STUDIES OF AVAILABLE LYSINE, AMINO ACIDS AND CHICK GROWTH TO EVALUATE PROTEIN QUALITY OF MENHADEN, WHITE AND PERUVIAN FISH MEALS

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Modestus Xavier Gomez

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by

Modestus Xavier Gomez

AN ABSTRACT OF A THESIS

Submitted to
Michigan State University
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STUDIES OF AVAILABLE LYSINE, AMINO ACIDS AND CHICK GROWTH TO EVALUATE PROTEIN QUALITY OF MENHADEN, WHITE AND PERUVIAN FISH MEALS

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Menhaden, white and Peruvian fish meals are three high quality fish products used regularly as protein supplements in chick rations. There has been considerable doubt as to the relative nutritional worth of the proteins of these meals and these studies were undertaken primarily to obtain a nutritional evaluation of these meals from the standpoint of their protein quality.

The fish meals were fed in two low protein starter and finisher rations as the only protein supplement and growth responses estimated. An attempt was then made to correlate the results obtained with determinations of the available lysine content and essential amino acid composition in the fish proteins.

In the chick bioassay experiments, menhaden fish meal showed consistently better growth response and feed efficiency particularly during the first three weeks. The Peruvian fish meal showed equally good results. The white fish meal, although fed in a slightly lower protein-containing meal produced comparatively good growth response, especially at the fourth and fifth week stages.

Available lysine estimations were found to have good correlations to growth response obtained from the chick studies. Available lysine estimations proved to be a useful indication of fish meal quality. The composition of essential amino acid in menhaden and Peruvian fish

meals were superior to that of white fish meal.

From a practical standpoint, the studies revealed that there was little to choose in terms of protein quality between menhaden, Peruvian and white fish meals.

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INTRODUCTION

The use of fish meals as a source of protein in poultry rations is now a common practice. It is generally accepted that fish meal is desirable not only because of its high quality protein but also because it provides essential vitamins, minerals and unidentified growth factors. Since the demand for fish meal has increased considerably, production and the marketing of a wide variety of fish meals is practiced.

A high quality fish meal is desired and one of the problems facing the feed manufacturer is its variable protein quality. Fish meal manufacturers experience great difficulties in controlling the quality of the meal due to a number of factors such as differences in raw material, processing techniques, and post-processing conditions. For instance, it is known that deterioration of fish meal takes place after processing, during storage.

Crude protein values and bio-assay methods, though reasonably reliable, to evaluate the protein content and overall quality of fish meals are, however, limited in usefulness for the purpose of determining protein quality. The first method yields values for only the total nitrogen content, and the second method is obviously too tedious and expensive to be adapted for any type of routine quality estimation.

In experiments to be reported, an attempt has been made to correlate the nutritional quality of the protein with its available lysine content using a method recently developed by Carpenter (1960). Some researchers believe that available lysine can provide a reliable

indication of the protein quality of a fish meal. If this hypotheses is valid, it would provide a rapid and practical quality control technique.

Of the fish meals produced in the United States, menhaden fish meal is one of the few fish meals produced from whole fish. For obvious reasons, this fish meal is held in high regard and frequently commands a premium price. Besides menhaden fish meal there are two other fish meals marketed to the feed formulators - white and Peruvian fish meals. White fish meals is processed from the trimmings from filleting operations of the cod, flounder and haddock. This meal also contains 5 to 10 percent of whole fish of small size. The Peruvian fish meal is similar to menhaden in that it is processed from the whole fish, "anchovetta" (a herring-like fish belonging to the Clupeoid family) and is the product of a recently developed industry in Peru. With the large influx of Peruvian fish meal into the United States, feed manufacturers and poultrymen have been questioning its nutritional quality. There is very little knowledge of the value of this fish meal and hardly any publicized work on its protein quality.

In this study, therefore, an effort has been made to evaluate and compare the protein qualities of menhaden, white and Peruvian fish meals. The studies have been made using protein quality estimation techniques, such as chick growth studies, available lysine and amino acid assays.

REVIEW OF LITERATURE

It has been recognized that there are wide variations in nutritive values among the grades of commercial fish meals, attributed generally to varying protein quality. Bender and Haizelden (1957) and McIntyre (1957) studied a wide variety of fish meals and demonstrated large differences in protein quality. Schneider (1932) showed that the protein of vacuum dried fish meal was more digestible than that of steam dried menhaden meal and the latter, in turn, was more digestible than the flame dried meal. Record, Bethke and Wilder (1934), found that meal prepared from waste of the edible portion of haddock was superior to meals prepared either from the heads and tails alone or from the entire waste material. This study led many to believe that meal from whole fish, like menhaden or Peruvian fish meal was superior to others from byproducts of filleting operations like white fish meal.

Besides the quality of the raw material and the method of processing, many other factors such as pre-processing and storage conditions (Lea et al., 1960) affect the protein quality of the meal. Almquist stated that fish meal made from whole sardines held 24 hours at room temperature before processing contained less arginine than one from fresh fish of the same catch. Later workers, Clandinin (1949) and Carpenter (1960), established conclusively that much damage to protein quality takes place during processing of fish meals.

There have been many efforts to develop techniques by which the biological value of a fish meal could be determined and thus enable manufacturers of fish meal to have better quality control. The earliest, and possibly still the most reliable methods for the evaluation of protein quality has been by bioassay. Osborne et al. (1919)

and Mitchell (1924) recognized that the feeding of protein concentrates as sole sources of dietary nitrogen would be the most sensitive method of detecting the amino acid adequacy of a protein. Almquist (1932), St. John et al. (1932, 1934) and Evans et al. (1944), in a series of experiments employed the chicken to evaluate the nutritional quality of the protein and suggested that it was necessary to use the test protein as the only source of protein in the bio-assays. Grau and Williams (1955) used a short-term chick growth bioassay effectively to classify over a hundred samples of fish meal on the basis of their nutritive value. They showed wide variations and concluded that the protein quality of a fish meal was not closely related to its crude protein content.

Hinners and Scott (1957) recognized that the sensitivity of the chick bio-assay would be enhanced if the rations contained sub-optimal protein levels and suggested feeding diets containing 10 to 15 percent protein. The sensitivity of the assay was greater at the lower level, suggesting that as the dietary protein is increased it becomes more difficult to demonstrate amino acid inadequacies. In their studies, they used supplemented egg protein as reference standard. Rand et al. (1959) used a modification of the Hinners-Scott method on fish meals from different species and organs, as well as to examine the effects of composition, heating, fat removal and storage period. Significant differences were noted in the quality of the protein which they translated into descriptive terms good, fair, etc. Meal from fish muscle had a higher protein quality value than that from whole fish. Meals from scales and skin and bones had poor protein value.

Osterhout and Snyder (1960) used modifications of the short-term methods of Grau and Williams (1955) and concluded that the nutritive value of a fish meal can only be obtained by the use of several test conditions since differences may be due to various factors. In the experiments reported on the bio-assay of protein quality, the fish proteins were evaluated for total, rather than a specific, amino acid availability.

