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Application of Chemical and Biological Assay Procedures for Lysine to Fish Meals

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Eight samples of capelin meals differing in freshness of raw material, treatment with formaldehyde, and proportion of solubles were used for this collaborative study in four laboratories. They were subjected to two procedures of bioassay for lysine based on chick growth rates and the results gave similar ranking of the samples, though absolute values differed. The chick results correlated closely with results from a rat assay and with fluorodinitrobenzene (FDNB)-reactive lysine, dye binding with Acid Orange 12, and total lysine values. The potency of different samples as sources of lysine for chicks was mainly determined by their total content of lysine, but the addition of excess levels of formalin to the fish immediately prior to processing reduced potency about 13% and this reduction was not demonstrated by any of the chemical methods used. There were considerable differences between laboratories in the absolute values for FDNB-reactive lysine.

Fish meal usually commands a higher price than other high protein feedstuffs, and a large part of this premium is explained by its amino acid composition being suited to balance the relative deficiencies of cereal proteins for monogastric animals. In particular, the high lysine content of fish protein compensates for the relatively low lysine in cereal proteins.

Carpenter et al. (1957) suggested that the chemical measurement of lysine reactive to fluorodinitrobenzene (FDNB) provided a better, and more convenient, measure of the value of processed animal proteins as a source of lysine than did total lysine as measured by either column chromatography or microbiological assay, after acid hydrolysis. Since then many procedures for measuring reactive lysine (using FDNB or other reagents) have been devised and applied to animal protein feedstuffs (cf. review by Carpenter, 1973).

In many studies a high correlation has been found between the ranking of samples according to FDNB-reactive lysine and according to feeding tests, in particular when the samples had been subjected to extreme temperature treatments. But in some other studies, with commercial fish meals, there was not a good correlation (see Carpenter, 1973). A lack of correlation between FDNB-reactive lysine and feeding tests could be due to the biological assay in question not being sensitive to variations in available lysine perhaps because the first limiting amino acids in the study were methionine and cystine, rather than lysine. Furthermore, it may be that while some reactions (e.g., those occurring at high temperatures) affect FDNB reactivity and biological availability to the same extent, other reactions do not. Finally, a lack of correlation between FDNB–lysine and feeding tests may be due to unrecognized sources of error in the use of either procedure.

These questions have an immediate practical interest as regards the methods to be used for quality control by both processors and users of animal products. They are also of more fundamental interest: the ability to predict nutritional value from chemical tests is some measure of our understanding of the reasons for the differing nutritional values of individual materials. To throw light on both these aspects a collaborative study was undertaken involving commercial meals with different contents of FDNB-reactive lysine as well as samples prepared in a pilot plant using variables expected to have an influence on the measured lysine values. In order to facilitate the statistical comparisons between the various methods, the various treatments were chosen so as to represent extremes. In addition to the work of the authors of the paper, who are also responsible for the collation of the results, further tests were run in Copenhagen by Dr. B. O. Eggum at the Institute of Animal Science and by Ing. W. Schmidtsdorff at the Ministry of Fisheries Laboratory, and in Bergen by Dr. J. Njaa at the Government Vitamin Laboratory. The test samples were prepared on the pilot plant at SSF and characterized by Mr. N. Urdahl and Mr. E. Nygard. The laboratories are coded V–Z in the tables of results.

MATERIALS AND METHODS

Test Materials. A description of the samples is given in Table I. Fish meals A–F were prepared in a pilot plant,

Department of Applied Biology, University of Cambridge, Cambridge, CB2 3DX, England (K.J.C), and the Norwegian Herring Oil & Meal Industry Research Institute, Bergen, Norway (J.O.).

while samples G and H were commercial capelin meals. Frozen capelin *Malotus vallosus* was thawed at room temperature before being subjected to the treatments summarized in Table I followed by processing in a pilot plant equipped with an indirect steam heated cooker, a single screw press, and a direct air dryer run at inlet and outlet temperatures of 500 and 90–100 °C, respectively; the meal left the drier at about 80 °C. The oil was separated by decanting and the aqueous layer concentrated to "solubles" (30–35% dry matter) at 100 °C. When combined the presscake and the solubles were mixed in appropriate portions in a meat mincer before drying. After drying 400 ppm of Ethoxyquin was added to all meals and they were then ground in a mill before being packed in portions of 500 g in bags of aluminum foil laminated with polyethylene, flushed with nitrogen, and stored at −18 °C.

