Chemical and nutritional changes in stored herring meal

3.* Effect of heating at controlled moisture contents on the binding of amino acids in freeze-dried herring press cake and in related model systems

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In Part 2 of this series (Lea, Parr & Carpenter, 1960) it was reported that appreciable losses of available lysine occurred when fresh herring meal in air, or pre-oxidized meal in nitrogen, was heated for 30 h at temperatures below 100°, whereas fresh meal in nitrogen could be subjected to similar heating without loss. It was suggested that this difference in behaviour was due to a 'browning'-type reaction between carbonylic secondary decomposition products of the autoxidizing oil and the ε-amino groups of the lysine side-chains, analogous to the well-known reactions between reducing sugars and amino acids or proteins.

When the temperature of heating was raised to 115–130° binding of the lysine ε-amino groups was greatly increased and occurred in the unoxidized as well as in the oxidized meal, the difference between the two becoming small at the highest temperature. Biely, March & Tarr (1951) had previously made a somewhat similar observation, that heating at 149° for 2 h appreciably damaged the nutritive value of herring meal containing (presumably oxidized) oil but did not damage defatted meal, whereas heating for 3 h caused more severe and similar damage to both.

Apparently, therefore, at least two lysine-binding reactions can occur in heated herring meal, the oxidized fat-dependent type becoming significant under less severe time–temperature conditions than the non-oxidative, fat-independent type.

The purpose of the work now reported has been to study the effects of temperature and of moisture content on the high-temperature, non-oxidative lysine-binding reaction, which must be largely responsible for the very low values for available lysine found for some 'scorched' commercial meals.

Part of the work has been done with freeze-dried herring press cake and part with bovine plasma albumin 'model' systems, the latter with and without small additions of ribose. A few observations have been recorded also on the binding of methionine, arginine and tryptophan in the heated press cake and on colour changes in both systems.

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Much work has been published previously on the damaging effects of heat on food proteins in respect of their biological value or in vitro digestibility by enzymes (see reviews by Rice & Beuk, 1953, and Liener, 1958), but available lysine has not usually been determined chemically and the carbohydrate and moisture contents of the proteins used were often not controlled or recorded, making comparison of the results difficult.

EXPERIMENTAL

Preparation of the materials

Freeze-dried press cake. The herring were cooked with steam in a commercial plant and pressed to remove most of the oil and the aqueous 'stick' liquor. A sample of the moist cake was then packed into Polythene bags with solid carbon dioxide and transported in a refrigerated container to the laboratory where it was freeze-dried, milled, gas-packed in 'oxygen-free' nitrogen and held at $-20°$ until required. The pH was 6·6.

A sample of the meal (no. 7), produced commercially from the same batch of press cake by drying in a hot-air tunnel, was similarly transported to the laboratory, milled, gas-packed and held at $-20°$.

Defatted press cake. Part of the ground, freeze-dried press cake was extracted with boiling 2:1 (v/v) chloroform–methanol, and then twice with diethyl ether. The combined extracts were evaporated to dryness under reduced pressure and the lipid was taken up in chloroform, residual traces of insoluble material being returned in diethyl ether to the extracted meal. The ether was then removed from the defatted meal under reduced pressure at $50–60°$.

Adjustment of the moisture content. The freeze-dried cake, as prepared, contained 4·6 % moisture and the defatted material somewhat less. Portions of both meals were adjusted to lower moisture content by further drying, the 'anhydrous' samples being prepared by heating for 24 h at $37°$ and then for 18 h at $70°$, both over P_2O_5 under reduced pressure. For adjustment to higher moisture content the required quantities of water were sprayed on to the meals as they were 'tumbled' in a rotating tilted vessel, and this procedure was followed by equilibration for 24 h at reduced pressure at $0°$.

Heating the moisture-adjusted press cake

To avoid serious losses of water from the moisture-adjusted press cake at the high temperatures used in the heating experiments it was necessary to heat the press cake in vessels with minimal head space. This was done by compressing the material into the closed end of a 6 in. × 0·5 in. Pyrex tube by means of a glass rod. A piece of similar rod was then inserted, over a plug of glass wool, to occupy most of the remaining free space, and the tube was drawn off, flushed with nitrogen and sealed at a residual pressure of from 2 to 3 cm mercury. Heating was at controlled temperatures ranging up to $145°$ for periods up to 27 h, a silicone-oil bath being used for the shorter (up to 3 h) and an oven for the longer periods.

Experiments with bovine plasma albumin

Crystallized bovine plasma albumin (BPA) (Armour Laboratories Ltd, Hampden Park, Eastbourne), stated to contain < 0·1 % protein-bound hexose as determined by an orcinol method, was used. Determination of sugar, by an anthrone method (Bailey, 1958) in the filtrate after an aqueous solution of the protein had been boiled for 45 min with 5 % (w/v) trichloroacetic acid, indicated < 0·025 % total sugar. All the ϵ-amino groups of BPA are reactive (Porter, 1948).

