very soluble in either hot or cold water, are formed by the reaction between monocalcium phosphate and potassium sulfate. The fact that triple superphosphate is very high in monocalcium phosphate supports this view. The reaction may be expressed as follows:

$$Ca(H_2PO_4)_2 + K_2SO_4 = CaSO_4 + 2KH_2PO_4$$

A weighed portion is boiled with ammonium oxalate according to the official method to prevent loss by the action of the colloids, but in the case of high-analysis base goods, the potassium sulfate has the same effect, and complete recovery of the potash is obtained.

In making up the 20 per cent base mixtures, the same kind and amount of potassium sulfate were used in all cases. The slightly different results may largely be ascribed to differences in composition of the phosphate rock deposits. According to Jacob *et al.* (*1*), Florida land pebble phosphate contains 0.19 per cent K_2O (average of 11 analyses), Tennessee brown rock phosphate 0.435 per cent (average of 6 analyses), and Idaho phosphate 0.44 per cent (average of 3 analyses).

While the potash is originally present mainly as silicates, a small amount is probably decomposed by the action of fluorine in the mixing pan and during subsequent curing.

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Fluorometric Determination of Riboflavin in Pork Products

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A method developed for the fluorometric determination of riboflavin in pork and pork products has been applied successfully to ham, pork muscles, fat, liver, heart, lung, and spleen. It is in close agreement with other fluorometric procedures and the results do not differ greatly from values reported for the same tissues when analyzed by bioassay or microbiological procedures. The adsorption procedure of Conner and Straub and the permanganate oxidation step have been eliminated, thus shortening the time required for analysis.

Large differences were found in the riboflavin content of different muscles of the same pig. Appreciable amounts of riboflavin were found in various pork fats from which all lean had been removed.

THE most widely used procedures for the determination of riboflavin in meats and meat products have been the biological rat growth assay (*3, 7, 8, 10*) and the microbiological procedure of Snell and Strong (*15*). The latter method measures the influence of riboflavin on both the cell growth and the acid production of *Lactobacillus casei* ε grown on a synthetic medium free of riboflavin.

The fluorometric method, which measures the degree of fluorescence given by riboflavin in violet light, is being used with success in the analysis of many foods (*6, 9, 12, 16*), but its application to animal tissue has been only partially successful (*14, 18*). For many reasons, the application of fluorometric methods to all types of foods and biological materials seems desirable. All the proposed methods have certain limitations: The biological procedure is expensive and time-consuming and in some food materials there is the question of the availability of riboflavin (*11*). In the fluorometric method, excessive turbidity and the presence of extraneous fluorescent materials in solution are troublesome features. Extracts of certain biological material may exert either inhibitory or stimulating effects (*17*) in the microbiological procedure. Both the fluorometric and microbiological methods have in common the problem of bringing the vitamin into solution. As existing methods become refined, these and other difficulties will be overcome.

Mickelsen, Waisman, and Elvehjem (*13*), using the microbiological method, have made extensive investigations of the riboflavin content of meat and meat products. These workers point out that the values in the literature previous to 1934 on the distribution of riboflavin are not of a quantitative nature. Williams and the University of Texas group (*17*), using the same method, have made important contributions in studies of the vitamin content of tissues and foods. Of particular value to workers in this field is their demonstration of the variability in efficacy of extraction of the different vitamins depending on the nature of the food, the enzyme used, and the heat treatment involved.

Of the fluorometric procedures reported, that of Conner and Straub (*4*) appears to give reliable results with many plant products. This procedure was, therefore, taken as a starting point in the present studies, and the optimal conditions were determined for its application to pork products. The method which was finally devised differs from the Conner and Straub method in the following points: The adsorption procedure and permanganate oxidation steps have been omitted; the incubation period with clarase has been increased from two hours to 24 hours; and "blank" values are obtained by reduction of the riboflavin of the extracts with sodium hydrosulfite.

Description of Method

Because of the sensitivity of riboflavin to light, all manipulations are conducted in a semidarkened laboratory; and amber glassware is used throughout.

