certain of our treatments were more severe than some of those used by Eggum (1969), the failure in the present work to show any consistent loss of total amino acids (apart from a small loss of arginine) is surprising. It may be accounted for by the fact that both the previous workers estimated total amino acids with an autoanalyser using a method based on a chromatographic separation of amino acids and their colorimetric determination, but our total amino acid estimations were done microbiologically.

The decrease in NPU after autoclaving might have been caused by modification to the protein, resulting in slower release of some amino acids within the gut, and hence in their delayed arrival at the sites of protein synthesis within the body. Alternatively, it might reflect the uptake of biologically unavailable peptide residues. In either instance the decreased utilization of absorbed N for protein synthesis would be reflected in a lower BV. With more severe treatment the release of amino acids might be prevented entirely, or at least delayed until after the absorptive area of the gut has been passed, the result being a reduced TD. The results obtained in Expt 1 support this suggestion, the BV being affected to a greater extent by autoclaving at 121° for 60 min than at 134° for 3 min, while TD was not significantly affected by the second treatment but was lowered by the first.

The effects of the treatments in Expt 1 on BV, TD and hence NPU are reflected in the results of the available amino acid assays and RNV (a measure suggested by Ford (1960) to estimate the value of protein microbiologically without animal tests). In the first experiment although RNV correlates closely with TD, BV and hence with NPU, it consistently over-estimated NPU. The results of the available amino acid assays suggested that the lowered protein quality, as measured both in the rat tests and as RNV, was due to the reduction in availability of some or all of the amino acids and not of one limiting amino acid alone. The hypothesis agrees with that of Ford (1960) who found that supplements of all the essential amino acids were required to restore the RNV of an autoclaved chick mash to its pre-autoclaved level.

These findings have important practical applications in laboratory animal nutrition as, if only one or two amino acids were rendered unavailable by treatment, it might have been economically preferable to add quantities of these, in the purified form, to the diet before autoclaving. However, the results appear to preclude this course and suggest that the only practical way of maintaining a required utilizable protein level in an autoclaved diet is to add extra protein during compounding.

It is difficult to draw definite conclusions from these results as to the effects of autoclaving on the availability of the amino acids relative to each other because the pattern is slightly different in each experiment. However, the results of both experiments indicate that availability of lysine was most affected, and that of leucine among the least affected by autoclaving. In Expt 2 methionine was as severely affected as lysine.

The lack of effect of irradiation on crude protein or total amino acids is in agreement

with similar results obtained for diets irradiated at between 2·5 and 10 Mrd by Eggum (1969), Schoen & Hiller (1971) and Udes *et al.* (1971).

The results reported here, indicating that irradiation had little effect on TD and BV, agree with those of Eggum (1969), but Schoen & Hiller (1971) reported a small but significant increase in digestibility of the protein of a commercial diet after irradiation.

Although irradiation had no obvious effect on the availability of other amino acids, that of lysine was lowered in both experiments. This decrease was not significant in the second experiment where results were statistically analysed, but it was consistently found in three different batches of irradiated diet PRD.

It is perhaps rather surprising that in Expt 1, irradiation at 10 Mrd had no greater effect than 2·5 Mrd on lysine availability, but no reason for this can be proposed.

The destructive effect of treatment with ethylene oxide in contrast to that of auto-claving was confined to only three of the six amino acids estimated. It has been found that ethylene oxide reacts with carboxyl, hydroxyl, sulphydryl, phenolic and amine groups (Fraenkel-Conrat, 1944) and reduction in availability of histidine and methionine has been reported by Windmueller *et al.* (1956). Windmueller, Ackerman & Engel (1959), however, also reported considerable destruction of lysine in ethylene-oxide-treated proteins, something not found in the present study to any appreciable extent.

The results in these experiments have been obtained for a typical rodent diet, but stability of nutrients in a diet may be influenced by other ingredients, and they may, therefore, not be directly applicable to diets of different formulations. For example, in contrast to PRD, in two other rodent diets the availability of lysine was essentially unchanged after irradiation (Table 7).

The conclusions to be drawn from these experiments are that irradiation had little effect on protein quality of PRD, whereas autoclaving caused some deterioration of protein quality which increased with increasing severity of treatment, and ethylene oxide rendered some amino acids unavailable, with probable accompanying reduction in protein quality.

From a practical point of view therefore, diets which are autoclaved should be given the minimum time and temperature treatment necessary to achieve sterilization. Our results also indicated that high temperature–short period autoclaving caused similar reduction in amino acid availability, and therefore in protein quality, as the commonly used treatment of 121° for 30 min. These tests were done using a modern, high-vacuum autoclave with small quantities of diet allowing fast steam penetration, and therefore the time spent reaching the required temperature and cooling down again were short. With less efficient autoclaves and larger loads, longer will be spent reaching the required temperature and cooling after sterilization, consequently damage to diet is likely to be increased.

REFERENCES

Bender,. A E. (1972). *PAG Bulletin* **13**, 1.

Booth, V. H. (1971). *J. Sci. Fd Agric.* **22**, 658.

Bradley, J. V. (1968). *Distribution – Free Statistical Tests.* New Jersey: Prentice-Hall Inc.

Carpenter, K. J. (1960). *Biochem. J.* **77**, 604.

Eggum, B. O. (1969). *Z. Tierphysiol. Tierernähr. Futtermittelk.* **25**, 204.

Ford, J. E. (1960). *Br. J. Nutr.* **14**, 485.

Ford, J. E. (1962). *Br. J. Nutr.* **16**, 409.

Ford, J. E. & Salter, D. N. (1966). *Br. J. Nutr.* **20**, 843.

Fraenkel-Conrat, H. L. (1944). *J. biol. Chem.* **154**, 227.

Mitchell, H. H. (1924). *J. biol. Chem.* **58**, 873.

Porter, G. & Lane-Petter, W. (1965). *Br. J. Nutr.* **19**, 295.

Schoen, A. & Hiller, H. H. (1971). *Z. Tierphysiol. Tierernähr. Futtermittelk.* **27**, 338.

Snedecor, G. W. (1957). *Statistical Methods.* Ames, Iowa: Iowa State College Press.

Udes, H., Hiller, H. H. & Juhr, N. C. (1971). *Z. Versuchstierk.* **13**, 160.

US Pharmacopeia, XIV (1950). p. 789.

Windmueller, H. G., Ackerman, C. J. & Engel, R. W. (1956). *J. Nutr.* **60**, 527.

Windmueller, H. G., Ackerman, C. J. & Engel, R. W. (1959). *J. biol. Chem.* **234**, 895.