Within the last decade, interest has developed to find which particular amino acid is responsible for the differences in biological value of the protein. Certain basic amino acids are quite susceptible to heat. Among the first workers to investigate the effects of heat on one of the most critical amino acids, lysine, were Eldred and Rodney (1940). They demonstrated a decrease in biologically available lysine in heated casein using the enzyme lysine decarboxylase. Pader et al. (1948) studied factors affecting the availability of lysine by in vitro digestion trials and concluded that in heated casein, the lysine is liberated by digestion at a rate too slow to allow effective supplementation of the other amino acids absorbed earlier in digestion. By this time, it was becoming clear that the variability in protein quality could be traced to variability in the availability of certain critical amino acids like lysine.

Kuiken and Lyman (1948) and Kuiken (1952) determined the available lysine content in a few proteins by measuring the quantity of ingested lysine that was excreted. Gupta (1957) measured the amount of test material required to supplement a wheat gluten diet low in lysine. Meanwhile, Clandinin (1949) studied the effects of processing in protein hydrolysates. He showed that, when herring meal was

over-heated or scorched, the arginine, lysine and threonine contents were low. He observed that the microbiological assays were of little value in predicting the relative nutritive worth of a protein, as they gave no indication of availability of any particular amino acid. Clandinin's findings that overheating depresses availability were confirmed by Tarr et al. (1953) who further demonstrated that chicks fed heat-treated protein plus a lysine supplement grew better than chicks fed the heat-treated protein alone.

In 1955, Carpenter and Ellinger developed an efficient chemical technique to determine available lysine based on the reaction (Sanger, 1945) of free amino groups with 2, 4 dinitro fluoro benzene to form stable dinitro phenyl compounds, identifiable colorimetrically. Carpenter's available lysine was that proportion of the lysine molecules having their ϵ-NH_2 group free or unbound. Carpenter and Ellinger (1955) also conducted biological tests for available lysine using chickens and showed a highly significant correlation between gross protein value and available lysine value. Their findings indicated that lysine was usually the critical amino acid.

Bruno and Carpenter (1957) and Carpenter (1960) modified their earlier technique by using the reaction of methoxy carbonyl chloride with ϵ DNP lysine to form an ether soluble derivative without change in color. This helped to eliminate any interference from α DNP arginine. Since Carpenter's work, Kellenbarger (1961) showed the available lysine content of a number of fish meals to have good correlation with chick growth ratings.

The free ϵ NH_2 lysine is admittedly only one aspect of the gross protein quality. Presently, there is little evidence on the influence

of the availability of other amino acids, except for a recent effort by Osterhout et al. (1959). These workers proposed a new chick assay method to determine some of the available amino acids. However, it is known and accepted that certain minimum levels of other amino acids, especially the class defined as "essential" should be present in a protein and the variability of their content would affect protein quality. At the present time, therefore, a knowledge of amino acid pattern in a protein, at least in the gross form would be a valuable indication of protein quality.

One of the best known techniques for determining quantitatively the amino acids of a protein is by the conventional microbiological assays. The first workers to apply this technique as a measure of determining amino acid content were Snell, Strong, and Peterson (1937) and Wood, Geiger and Werkman (1940). They showed that lactic acid-producing bacteria required over 17 essential amino acids for their normal functioning and suggested that they might be used in the assay of amino acids. The currently accepted techniques are those described by Barton-Wright (1952). Interest in microbiological tests have recently increased and the application of new or modified procedures are being studied by various workers.

OBJECTIVES

Interest in setting up these experiments was stimulated by the numerous queries raised by feed manufacturers and practical poultrymen on the quality of the better known fish meals, namely, menhaden fish meal, white fish meal and Peruvian fish meal.

In this study on the above three grades of fish meal, an attempt has been made primarily to show the relative position of these fish products from the standpoint of their nutritional quality. In addition, the experiments were conducted and designed to produce evidence on the following:

a. Is the premium paid for menhaden fish meal justifiable.

b. Is the quality of Peruvian (source - whole "Anchovetta") fish meal questionable as some claim.

c. Could the recently developed method by Carpenter (1960) for the estimation of the available lysine content be adapted for estimating the relative nutritional value of fish proteins.

d. Could Carpenter's method be used as a quality control test so that a precise criterion would be available for labeling a fish meal.

e. Would microbiological assays of the essential amino acids of the above three fish meals help confirm their relative nutritional values.

GENERAL EXPERIMENTAL PROCEDURE

The experimental approaches employed were by chick growth bioassay, microbiological assays for amino acids and estimation of available lysine.

The fish meals used in this study were representative lots as offered to feed manufacturers. Being commercial products, the fish meals were not very uniform in texture.

In the formulation of the diets, the fish meals were used as a certain percentage of the diets - the way most feed manufacturers would use the product - although use on a pound to pound basis may not be the most desirable for fundamental evaluation. Thus, the experimental design was of a practical nature such as small feed manufacturers with limited laboratory facilities and dependent upon "guaranteed" analyses of ingredients would be forced to operate.

EXPERIMENT I

Procedure -- In this experiment, the three fish meals, menhaden, white and Peruvian were fed at two low-protein levels as the protein supplement in iso-caloric diets. The chicks used in the experiment were commercially hatched U. S. typhoid clean White Plymouth Rock cockerels. Two hundred and forty chicks were weighed, wing-banded and randomized on a weight basis into 24 pens in modified Petersime brooder units.

The three fish meals were fed in approximately 16 and 13 percent protein diets. The fish meals were added at levels of 15.5 and 9.5 percent of the total diet, assuming that they contained 60 percent protein (Tables 1 and 2). A proximate analysis conducted later showed some variation from this figure. The rations were made adequate with respect to vitamin and mineral requirements based on data reported in the literature for the various ingredients.

The 40 chickens in each of the six treatments were allotted to four replicate pens of 10 chicks each. The chicks were fed the experimental diets from 0 to 5 weeks of age. Growth rates were obtained from pen weights recorded weekly (Table 3). Individual weight records were, however, made at the end of the fifth week and the growth rates were analyzed statistically (Table 4). Feed efficiency figures were determined from weight of feed consumed during the period of the experiment.

Results and Discussion -- On a weekly weight basis, up to the end of the first three weeks, the average weight of the chicks on the menhaden meal showed the best growth response at both levels of protein. The growth response from the Peruvian meal was close to that of menhaden while that of the white fish meal was the lowest. These differences were more pronounced at the lower protein level.

At the end of the fourth and fifth weeks, a marked change in the growth rates occurred. At the higher protein level, chicks on white fish meal gained weight rapidly and over-reached those on menhaden and Peruvian meals. However, at the lower protein level the chicks on the menhaden treatment continued to maintain their leading position although the differences in chick weights were again not very marked.

The menhaden meal at both levels of protein gave comparably better feed efficiency, followed closely by the Peruvian and white fish meal treatments.