A 10- to 20-gram sample is dropped into 100 cc. of 0.04 *N* sulfuric acid in a Waring Blendor and macerated for 2 minutes at high speed. The creamy mixture is then transferred quantitatively to a 250- or 300-cc. Erlenmeyer flask, using a minimum amount of water from a wash bottle to effect the transfer. The flask is plugged with cotton and autoclaved 15 minutes at 6.8 kg. (15 pounds) pressure. As soon as the flask has cooled, 20 cc. of a 2.5 per cent solution of clarase (*4*) freshly prepared in a sodium acetate–acetic acid buffer are added. [The buffer solution (pH 4.5) is prepared by adding 54.4 cc. of glacial acetic acid to 66.9380 grams of anhydrous sodium acetate together with sufficient distilled water to obtain a solution of the reagents, and then transferred to a 1-liter volumetric flask and made up to volume with distilled water.] The contents of the flask are mixed thoroughly

and then incubated at a temperature of 45° C. for 24 hours. The flask is agitated two or three times during the incubation.

Following the incubation period, the extract is brought to a volume of 200 cc. At this point the contents of the flask should be thoroughly mixed, either by stoppering the flask and shaking thoroughly, or transferring the contents to a Waring Blendor and mixing for 30 seconds. The solution is then filtered and approximately 20 cc. of filtrate are collected. Clarification by centrifugation may be used instead of filtration, if preferred.

Into each of two test tubes, A and B, are pipetted 5 cc. of filtrate. Five cubic centimeters of water are added to tube A, and 5 cc. of an aqueous riboflavin solution containing 0.2 microgram per cc. to tube B. The solutions are mixed and fluorescent readings are made after adjusting the instrument. With the Coleman electronic photometer (Model 12), the instrument was adjusted each day with a sodium fluorescein solution of such strength that when its reading was 100, an aqueous solution containing 0.2 microgram of riboflavin read 70. This range was suitable for most pork extracts prepared in the above manner. Having obtained readings for A and B, 0.5 cc. of sodium hydrosulfite solution is added to each tube and readings are taken again. The average of these two readings constitute the reading of the sample blank, or C reading.

The hydrosulfite solution (*1*) was prepared by dissolving 5 grams of sodium hydrosulfite in 100 cc. of an ice-cold sodium bicarbonate solution (2 grams of sodium bicarbonate per 100 cc.).

To determine the concentration of riboflavin per cubic centimeter of extract, the following formula was used:

$$\frac{A - C}{B - A} \times 0.1 \text{ microgram per cc.} = \text{micrograms of riboflavin per cc.}$$

It is important that a "complete blank" containing all the reagents used in the method be run through the entire procedure, and the resulting concentration subtracted from the value of the sample extract. The high riboflavin content of clarase has not been sufficiently emphasized, nor has the fact that a considerable portion of the blank reading is due to the enzyme. Cheldelin, Eppright, Snell, and Guirard (*2*) have determined the riboflavin content of many of the enzymes. Clarase was not included in this group. In the proposed procedure, this enzyme contained 8.0 micrograms per gram. For pork muscle tissues its fluorescence reading represented 15 to 25 per cent of the total sample reading (A). Its blank reading (C), after reduction with hydrosulfite was large, representing 50 to 80 per cent of the values obtained for C readings of pork muscle extracts carried through the above procedure with 20-gram samples. In Table I are shown typical readings for pork muscle extracts.

Because of the remarkable clarity of the extracts, the time-consuming adsorption step of Conner and Straub was not used. Instead, aliquots of the extract were prepared by the procedure of Bailey and Thomas (*1*). The general practice of destroying colored impurities by oxidation with potassium permanganate was not resorted to, since independent experiments indicated that it was not permissible to assume that the blank value of any two permanganate-treated eluates would necessarily be identical, nor that they would necessarily be small and negligible. This is typical of many fluorometric procedures, as is also the assumption that the reading "increment" for a given amount of pure riboflavin will be the same for all permanganate-treated extracts of animal and plant tissues. The use of permanganate would also interfere with the sodium hydrosulfite reducing treatment subsequently used.

As emphasized by Bailey and Thomas (*1*), this method renders unnecessary the preparation of a standard curve of reference of photometer response unless one desires to know the extent to which the intensity of fluorescence of riboflavin is quenched by substances in the solution analyzed. The average quenching value of pork muscle extracts was 11.5 per cent, the low value of which shows the clarity of the extracts obtained by this method.