Meal D, with no solubles added back, is termed presscake meal. Samples A, B, C, and E, for which all the solubles from a batch of fish were added back to the presscake, are termed whole meals. Meal F had twice the equivalent quantity of solubles added back to the presscake and is termed double solubles meal.

Biological Assays. Two procedures were used to estimate the potency of test materials as sources of lysine for chicks, each in a different laboratory. Both use lysine-deficient basal diets and measure the weight gain and feed consumption in young chicks receiving dietary supplements of test material or pure lysine. That used in laboratory W was based on the procedure of Combs et al. (1968) as described by Opstvedt (1975) and used total lysine consumption vs. growth as criteria, while the method used in X was that of Carpenter et al. (1963) with values calculated from "% lysine in the diet" in relation to "gain/feed eaten". Two independent assays were run in each laboratory at intervals of several months, with new diets prepared for each.

A single rat NPU test using a modification of the Mitchell balance technique (Eggum, 1973) was run with each fish meal providing 2.7%, and wheat gluten (of low lysine content) a further 7.3% crude protein. Each diet was further supplemented with threonine. There were 5 rats per treatment.

Chemical Analyses. The raw fish were analyzed, immediately prior to processing in the pilot plant, for volatile N, trimethylamine, and trimethylamine oxide (Conway and Byrne, 1933) and the oil expressed during processing was analyzed for its free fatty acid content as an indicator of freshness. The fish meals were analyzed for their proximate constituents, for volatile and water-soluble N and for chloride content (AOAC, 1960).

Analyses for total lysine were carried out essentially by the procedure of Spackman et al. (1958) or, in laboratory X, with a shorter ion-exchange column (Roach et al., 1967). Fluorodinitrobenzene (FDNB)-reactive lysine was determined directly by the procedure of Carpenter (1960), or in the case of laboratory X, with the slightly modified conditions of Booth (1971). It was also determined by difference (Roach et al., 1967).

Dye-binding measurements with the azo dye Acid Orange 12 in a pH 1.25 buffer containing oxalic acid were carried out in an automated commercial apparatus (Jacobsen et al., 1972), using 1.0 g of test material (or 0.8 g where the higher weight gave an off-scale reading) and 40 ml of buffered dye solution. The results are expressed as millimoles of dye bound per gram of crude protein.

Complete amino acid analyses were carried out but only total methionine values will be referred to. The values given represent the means of two series, one obtained by

Table I. Source of the Samples and Some Analytical Characteristics of the Raw Materials and Products

Source of sample	Processing variables: Period of storing fish before processing, days[a]	Processing variables: Level of solubles included	Raw fish at time of processing, mg of N/100 g: Total volatile N (Conway)	Raw fish at time of processing, mg of N/100 g: Trimethylamine	Raw fish at time of processing, mg of N/100 g: Trimethylamine oxide	Oil expressed in processing: % free fatty acids	Test sample of mean, % composition: H_2O	Crude fat	Ash	Cl (as salt)	N × 6.25	H_2O-soluble N × 6.25	NH_3-N (Bollman)
Pilot plant	A. 8	Normal	108	61	7	4.0	6.8	11.6	10.4	1.7	70.1	20.8	0.19
	B. 23 (+ formalin)[b]	Normal	108	73	4	4.3	7.3	7.9	10.3	1.6	73.0	17.5	0.21
	C. Fresh (+ formalin)[b]	Normal	11	1	67	1.7	7.6	9.2	10.4	1.5	74.5	13.6	0.11
	D. Fresh	None	11	2	67	1.6	8.1	7.8	8.8	1.0	75.8	7.2	0.10
	E. Fresh	Normal					7.9	16.1	9.9	1.6	70.2	16.4	0.11
	F. Fresh	Twice normal					6.0	8.9	11.8	2.5	73.7	23.1	0.14
Commercial	Unknown:	selected for high FDNB-lysine value	...	...	...	...	6.3	10.5	10.4	1.2	70.4	16.4	0.21
	Unknown:	selected for low FDNB-lysine value	...	...	...	...	7.0	8.2	11.7	1.9	71.0	18.3	0.23

[a] The fish was stored at 6 °C. [b] In each case 346 ml of formalin solution (40% w/v formaldehyde) was added per 100 kg of fish. For sample B this was for the whole storage period, and for sample C it was added to the fresh fish just before the processing began. In periods when formalin is used as a preservative this level is the highest permitted in the Norwegian fish meal industry.