A statistical analysis of variance followed by Duncan's multiple range test, revealed significant differences only with respect to protein levels. The apparent differences in growth response observed among the different fish meals at each protein level were not statistically significant, indicating that there is little difference between the fish meals with respect to growth response.

However, special note could be made of the remarkable increase in growth shown by the treatments on white fish meal during the last two weeks. The white fish meal on analysis for amino acids (Experiment IV) was found to have the highest lysine content per percent of protein. It is possible that this high lysine was contributory as a booster for growth at the four and five-week stage. The total analysis of this fish meal showed no other characteristic that would explain this advantage over the other two meals.

Table 1. Percentage Compositions of Rations in Experiment I - (0 - 5 weeks)

Ingredients	Calculated level of total protein in ration	
	16%	13%
Corn, yellow ground	77.50	84.25
Fat, stabilized animal	2.50	1.75
Alfalfa meal (17% prot.)	1.75	1.75
Limestone, ground	1.50	1.50
Dicalcium phosphate, feed grade	0.50	0.50
Salt	0.50	0.50
Vitamin-trace mineral suppl.*	0.25	0.25
Fish meal**	15.50	9.50
Total	100.0	100.0

*Nopcosol M-5, Nopco Chemical Co.

**Menhaden, white or Peruvian

Table 2. Analysis* of the Rations in Experiment I

	Treatments					
	1	2	3	4	5	6
Fish meal	Menhaden	White	Peruvian	Menhaden	White	Peruvian
Protein	17.34	15.89	17.48	13.86	12.98	13.95
Fat	7.25	6.24	6.00	6.11	5.49	5.49
Calcium	1.47	2.15	1.50	1.19	1.62	1.21
Phosphorus	0.80	0.98	0.79	.60	.71	.61
Calories/lb**	1103	1103	1103	1103	1103	1103

*Calculated productive energy

**Calculated except for actual analysis of the fish meals

Table 3. Average Chick Weights and Feed Efficiency for the Six Treatments at Five Weeks of Age -- Experiment I

Treatment	Fishmeal	Approx. protein level in ration %	Average wt. of chicks in grams					Lbs. feed/ lb. gain
			1st. wk.	2nd. wk.	3rd. wk.	4th. wk.	5th. wk.	
1	Menhaden	16	83	160	271	397	494	2.17
2	White	16	77	151	267	412	519	2.28
3	Peruvian	16	80	152	253	353	488	2.25
4	Menhaden	13	74	134	227	343	452	2.47
5	White	13	70	127	212	329	425	2.60
6	Peruvian	13	74	132	215	328	429	2.45

Table 4. Analysis of Variance of Weights of Chicks at the End of Five Weeks - Experiment I

Source of variance	Sum of squares	Degrees of freedom	Mean square	F Value (calculated)	F value P = 0.01	F value P = 0.05
Total	2,233,102	209	10,680			
Subclass	429,102	23	18,656	1.92**	1.90	1.57
Treatments	226,905	5	45,381	4.67**	3.12	2.26
Protein levels	188,745	1	188,745	19.46**	6.77	3.89
Fish meals	923	2	461	0.05	4.72	3.04
P X F (Int.)	414,727	2	207,363	21.3**	4.72	3.04
Replicates	10,101	3	3,367	0.34	3.89	2.65
T X R (Int.)	192,096	15	12,086	1.32	2.10	1.70
Error	1,804,000	186	9,699			

Comparison of treatments. Mean weights underscored by the same line are not significantly different

Treatment	5	6	4	3	1	2
5% Level of probability Av. wt. (gm.)	425	430	452	488	494	519
At 1% level of probability Av. wt. (gm.)	425	430	452	488	494	519

**Significant at the .01 level of probability

EXPERIMENT II

Procedure -- The three fish meals were fed at lower protein levels as the only protein supplement in isocaloric diets. The protein level for the first three rations was approximately 13 percent and for the next three rations 11 percent. The respective fish meal levels were 9.5 and 5.5 percent of the total ration (Tables 5 and 6). The rations were again made adequate with respect to vitamin and mineral requirements.

The chicks from Experiment I were fed these diets from 5 to 9 weeks of age, in finisher batteries. The original experimental design was maintained. Growth rates were obtained from individual chick weights made before and after the experiment (Table 7) and the results were analyzed statistically (Table 8). Feed efficiency figures were determined from weight of feed consumed during the period of the experiment.

Results and Discussion -- The growth responses observed in this experiment were notable primarily for the marked improvement shown by the higher protein treatments over the lower. This was confirmed statistically as seen in Table 8.

At the higher protein level, menhaden fish meal showed the best weight record, both from the average gross weight of the chicks and the average gain in weight during the period of the experiment; Peruvian fish meal, again was a close second. Feed efficiency values for all treatments for the duration of the experiment were hardly indicative of any protein advantage as they were very closely similar.

At the lower protein level, the chicks on the Peruvian fish meal showed a slight improvement over those on menhaden both in gross average weights of chicks and average increase in weight during the four weeks of the experiment. In terms of feed efficiency, too, the Peruvian

fish meal treatment proved to be the better. An analysis of variance of the chick weights showed that there was a significant difference at the one percent level of probability where treatments were concerned but this was attributed to differences in protein levels and not treatments within each protein level. That is to say, there was no significant difference contributed by the different fish meals in terms of growth response.

On examining the weight records for the Menhaden treatments in comparison with the Peruvian treatment, it is seen that chicks on the menhaden fish meal fared better even though they were fed a slightly lower protein ration. The chicks on the white fish meal treatments, after the improved growth in the later stages of Experiment I, dropped considerably in performance, especially at the lower protein level. However, considering the fact that the white fish meal used in these experiments had a protein level lower by approximately 10 - 12 percent from that of the menhaden and Peruvian fish meals, due recognition should be given to its very close performance to that of menhaden and Peruvian fish meals.

Table 5. Percentage Composition of Rations in Experiment II (6 - 9 weeks)

Ingredients	Calculated level of total protein in ration	
	13%	11%
Corn, yellow ground	84.25	88.75
Fat, stabilized animal	1.75	1.25
Alfalfa meal (17% prot.)	1.75	1.75
Limestone, ground	1.50	1.50
Dicalcium phosphate, feed grade	0.50	0.50
Salt	0.50	0.50
Vitamin mineral suppl.*	0.25	0.25
Fish meals**	9.50	5.50
Total	100.0	100.0

*Nopcosol M-5, Nopco Chemical Co.

