2-Chlorophenazine—To the solution of 2-aminophenazine (0.2 g.) in conc. HCl (4 cc.), sodium nitrite (0.18 g. in 2 cc. water) was added and diazotized at 0°. To the mixture, freshly prepared cuprous chloride (0.4 g.) was added in portions. After standing for 20 minutes at room temperature, the reaction mixture was warmed on a water bath at 60°. This was neutralized with ammonia water, the precipitate that separated was dissolved in benzene, and chromatographed on alumina. It gave pale yellow flat needles, m.p. 135~136° (from ligroine), not depressed on admixture with the authentic specimen of 2-chlorophenazine. Pachter, *et al.*[4] and McCombie, *et al.*[9] recorded m.p. 138~139°. Yield, 0.14 g. *Anal.* Calcd. for $C_{12}H_7N_2Cl$: N, 13.05. Found : N, 13.17.

Summary

1) Phenazine and some of its derivatives were nitrated under the same conditions and the position of the nitro groups substituted was determined.

2) Phenazine was not nitrated easily at 0°, but when the reaction temperature was raised to 60°, formed 1,3-dinitrophenazine.

3) Phenazine mono-N-oxide was nitrated to form 3-nitrophenazine 5-N-oxide in a good yield, and 1-nitro compound as a by product.

4) Phenazine di-N-oxide was not nitrated smoothly and only a small amount of 3-nitrophenazine 5-N-oxide was obtained.

5) 2-Methoxyphenazine 10-N-oxide was nitrated to form 1-nitro derivative.

6) From 2-methoxyphenazine 5-N-oxide, 1,3-dinitro-2-methoxyphenazine 5-N-oxide was obtained.

7) 1-Methoxyphenazine and its 5-N-oxide were substituted both at 1-position by the nitro group.

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67. Toru Masuda and Mitsuko Asai : Application of Chromatography. XX[1]. Quantitative Determination of Cocarboxylase Preparations.

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In general, the synthesis of pure cocarboxylase (TDP) in various methods is attended with some difficulty. From the practical point of view, however, less pure preparations will do, so long as they have a constant purity and have no unwanted activities. A method, therefore, is needed which can determine exactly the purity of the preparations.

The purity of cocarboxylase preparations has hitherto been determined by measuring their biochemical cocarboxylase activity. Since, however, the method is conducted using apocarboxylase isolated, for example, from yeast, the values always fluctuated depending on the purity of the apocarboxylase used. Such circumstances necessitated the authors to find a chemical method which can be carried out easily and which gives exact values.

The crude TDP may be contaminated by monophosphate (TMP), triphosphate (TTP), or by free thiamine in view of the mode of its preparation.

The molecule of thiamine essentially has a positive electric charge. If phosphoric acid combines with the molecule to form an ester, the positive electric charge ought to decrease in proportion to the increase of phosphoric acid radical. Different esters, therefore, would

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1) Satoru Kuwada : Application of Chromatography. XX. Parts I~XIX by S. Kuwada and his co-workers were published in Vitamins, 1950~1954 (in Japanese).

show different ionophoreses, and they would be separated from each other by ionophoresis when they are mixed with each other. If TDP is separated without decomposition its quantity will be calculated from the values of thiamine, N, and P in the extract of its band of ionopherogram.

As a preliminary experiment the authors subjected crude TDP to paper ionophoresis in buffer solutions of various pH's. The pherograms were dried and observed under ultraviolet rays of $\lambda=240\sim280$ mμ. As a result, several absorption bands were detected which gave the color reaction of N-compound with the Dragendorff reagent. From the result of the qualitative tests, Theorell buffer solution of pH 3 was found to be the most excellent for the separation.

On the other hand, when TDP of Nutritional Biochemical Corporation and TMP reprepared from this TDP were chosen as standard samples and subjected to paper ionophoresis under the same conditions as above, each pherogram gave only one absorption band. From this result, the bands of TDP and TMP in the pherogram of the crude TDP were located definitely.

