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EFFECT OF HEAT ON THE EXTRACTABILITY OF LIPID FROM LEAF PROTEIN MEAL

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Drying of leaf proteins at elevated temperature results in a decrease in the extractability of the lipids. This seems to be due to oxidation of free lipids, and formation of insoluble complexes by phospholipids and oxidation products of the lipids with proteins. Liberation of bound lipids by 0.2N HCl or 90% phenol suggests that the lipids are not bound to proteins by covalent linkages and the decrease was due to a physical change in the protein.

Preliminary experiments indicated that the percentage of extractable lipids decreased when leaf proteins were stored in air for a long time. The decrease in the extractability of lipids from a system containing protein is generally attributed to the formation of insoluble complexes between the autoxidized lipids and the protein.

Formation of brown complexes between carbonyl groups, as found in many sugars, and free amino groups of proteins and amino acids was reported by Maillard.[1] Davies and Gill[2] suggested that phospholipids containing free amino groups can also form carbonyl-amine complexes with sugars and aldehydes. According to Lea *et al.*.[3] this 'browning' reaction (carbonyl-amine reaction) takes place between the secondary oxidation products of lipids and the α-amino groups of amino acids. Biely, March and Tarr[4] reported that heating for 2 hr at 149°C appreciably reduced the nutritive value of whole herring meal but did not affect the defatted product and suggested that oxidation of the lipids might be responsible for this.

Tappel[5] suggested that the brown polymeric substances formed when highly unsaturated fat plus protein was oxidized in aqueous emulsion, were partly due to the aldehyde-amine condensation between the oxidation products and protein. However, Venolia and Tappel,[6] and Narayan and Kummerow[7] concluded that the insoluble complexes formed between autoxidized fat and protein at 37°C do not involve covalent bonding, but are probably due to the physical adsorption of the oxidized fat on to the protein. Lea, Parr and Carpenter,[3] while studying chemical and nutritional changes in herring meal, reported the destruction of lysine during the reaction between fat oxidation products and proteins. Miller[8] and Tarr[9] have attributed the loss in nutritive values of strongly heated fish meals to the carbonyl-amine (Maillard) reactions. Bisset and Tarr[10] suggested that liberation of pentoses from nucleic acid might be responsible for the reduced availability of the amino acids when herring meal was heated for 3 hr at 149°C.

Carpenter *et al.*[11] compared the development of brown colour in bovine plasma albumin (BPA) and BPA+ribose systems. At 85–155°C addition of small amounts of ribose to BPA caused a marked increase in browning, but in sugar-free systems this only occurred at 145°C. This was accompanied by a decrease in the availability of lysine and it was attributed to the formation of cross-linkages between the side-chains of the proteins.

Almquist[12] reported a reduction in the percentage of fat extracted by solvents from fish meals stored in air, but little or no decrease was observed in case of samples kept in sealed glass tubes. Lea *et al.*[13] studied the effects of storage conditions on herring meals and reported a rapid oxidation of the lipids of meals stored in air.

Thus, it is generally assumed that the nutritive value of proteins decrease both on heating and during storage. The following experiments were made to study the effects of drying on leaf proteins and their lipids.

Materials and Methods

Preparation of Leaf Protein.—The proteins used in these experiments were extracted from barley (*Hordeum vulgare*) and red clover (*Trifolium pratense*) leaves by the method of Morrison and Pirie.[14] The proteins were suspended separately in 20 times their weight of water and the pH of the suspensions was adjusted to 4.0 by the addition of dilute HCl. The acid-washed protein was filtered and pressed to remove excess water. The pH of the barley and clover cakes was 3.98 and 4.0 respectively.

Drying of Protein.—1-kg lots of coarsely sieved protein cake were spread in thin layers on fine sieves and dried by blowing hot air, at various temperatures, under the sieves. After drying,

duplicate samples were mixed before grinding; particles which passed through a 40-mesh sieve were used in the experiments.

