QUANTITATIVE DETERMINATION OF COCARBOXY- LASE BY ION EXCHANGE CHROMATOGRAPHY

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Cocarboxylase (thiamine diphosphate, TDP), an active form of thiamine, is widely distributed in animal and plant kingdoms, participating with many important physiological reactions such as carbohydrate metabolism and nervous activity.

The assay of TDP has been established by the following three methods: (a) Enzymatic decarboxylation using apocarboxylase; (b) Chromatography; (c) Determination of thiamine after hydrolysis by phosphatase. (a) has been proposed by Lohmann and Schuster (1) and improved by Goodhart, Sinclair (2) and Goodhart (3). It is highly specific for TDP and very sensitive, enabling the determination of as little as 1.0—0.1 μ g of TDP. But it necessitates some skill and the error is somewhat great.

The method (b) has been reported by Siliplandi *et al.* (4). In case of paper chromatography 10—30 μ g of TDP is used with an error of about 5 per cent. It is suitable for determining TDP in a somewhat purified preparation. It seems to be unsatisfactory for separating TDP from thiamine triphosphate (TTP). Thereafter, Siliplandi *et al.* (4) have reported to have successively determined TDP after separating it from TTP by paper electrophoresis or starch column chromatography. These methods seem to be somewhat troublesome for determining TDP in synthetic TDP preparations. The method (c) has been proposed by Kinnesky and Peters (5), but it is impossible to determine TDP in a sample containing TTP and thiamine monophosphate (TMP).

The present authors have tried to establish a simpler and exacter method for determining TDP in synthetic preparations using ion exchange chromatography. The method established by the authors will be described in this paper.

EXPERIMENTAL AND RESULTS

1. Principle

A sample is treated with ion exchange resin (Amberlite IRC-50, 100—200 mesh, H⁺-form) and TDP thus separated is determined photometrically by estimating the optical densities at 270, 246 and 237 m μ .

2. Determination of Experimental Conditions

As Siliplandi *et al.* (4) has reported, TTP and TLP were completely

FIG. 1 Fractional Separation of TTP and TDP by Amb. IRC-50

Resin 20 ml (15 cm high), 100–200 mesh, H^+ -form. 1.0 mg/ml of crude sample was used. Eluted with water (10.0 ml/hr).

separated by passing a crude TDP preparation through a COOH-form cation exchange resin column with a capacity of less than 1 mg TDP/1 ml resin and eluting with water alone, whereby TTP and thiamine were not eluted at all (Fig. 1). Therefore, if TDP are completely eluted from the resin without any degradation during chromatography, the amount of TDP can easily be determined by estimating the optical density of the TDP fraction separated. For testing the elution and degradation of TDP, the following experiments were carried out: 350 mg of crude TDP was passed through a 400 ml resin column (Amberlite IRC-50, 50–100 mesh, H^+ -form), and eluted with water. TTP and TDP fractions were separated using thiochrome reaction.

After the eluate became negative for thiamine, the column was eluted with 0.02 N HCl, whereby TTP and thiamine fractions were obtained.

Each of the three fractions was freeze-dried and subjected to paper chromatography using n -propanol- $M/5$ acetate buffer (pH 5.0)–water (13:4:3) as a developing solvent system. After development the spots were located by spraying alkaline ferricyanide solution and by testing the fluorescent spots. The results given in Fig. 2 show that TTP and TDP were separately found in the first and second fractions respectively, and in the third fraction TTP and thiamine were detected. TTP and TDP were thus proved to be completely separated by eluting with water alone from a Amberlite IRC-50 column.

For examining the stability of TDP during the course of chromatography the following test was performed. A suitable quantity of TDP hydrochloride (0.953 mg) which had given only one spot on a paper chromatogram, was dissolved in 0.3 ml of water and made up to 50 ml with 0.01 N HCl. On the other hand, the same quantity of TDP was dissolved in water, passed through a resin column, and the TDP fraction was collected. It was filled with dilute HCl to 50 ml to give a final concentration of 0.01 N HCl. The optical densities of both solutions were determined with the results given in Table I, which indicates that

FIG. 2 Paper Chromatograms of the Fractions

TABLE I
Effect of Resin Treatment on TDP

Treatment	Optical densities at $m\mu$		
	270	246	237
Without resin	0.361	0.554	0.452
With resin	0.368	0.550	0.452

the TDP of the sample was quantitatively recovered after resin treatment without any destruction or oxidation.

Other compounds which may occur in this fraction are HCl and phosphoric acid. Since they have no absorption in ultraviolet region, TDP may be determined by estimating the optical densities at 270, 246 or 237 $m\mu$. The authors have therefore tested the effect of pH on the optical densities in ultraviolet region. The optical densities of 20 ml each of TDP fraction with the pH of 6.6, 4.4, 4.0, 3.6, 3.2 and 1.0 respectively and that with 0.02 *N* HCl were determined and the results shown in Fig. 3 were obtained. The spectrum of TDP was similar to that of thiamine, depending markedly upon pH. An absorption maximum