Table II. Relative Values of the Materials in Tests for Lysine Content and Dye Binding Capacity

Test material	Lab.	Total Y	Total Z[a]	Total X	Unreactive, X[b]	FDNB reactive: By diff., X	FDNB reactive: Direct X	Direct V	Direct W	Chick growth assays W.1[c]	W.2	X.1	X.2	Dye binding V	Dye binding X
A. Unpreserved		93	96	101	(7)	102	99	96	96	99	94	89	99	100	98
B. HCHO preserved		100	101	99	(10)	98	97	98	99	96	96	100	103	99	98
C. HCHO firmed		96	98	99	(12)	96	96	94	101	90	89	87	90	97	98
D. Presscake		121	117	115	(8)	117	113	116	111[a]	123	128	117	119	112	116
E. Standard		102	102	100	(8)	102	102	104	105	102	98	106	102	101	101
F. Double solubles		99	95	95	(7)	97	98	98	97	92	97	101	95	95	96
G. Comm. high		95	98	98	(9)	97	102	100	102	98	104	104	98	100	100
H. Comm. low		94[a]	94	92	(10)	90	93	92	89	96	94	96	94	96	95
Absolute overall means, g/16 g of N		7.52	7.36	7.39		6.77	7.09	6.49	6.30	7.32	6.80	8.15	7.88		(1004)[d]

[a] A revised value, or series of values (see text). [b] Expressed as a percentage of the mean total lysine value in laboratory X, i.e. 7.39 g/16 g of N. [c] W.1 means the first assay at laboratory W, W.2 the second assay. [d] Millimoles of dye bound/16 g of N.

Table III. Between-Assay Analysis of Variance for Chick Growth and FDNB-Lysine (Direct) Results

Source of variation		df	Mean squares	Components of mean squares	Estimates of components of variation
Chick assays					
Labs		1	7.354[b]	$\sigma_E^2 + 16\sigma_L^2$	0.455 (σ_L^2)
Times		1	1.248[b]	$\sigma_E^2 + 16\sigma_T^2$	0.074 (σ_T^2)
Samples		7	2.148[b]	$\sigma_E^2 + 4\sigma_S^2$	0.520 (σ_S^2)
Interactions					
Lab × time					
Lab × sample	Error[a]	22	0.0686	σ_E^2	0.0686 (σ_E^2)
Time × sample					
Residual					
FDNB-lysine (direct):					
Labs		2	1.366[b]	$\sigma_E^2 + 8\sigma_L^2$	0.166 (σ_L^2)
Samples		7	0.461[b]	$\sigma_E^2 + 3\sigma_S^2$	0.140 (σ_S^2)
Error (i.e. Lab × sample)		14	0.040	σ_E^2	0.040 (σ_E^2)

[a] All three interactions gave mean square figures similar to that of the residual, so that all four were pooled to give a mean square with 22 df as an estimate of error. [b] Statistically highly significant ($P < 0.01$).

ion-exchange chromatography (Spackman et al., 1958) and the other by microbiological assay (Henry and Ford, 1965).

RESULTS

The analyses used to characterize the samples are given in Table I. The raw materials used for samples A and B, and the oil expressed from them, show the changes associated with bacterial and enzymic attack in stored fish. Also, in samples D, E, and F the levels of chloride and of water-soluble N reflect, as expected, the ratio of solubles added back to the presscake. The two commercial samples, G and H, appear generally similar, as regards these analyses, to the whole meal E, with the normal level of solubles, prepared in the pilot plant.

The results of the lysine analyses and assays are given in Table II. In order to facilitate the comparison between the different methods and because some tests (and some laboratories) seemed to be giving systematically higher values than others, the mean value obtained for each series of tests is given together with the individual values as percentages of that mean. When the results of the chemical determinations were first compared, a few appeared very much out of line with the rest. These analyses were then repeated and changed values are indicated on the table. In each case the repeat analyses were also carried out on samples that had given apparently reasonable results, to check that a new value was not due to some systematic lowering or raising of results with time. In addition, one complete series of total lysine results was repeated because of an apparent confusion of samples in the first series. The generally good replication in ranking between laboratories does not therefore provide evidence of the reliability of otherwise unchecked results.

The results for direct FDNB-reactive lysine from the three laboratories have been subjected to an analysis of variance (Table III) and it is seen from the magnitudes of the components of variance that the laboratory in which the analysis was done has had as big an effect on the result as which sample was taken for analysis. However, the laboratory effect appears to have been a consistent one as the residual standard deviation of an analytical result is only 0.2 g/16 g of N.