Extraction of Lipids.—Procedure for extraction of the lipids was the same as mentioned in a previous communication.[15]

Release of Bound Lipids.—(a) 2.0 g protein was soaked overnight in 10 ml 90% phenol before extraction with $CHCl_3$–CH_3OH. The extract was washed with water to remove phenol and nonlipids contaminants. The traces of phenol, left after washing, were removed by concentrating the extract under reduced pressure.

(b) 2.0 g protein was soaked in 10 ml 0.2N HCl overnight before extraction with $CHCl_3$–CH_3OH. The extract was washed to remove nonlipid contaminants.

Extraction of Nucleic Acid.—0.5-g samples of protein were extracted three times with 25 ml 5% trichloroacetic acid (TCA) at room temperature. After acid treatment, the residues were extracted three times with 2:1 $CHCl_3$–CH_3OH mixture to remove lipids. The lipid-free protein was then digested in 25 ml 0.5N $HClO_4$ at 100° for 20 min. The undigested residue was removed by centrifugation, washed with 25 ml 0.5N $HClO_4$ and again centrifuged. The extract and the washing were combined and used for spectrophotometric estimation of nucleic acid phosphorus.

Estimation of Carbohydrates.—50–70 mg protein samples were hydrolysed with 10 ml 2N H_2SO_4 at 100°C (in a water bath) for 2 hr. The hydrolysate was centrifuged and the sugar content of the supernatant was estimated by the orcin method of Pirie.[16]

Estimation of Reducing Sugars.—10 g of the leaf protein sample was extracted with 125 ml 80% ethanol for 1 hr at 80–85°C. After filtration the alcohol was removed from the filtrate by concentrating on a water bath. 5 ml of the alcohol-free extract was deproteinized by mixing with an equal volume of a solution containing 0.3N NaOH and 5% $ZnSO_4$. The amount of reducing sugars present in the deproteinized extract was estimated by the method of Somogyi.[17]

Analysis.—The dry matter content of leaf protein preparations was determined by drying the samples at 100°C for 40 hr. Lipid extracts were concentrated at 40°C under reduced pressure and dried over H_2SO_4 in vacuum to a constant weight.

Nitrogen was determined by a microkjeldahl method using K_2SO_4: $CuSO_2$: SeO_2 (9:1:0:02) catalyst. Total phosphorus was determined by the method of Holden and Pirie[18] and nucleic acid phosphorus by the method of Spirin.[19] The chlorophyll contents of the protein samples were determined by the method of Arnon[20] (using filter 607 in an EEL colorimeter). Iodine value of the lipid extracts was determined by the method of Trappe.[21] Peroxide value of the lipid extracts was determined by TBA method.[22]

Results

Proteins extracted from leaves generally contain substantial amounts of chlorophyll, unsaturated fatty acids, phospholipids, carbohydrates, and small amounts of minerals. The presence of these substances in the protein make it vulnerable to damage.

Red clover protein when dried at various temperatures to 90±2% dry matter (Table 1) showed a decrease in the extractability of $CHCl_3$–CH_3OH soluble lipids from the samples dried at high temperature. The fresh and the air-dried (100°C) samples when soaked in 90% phenol, before extraction with $CHCl_3$–CH_3OH, gave the same amount of extractable lipids. This suggested that the lipids which could not be extracted after heating were not bound to the protein by covalent linkages. Table 2 shows changes in the amounts and composition of the lipids extracted from barley protein dried at 100°C for various lengths of time. In all cases the amount of water left in the samples was sufficiently low to avoid spoilage during storage.

Air Drying.—The amount of lipid (mg/g. of protein) extracted from fresh, 1, 2 and 3-hr heated protein sample showed little variation (Table 2). However, the lipid extracted from samples heated for 12 and 24 hours fell to 174 and 172 (mg/g of protein) as compared with 190 in unheated protein.

The total lipid nitrogen extracted decreased gradually from 2.6 to 1.9 (mg/g of protein), during 24 hr of heating. Because the amount of chlorophyll extracted also decreased, the loss in the amount of nitrogen due to unextractability of

TABLE 1.—EFFECT OF DRYING ON THE EXTRACTABILITY OF LIPIDS FROM CLOVER PROTEIN.