FIG. 3 Absorption spectra of TDP at different pH's

lies at 246 $m\mu$ at pH's lower than 4, the optical density remaining at a constant level. With the rise of pH above 4, absorption maxima at 230 and 260 $m\mu$ made an appearance which shifted further with the rise of pH. Therefore it is possible to determine TDP after the pH of the test solution is adjusted to below 4. The wavelength of 246 $m\mu$ may be chosen for determination, but it would be safer to determine at 270, 246 and 237 $m\mu$ and to ascertain the agreement of relative values with an authentic TDP solution. Further, the time course of ultraviolet absorption spectra was tested. Two solutions of 50 ml each of the TDP fraction obtained by ion exchange chromatography were made up to 200.0 ml with HCl and water. One was adjusted to pH 2.0 and the other was made up to 0.02 *N* HCl in final concentration. They were kept at room temperature and the optical densities were determined at 270, 246, 256, and 237 $m\mu$ after varying time intervals. The results were given in Table II, which indicates that both solutions remain unchanged at least for five days. For obtaining a standard sample of TDP the authors purified it by ion chromatography and obtained pure samples of both TDP·HCl (mp. 227–8° with decomposition, needles, agreeing in elementary analyses, giving one spot paper-chromatographically) and crystals presumed to be free TDP (mp. 127° with de-

TABLE II
Time Course of Ultraviolet Absorption Spectra of TDP Solution

<i>hr</i>	pH	Optical densities at $m\mu$			
		237	246	256	270
0	2.0	0.271	0.334	0.304	0.225
	0.02 <i>N</i> HCl	0.275	0.338	0.304	0.225
24	2.0	0.277	0.337	0.305	0.225
	0.02 <i>N</i> HCl	0.279	0.335	0.307	0.225
72	2.0	0.270	0.332	0.302	0.222
	0.02 <i>N</i> HCl	0.271	0.335	0.304	0.225
120	2.0	0.274	0.338	0.307	0.225
	0.02 <i>N</i> HCl	0.272	0.338	0.308	0.223

composition, short rods agreeing in elementary analyses, giving one spot paper-chromatographically). The optical densities at various wave lengths are given in Table III. They were not completely the same according to preparations. The highest values of TDP•HCl were chosen tentatively as a standard.

TABLE III
Standard Absorption Spectra of TDP

Substance	Concentration	Optical densities at $m\mu$		
		270	246	237
TDP•HCl	$\mu\text{M/ml}$			
	0.03717 (Calc. as 0.0300)	0.3717 (0.3000)	0.5667 (0.4575)	0.4630 (0.3745)
Free TDP	0.03776 (Calc. as 0.0300)	0.361 (0.286)	0.554 (0.447)	0.452 (0.361)
Thiamine•HCl	0.02674 (Calc. as 0.0300)	0.2648 (0.278)	0.3825 (0.430)	0.3172 (0.355)

TDP•HCl solutions of varying concentrations were made by diluting an original solution with 0.01 *N* HCl and the optical densities *versus* concentrations were plotted in Fig. 4, showing a proportionality of the optical densities to the concentrations within 0.008–0.065 $\mu\text{M/ml}$.

3. Method of Determination

All the experiments are conveniently performed in a dark room provided with an ultraviolet lamp. For ion exchange chromatography a 25 ml burette is used. The burette is plugged with some glass wool in the bottom upon which ca. 20 ml of Amberlite IRC-50 (100–200 mesh, H⁺-form) is filled and the resin was thoroughly washed with water till the washings showed no more ultraviolet absorption at pH above 4.

Prior to the determination, the water above the resin is entirely flowed

through and 0.5–10 ml of a test solution containing 0.1–1.5 mg of TDP is added and allowed to pass through the column at the rate of 10 ml/hr. After the solution has flowed out, the inner wall of the tube is washed with 2–3 ml of water and this procedure is repeated once more. TDP is then eluted with a large amount of water maintained at a constant level with a siphon at a flow rate of 10 ml/hr. By checking with thiochrome reaction, TTP is shown to flow out with up to 8–10 ml of water. After disappearance of the thiochrome-positive substance a thiochrome-positive substance makes an appearance again. The fraction is collected till the reaction becomes negative. TDP is contained in this fraction. It appears quantitatively in the fractions between 12 to 18 ml. The TDP fraction is made with dilute HCl to 0.01 *N* HCl with the total volume of 50–100 ml. An aliquot is taken and the optical density at 270 $m\mu$ is determined. The amount of TDP is calculated, comparing with the value of a standard.

FIG. 4 Standard Curve of TDP hydrochloride

4. Recovery and Specificity

The recovery of the added TDP in this method was shown in Table IV.

TABLE IV
Recovery

Sample	Amount	Volume	Opt. density at 270 $m\mu$	TDP		Purity
				as free	as phosphate	
	μg	ml		μg		per cent
Free TDP (purified)	802.4	50	0.368	781.5		97.5
	401.2	50	0.182	386.5		94.4
Samples treated with resin	827.2	50	0.272	578.0		70.0
	827.2	50	0.275	584.0		70.5
	413.6	25	0.270	287.8		69.5
	413.6	25	0.275	292.0		70.5
	206.8	25	0.137	145.5		70.4
Crude sample I (phosphate)	1272.0	50	0.133		345.5	27.0
	1272.0	50	0.135		351.0	27.5
	636.0	25	0.138		179.2	28.2
Crude sample II (phosphate)	1584.0	50	0.133		345.5	21.8
	1584.0	50	0.130		340.0	21.4
	792.0	50	0.0693		181.0	22.8
	792.0	50	0.0688		178.0	22.5

The values seem to be stabler than other methods. Specificity of the method has not been thoroughly tested, but it seems useful for the determination of TDP in synthetic preparations. The existence of inorganic phosphoric acid, HCl, Na⁺, Cu⁺⁺, TTP, TMP and thiamine do not disturb the determination.

SUMMARY

A simple and exact determination method of cocarboxylase is reported. A sample is subjected preliminarily to ion exchange resin chromatography and the cocarboxylase fraction is separated from thiamine esters and other impurities. The pH of the fraction is brought to less than 4 and the optical density is determined at 270 m μ . The recovery is proved to be satisfactory.

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