Preparation and heating of BPA–ribose mixtures. Ribose was added to aqueous solutions of BPA to give concentrations corresponding to 0·3 and 1·0 % ribose calculated on the dry weight of the protein. The solutions were immediately freeze-dried and equilibrated under reduced pressure at 0° over a solution of H_2SO_4 giving a relative humidity of 65 %. The protein without ribose then contained 14·1 % moisture. The moisture contents of the ribose-containing samples, which could not be determined directly, were assumed to be similar.

Portions (0·5 g) of the three products were sealed off in small tubes with minimal head space, as with the herring press cake, and heated under controlled conditions in a similar way.

Analytical procedures

Lipid content. The dependence of the amount of lipid extracted from herring meals on the extraction procedure used has already been discussed (Lea, Parr & Carpenter, 1958). In this study the 'fat' content of the materials is defined as the chloroform-soluble portion of the extract obtained after digestion for 3 h with hot chloroform–methanol (2:1, v/v). When the lipids were to be used, after purification, for determination of their iodine values, the extraction was made under inert gas with de-aerated solvents. Without these precautions the iodine values obtained were erratic and lower by from 6 to 18 units.

Water content. The moisture contents of the press-cake samples were determined by heating to 'constant weight' (5–7 h) under greatly reduced pressure (oil pump) over P_2O_5 at 70°. This treatment was considered to be similar to that for 18 h at 70° and 1 in. pressure with dry air stream, found adequate for casein by Mellon, Korn & Hoover (1947). Continuation of the heating for a further 18 h increased the apparent moisture content by 0·2–0·3 %, and raising the temperature to 110° for a further 2–4 h increased the loss of weight by a further 0·3 %, but it seems likely, in view of the 'charring' which occurred at the surface of the P_2O_5, that much of the extra volatile material lost under these severe conditions was organic matter rather than moisture. The defatted cake showed a similar difference in apparent moisture content between the two extremes of treatment.

Determination of 'available' amino acids

The method used for determining available lysine was based on the Sanger reaction between free lysine ϵ-NH_2 groups and fluorodinitrobenzene, and followed the published procedure of Carpenter (1960). Duplicate assays were always made and a control

sample of press cake was included in each run. Reproducibility of the results was estimated to be such as to give a 95 % probability level of significance to differences of 5 % in mean values obtained within the same run, and of 8 % for differences obtained in different runs.

The values for the defatted materials as sources of the amino acids methionine, arginine and tryptophan were determined with a proteolytic strain of *Streptococcus zymogenes* after a mild digestion with papain. The technique of Ford (1962) was applied except that double the standard quantity of papain (British Drug Houses Ltd, Poole; non-crystalline grade) was used. It was not possible to assay lysine also in this way, because it is not an essential nutrient for this organism.

Measurement of colour. The defatted press-cake samples and the BPA with and without ribose were examined for colour before and after heating by means of a spectrophotometer with reflectance attachment, the extinction being measured at wavelengths of 400 and 500 mμ as compared with a standard white magnesium carbonate. The brown discoloration resulting from heating caused general absorption in the blue end of the visible spectrum which decreased fairly regularly with increasing wavelength. The figures at 500 mμ are given rather than the somewhat higher values at 400 mμ, as being more representative of the visible change. In a few tests colour was also measured with a Lovibond Tintometer but reproducibility by this method was less satisfactory.

<div align="center">RESULTS</div>

<div align="center">*Composition of the press cake and of the commercial meal*</div>

The analytical values summarized in Table 1 show only very small differences between the compositions of the two materials. As usual, the three major components totalled more than 100 when the conventional 'N × 6·25' was used as the measure of 'crude protein' content and when an efficient extraction procedure was used for the measurement of fat content.

Table 1. *Composition of the freeze-dried herring press cake and of the commercial herring meal (no. 7) prepared from the same batch of press cake*

	Freeze-dried press cake	Herring meal no. 7
Lipid* (g/100 g solids)	20·6	20·2
Crude protein (N × 6·25) (g/100 g solids)	67·2	66·0
Ash (g/100 g solids)	17·8	17·6
Total†	105·6	103·8
Iodine value of the lipid	139·8	138·8
Available amino acids‡ (g/100 g fat-free solids):		
Lysine	5·5	5·5
Methionine	1·6	1·6
Arginine	2·9	—
Tryptophan	0·85	—

* Defined here as material removed by extraction for 3 h with hot chloroform–methanol (2:1, v/v) and soluble in chloroform.

† See above.

‡ Available lysine was measured by the reactivity of the sample with fluorodinitrobenzene, and the other amino acids by the assay of papain digests of defatted samples with *Streptococcus zymogenes*.