Sample	Drying temp °C	Drying time (min)	Dry matter (%)	% Extraction Lipid	% Extraction Non-lipid
Fresh	—	—	34.4	22.6	0.87
Freeze-dried	—	—	97.1	22.2	0.86
Air-dried	40	120	91.4	22.3	0.92
	53	105	88.6	20.9	0.95
	64	85	88.6	20.3	0.88
	70	70	88.6	21.2	1.01
	83	60	90.0	20.6	0.83
	100	60	92.0	20.4	0.32

TABLE 1b.—EFFECT OF SOAKING IN 90% PHENOL, ON THE EXTRACTABILITY OF LIPIDS FROM CLOVER PROTEIN.

		% extraction of unwashed lipid
Freeze-dried sample	=	24.2
Air-dried 100°C	=	24.2

Table 2.—Effect of Drying on the Extractability and Composition of the Lipids Present in Barley Protein

Time of drying in air (hr)	D.M. of sample (%)	mg/g of protein				Iodine value	Peroxide 10–4M malondialdehyde	Lipid N as mg/g of lipid	Nonlipid fraction mg/g of protein		Loss of N due to decrease in chlorophyll (mg)
		extractable lipid	N	P	Chl				*N*	*P*	
Fresh cake	46.6	190	2.58	0.68	12.8	104	3.65	13.6	0.22	0.14	—
1	96.6	191	2.56	0.6	9.7	113	5.6	13.5	0.30	0.23	0.19
2	97.6	188	2.22	0.6	9.6	111	6.3	11.9	0.31	0.25	0.20
3	98.5	190	2.20	0.6	8.5	108	6.1	11.6	0.30	0.25	0.27
12	98·8	174	1.90	0.4	1.8	85	5.2	10.9	0.16	0.07	0.68
24	98.8	172	1.90	0.38	1.2	53	2.5	11.0	0.16	0.04	0.72

chlorophyll was calculated on the basis of a 6.2% nitrogen content in chlorophyll. Table 2 shows the results from samples heated for2,3, 12 and 24 hr.

A small increase in the non-lipid nitrogen extracted (methanol–water soluble protion of the chloroform–methanol extract) was observed in the samples heated for 1,2 and 3 hr but the samples heated for 12 and 24 hr showed a decrease.

Lipid phosphorus extracted decreased from 0.7 to 0.6 (mg/g of protein) when the protein was dried for 1 hr, but this was accompanied by an equivalent increase in the nonlipid phosphorus. Samples heated for 2 and 3 hr gave the same results, but heating for 12 and 24 hr resulted in a decrease in both lipid and nonlipid phosphorus.

The amount of chlorophyll in the lipids extracts of the samples kept for 24 hr decreased from 12.8 to 1.2. (mg/g of protein), About 34.0% of the chlorophyll could not be extracted from the sample heated for 2 hr and 86.0% from that for 12 hr.

There was no change in the iodine value of the lipids extracted from protein heated for 1, 2 or 3 hr but it fell from 104 to 85 and 53 in the lipids obtained from the 12 and 24 hr samples.

The nucleic acid contents of the samples heated for 1, 2 and 3 hr were the same as that of the fresh sample, but a decrease was observed in the samples heated for 12 and 24 hr (Table 3).

Overnight soaking of barley protein in 0.2N HCl, before extraction with $CHCl_3$–CH_3OH resulted in the release of more lipids from both the samples dried at 100°C for 1 hr and the fresh protein (Table 4). The phosphorus content of these lipids were also higher than those extracted from unsoaked samples.

Carbohydrates.—There was no appreciable variation in the amount of sugars present in the hydrolysates of all the protein samples (Table 5).

Peroxides.—The peroxide value of the lipids extracted from barley protein increased when the sample was heated for 1, 2 and 3 hr, but when the sample was heated for 12 or decreased 24 hr.

Table 3.—Effect of Drying at 100°C on the Nucleic Acid Contents of Barley Protein.