TABLE I. TYPICAL FLUOROPHOTOMETER READINGS AND CALCULATED RIBOFLAVIN CONTENT OF PORK MUSCLE EXTRACTS

Sample	Fluorophoto meter Readings[a] A	B	C	A-C	B-C	Calculated Riboflavin Content: Extract	Muscle
						Microgram/cc.	*Microgram/gram*
1	58	91	12	46	33	0.139	2.58
2	53	86	10	43	33	0.130	2.40
3	55	87	11	44	32	0.138	2.56
4	67	97	11	56	30	0.186	3.52
5	40	72	10	30	32	0.094	1.68
Enzyme blank	10	40	7	3	30	0.010	....

[a] A, reading of extract.
B, reading of extract containing 1 microgram per cc. of added riboflavin.
C, reading of extract after reduction with sodium hydrosulfite.

TABLE II. EFFECT OF TIME OF INCUBATION WITH CLARASE ON YIELD OF RIBOFLAVIN FROM PORK MUSCLE

Sample	2 Hours	24 Hours
	(*Micrograms/gram of fresh pork tissue*)	
A	1.67	2.26
	1.98	2.28
B	1.57	2.12
	1.70	2.34
C	1.58	2.24
	1.74	2.34
Average of 12 loin samples	1.32	1.78

Critical Study of Steps in Procedure

EXTRACTION METHOD AND ENZYMATIC HYDROLYSIS. In preliminary determinations, the method of extraction by Conner and Straub (*4*) was used with modifications. In all direct comparisons adjacent slices of a single, separated muscle were used for samples. Samples of pork muscle which had been mixed in 0.04 *N* sulfuric acid with a Waring Blendor were digested for varying lengths of time on a boiling water bath previous to incubation with clarase. These results were compared with analyses of the same samples which had been autoclaved for 15 minutes at 15 pounds' pressure. The results of these experiments indicated that the length of heating was not so critical a factor as the length of time of incubation with enzyme subsequent to heating. Thus, 12 duplicate samples of loin gave an average value of 1.75 micrograms of riboflavin per gram when heated for 1 hour on a boiling water bath, as compared to an average value of 1.78 micrograms per gram when the samples were autoclaved. (These samples were subsequently incubated 24 hours with clarase.) In the routine determination of a large number of samples it proved to be more convenient to use the autoclave throughout, with results from check samples agreeing more consistently.

Incubation of samples with clarase for 2 hours as in the method of Conner and Straub gave erratic results and the values were frequently considerably lower than those which had been reported in the literature (*13*) for similar products analyzed by the microbiological method. Also, the exhaustive investigations on the enzymatic liberation of B vitamins from plant and animal tissues by Cheldelin *et al.* (*2*) suggested that a 2-hour incubation might not be sufficient. This proved to be the case. In Table II are shown the values for a 2-hour and a 24-hour incubation with 2.5 per cent clarase (incubation for more than 24 hours did not improve the yield). Values range from 15 to 35 per cent greater for the 24-hour incubation. These figures also show the duplicability of results by this method for pork products.

Since Cheldelin *et al.* (*2*) had demonstrated that enzymes may differ widely in the efficiency with which they will liberate riboflavin from foods, it seemed desirable to compare the effectiveness of other enzymes with that of clarase. Solutions (2.5 per cent) of clarase, papain, takadiastase, and mixtures of the latter two were used on aliquot samples of pork muscle exactly as described for clarase in the original method. Relative yields for these enzymes were: clarase 100 per cent, papain 49 per cent, takadiastase 81 per cent, and papain and takadiastase 54 per cent. It is not intended to imply that these are the maximum yields obtainable with the enzymes other than clarase. A variation in conditions might easily change the order of efficacy. These results do, how-

TABLE III. COMPARATIVE RESULTS OBTAINED BY PROPOSED AND CONNER AND STRAUB ADSORPTION PROCEDURES

Sample No.	Proposed Method *Micrograms/g.*	Adsorption Method *Micrograms/g.*
12	2.54	2.50
13	1.82	1.96
16	1.74	2.08
20	1.82	1.90
28	1.92	2.00

ever, represent the values obtained under the conditions of the proposed method. The Texas group (*17*) usually obtained maximum yields of riboflavin from tissues with papain or combinations of papain and takadiastase.