The composition of the fresh commercial meal (no. 7) was generally similar to those recorded for meals nos. 1, 3 and 4 used previously in these studies, though the available lysine value, 6·8 g/16 g N, was rather lower than the previously obtained values of 7·2, 7·0 and 7·9 g/16 g N respectively (Lea *et al.* 1958, 1960; Carpenter, Lea & Parr, 1963.)

The compositions of the heated, moisture-adjusted press-cake samples are given in Table 2.

Table 2. *Moisture and fat-free solids in the samples of moisture-adjusted heated freeze-dried herring press cake*

| | Press cake† | | | Defatted press cake† | |
| | Moisture (%) | | | | |
Fat* (%)	In whole cake	On fat-free basis	Dry fat-free solids (%)	Moisture (%)	Dry fat-free solids (%)
20·6	'0'	'0'	79·4	'0'	100
19·8	3·9	4·9	76·3	4·9	95·1
19·3	6·3	7·8	74·4	7·8	92·2
18·8	8·6	10·6	72·6	10·6	89·4
18·1	11·8	14·4	70·1	14·4	85·6
15·4	25·0	29·6	59·6	29·6	70·4
10·3	50·0	55·8	39·7	55·8	44·2

* Defined here as material removed by extraction for 3 h with hot chloroform–methanol (2:1, v/v) and soluble in chloroform.
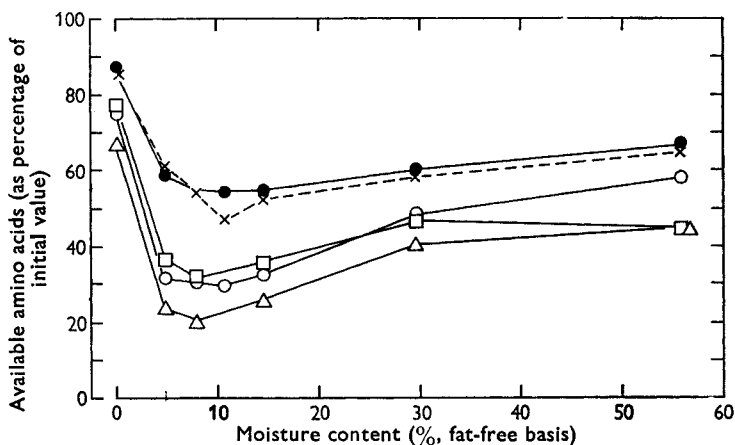† Prepared as described on p. 452.

Fig. 1. Effect of heating at 130° for 27 h on the availability of amino acids in defatted herring press cake of different moisture content. ●——●, lysine (reactivity with fluorodinitrobenzene); ○——○, methionine (*Strep. zymogenes*); △——△, tryptophan (*Strep. zymogenes*); □——□, arginine (*Strep. zymogenes*). Available lysine was determined also in whole press cake (20·6 % lipid, dry basis) similarly treated (× --- ×).

Effect of lipid on the lysine-binding reaction

The available lysine contents of the whole press cake and of the defatted samples, after heating for 27 h at 130°, are compared in Fig. 1. Over the wide range of moisture contents covered there was no evidence of any significant participation of lipid (which was, of course, unoxidized) in the lysine-binding reaction.

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Effect of moisture content on amino acid binding

Drying the press cake to 'zero' moisture content by the method described on p. 452 greatly reduced the amounts of the amino acids rendered unavailable by heating (Fig. 1). These extremely hygroscopic fully dried materials, with their sponge-like structure and large surface:weight ratio, undoubtedly reabsorbed some moisture from the air of the laboratory during manipulation, and it is possible that complete inhibition of the reaction might have been obtained had greater precautions been taken at this stage.

On heating the press cake for 27 h at 130°, amino acid binding was found to be at a maximum in the region of 4–12 % moisture content (5–14 % on the fat-free basis),
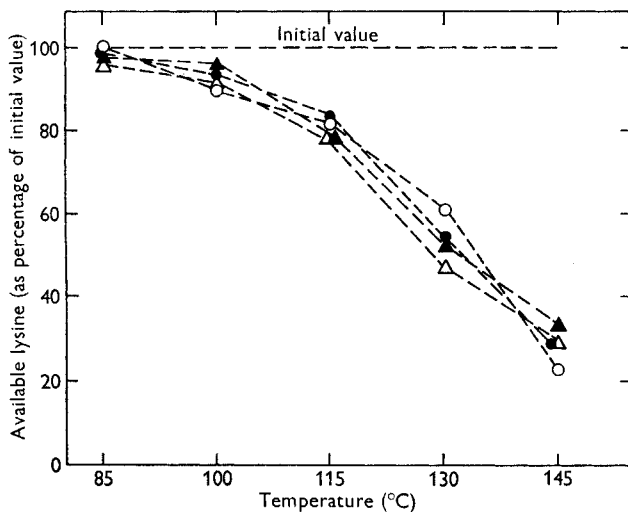

Fig. 2. Available lysine in freeze-dried herring press cake at different moisture contents after heating at 85–145° for 27 h. ○---○, 3·9 % moisture; ●---●, 6·3 % moisture; △---△, 8·6 % moisture; ▲---▲, 11·8 % moisture.

the decrease on heating being appreciably less for the very moist as well as for the driest samples. However, the effect of moisture content over the range usual for herring meals was small.