Time of drying hr	mg/g of protein	
	Nucleic acid P	Nucleic acid*
Fresh cake	0.38	3.91
1	0.38	3.91
2	0.37	3.81
3	0.37	3.81
12	0.29	2.98
24	0.29	2.98

* Nucleic acid=10.3×nucleic acid phosphorus.

Table 4.—Effect of Soaking in 0.2N HCl on Extractability and Composition of the Lipids Present in Barley Protein.

Time of drying at 100°C hr	mg/g of protein		
	extractable lipid	Lipid N	Lipid P
Fresh cake	218	2.86	0.90
1	203	2.40	0.79
2	190	2.22	0.65
3	194	2.24	0.64
12	171	1.98	0.57
24	160	2.00	0.56

Table 5.—Effect of Heat on Carbohydrate Contents of Barley Protein.

Drying time (hr)	Carbohydrates* (%)
Fresh	4.59
1	4.42
2	4.29
3	4.26
12	4.26
24	4.37

* By orcin method of Pirie

Discussion

Drying of leaf protein at high temperature results in a decrease in the extractability of the lipids (Table 2) and affects the solubility of the protein. The reduction in the amount of extractable lipids from stored herring meal was observed by Almquist[12] and Lea *et al.*,[18],[13]., and was attributed to the oxidation and copolymerization of the lipids present in the meal. According to Vinolia and Tappel[6] and Narayan and Kummerow[7] the oxidised lipids present in an emulsion containing protein are adsorbed on the surface of the protein. However, the work of Lea *et al.*[7] indicates that 8% of the lysine present in the fish meal is lost after storage for one year, suggesting that a reaction between the lipid oxidation products and protein takes place, although only to a limited extent.

Oxidation appears to be responsible for the reduced extraction of lipids from the air-dried barley and clover proteins. The lipids which could not be extracted from clover protein after air-drying at 100°C for 1 hr were completely recovered on soaking the protein in 90% phenol (Table 1); this suggests adsorption of the lipids on the surface of the protein.

Soaking of the barley protein in water before extraction with $CHCl_3$–CH_3OH resulted in the total recovery of the lipids from 1, 2 and 3-hr dried samples, but there was incomplete recovery from 12 and 24 hr samples (Table 2).

This decrease in extractability was accompanied by a drop in the chlorophyll and nitrogen contents of the lipid. The loss of nitrogen would appear to be related to the decrease in extractable chlorophyll (chlorophyll contains 6.2% of nitrogen).

Pretreatment of the fresh barley protein cake with dilute acid, before extraction with $CHCl_3$–CH_3OH, released additional lipids. The increase in the phosphorus content of this lipid fraction suggests that considerable amounts of phosphatides are released. Channon and Chibnall,[23] and Smith and Chibnall[24] reported that most of the phospholipids present in plant tissue are combined with carbohydrates or proteins. Lovern[25] and Holden[26] reported that the 'bound' lipids present in plants can often only be extracted after treatment of the tissues with mineral acids, and this would appear to be the case here.

Some of the additional lipids released from fresh protein by acid treatment could not be extracted if protein heated for 1 hr was used, and none was extracted from samples heated for 2 hr. The lower phosphorus content of the lipids obtained from heated samples (Table 4) suggests that the bound phospholipids cannot be extracted after heating.

Buchanan[29] reported extensive oxidation of the lipids present in wheat leaf protein when stored at various temperatures. This was accompanid by a decrease in extractability of the lipids. The decrease in nitrogen and phosphorus contents of the lipids was more at 100° and 60°C than at 4° or 28°C. The losses in the nutritive value of the wheat leaf protein, due to oxidation and copolymerisation of the lipids with proteins and due to heat damage to the protein, were also discussed by Buchanan.[30]

Bisset and Tarr[10] have suggested that heating liberates pentoses from nucleic acid which subsequently undergo Maillard reactions with the fish meal protein and lower its nutritive value. Miller[8] and Tarr[9] also state that Maillard reactions are responsible for the loss in the nutritive value of strongly heated fish meals. Formation of aldehyde-amine compounds by free amino groups of phospholipids with sugars present in eggs was reported by various workers. Lea[27] suggested that similar reaction can take place in other foodstuffs.

