Summary

It was found that 16β,20α-dihydroxysteroids react with acetone in the presence of a small quantity of boron-trifluoride etherate giving the 16β,20α-isopropylidenedioxy compounds in good yield, the method being useful for a selective protection of the 16β,20α-dihydroxyl groups.

By this method a number of 16β,20α-isopropylidenedioxysteroids, which exhibit a characteristic infrared bands at 9, 10, 11 and 11.8 μ, were prepared.

(Received February 19, 1962)

UDC 615.398.19

25. Tsutomu Unemoto, Keiko Ikeda, Makoto Hayashi, and Komei Miyaki : Studies on Polyamines. I. A New Fluorometric Determination of Spermine and Spermidine.

(*Laboratory of Prof. K. Miyaki, Institute of Food Microbiology, Chiba University*[*1])

There are several methods to determine various amines in biological materials. They involve separation of amines by paper chromatography,[1,2] by paper electrophoresis[1,2] or by cation exchange resin,[3,4] followed by determination of each amine by Ninhydrin[1,2] or dinitrofluorobenzene[3,4] procedure. In these cases, colorimetric assays are non-specific and specificity of these methods depends on separation procedure. In general, satisfactory separation of amines demands a complicated and time-consuming technique.

In our study on the oxidation of spermine or spermidine by beef plasma amine oxidase,[5,6] it was found that one of the oxidation products reacts with resorcinol to produce a fluorescent compound that has an absorption maximum at 520 mμ (Fig. 1), the intensity of which was proportional to the amount of the amine oxidized. As the formation of the fluorescent compound was quite specific to spermine and spermidine, it was attempted to devise a specific fluorometric method for the estimation of these amines.

Reaction of resorcinol with the oxidation products proceeded fast at a neutral or slightly alkaline pH and the fluorescent compound formed was stable at an acidic pH. We oxidized spermine or spermidine in the presence of resorcinol at pH 7.0, since it did not affect the activity of the amine oxidase. After that the reaction mixture was heated at the same pH to enhance the formation of the fluorescent compound and acetic acid was added to stabilize its intensity. Whether or not the fluorescent compound obtained from spermine or spermidine is the same compound is under investigation.

---

*1 Okubo, Narashino, Chiba-ken (畝本 力, 池田恵子, 林 誠, 宮木高明).

1) F. G. Fischer, H. Bohn : Z. physiol. Chem., **308**, 108 (1957).
2) E. J. Herbst, D. L. Keister, R. H. Weaver : Arch. Biochem. Biophys., **75**, 178 (1958).
3) S. M. Rosenthal, C. W. Tabor : J. Pharmacol. Exptl. Therap., **116**, 131 (1956).
4) H. Tabor, S. M. Rosenthal, C. W. Tabor : J. Biol. Chem., **233**, 907 (1958).
5) J. G. Hirsch : J. Exptl. Med., **97**, 327 (1953).
6) C. W. Tabor, H. Tabor, S. M. Rosenthal : J. Biol. Chem., **208**, 645 (1954).

As to the estimation of spermine, Rosenthal *et al.*[3] reported a rapid method that consists of the separation of spermine from other amines by Amberlite XE-64 and the assay of this amine by the dinitrofluorobenzene procedure. However, the estimation of spermidine was not so simple as that of spermine because of the difficulty of its separation from other amines. Therefore, an attempt was made to separate various amines in biological materials into two fractions, i.e. spermine and spermidine fractions, by the procedure of Rosenthal *et al.*[3] and then analyze each fraction by the present specific assay method in order to estimate spermidine rapidly.