The influence of moisture content showed the same general trend for each of the four amino acids investigated, and at each moisture content the proportions of 'available' methionine, arginine and tryptophan lost were similar. The relative losses of available lysine, as measured by the presence of reactive ε-NH_2 groups, however, were only about 70 % of those for the three amino acids assayed by *Strep. zymogenes.*

At 85°, when the lysine-binding reaction was very much slower, the influence of moisture content was perhaps a little more marked. After 27 h (mean binding 2 %) differences were not significant in relation to the experimental error of the determination, and even after 243 h the two samples examined (4·9 % and 10·6 % moisture, fat-free basis) showed approximately the same loss of available lysine (9 %). But after

729 h lysine binding showed an apparently significant gradation from 16 % at 4·9 % moisture through 21 % at 7·8 % moisture to 26 % at 10·6 % and 14·4 % moisture (fat-free basis).
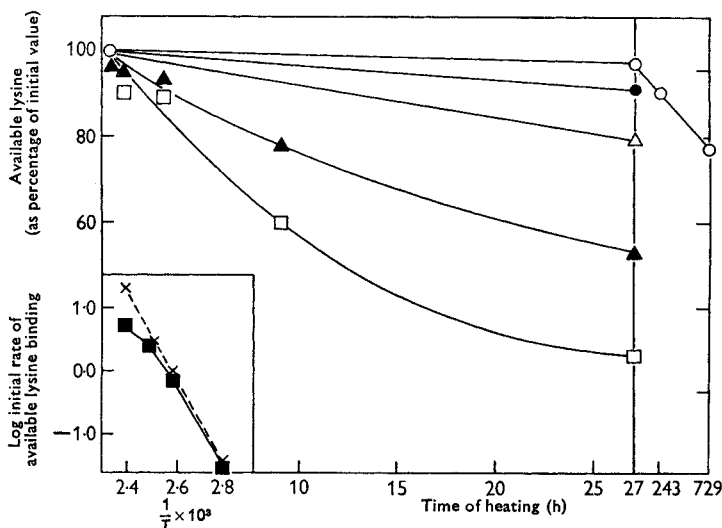

Fig. 3. Effect of temperature on the lysine-binding reaction: mean values for samples of herring press cake containing 3·9–11·8 % moisture. o——o, 85°; ●——●, 100°; △——△ 115°; ▲——▲, 130°; □——□, 145°. In the inset figure the initial rates of reaction have been plotted as described on p. 458, the corresponding values for bovine plasma albumin (BPA) being included for comparison. ■——■, press-cake; × - - -×, BPA. T, absolute temperature.
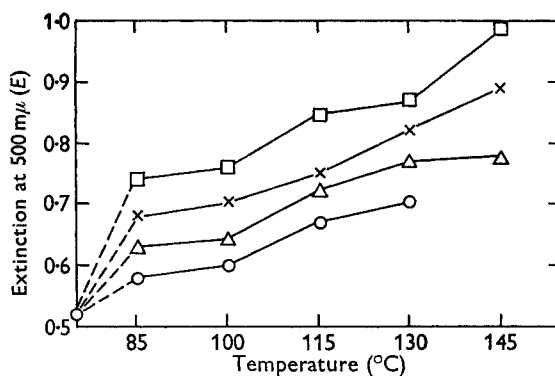

Fig. 4. Darkening of defatted herring press cake (4·9–14·4 % moisture) on heating for 27 h at 85–145°. o——o, 4·9 % moisture; △——△, 7·8 % moisture; ×——×, 10·6 % moisture; □——□, 14·4 % moisture.

Effect of temperature

The effect of heating press-cake samples containing 3·9–11·8 % moisture (4·9–14·4 % on the fat-free basis) at 85–145° for 27 h is shown in Fig. 2. The influence of moisture content under these conditions was comparatively small and erratic.