Biological Assays. The four chick assays were carried out as planned, and statistical checks indicated that each assay was valid. The results of each assay are summarized in Table II. The method of calculating the standard errors of the estimates from the procedure of Combs et al. (1968), as used in laboratory W, gave pooled estimates of 0.17 and 0.21 g/16 g of N for the first and second assays, respectively. These estimates do not take into account variability in the response to the standard lysine doses, but are relevant for use in testing the significance of differences between the test samples. In laboratory X which used the procedure of Carpenter et al. (1963), the estimates of standard errors calculated by the slope–ratio procedure were 0.37 and 0.36 g/16 g of N for the two assays. All the above estimates were within-assay measures. A further analysis was made of the four sets of estimates considering just between-assay variability, with the results shown in Table III. It is clear that the results from laboratory X are systematically higher than those from laboratory W by 1.0 g/16 g of N, and higher on the average than the total

Table IV. Mean Results from Different Tests, with the Samples Ranked According to Their Potency in the Chick Assays, and Their Intercorrelation

Test material	Relative lysine values: Chick assay	FDNB reactive: Direct	FDNB reactive: Diff.	FDNB reactive: Total	Rel. dye binding	Rat NPU (+ gluten)	Total Met g/16 g of N
D. Presscake	122	113.5	117	117.5	114	67.9	3.33
E. Standard	102	103.5	102	101.5	101	62.6	3.05
G. Comm. high	101.5	101.5	97	97	100	62.6	2.88
B. HCHO preserved	99	98	98	100	98.5	60.1	3.05
F. Double solubles	97	97.5	97	96.5	95.5	61.7	2.74
A. Unpreserved	95	97	102	96.5	99	61.7	3.06
H. Comm. low	95	91.5	90	93.5	95.5	63.9	2.89
C. HCHO firmed	89	97	96	97.5	97.5	58.0	3.13
Correlation between results							
FDNB direct	0.910 (0.645)[a]						
FDNB diff.	0.867 (0.357)	0.931 (0.741)					
Total lysine	0.921 (0.467)	0.936 (0.743)	0.942 (0.716)				
Dye binding	0.933 (0.562)	0.944 (0.838)	0.945 (0.736)	0.970 (0.720)			
Rat NPU	0.863 (0.584)	0.640 (−0.057)	0.636 (−0.174)	0.678 (−0.320)	0.742 (0.010)		
Methionine	0.545 (−0.308)	0.655 (0.162)	0.753 (0.393)	0.776 (0.477)	0.784 (0.505)	0.303 (−0.565)	

[a] The correlation coefficients in parentheses are calculated for seven samples only, omitting D.

lysine content of the samples. Also, it is clear that the mean results from the second set of assays were lower than those from the first by 0.4 g/16 g of N (both differences being significant). However, there was no evidence of differences in the relative value of the samples from one assay to another.

The NPU results obtained with rats for the wheat gluten–capelin meal mixtures are summarized in Table IV. The overall mean value for true digestibility of the total N in the eight diets was 96% with a pooled estimate of standard error for treatment means of 0.56, and the analysis of variance gave a nonsignificant F value of 1.90 for treatment differences. There were highly significant differences in NPU for which the standard error of treatment means was 0.70.

Correlation between Tests. These, including values for total methionine, are summarized in Table IV. When all eight samples are considered, each of the lysine procedures and also the dye binding test have shown a similarly highly significant degree of correlation with the results of the chick assays. However, much of the variation between values comes from the considerable superiority of sample D in all the tests, and when this sample is omitted from the comparisons the coefficients of correlation are considerably reduced. However, one can make approximate estimates that with the chick assay values having the errors calculated in Table III, and with the perfect chemical test that gives unbiased estimates of the true chick values, but with a coefficient of variation of 3%, the average correlation coefficient would be 0.940 for all eight samples and, with sample D omitted, would be only 0.744, a value lower than the minimum required to reach statistical significance. Methionine analysis has not proved to be a useful predictor of chick lysine values.

DISCUSSION

The prerequisites of the study were achieved; that is to say, a series of test samples was obtained which gave significantly different values in biological assays, and there was acceptable agreement between laboratories as to the relative assay values of these materials. It was therefore possible to study the correlation of FDNB-reactive lysine values with the results of the chick assays, and the overall correlation of 0.910 has indicated a generally good predictive value for the test. However, three qualifications should be added to this generalization.

First, the FDNB direct procedure gave considerably different absolute values in the different laboratories, and the cause of this has not yet been traced. It is not explained by differences in the standards used, but may be partly related to destruction of DNP-lysine in contact with ether which contains peroxides, or to loss of aqueous solution when the ether layer is removed, following extraction of interfering materials. Until the problem of interlaboratory differences is overcome the test can really only be relied on to give relative values for quality control.