**Menhaden, white or Peruvian

Table 6. Analysis* of the Ration in Experiment II

	Treatments					
	1	2	3	4	5	6
Fish Meal	Menhaden	White	Peruvian	Menhaden	White	Peruvian
Protein	13.86	12.98	13.95	11.60	11.03	11.60
Fat	6.11	5.49	5.49	5.35	4.99	4.90
Calcium	1.19	1.62	1.31	1.00	1.25	1.01
Phosphorus	0.60	0.71	0.60	.49	0.55	0.48
Calories/lb*	1103	1103	1103	1103	1103	1103

*Calculated productive energy

**Calculated except for actual analysis of the fish meals

Table 7. Average Chick Weights and Feed Efficiency for the Six Treatments Ending at Nine Weeks of Age - Experiment II

Treatment	Fishmeal	Approx. protein level in ration %	Av. weight of chicks at 9 wks. (gm.)	Av. weight increase 6 - 9 wks. (gm.)	Lbs. feed/ lb. gain 6 - 9 wks.
1	Menhaden	13	1455	960	2.86
2	White	13	1434	915	2.81
3	Peruvian	13	1444	957	2.85
4	Menhaden	11	1259	804	3.20
5	White	11	1151	727	3.27
6	Peruvian	11	1260	827	2.99

Table 8. Analysis of Variance of Weights of Chicks at the End of Nine Weeks - Experiment II

Source of variance	Sum of squares	Degrees of Freedom	Mean square	F value (calculated)	F value P = 0.01	F value P = 0.05
Total	9,944,686	190	52,540			
Subclass	2,613,262	23	113,620	2.59**	1.90	1.58
Treatment	2,011,028	3	402,105	9.16**	3.90	2.67
Replicates	68,655	5	22,885	0.52		
T X R	533,579	15	35,572	0.81		
Error	7,331,424	167	43,900			

Comparison of treatments. Mean weights underscored by the same line are not significantly different.

Treatment	5	4	6	2	3	1
At 5% level of probability Av. wt. (gm.)	1151	1259	1267	1434	1444	1455
At 1% level of probability Av. wt. (gm.)	1151	1259	1267	1434	1444	1455

**Significant at 1% level of probability

EXPERIMENT III

Procedure -- A chemical determination was made of the available lysine content in each of the fish meals using the technique developed by Carpenter, 1960.

The procedure was carried out in duplicate and the entire experiment repeated with three different samples after a pilot run. The tests were conducted away from direct sunlight.

Stage I: The fish meals used were carefully sampled and ground finely. As the assay sample should contain 30 - 50 mg. N for results within the standard range, 500 mg. of each of the meals were weighed. Each sample was treated as follows: added 8 ml. of eight percent (w/v) $NaHCO_3$ and gently shook for ten minutes. Added 12 ml. of FDNB* solution in ethanol, stoppered and set on mechanical shaker for two hours. Evaporated the ethanol on a water bath, added 24 ml of 8.1 N HCl and autoclaved for 10 hours at 15 pounds pressure. Cooled flask in ice water and filtered the solution, made the filtrate up to 500 ml. using water washings. The solution was made up to 500 ml. so that 2 ml. aliquots would contain an estimated 35 to 50/mcg. of available lysine.

Stage II: Transferred 2 ml. aliquots of diluted filtrate into glass-stoppered, graduated cylinders A and B and into a conical flask C. The contents of the tubes were extracted twice with 5 ml. portions of ether to remove colored α- DNP amino acids (All α DNP amino acids are ether soluble, except ϵ-DNP lysine and αDNP arginine). The ether was evaporated by standing the tubes in hot water. Cooled cylinders and made up A to 10 ml. with 1.N HCl.

* FDNB fluoro-dinitro benzene

Stage III: The contents of flask C was titrated with ten percent NaOH using phenolphthalein as indicator, and then discarded. The same volume of ten percent NaOH was then added to tube B, and also 2 ml. of pH 8.5 buffer solution. Methoxy carbonyl chloride (0.045 to 0.055 ml.) was then added and the tube shaken vigorously to dissolve the compound. After ten minutes, 0.75 ml. of conc. HCl was added cautiously. The contents were extracted twice with 5 ml. of ether and the total volume was made up to 10 ml. with distilled water.

During the preparation of tubes A and B, four 2 ml. aliquots of the standard ϵ-DNP lysine HCl solution (6 mg/100 ml.) in four graded concentrations were given the same treatment from stage II onwards with omission only of ether washings.

Stage IV: Contents of tubes A and B were transferred to 1 cm. cells and their optical densities measured at 435 μ using a Beckmann B spectrophotometer.

Note: The standard solution of 6 mg/100 ml. was prepared so as to contain 40 - 50 mcg. of available lysine per 2 ml. aliquot.

$$\frac{146.1}{366.5} \times 120 = 46.8 \text{ mcg. of av. lysine per 2 ml.}$$

Calculation: A calibration curve was plotted from the optical densities obtained for the four concentrations. The optical densities of the samples were then related to their concentrations from the curve.

Results and Discussion -- The mean values for available lysine as a percentage of the total sample showed Peruvian and menhaden fish meal to have a higher content than that in the white fish meal. This is consistent with the better growth response shown by these fish meals.

However, on the basis of the percentage of available lysine in the fish protein, white fish meal proved superior. This would help explain why the white fish meal gave comparable results to the menhaden and Peruvian fish meals in the chick growth tests.

The Peruvian fish meal sample had a much higher percentage of available lysine in its total lysine content than menhaden or white. This indicates less damage to the lysine during processing and post-processing operations.

From the results, there is no doubt that much valuable information can be obtained with respect to protein quality of a fish meal by estimating the available lysine content. This provides an excellent index to protein quality, which is confirmed by growth response in Experiments I and II, as well as a precise indication as to the extent of damage to nutritive value of the protein during processing. On the basis of percentage available lysine in the total protein, figures for the three samples gave a valuable assessment of the nutritive value of the protein. The entire procedure in the determination of available lysine could well be adapted as a routine method for quality control on an industrial scale.

Table 9. Available Lysine Content in the Fish Meals*

Sample No.	Fish Meal	Available lysine in sample		Lysine content (% in protein)	% Available lysine of total lysine content
		% of total	% of protein (g/16 gms of N)		
I	Menhaden	3.55			
	White	3.22			
	Peruvian	3.73			
II	Menhaden	3.22			
	White	3.16			
	Peruvian	3.83			
III	Menhaden	3.33			
	White	3.09			
	Peruvian	3.44			
Mean values					
	Menhaden	3.36	4.98	10.09	49.35
	White	3.16	5.43	11.35	47.90
	Peruvian	3.66	5.36	9.95	53.86

*Gross protein content of the fish meals: menhaden 67.4%, white 58.1%, Peruvian 68.3%

EXPERIMENT IV

Procedure -- As a final effort to evaluate the relative nutritional quality of the proteins of the three fish meals, a microbiological assay was conducted to estimate quantitatively the ten essential amino acids. The procedures adopted in this experiment were by Barton and Wright (1952).

The fish meals were carefully sampled, ground, defatted and dried, then 1 gram of each sample was weighed, digested with 6 N HCl, autoclaved for six hours at 15 pounds pressure, boiled free of excess HCl on a water bath and made up to 100 ml. with distilled water. The solution was filtered and 5 ml. of the filtrate was removed and made up to 100 ml. The concentration of the test material prepared was 500 mcg. per ml.