For a more quantitative measure of the influence of temperature on the rate of the reaction, values for available lysine were determined on press-cake samples containing

3·9–11·8 % moisture (4·9–14·4 % on the fat-free basis) after heating for 0·3, 1, 3, 9 and 27 h at 145° and 130°, for 27 h at 115 and 100°, and for 27, 243 and 729 h at 85°. The mean values for the available lysine at the several moisture contents (usually four, but in a few instances only two) after each treatment are given in Fig. 3 together with an Arrhenius plot of the logarithm of the initial reaction rates (calculated for the binding of the first 20 % of the available lysine), against the reciprocal of the absolute temperature × 1000.

Colour changes on heating

Over the range from 4·9 to 14·4 % moisture the initial colour of the unheated, defatted press cake was the same ($E = 0·52$ at 500 mμ). On heating, all the samples darkened, the discoloration being progressively greater the higher the moisture content (Fig. 4), in contrast to the amount of amino acid binding which remained approximately the same over this range of moisture content. Since the measurements at 400 mμ showed a generally similar picture they have not been reported.

Table 3. *Binding of lysine in bovine plasma albumin* caused by heating with and without added ribose*

Temperature (° C)	Ribose added (%)	Available lysine† after heating for		
		1 h	27 h	729 h
85	0·0	100	100	75
	0·3	98	98	73
	1·0	93	93	68
115	0·0	95	74	—
	0·3	94	72	—
	1·0	93	69	—
145	0·0	80	23	—
	0·3	75	22	—
	1·0	74	21	—

* Previously equilibrated to 65 % relative humidity at 20° and containing 14·1 % moisture and, initially, 10·8 % available lysine. Available lysine values in the ribose-containing samples have been corrected to the ribose-free basis.

† Determined by the fluorodinitrobenzene method, and expressed as a percentage of the corresponding value for the unheated material.

Bovine plasma albumin 'model' systems

Changes in available lysine. The results summarized in Table 3 show that BPA suffered no detectable loss of available lysine when heated alone for 27 h at 85°. But very prolonged exposure to this temperature, or exposure of an hour or more to a temperature of 115° or above, produced progressively more severe losses until after 27 h at 145° more than three-quarters of the lysine originally available had been bound.

In the presence of small quantities of added ribose the reaction between the reducing sugar and the free amino groups of protein (Maillard or 'browning' reaction) had already produced its maximum lysine-binding effect within the shortest exposure tested (1 h at 85°), 0·3 % of ribose binding approximately 2 % of the available lysine and 1·0 % of ribose approximately 7 % of the available lysine. These figures correspond to about 0·8 mole of lysine bound per mole of ribose added.

Changes in colour. The protein without added sugar darkened only very slightly on heating at 85°, but quite markedly at 145° (Fig. 5). In the presence of added ribose the extent of the change was greatly increased, 1 % of ribose causing nearly as much discoloration in 1 h at 85° as developed in the protein alone in 27 h at 145°. A large proportion of the observed change usually occurred within the 1st h of heating.
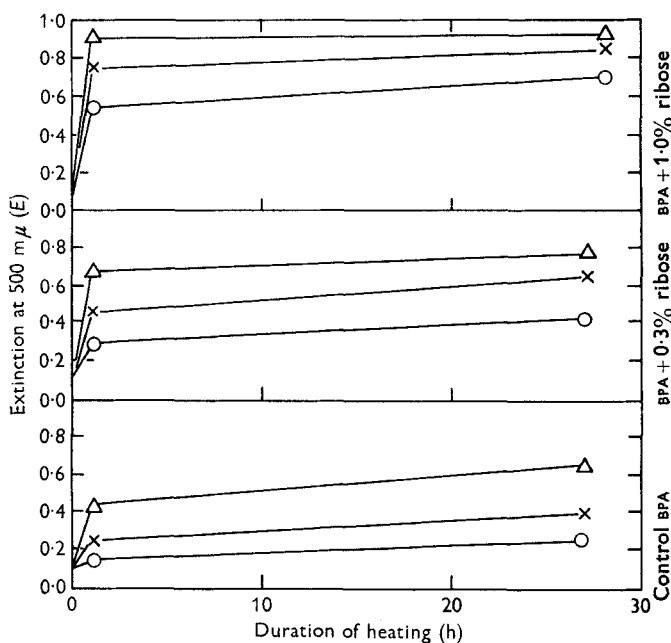

Fig. 5. Darkening of bovine plasma albumin (BPA) (14·1 % moisture) on heating with and without added ribose. ○—○, 85°; ×—×, 115°; △—△, 145°.

DISCUSSION

Reactions of proteins or amino acids with free reducing hexoses, and even more so with pentoses, are normally much more rapid than the main lysine-binding reaction studied in our heating experiments with freeze-dried herring press cake. Lea & Hannan (1949), for example, found that 30 % of the free amino nitrogen in a casein–glucose mixture (10–14 % moisture) was bound in about 24 h at 40° (1 min at 100°), whereas more than 27 h at 115° was required for a similar loss of available lysine in the press cake (Fig. 2). It was so also in the experiments with freshly prepared commercial herring meal previously reported (Lea *et al.* 1960).