The absorption bands were cut out of the pherograms, extracted with hot water, and the extracts were treated as follows.

a) The extract was treated with Takadiastase to decompose the ester in it to thiamine, which was then determined by the cyanogen bromide method. From the value the corresponding amount of TDP or TMP was calculated (cyanogen bromide method).

b) The extract was decomposed by the micro-Kjeldahl method, and the nitrogen in the reaction mixture was determined by azotometry. From the value of nitrogen obtained, the corresponding amounts of thiamine and the esters were calculated (azotometric method).

c) The extract was hydrolyzed with hydrochloric acid, and the phosphorus in the resulting phosphoric acid was determined. From the value of phosphorus obtained, the corresponding amount of TDP was calculated (determination of phosphorus in hydrolyzed phosphoric acid solution).

If the values of TDP and TMP thus obtained agree with each other they may be regarded as being correct.

With the pure cocarboxylase preparation of Nutritional Biochemical Corporation, each of the values of TDP in tests (a) and (b) was nearly 100%. With the preparation of Fallek Products Co., the pherogram showed two absorption bands corresponding to TDP and TMP, and their values were 90% and 14.5% in test (a), 85.4% and 15.7% in test (b), respectively, and in test (c) the value of TDP was 91~93%. Thus, the total of the values of TDP and TMP showed about 100% each.

The authors synthesized cocarboxylase according to the original report of Tauber[2] and subjected the product in the course of purification to paper ionophoresis. The pherogram showed two absorption bands corresponding to TDP and TMP. The bands were cut out and tested as above, but the total of the values did not reach 100%. From the value of phosphorus the product seems to contain some inorganic phosphorus compounds besides moisture.

For valuation of this method it is important to compare the result of this method with that of enzymatic test. The results obtained by the authors almost agreed with those of the enzymatic tests kindly conducted by Dr. Uehara, Assistant Professor of the Osaka University.

Note : The above results were reported at the meeting of the Vitamin Committee on May 1, 1954, but their publication was delayed for certain reasons. Just before contribution of this manuscript, the authors received Biochimica et Biophysica Acta [**14**, 52 (1954)] in which D. Siliprandi reported on a similar subject under the title of "Separation and Quantitative Determination of Thiamine and

2) Tauber : J. Am. Chem. Soc., **60**, 730, 2263 (1938).

Thiamine Phosphoric Esters and their Preparation in pure Form". They stated that the phosphoric esters can successfully be determined by applying paper- or column-ionophoresis, and this view well agrees with that of the present authors.

The authors wish to express their appreciation to Mr. M. Yamagishi and Mr. M. Yokoo in this Laboratory for carrying out the azotometry.

Experimental

Paper Ionophoresis of Thiamine and Its Phosphates—The samples are subjected to ionophoresis for 3 hrs. in the Theorell buffer solution of various pH's applying an electric field of 300 V, and the pherograms are observed under ultraviolet rays of $\lambda=240\sim280$ mμ. On the other hand, the pherograms are sprayed with the Dragendorff reagent to detect the reddish brown band. The two results well agree with each other in location. The direction and distance of ionophoresis are shown in Table I, (−) represents migration towards negative electrode, and the figures show the distance (cm.). Of the samples, TDP is the pure preparation of Nutritional Biochemical Corporation, and TMP is also a pure product prepared from the TDP by hydrolysis with HCl.