The organisms used in the assays were Leuconostoc mesenteroides P60 (ATCC No. 8042) for the assay of arginine, histidine, lysine, methionine and phenylalanine and Streptococcus faecalis R (ATCC No. 8043)* for the assay of isoleucine, leucine, threonine, tryptophane and valine (See Appendix I for preparation of inoculum.)

For the assays with Streptococcus faecalis, Stokes (1945) medium was used and for assays with Leuconostoc mesenteroides, the lysine assay medium described by Barton-Wright was used. Note was made to include all amino acids, except the assayed amino acids in the preparation of the basal media. (See Appendix II for composition of basal medium.) Standard solutions for the assay were prepared as recommended by Barton-Wright. Due allowances were made when the salt of the amino acid was used as standard instead of the free acid. (See Appendix III for concentration of standard solutions prepared).

* American type culture collection (No. 8043)

The assays were made with 2 ml. aliquots and each assay tube was set up in duplicate. All tubes had 1 ml. of the basal media and 1 ml. made up of graded increments of sample and distilled water. The sample was dispensed at four concentrations; 0.25 ml., 0.5 ml., 0.75 ml., and 1.0 ml. In the tubes set for the standard solution, six graded concentrations of standard were used; 0 ml. (blank), 0.2 ml., 0.4 ml., 0.6 ml., 0.8 ml. and 1.0 ml. The assay solutions were all sterilized, inoculated with assay organisms and incubated at 37° C. At the end of 60 hours in the case of L. mesenteroides and 72 hours in the case of S. fecalis, the lactic acid produced by the organisms were determined by titration against 0.02 N NaOH. Bromothymol blue was used as an indicator. As S. fecalis is a poor acid producer, 0.01 N NaOH was used in the final titration.

For each assay the concentrations of standard were plotted against burette readings of NaOH and a standard curve obtained. The concentration of the amino acid in the test material was then calculated from the standard curve.

In the assay for tryptophane the test material was prepared by hydrolysis with 5 N NaOH, as acid hydrolysis destroys this amino acid. Since alkali hydrolysis causes racemization, note was made to double the final result.

Results and Discussion -- The values obtained for the amino acid content of each of the fish meals are shown in Tables 10 and 11. The results are expressed both as percentage of the fish meal and of the crude protein content. The following observations have been made on the percentage of amino acids in the protein (Table 11).

Lysine - the fish protein of white fish meal showed a higher value for percentage lysine in the protein than the other fish meals. In

general, however, there was little difference between the three meals in this respect. The values obtained for lysine are sufficiently high to provide at least one-third of the optimum lysine requirements, if the fish meal is used at the level of five percent of the diet.

Methionine - menhaden fish meal showed a distinctly higher methionine content than the white and Peruvian meals. The high percentage apparently contributed a large part of the methionine requirements of the chick and it may even be concluded here that the better growth response seen in the chicks on the menhaden treatments in Experiment I during the first four weeks could be clearly attributed to this high methionine content. The methionine content of Peruvian fish meal was lower than that of white fish meal on a percentage of protein basis.

Arginine - menhaden and Peruvian had an almost similar arginine value with Peruvian having a slight edge over the menhaden fish meal.

Histidine and Valine - menhaden and Peruvian fish meals shared a higher content of these amino acids with white fish meal having much lower values.

Isoleucine and Leucine - menhaden and Peruvian fish meals shared the better figures in the content of these amino acids with white fish meal having a much lower value.

The true biological value of the protein depends primarily on this balance. Careful examination of the amino acid structure of each of the fish proteins show that the meals are adequately fortified to substantially contribute to the total minimum requirements of essential amino acids in a chick ration if provided at suitable levels.

Table 10. Results of Microbiological Assay for Essential Amino Acids Expressed as Percentage of Feedstuff

Amino acid	Menhaden %	White %	Peruvian %
Arginine	3.98	3.25	4.12
Histidine	1.62	1.08	1.62
Isoleucine	4.20	3.28	4.12
Leucine	5.21	4.12	5.32
Lysine	6.80	6.60	6.80
Methionine	1.81	1.45	1.68
Phenylalanine	1.75	1.75	2.08
Threonine	3.02	2.92	3.60
Tryptophane	0.64	0.64	0.90
Valine	3.62	2.64	3.04
Crude protein	67.4	58.1	68.3

Table 11. Results of Microbiological Assay for Essential Amino Acids Expressed as Percentage of Protein Content

Amino Acid	Menhaden %	White %	Peruvian %
Arginine	5.90	5.60	6.03
Histidine	2.40	1.85	2.37
Isoleucine	6.23	5.64	6.03
Leucine	7.72	7.03	7.78
Lysine	10.08	11.35	9.95
Methionine	2.69	2.49	2.45
Phenylalanine	2.59	3.01	3.40
Threonine	4.48	5.02	5.27
Tryptophane	0.94	1.10	1.40
Valine	5.37	4.54	4.45

GENERAL DISCUSSION

The fish meals were considered typical, average and representative products offered to the feed manufacturing industry. Some information on the source of the meal, the type of fish, and the processing conditions used were available but were not complete. The results obtained, therefore, are limited to the particular samples obtained and cannot be accepted as completely final and conclusive for all fish meals.

The information obtained, however, may be of interest in assessing the nutritive value of fish meals. Menhaden fish meal is the product of a leading fish meal industry. Since the meal is prepared from a herring-type fish which is small, oily and a variety not used for human consumption, it is caught especially for the purpose of reduction to meal and oil. As the enormous catches of menhaden usually are composed of uniform size fish, the fish meal produced is generally of rather uniform quality. Being produced from whole fish entirely, menhaden fish protein has an excellent and balanced distribution of amino acids besides being rich in vitamin and mineral content.

Clandinin (1949) and Bender and Haizelden (1957) showed that the nutritive quality of protein in herring-type fish is extremely variable. One factor affecting quality is the damage to the protein by the heat of processing. Therefore, a poorly processed menhaden meal may not measure up in quality to a meal produced from left-overs of filleting operations which is carefully processed. White fish meal is an example of the latter and being produced from fish scraps, heads and fins will not necessarily contain a high percentage of high protein muscle tissue. The only source of muscle tissue in this meal is from

the small percentage of small sized, inedible whole fish incorporated in the meal.

Of the third type of fish meal studied here, Peruvian fish meal, we obtained comparatively much less knowledge. This meal, like the menhaden, is processed from whole herring-like "anchovetta." These are a very small variety of fish (a few inches long), non-oily, and hence should produce a high quality fish meal. But here again, tests have to be made to assess the nutritive value of the protein before any judgment could be made. It is evident, therefore, that the three fish meals compared for their biological nutritional value are all excellent fish meals with only gross protein values supplied as an index of their quality.