The casein–glucose mixture of Lea & Hannan (1949) contained about 9 % glucose, an amount stoichiometrically equivalent to the total free amino groups of the protein, but only a very small fraction of this concentration could be present in herring meal made from press cake. Traces of free sugar, mainly glucose, are normally present in fresh fish and small amounts, mainly of enzyme-liberated ribose (Tarr, 1955), are likely to be present in stale fish. But a considerable proportion of the water-soluble free and combined sugar present would be rejected in the aqueous 'stick' liquor

pressed from the cooked fish during processing, so that levels in 'press cake' of the type used here would be considerably lower than in the original fish. The amount of protein-bound (nucleic acid) ribose, which might require a drastic heat treatment for release of the free sugar, appears to be only of the order of o·1 % of the dry weight of fish muscle (Bluhm & Tarr, 1957), although quantities several times larger would be present in organ tissues (offal) included in the meal.

Maillard reactions involving the small quantities of free reducing sugars and sugar phosphates present are considered to be mainly responsible for the browning of fish flesh which is sometimes observed during sterilization procedures (Tarr, 1954; Jones, 1959). Degradation products of 1-methylhistidine, however, may also make some contribution (Jones, 1956) and the degree of darkening of different species of fish during autoclaving is not necessarily proportional to their free reducing-sugar content since other factors may be limiting (Nagayama, 1960).

In the experiments with BPA at 85 and 115° small additions of ribose brought about a marked increase in browning (Fig. 5), but at 145° discoloration was becoming fairly marked even in the sugar-free protein. Though browning and lysine binding both increased with increasing temperature there was no consistent relationship between the two.

Bissett & Tarr (1954) have suggested that a liberation of pentose from nucleic acid might be responsible for the reduced availability of essential amino acids which they observed when herring meal was heated for 3 h at 149°, and Tarr (1960) has stated that there is little doubt that Maillard (carbonyl–amine) reactions are responsible for the loss of nutritive value of some strongly heated fish meals. Miller (1956) has expressed a similar opinion. The very large amount of lysine and of other amino acids that can be bound in relation to the small quantities of free or even of combined sugar likely to be present in dried press cake militates against this view. The more strongly heated samples of press cake referred to in Fig. 2, for example, lost more than 4 g lysine/100 g of dry fat-free solids. It is known that carbonyl–amine 'browning' reactions are not stoichiometric, in that the first-formed condensation product, after rearrangement or degradation, can react with further molecules containing either carbonyl or amine groups, but the discrepancy between the concentrations of active carbonyl and potential amino acid reactants in herring press cake is too large for this explanation to be feasible.

Fig. 6, in which the binding of lysine in press cake and in model BPA and BPA + ribose systems is compared, indicates strongly that amounts of sugar such as might be present in herring meal made from press cake are insufficient to make more than a minor contribution to the losses of available lysine observed on severe heating, and that most of the binding of lysine (and of other amino acids) under these circumstances is likely to result from some form of internal reaction, or cross-linking, between the side-chains in the proteins themselves.

On the other hand, it is also clear that even a very mild degree of heating is sufficient to allow any small quantities of reducing sugar, or of carbonylic products of fat oxidation that may be present, to take their toll of the available lysine by way of a carbonyl–amine 'browning' reaction. Other amino acid side-chains can also become involved in

reactions with reducing sugars, but lysine is usually the first to be affected (Lea & Hannan, 1950).

The rates of lysine binding in heated BPA (without ribose) and in the herring press cake were approximately the same, on a percentage basis, at the lower end of the temperature scale (Table 4, Fig. 3). At the highest temperature (145°) the reaction was considerably faster in the pure protein than in the press cake, which contained much non-protein material and had a lower initial concentration of lysine. However, the rate of reaction of the lysine ε-amino groups fell off rather sharply as the reaction
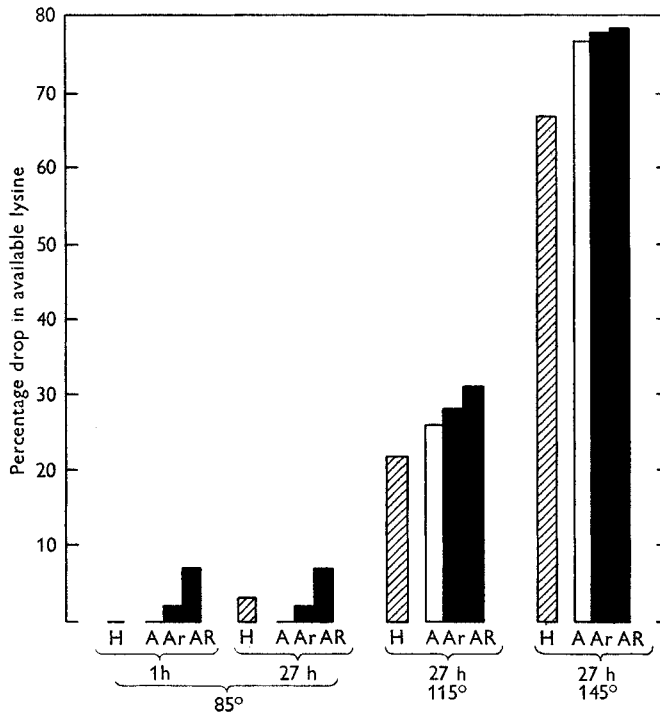