Table I.

pH of buffer \ Sample	TDP	TMP	Thiamine
3	(−) 1.0	(−) 3.0	(−) 6.0
4	(−) 0.5	(−) 2.5	(−) 5.5
5	(−) 0.4	(−) 2.5	(−) 6.0
6	0.0	(−) 1.5	(−) 5.0

Quantitative Determination of Thiamine Phosphates— A definite amount between 50~100 mg. of the sample is weighed accurately and dissolved in 5 cc. of water in a measuring flask. A definite amount (0.05~0.1 cc.) of the solution is applied on a filter paper strip, 8×42 cm., and the paper strip, after being moistened with the Theorell buffer solution of pH 3, is subjected to ionophoresis for 3 hrs., applying an electric field of 300 V. The pherogram is dried and observed under ultraviolet rays of $\lambda=240\sim280$ mμ. The detected absorption bands are cut out, extracted with 10 cc. of an acetate buffer solution of pH 4 at 100° for 30 mins., separately, and each of the extracts is tested as will be described later. On the other hand, a piece of filter paper of nearly the same dimensions as that of the absorption band is extracted with 10 cc. of the acetate buffer solution under the same conditions as above, and the extract is tested to obtain the value of blind test.

a) Determination of thiamine by the cyanogen bromide method: To 1 cc. of the extract are added 1 cc. of 10% Takadiastase solution and 2 cc. of an acetate buffer solution of pH 4, the whole is diluted with water to 10 cc., and warmed at 40~50° for 3 hrs. One cc. of the reaction mixture is treated according to the Fujiwara's method[3] to determine the quantity of thiamine. The quantities of TMP and TDP are obtained by multiplying the value of thiamine by 399/337.28=1.18 and by 478.76/337.28=1.42, respectively (In this case both TMP and TDP are calculated as a monohydrate).

b) Determination of N by Azotometry: Two cc. of the extract is decomposed by micro-Kjeldahl method, and the nitrogen in the reaction mixture is determined by azotometry. The quantities (%) of TDP and TMP are calculated from the following equation:

$$N_2(mm^3)\times\frac{\text{Gram molecule of TDP or TMP}/44.8}{\text{Quantity of sample }(\gamma)}\times500=\%$$

c) Determination of phosphorus: A definite quantity between 100~150 mg. of the sample is weighed accurately, dissolved in 5 cc. of water, and the solution is subjected to ionophoresis in acetate buffer of pH 3.0 under the same conditions as before. The absorption band corresponding to TDP is cut out, extracted with 5 cc. of water at 100° for 30 mins., and 2 cc. of this extract is heated with 1 cc. of 3 *N* HCl at 100° for 1 hr. After cooling, the solution is mixed with 5 cc. of saturated aq. solution of NH_4Cl (to inhibit precipitation of thiamine by ammonium molybdate), subjected to the color reaction according to Fiske and Subbarow's method[4], and after standing for 20 mins., its extinction, E, at $\lambda=740$ mμ is measured by the Beckman spectrophotometer. For blind test a piece of filter paper of nearly the same dimensions as above is extracted with 5 cc. of water at 100° and the extract treated as above to obtain its extinction value, E_o.

A standard solution of KH_2PO_4 containing 50 γ/cc. of P is treated as above to obtain its extinction value, E_s. A blind test, E_b, is obtained in the same manner. The quantity of P in the sample is calculated from the following equation:

$$50(\gamma)\times\frac{E-E_o}{E_s-E_b}\times25=P(\gamma)$$

3) Fujiwara: Anal. Chem., **25**, 810 (1953).

The quantity (γ) of TDP is obtained by multiplying the value of P by 478.76 (TDP)/30.98 (P) = 15.45.

Result of Determination of Cocarboxylase Preparations—1) Preparation of Nutritional Biochemical Corporation: After dissolving 91.4 mg. of the preparation in 5 cc. of water, 0.1 cc. of the solution (1.83 mg. of sample) is subjected to ionophoresis. The bands of TDP and TMP are cut out, and the TDP and TMP are determined separately by the methods (a) and (b). On the other hand, the quantity of thiamine in 1 cc. of the solution is determined by the direct method.

Table II.