The experiments conducted proved menhaden and Peruvian fish meals superior to white fish meal. Menhaden could be regarded as having a slight edge over Peruvian, in that this meal performed better than Peruvian in the chick bio-assays in spite of the fact that it was being included in the rations at a lower protein level, and that it had a lower available lysine content per 16 grams of N. The extra damage to the available lysine in the menhaden meal is probably due to its exposure to a double heat treatment, i.e., treatment for oil extraction and treatment for drying the meal. It appears, therefore, that the protein value of a menhaden meal carefully processed would be much superior to that of the Peruvian "anchovetta" meal. The overall essential amino acid constitution of menhaden and Peruvian fish proteins are nearly the same and the differences are equally distributed. Five of the essential amino acids -- histidine, isoleucine, lysine, methionine and valine -- have higher values in menhaden. The white

fish meal apparently made from poorer protein quality raw material fared reasonably well, especially in Experiment I at the higher protein level. As explained earlier, this may very well have been due to the higher lysine content in this meal. The performance of white fish meal was remarkable in view of the fact that the sample used in these experiments had a nearly ten percent lower protein content than the menhaden and Peruvian meals.

In the amino acid assay for white fish meal, it was observed that the amino acid distribution in the protein of the meal was extremely well balanced. Although the amino acid values were lower than those for menhaden and Peruvian meals, they were not too low. Of the critical amino acids, lysine and tryptophane, values were higher than those of the whole fish meals. This is significant as one would not have expected any superiority with respect to lysine of a fish meal made from fish scraps. It may, therefore, be worthwhile to make an analyses of all the different components of fish scraps before any understatement as to their protein quality is made.

In a proximate analysis of the white fish meal, it was observed to contain high calcium and phosphorus levels and this may suggest itself for use preferably in layer rations. Judging from figures obtained for feed efficiency, there seems to be little to choose between the three fish meals compared.

According to Carpenter (1960) and Kellenbarger (1961), there is a positive correlation between the content of available lysine per 16 grams of N and the growth response of chicks. White fish meal had the highest available lysine content in the fish proteins and this may explain adequately the close performance of white fish meal with that of menhaden and Peruvian meal treatments, even though the protein level

in the white fish treatments were low. Carpenter's technique for determining available lysine as an index of fish meal quality has, therefore, proved to be most valuable in estimating the relative nutritive quality of a protein. This technique in the laboratory presented no difficulties and is adaptable for use in the quality control of fish meals, because it yields a valid reflection of damage to a critical amino acid lysine during processing.

CONCLUSIONS

The relative nutritive values of the fish proteins of single lots of menhaden, white and Peruvian fish meals were studied by feeding these meals at low levels in chick starter and finisher rations. An attempt was then made to correlate the results obtained with determinations of the available lysine and essential amino acid contents of the fish proteins.

The observations made indicate the following for the specific samples of fish meal studied:

1. In the chick bioassay experiments, menhaden fish meal showed consistently better growth response and better feed efficiency figures. The Peruvian fish meal fared very close to menhaden in the starter rations and better than menhaden in the lower protein finisher ration. The white fish meal although fed in a lower protein containing meal produced comparatively good growth response, especially at the fourth and fifth week stage.
2. Results obtained for the content of available lysine in the fish meals showed good correlation to the growth response in chicks and Carpenter's technique proved an excellent and useful indicator of fish meal quality.
3. Judging from the samples analyzed and used in these experiments, the Peruvian fish meal appeared to have suffered less damage to its content of biologically available lysine during processing. This observation may not be taken as a general rule as processing operations are so variable.
4. The general amino acid composition of menhaden and Peruvian fish meal samples proved superior to that of the white fish meal.

5. The Peruvian fish meal showed itself to be of high quality and closely matched menhaden fish meal as an excellent fish meal.
6. In general, there was little to choose between menhaden, Peruvian and white fish meals from the standpoint of protein quality.

LITERATURE CITED

Almquist, H. J., E. L. R. Stokstad and E. R. Holbrook, 1935. Supplementary values of animal protein concentrates in chick rations. J. of Nutr., 10: 193-211.

Barton-Wright, E. C., 1952. The Microbiological Assay of the Vitamin B-Complex and Amino Acids. Pitman Publishing Corporation.

Bender, A. E. and S. Haizelden, 1957. Biological value of proteins of a variety of fish meals. Brit. J. of Nutr. 11: 42-43.

Bisset, H. M. and H. L. A. Tarr, 1954. The nutritive value of herring meals. 2. Availability of essential amino acids. Poultry Sci. 33: 250-254.

Boyne, A. W., K. J. Carpenter and A. A. Woodham, 1961. Progress Report on an Assessment of Laboratory Procedures Suggested as Indicators of Protein Quality in Feedingstuffs. J. Sci. Food Agric. 12: 831-843.

Bruno, D. and K. J. Carpenter, 1957. A modified procedure for the estimation of available lysine in food proteins. Biochem. J. 67: 13.

Carpenter, K. J. and G. M. Ellinger, 1955. The estimation of available lysine in protein concentrates. Biochem. J. 61: 11.

Carpenter, K. J. and G. M. Ellinger, 1955. Protein quality and available lysine in animal products. Poultry Sci. 34: 1451.

Carpenter, K. J., 1958. Chemical methods of evaluating protein quality. Proc. Nutr. Soc. 17: 91-100.