Fig. 6. Binding of lysine in herring press cake (14·4 % moisture, fat-free basis) and in bovine plasma albumin (BPA)–ribose model systems (14·1 % moisture). H, herring press cake; A, BPA; Ar, BPA + 0·3 % ribose; AR, BPA + 1·0 % ribose.

Table 4. *Comparison of the effects of temperature on lysine binding in herring press cake* and in bovine plasma albumin† (BPA)*

Material	Initial‡ rate of lysine binding (%/h) at			
	85°	115°	130°	145°
Herring press cake (mean for four samples containing 4·9–14·4 % moisture)	0·030	0·74	2·5	5·0
Herring press cake (14·4 % moisture)§	0·036	0·82	2·3	5·0
BPA (14·1 % moisture)	0·034	1·02	—	20·0

* Lipid content 20·6 % of the dry solids. Initial available lysine 5·5 % of the dry, fat-free solids.
† Initial available lysine 12·6 % of the dry solids.
‡ Calculated from the time required for 20 % lysine binding at the various temperatures.
§ Fat-free basis.

proceeded (Table 3, Fig. 3), probably as a result of a reduced accessibility of the remaining reactant groups to one another, and after 27 h at 145° there was little difference between the extent of the reaction in the two materials (Fig. 6).

The apparent activation energies for the binding of lysine in BPA (31 kcal/mole) and in herring press cake over the lower part of the temperature range (29 kcal/mole) are similar to values previously reported for protein–sugar 'browning' reactions by Mohammad, Fraenkel-Conrat & Olcott (1949) and by Lea & Hannan (1949). They correspond to the high average temperature coefficient per 10° of 2·9–3·1 over the range 85–115°.

Discoloration also developed at fairly similar rates in the press cake and BPA systems, increases in extinction at 500 mμ after heating for 27 h at 85–145° being 0·22–0·47 for defatted press cake (14·4 % moisture) compared with 0·13–0·52 for BPA without ribose and 0·30–0·64 for BPA with 0·3 % added ribose. On the Lovibond scale, darkening in the defatted press cake (10·6 % moisture) corresponded to 2·6 and 3·4 red plus yellow units after 27 h at 130° and 145° respectively, considerably less than the browning observed by Carpenter, Ellinger, Munro & Rolfe (1957) in cod fillets of similar moisture content (5·5 units after 24 h at 105°). This difference was probably attributable in part to a lower free-sugar content in the press cake. However, it is also possible that the heating conditions referred to by these authors were not precisely controlled in the equipment used, and the effective temperature may have been higher than that stated. This view is supported by the fact that, whereas the heated fillets in the earlier experiment showed a 30 % decrease in available lysine content, a recent repetition of the treatment with similar material but precise temperature control gave a decrease of only 17 % (March & Carpenter, unpublished results). This last figure indicates a rate of binding of lysine of 0·46 %/h, which is rather less than twice as fast as that expected for press cake at 105° on the basis of the work now described (Table 4).

The degree of darkening in defatted cake is, of course, much less than that developed in heated herring meals containing oxidized oil, in which most of the colour produced is attributable to interaction between oxidized fat and non-fatty constituents of the meal (Lea et al. 1960).

The effect of variation of moisture content on the reactions causing binding of amino acids at high temperatures in the absence of appreciable amounts of reducing sugar, was qualitatively similar (Fig. 1) to that reported for the reaction causing binding of sugars with lysine at lower temperatures (Lea & Hannan, 1949; Lea, Hannan & Greaves, 1950; Schwartz & Lea, 1952). In both instances there was a region of moisture content giving maximum damage, with less in both drier and moister samples, but the effect appeared to be smaller in our work and the moisture content for maximum effect was less clearly defined. Miller (1956), who examined the effect of heating for 24 h at controlled moisture content or relative humidity (r.h.) on the net utilization by rats of extracted fish protein, also found that damage was least in the completely dry sample. Heating at a moisture content of 17 % caused slightly more damage than at 9 %, and at 97 % r.h. the effect was slightly worse than at 69 % r.h., but both differences were little larger than the standard error of the assay.