It is known that the nucleic acid content of proteins extracted from cereal leaves and precipitated by heat is very small.[27] Since there is no decrease in the nucleic acid content of the samples heated for 1, 2 and 3 hr (Table 3) the decrease in the extractability of the lipids from these samples cannot be attributed to the combination of the phospholipids with pentoses released from nucleic acid. Maillard reactions involving reducing sugars and phospholipids are not very likely to occur as most of the water-soluble substances present in the protein are removed during the precipitation and subsequent washing. The tests for reducing sugars (Somogyi's method) were negative and the carbohydrate contents of the samples, after heating, remained constant.

Therefore, it must be assumed that when leaf proteins are heated in air at 100°C at least two types of reactions are taking place: (1) 'free' lipids, extracted by $CHCl_3$–CH_3OH oxidize, and these free lipids and their oxidation products are adsorbed on the surface of the protein; (2) tht 'bound' lipids extracted after acid treatment, form complexes with the protein present.

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References

1. L.C. Maillard, Compt. Rend., **154,** 66 (1912).
2. W.L. Darruesm and E. J. Gills, J. Soc. Chem. Ind., **55,** 141T (1936).
3. C.H. Lea, L. J. Parr, and K. J. Brit, J. Nutr., **14,** 91(1960).
4. J. Biely, B.E. March and H.L.A. Tarr, Prog. Rep. Paciff. Biol. Stas., **89,** 79(1951).

5. A.L. Tappel, Arch. Biochem. Biophys., **54,** 266 (1955).
6. A.W. Venolia and A.L. Tappel. J. Am. Oil Chemists Soc., **35,** 135 (1958).
7. K.A. Narayan, and F.A. Kurmmerow, J. Am. Oil Chemists Soc., **35,** 52 (1958).
8. D.S. Miller, J. Sci. Food Agr., **7,** 737 (1956).
9. H.L.A. Tarr, Circulation, Fush Res. Board, Canada, No. 24.
10. H.M. Bisset and H.L.A. Tarr, Poultry Sci., **33,** 250 (1954).
11. K. J. Carpenter, C.B. Morgan, C.H. Lea and L. J. Parr, Brit. J. Nutr., **16,** 451 (1962).
12. J.H. Almquist, J. Agr. Food Chem., **4,** 638 (1956).
13. C.H. Lea, L. J. Parr and K. J. Carpenter, Brit. J. Nutr., **12,** 297 (1958).
14. J.E. Morrison and N.W. Pirie, J. Sci. Food Agr., **12,** 1(1961).
15. F.H. Shah, J. Sci. Food Agr., **19,** 199 (1968).
16. N.W. Pirie, Brit. J. Exp. Pathol., **17,** 269 (1936).
17. M. Somogyi, J. Biol. Chem., **195,** 20 (1952).
18. M. Holden and N.W. Pirie, Biochem. J., **60,** 53(1955).
19. A.S. Spirin, Biokhimiya, **23,** 656 (19.58)
20. D.I. Arnon, Plant Physiol., **24,** 1(1949).
21. W. Trappe, Biochem. Z., **296,** 180(1938).
22. A. Ottolenghi, Arch. Biochem. Biophys., **79,** 355 (1959).
23. H. J. Channon and A.C. Chibnall, Biochem., J., **21,** 1112 1927).
24. J.A.B. Smith and A.C. Chibnall, Biochem. J. **26,** 219 (1932).
25. J.A. Lovern, Dep. Sci. Ind. Res. Food Invest. Spec. Rep. No. 52, (H.M.S.O., London 1942).
26. M. Holden, Biochem. J., **51,** 433 (1952).
27. C.H. Lea, J. Sci. Food Agr., **1,** 1(1957).
28. N. Singh, Biochim. Biophys. Acta, **45,** 422 (1960).
29. R.A. Buchanan, J. Sci. Food Agr., **20,** 359 (1969).
30. R.A. Buchanan, Brit. J. Nutr., **23,** 533 (1969).