	Cyanogen bromide method			Azotometric method				
	Thiamine found (γ)	TDP or TMP calcd. from thiamine value(γ)	Content (%)	N_2 found (mm^3)	N_2 in 1.83 mg. of sample (mm^3)	Thiamine calcd. from N value(γ)	TDP or TMP calcd. from thiamine value(γ)	Content (%)
Total value	1340			35.2	176.0	1332		
TDP	1340	1900	104	34.7	173.5	1275	1820	99.5
TMP	36	42	2	2.2	11.0	79.4	94	5.0

The same preparation (95.4 mg.) is treated according to the method (c) and 47.08 γ of P is detected in the hydrolyzed solution. This means the presence of 117.78 γ of P in 5 cc. of the sample solution (1.91 mg. of sample), i.e. 1818.5 γ of TDP, equivalent to 95.3%.

2) Preparation of Fallek Products Co.: The preparation (125.5 mg.) is treated as above. The results are shown in Table III. In this case, 0.1 cc. of the solution for determination contains 2.51 mg. of the sample.

Table III.

	Cyanogen bromide method			Azotometric method				
	Thiamine found (γ)	TDP or TMP calcd. from thiamine value(γ)	Content (%)	N_2 found (mm^3)	N_2 in 1.83 mg. of sample (mm^3)	Thiamine calcd. from N value(γ)	TDP or TMP calcd. from thiamine value(γ)	Content (%)
Total value	1920			51.8	259.0	1900		
TDP	1600	2270	90	42.5	212.5	1565	2200	84.5
TMP	310	365	14.5	9.1	45.5	335	396	15.7

The same preparation (91.6 mg.) is treated according to the method (c) and 108.1 γ of P is detected in 1.83 mg. of the sample. This corresponds to 1669 γ of TDP, equivalent to 91%. This value well agrees with that obtained by the cyanogen bromide method.

3) Preparation of the authors': Two kinds of the preparations were tested under the same conditions as in the cases of the imported preparations. The results are shown in Tables IV and V.

The values well agree with those of TDP calculated from the thiamine values obtained by the cyanogen bromide method.

Table IV.

Cyanogen bromide method

		Amount of sample (mg.)	Thiamine found (γ)	Thiamine in 3.14 mg. or 2.91 mg. of sample(γ)	TDP or TMP calcd. from thiamine value(γ)	Content (%)
(A)	Total value	3.14	18.8	1880		
	TDP	〃	15.8	1580	2240	71.5
	TMP	〃	3.09	309	366	11.6
(B)	Total value	2.91	20.4	2040		
	TDP	〃	15.9	1590	2250	77.0
	TMP	〃	3.9	390	460	15.8

Azotometric method

		Amount of sample (mg.)	N_2 found (mm^3)	N_2 in 3.14 mg. or 2.07 mg. of sample (mm^3)	TDP or TMP calcd. from N_2 value(γ)	Content (%)
(A)	Total value	3.14	53.2	266.0		
	TDP	〃	42.9	214.5	2330	72.8
	TMP	〃	10.2	51	450	14.4

4) C. H. Fiske, Y. Subbarow: J. Biol. Chem., **66**, 375 (1925).

(B)	Total value	2.07	42.0	210		
	TDP	〃	31.4	157.0	1676	81.0
	TMP	〃	9.6	48.0	426	20.6

TABLE V. Determination of Phosphorus in Hydrolyzed Phosphoric Acid Solution

		Amount of sample (mg.)	P found (γ)	P calcd. in 2.09 mg. or 2.91 mg. of sample (γ)	TDP Calcd. from. P value (γ)	Content (%)
(A)	Total value	2.98	56.6	141.5		
	TDP	〃	56.04	140.1	2164.5	72.7
(B)	Total value	2.91	59.0	147.5		
	TDP	〃	58.0	145.5	2248	76.5

Summary

In order to examine cocarboxylase preparations, they were subjected to paper ionophoresis. The resulting bands in the ionopherograms were identified by comparing them with those obtained by paper ionophoresis of pure TDP, TMP, and thiamine conducted under the same conditions. The bands were eluted, and from the quantities of thiamine, N, and P in the eluates, the quantities of the components of the preparations were calculated.

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