The nature of the chemical reactions responsible for the binding of amino acids in

strongly heated proteins does not seem to have been elucidated. A possibility with lysine would appear to be a condensation reaction between the lysine ε-amino groups and the carboxyl groups of glutamic and aspartic acid side-chains as suggested by Evans & Butts (1949 *a*, *b*). But this reaction has not, to our knowledge, been shown to occur in heated proteins although it apparently does proceed to some extent during the partial acid hydrolysis of proteins (Swallow, Lockhart & Abraham, 1958).

The three amino acids assayed with *Strep. zymogenes* all showed a greater drop in availability than was indicated for lysine by the decrease in its reactive ε-NH_2 groups, although the side-groups of these amino acid units are less reactive in casein–glucose systems (Lea & Hannan, 1950). It may be that these low microbiological values reflect a general difficulty of proteolytic attack on the peptide chain of such severely heated proteins, and, in other experiments with proteins receiving this type of treatment (Morgan & Carpenter, unpublished results), even leucine, with an unreactive paraffin side-group, has shown a similar decrease in availability. This view is supported by the further observation that the microbiological values for heated materials depend greatly on the concentration of papain used in the pretreatment; with a fourfold increase in the quantity of papain, the relative decline of availability was less and of the same order as the relative decline in reactive lysine groups. Which set of conditions more closely reflects the availability of particular amino acids for animals can only be determined from the results of biological assays with the species concerned.

Clandinin (1949) and also Bissett & Tarr (1954) subjected normal and overheated samples of herring meal to in vitro digestion with pancreatin plus 'hog intestinal mucosa', and assayed the digests for individual amino acids with non-proteolytic bacteria; they concluded that the relative decrease in the release of lysine from an over-heated meal was similar to that for other amino acids. (*See note*, p. 465.)

The very low values for available lysine found in occasional samples of commercial fish meals (Lea *et al.* 1960; Olley & Watson, 1961; Carpenter *et al.* 1963), and their generally inferior biological value as protein supplements in animal feeding, would seem to indicate that such meals have been subjected to very severe overheating, either during drying or, more probably, during autoxidation of their oil after drying. As indicated by microbiological assay and digestibility studies, these meals are poor sources of other essential amino acids as well as of lysine.

Though drying to a very low moisture content would certainly afford some protection against the damaging effects of subsequent overheating, it is doubtful whether sufficiently low values could be achieved commercially without causing damage during drying. Moreover, very dry meals are known to autoxidize more rapidly than those with higher moisture contents (Lea *et al.* 1958; Astrup, 1958), so that the problem of preventing oxidation-induced overheating in the freshly dried material would be accentuated. Although it has been observed that heat treatment may itself have some stabilizing effect on the lipid in fish meal (cf. Lea *et al.* 1960) it appears only to delay the onset of rapid oxidation for a limited period, and the conditions for producing even this effect seem difficult to define (Lea & March, unpublished results).

The most practicable means of preventing heat damage at present available would therefore seem to be to dry the meal to a normal moisture content, and to reduce oxidative

overheating to a minimum by utilizing the delaying action of antioxidants (cf. Meade, 1956; Astrup, 1958; Lea *et al.* 1960) and containers of low permeability to oxygen (Astrup, 1958), coupled with adequate cooling or ventilation between the bags.

SUMMARY

1. Freeze-dried herring press cake adjusted to various moisture contents was heated at 85–145° in sealed tubes. 'Available' lysine was determined by the fluorodinitrobenzene method, and in some instances 'available' methionine, arginine and tryptophan were determined by a microbiological method. Decreases of up to 75 % of the initial values for these amino acids were recorded.

2. Binding of amino acids was greatest at the intermediate range of moisture content of from 5 to 14 % on the fat-free basis, the rate falling off sharply in very dry samples and more slowly at high moisture contents.

3. The unoxidized lipid present in the fresh press cake played no part in the lysine-binding reaction.

4. Bovine plasma albumin lost its available lysine and discoloured on heating at rates similar to those observed for the press cake. Small additions of ribose increased both of these changes, but the effects were already largely complete after time–temperature treatments much milder than those required to produce corresponding changes in the press cake. Rather less than 1 mole of lysine was bound per mole of ribose added.

5. It is concluded that the small quantities of free and combined sugar present in herring meal made from press cake can make only a comparatively minor contribution to the major losses of available amino acids that result from severe overheating.

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Note added in proof. It has recently been shown by biological assay that the net protein value of heated pork fell by a greater extent than could be accounted for by losses of lysine, available lysine or the sulphur amino acids, the last being the limiting factor in both original and heated material (Donoso, G., Lewis, A. M., Miller, D. S. & Payne, P. R.(1962), *J. Sci. Fd Agric.* **13**, 192).