COMPARISON OF RESULTS BY DIFFERENT FLUOROMETRIC PROCEDURES. In order to test the method it seemed desirable to compare the results obtained with those of other methods which had been suggested. Good agreement was found with the Van Duyne (*18*) procedure in the case of pork heart or liver, but poor checks were obtained with muscle tissues. In the Van Duyne method the sample is digested for 24 hours with 1 per cent pepsin in hydrochloric acid at 37° C. The lack of provision for determining the size of the increment due to pure riboflavin in the Van Duyne procedure and the relatively low concentration of riboflavin in muscle tissue with its concomitant high blank value probably account for the lack of agreement.

A comparison of the proposed method with the results obtained by the adsorption procedure of Conner and Straub is shown in Table III. These values are averages of duplicate d terminations of pork tenderloin muscles. The extracts adsorbed on Supersorb were those obtained by the extraction procedure described.

Ordinarily the adsorption method yielded slightly higher results. It is possible that this can be accounted for by the assumption which is implied in the Conner and Straub procedure that the blank value of the test material extract after adsorption and permanganate treatment is negligible.

Good agreement was also obtained on pork extracts with the Najjar (*14*) method proposed for the fluorometric determination of riboflavin in urine and other biological fluids. The Najjar method involves treatment of the extract with permanganate, followed by peroxide and subsequent extraction of the riboflavin by use of butyl alcohol. Pyridine and sodium sulfate are added before extraction with the alcohol. The blank value is obtained by exposing the sample to a mercury vapor lamp (or direct sunlight) for an hour or two. Six cured ham samples gave an average value of 2.08 micrograms per gram by the proposed method and 1.85 micrograms per gram by the Najjar method.

TABLE IV. RIBOFLAVIN CONTENT OF PORK MUSCLES FROM THE SAME PIGS

(Micrograms per gram of fresh tissue)

Muscle	Riboflavin Content
Shoulder (triceps-Brachii)	3.0
Tenderloin (Psoas Major)	2.4
Loin (anterior end; Longissimus Dorsii)	1.3
Loin (posterior end; Longissimus Dorsii)	1.3
Ham (inside; adductor)	1.3
Ham (eye; Semitendinosus)	1.8

RIBOFLAVIN CONTENT OF PORK AND PORK PRODUCTS. The method has been applied to various pork muscles, fat, organs, and hams, both dry-cured and brine-cured.

In Table IV are shown riboflavin values for various fresh pork muscles. The muscles were obtained from two pigs which had been fed a standard fattening ration, and samples from the right and left sides of the carcass were taken as duplicates. Each value represents the average of two duplicate determinations (one duplicate for each pig). The high values for shoulder and tenderloin muscles, and the large differences between different muscles were unexpected. Such differences in the muscles themselves may be significant in evaluating published results.

Table V gives the values obtained from various pork organs compared with data available on similar organs from the work of Mickelsen *et al.* (*13*). Cheldelin (*2*) reports a somewhat lower value, 8.4 micrograms per gram for pork heart. Darby and Day (*5*), using the rat bioassay method, give values of 3.0 micrograms per gram for fresh ham and 23.0 micrograms per gram for pork liver. The high value of 0.9 microgram per gram reported by the latter workers for bacon suggested that either the bacon studied by them was unusually high in its content of lean, or pure pork fat itself contained significant amounts of riboflavin. This prompted a study of the various types of pork fat, the results of which are presented in Table VI. The relatively high values obtained are important in view of orthodox concepts regarding the solubility characteristics of riboflavin. These findings suggest a reconsideration of animal studies concerned with the "sparing" action of fats on the B vitamins.

TABLE V. RIBOFLAVIN CONTENT OF VARIOUS PORK ORGANS

(Micrograms per gram of fresh tissue)

Organ	Values by Proposed Method	Values by Mickelsen *et al.*
Liver	22.0	25.4
Liver	29.4	26.9
Liver	....	29.0
Heart	10.1	11.2
Heart	9.1	....
Lung	3.4	....
Lung	2.7	....
Spleen	3.6	....
Spleen	4.3	....

TABLE VI. RIBOFLAVIN CONTENT OF PORK FATS

Type of Fat	Riboflavin Content *Microgram/g.*
Lard	0.09
Ham facing	0.63
Back fat	0.59
Leaf fat	0.57

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