Secondly, the differences between these samples in their reactive lysine content seem to be almost entirely explained by their differences in total lysine content rather than in a difference of availability of the lysine. Total lysine values have shown as high a correlation with the chick assay values as did the direct FDNB values, and correction of the total for an FDNB-*un*-reactive fraction gave no improvement in correlation. This was perhaps to be expected when heat damage was excluded from the variable factors studied. It is in heat-damaged materials that FDNB tests have been shown to be a better indicator than total lysine of nutritional value.

Total lysine determination is no quicker or cheaper than FDNB analysis. But it is interesting that the rapid dye-binding test was as good a predictor of chick values in this study as the more specific lysine tests. It is, of course, a measure of histidine and arginine as well as of lysine (cf. Hurrell and Carpenter, 1975) and further work is needed to determine whether it will be equally useful for meals made from different species of fish, as well as with different processing procedures.

Lastly, much of the variation between the test materials came from a single sample, D (the presscake meal) having a considerably higher lysine value in all the tests. When that was eliminated from the calculations none of the correlations between chemical and biological tests remained significant. As explained above, with a relatively narrow range of samples and the standard errors in the

results from the procedures under test, this is not in itself proof that they were measuring different things.

It is, however, of interest that sample C gave a value in the chick assays consistently about 13% below that of the standard E, and it also gave the lowest value in the rat NPU test, while in the chemical tests its value ranged from only 4% (total lysine) to 6.5% (direct FDNB test) below the value for sample E. It does appear therefore that the application of formalin as a firming agent immediately prior to processing, to ease the process of pressing liquor out of the fish after cooking, may have a greater biological effect than is detected by any of the chemical tests used here. The addition of somewhat higher levels of formalin has been found to reduce FDNB-reactive lysine in previous studies (Wessels et al., 1973; Carpenter, 1973), and it would be of interest to study the reactions occurring under different conditions and their effects on nutritional value. Sample B, in which formalin was added as a preserving agent prior to storage of the raw fish, had a higher value than sample C in the chick assays; this may be due to the formalin having been largely degraded during the storage period.

In general the results have been encouraging as to the usefulness of laboratory measures for predicting the value of fish meals as a source of lysine. They do, however, bring out the need for further standardization of the methods.

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Soybean Trypsin Inhibitor Activity of Soy Infant Formulas and Its Nutritional Significance for the Rat

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The soybean trypsin inhibitor (SBTI) activity of five soy-based infant formulas was determined by a method specifically designed to test their relatively low SBTI levels. All formulas, except one, contained 15% or less of the SBTI activity of a soy-protein isolate source typically used in the manufacture of some formulas. Resterilizing two of these formulas did not significantly reduce residual SBTI activity. Weanling rats were fed adjusted diets containing an unprocessed soy formula, the formula after various stages of processing, the fully processed formula, and another commercially available soy formula. The rat diet groups did not differ from each other nor from the casein control in their caloric and protein utilization or pancreas weights. No pancreatic hypertrophy or hyperplasia was observed in any of the rats. Our results show that the level of SBTI in the soy infant formulas tested is low and of no nutritional significance for the rat.

Many years of clinical experience have demonstrated that soy-based and soy-protein-based infant formulas satisfactorily support the growth and development of infants (Cowan et al., 1969; Omans et al., 1963; Graham et al., 1970; Dean, 1973). Despite their apparent nutritional adequacy, it was reported that rats, when fed one such formula, showed poorer weight gain and caloric efficiency than rats fed other soy-based formulas (Theuer and Sarett, 1970). The relatively poor weight gain and caloric efficiency observed in the rats were attributed to the greater soybean trypsin inhibitor (SBTI) activity the investigators found in the formula in question than in the other formulas studied. SBTI activity is responsible, at least in part, for the poor growth, reduced protein and food efficiency, and pancreatic hypertrophy seen in rats which are fed raw soybean meal (Rackis et al., 1963; Booth et al., 1964; Rackis, 1965).

Appropriate heat treatment of soybean meal, however, readily inactivates SBTI, eliminating the antinutritional effects associated with the feeding of non-heat-treated soybean meal (Rackis, 1965; Leiner, 1962; Longenecker et al., 1964; Yen et al., 1969). The formula in question is heat treated during its manufacture. This formula also is manufactured from a soy-protein isolate which, due to the nature of its preparation, contains lower levels of SBTI

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