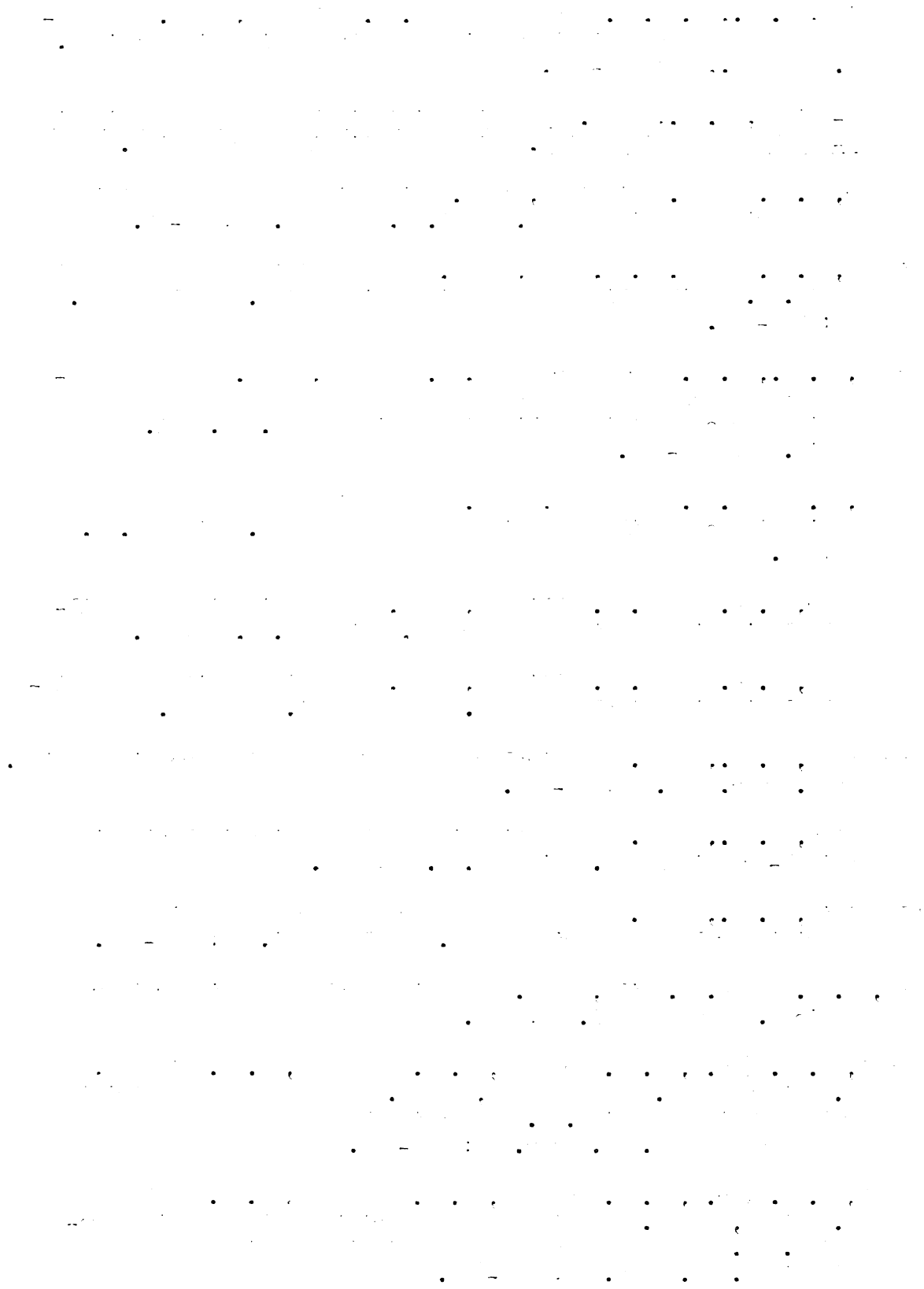
Carpenter, K. J., 1960. The estimation of the available lysine in animal-protein foods. Biochem. J. 77: 604.

Clandinin, D. R., 1949. The effects of methods of processing on the nutritive value of herring meals. Poultry Sci. 28: 128-133.

Day, R. J. and J. E. Hill, 1959. New fish meal tested in broiler rations. Poultry Sci. 38: 556.

Deuel, H. J. (Jr.), M. C. Hrubetz, C. H. Johnston, R. J. Wingler, E. Geiger and A. Schnakenberg, 1946a. Studies on the nutritive value of fish proteins. I. Evaluation by rat growth method and by Cannon method. J. Nutr. 31: 175-185.

Deuel, H. J. (Jr.), N. C. Hrubetz, C. H. Johnston, H. S. Rollman and E. Geiger, 1946b. Studies on the nutritive value of fish proteins. II. The use of mackerel protein in the bioassay test for vitamin A. J. Nutr. 31: 187-192.



Eldred, N. R. and G. Rodney, 1946. The effect of proteolytic enzymes on raw and heated casein. J. Biol. Chem. 162: 261.

Evans, R. J., J. S. Carver and Carroll I. Draper, 1944. A comparison of the chemical protein quality index with the gross protein value of fish protein concentrates. Arch. Biochem. 3: 337-343.

Ford, J. E., 1960. A microbiological method for assessing the nutritional value of proteins. Brit. J. Nutr. 14, 485.

Grau, C. R., N. L. Karrick, B. D. Lundholm, and R. N. Barnes, 1959. Nutritional values of fish meal proteins and their relation to processing variables. Comm. Fish. Rev. 21 (8): 7-12.

Grau, C. R. and M. A. Williams, 1955. Fish meals as amino acid sources in chick rations. Poultry Sci. 34: 810-817.

Gupta, J. D., A. M. Dakroury, A. E. Harper and C. A. Elvehjem, 1958. Biological availability of lysine. J. of Nutr. 64: 259-270.

Harms, R. H., Waldroup, P. W., and Douglas, C. R., 1961. The value of menhaden fish meal in practical broiler diets. Poultry Sci. 40: 1617.

Henderson, L. M. and E. E. Snell, 1948. A uniform medium for determination of amino acids with various micro-organisms. J. Biol. Chem. 172: 15.

Hieman, V., J. S. Carver and J. W. Cook, 1939. A method of determining the gross value of protein concentrates. Poultry Sci. 18: 464-474.

Hinners, S. W. and H. M. Scott, 1960. A bioassay for determining the nutritional adequacy of protein supplements for chick growth. Poultry Sci. 39: 176.

Kellenbarger, S., 1961. Available lysine as an index of fish meal quality. Poultry Sci. 40: 1756.

Kuiken, K. A. and C. M. Lyman, 1948. Availability of amino acids in some foods. Ibid. 36: 359.

Lea, C. H., L. J. Parr and K. J. Carpenter, 1960. Chemical and nutritional changes in stress herring meal. Brit. J. of Nutr. 14: 91.

Mitchell, H. M., 1924. A method of determining the biological value of a protein. J. Biol. Chem. 58: 873-903.

MacIntyre, T. M., 1957. Variation in the nutritive value of fish meals for growing chickens. Can. J. of Anim. Sc. 37: 58-63.

Osborne, T. B., L. B. Mendel and E. L. Ferry, 1919. A method expressing numerically the growth promoting value of protein. J. Biol. Chem. 58: 873-903.

Osterhout, L. E., B. D. Lundholm, 1959. Biological availability of amino acids in fish meals and other protein sources. J. of Nutr. 69: 65-73.

Osterhout, L. E. and D. G. Snyder, 1960. The nutritional evaluation of fish meals. Poultry Sci. 39: 1281.

Pader, M., D. Melnick and B. L. Oser, 1948. Factors affecting the availability of lysine in heat processed casein. J. Biol. Chem. 172: 763.

Rand, N. T., V. K. Collins, D. S. Varner and J. D. Mosser, 1960. Biological evaluation of the factors affecting the protein quality of fish meals. Poultry Sci. 39: 45-53.

Record, P. R., R. M. Bethke and O. H. M. Wilder, 1934. Effect of method of manufacture on nutritive value of fish meals as determined by growth studies on chicks. J. Agr. Res. 49: 715-722.

Sanger, F., 1945. The free amino groups of insulin. Biochem. J. 39: 507-515.

Schweigert, B. S., 1948. The value of various feeds as sources of arginine, histidine, lysine and threonine for poultry. Poultry Sci. 27: 223-227.

Stokes, J. L., L. M. Guiness, L. M. Dwyer, and M. C. Caswell, 1945. Microbiological methods for the determination of amino acids. II. A uniform assay for the ten essential amino acids. J. Biol. Chem. 160: 35-69.

St. John, J. L., J. S. Carver, O. Johnson, S. A. Moore and H. Gerritz, 1934. The biological value of rations containing fish meals. J. Nutr. 7: 13-26.

St. John, J. L., O. Johnson, J. S. Carver, S. A. Moore, 1932. A method of determining the biological value of protein in the study of avian nutrition. J. Nutr. 5: 267.

Schneider, B. H., 1932. Nitrogen balance studies with various fish meals. J. Agr. Res. 49: 715-722.

Tarr, H. L. A., J. Biely, and B. E. March, 1954. The nutritive value of herring meals. I. The effect of heat. Poultry Sci. 33: 242-250.

Wilgus, H. S., L. C. Norris, G. F. Heuser, 1935a. The relative protein efficiency and relative vitamin C content of common protein supplements used in poultry rations. J. Ag. Res. 51: 383-399.

APPENDIX I

Appendix I. Preparation of Inoculum

Ingredient		Amount
Glucose		1.0 Gm.
Yeast extract		1.5 Gm.
K_2 H PO_4		0.2 Gm.
$CaCO_3$		0.3 Cm.
Inorganic salt	"A"	0.5 ml.
Inorganic salt	"B"	0.5 ml.
Agar		2.0 Cm.

The above ingredients were dissolved in distilled water and the volume made up to 100 ml., warmed on a water bath, pH corrected to 6.8 and distributed into test tubes which were capped with aluminum thimbles and sterilized. The organisms were sub-cultured twice at intervals of 18 - 24 hours before they were ready for assays. The final inoculum was made in liquid medium (same as above with agar omitted) and prior to using the organisms were centrifuged aseptically and washed twice with 0.9 percent saline. The final suspension was made in 0.9 percent saline and the inoculation done using a sterilized pipette at the rate of a drop per test tube.

APPENDIX II

Preparation of basal media: For the two organisms used in the assays, the following basal media were prepared. In each amino acid assay the media was prepared omitting completely the assayed amino acid.

For 500 ml. of medium:

Amino acid	Basal medium for L mesenteroids*	Basal medium for S. fecalis**
DL Alanine	1000 mg.	200 mg.
L (+) Arginine HCl	250 mg.	200 mg.
DL Aspartic Acid	400 mg.	200 mg.
L (-) Cystine	100 mg.	200 mg.
L (+) Glutamic acid	500 mg.	200 mg.
Glycine	100 mg.	200 mg.
L (+) Histidine HCl	100 mg.	200 mg.
DL Isoleucine	200 mg.	200 mg.
L (-) Leucine	100 mg.	200 mg.
L (+) Lysine HCl	100 mg.	200 mg.
DL Methionine	100 mg.	200 mg.
DL Phenylalanine	100 mg.	200 mg.
L (-) Proline	100 mg.	200 mg.
DL Serine	100 mg.	200 mg.
DL Threonine	500 mg.	200 mg.
DL Tryptophan	200 mg.	200 mg.
L (-) Tyrosine	100 mg.	200 mg.
DL Valine	200 mg.	200 mg.

Other ingredients	Basal medium for L. Mesenteroids*	Basal medium for S. fecalis**
Glucose (anhydrous)	20.0 gm.	10.0 gm.
Sodium acetate (hydrated)	20.0 gm.	10.0 gm.
Sodium chloride	5.0 gm.	5.0 gm.
Ammonium chloride	6.0 gm.	--
+ Adenine, guanine, uracil sol	12.0 ml.	10.0 ml.
+ Xanthine sol	12.0 ml.	10.0 ml.
+ Inorganic salt sol A	5.0 ml.	5.0 ml.
+ Inorganic salt sol B	5.0 ml.	5.0 ml.
+ Vitamins		
Aneurine (thiamine)	1000 mcg.	2000 mcg.
Ca - d - pantothenate	1000 mcg.	4000 mcg.
Nicotinic acid	2000 mcg.	6000 mcg.
Riboflavin	2000 mcg.	200 mcg.
Pyridoxine	1600 mcg.	1200 mcg.
p-Aminobenzoic acid	50 mcg.	40 mcg.
Biotin	5.0 mcg.	0.4 mcg.
Folic acid solution (un)	--	2 mcg.
Water to	500 ml.	500 ml.

Correct pH to 6.8

*Barton - Wright

**Stokes

+See Appendix for composition of these stock solutions and the vitamin solutions.

APPENDIX III

Preparation of Stock Solutions:

Adenine, Guanine and Uracil Solution: 250 mg. of each were dissolved in water with a few drops of concentrated HCl and the solution made up to 250 ml.

Xanthine Solution: 250 mg. of xanthine were dissolved in 250 ml. water. A few drops of strong ammonia were used to effect solution.

Inorganic salt Solution A: 25 grams each of K_2HPO_4 and KH_2PO_4 are dissolved in water and the solution made up to 250 ml.

Inorganic salt Solution B: 10 grams $MgSO_4.7H_2O$, 0.5 grams $MnSO_4.4H_2O$ and 0.1 $FeCl_3$ (anhydrous) are dissolved in water and the solution made up to 250 ml. Added a few drops of conc. HCl to prevent precipitation.

Aneurin Solution: Aneurin 0.1 gram was dissolved in a little water and the solution made up to 100 ml. with 2 percent HCl.

Calcium d-pantothenate Solution: Calcium d-pantothenate 0.1 gm. is dissolved in 100 ml. water.

Pyridoxine Solution: Pyridoxine hydrochloride 0.244 grams is dissolved in 100 ml. water.

Nicotinic acid Solution: Nicotinic acid 0.1 grams is dissolved in 100 ml. water.

Riboflavin Solution: 25 mg. of riboflavin are mixed in a little water and a few drops of glacial acetic acid. The total is made up to 1000 ml. with water.

P-Aminobenzoic acid Solution: P-aminobenzoic acid 0.1 gram is dissolved in 100 ml. of water containing 1 ml. glacial acetic acid.

Biotin Solution: The contents of an ampule (containing 25 mcg.) are dissolved in 200 ml. water and the final solution made up to 250 ml. with ethanol.

Folic acid Solution: 1 mg. of folic acid is dissolved in 250 ml. of water and the volume made up to 500 ml. with 90 percent ethanol.

The above stock solutions were stored in a refrigerator during the course of the experiments. The vitamin solutions were prepared afresh at weekly intervals.

APPENDIX IV

Appendix III. Concentration of standard solutions prepared

Standard solutions		Concentration of free amino acid
L Arginine HCl	3.6 mg/250 ml	12 mcg/ml
L Histidine HCl	6.8 mg/1000 ml	5 mcg/ml
DL Isoleucine	6.0 mg/250 ml	24 mcg/ml
L Leucine	3.0 mg/250 ml	12 mcg/ml
L Lysine	5.0 mg/100 ml	40 mcg/ml
DL Methionine	4.0 mg/250 ml	16 mcg/ml
DL Phenylalanine	5.0 mg/250 ml	20 mcg/ml
DL Threonine	6.0 mg/250 ml	24 mcg/ml
DL Tryptophane	2.5 mg/100 ml	2.5 mcg/ml
DL Valine	6.0 mg/250 ml	24 mcg/ml