In our experiments, the separation of spermine and spermidine by the Rosenthal *et al.*'s procedure was not complete, probably because of the difference of the capacity of Amberlite XE-64 used, and 40 to 50 per cent of spermine went to the spermidine fraction. Therefore, conditions of the separption of both amines by this resin were examined and as a result, the following modification was found to give much better results. These amines were adsorbed on the Na-form of the resin without bringing it to pH 7.0 and spermidine was eluted by 0.05*N* acetic acid in place of 0.1*N* acetic acid by the original method. Fig. 2 shows a chromatogram of rat liver extract by this procedure. Electrophoresis of each fraction (Fig. 3) indicates that the separation of spermine and spermidine is satisfactory, although there are still present other Ninhydrine positive substances in the spermidine fraction. Data on the recovery of spermine and spermidine added to a rat liver extract are given in Table I. Evidently the recovery of these amines is quantitative. Analytical data on rat liver by the present

Table I. Recovery of Spermine and Spermidine

		Rat liver extract (0.5 g. of tissue)	After the addition of both amines	Recovery (%)
Sp	DNFB method	0.65	1.63	98
	fluorometric method	0.55	1.50	95
Sd	DNFB method	0.72	1.76	104
	fluorometric method	0.24	1.20	96

1.0 μmole of spermine and spermidine were added to the rat liver extract, corresponding to 0.5 g. of wet tissue. These amines were separated by the present procedure and each fraction was analyzed by the dinitrofluorobenzene (DNFB) method and by the fluorometric method. All figures are in μmole.

Table II. Analytical Data on Rat Liver

		Exp. 1	Exp. 2
Sp	DNFB method	0.84	1.03
	fluorometric method	0.85	1.02
Sd	DNFB method	0.89	0.94
	fluorometric method	0.76	0.80

All figures are in μmole per 1.0 g. of wet tissue.

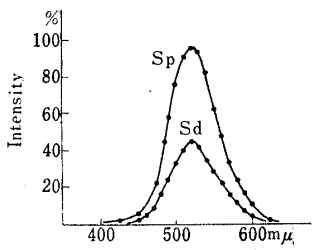

Fig. 1. Fluorescence Spectra
Sp : spermine
Sd : spermidine

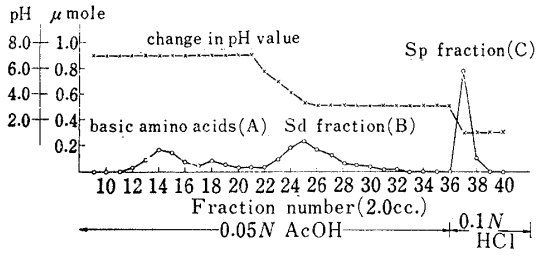

Fig. 2. Chromatogram of Rat Liver Extract on Amberlite XE-64
The extract corresponding to 1.0 g. of wet tissue was submitted to chromatography according to the procedure described in the text and each fraction was analyzed by dinitrofluorobenzene method.

technique are shown in Table II. In the case of spermidine, the value obtained by the dinitrofluorobenzene method, was larger than that obtained by the fluorometric method, presumably owing to the contamination of other amines in the spermidine fraction.

From these facts, we concluded that the estimation of spermidine can be made rather rapidly by the combination of a simple separation procedure with the specific assy method presented here.

Experimental

Instruments——For photometry Hitachi Photo-electro Spectrophotometer, type EPU-2, was used and for fluorometry Hitachi Attachment for Flurometry, type L-3, was attached to the above instrument. Its exciting wave length was 366 mμ group of mercury lines.

Reagents——0.5*M* phosphate buffer, pH 7.0
0.1*M* resorcinol
1.0*M* acetic acid

Preparation of Enzyme——Fresh beef plasma is fractionated by $(NH_4)_2SO_4$ and the fraction that precipitates between 30 and 50 per cent of saturation of the salt is collected, dialyzed against 0.05*M* phosphate buffer of pH 7.0, and made up to a half volume of the original plasma with distilled H_2O. It can be stored for months in the deep-freeze. Usually 0.2 cc. of the enzyme preparation is employed in the assay. However, the activity of the enzyme is variable according to the preparation and an excess of contaminating proteins interferes with the intensity of fluorescence. It is, therefore, advisable to determine a suitable amount of enzyme preparation beforehand.

Procedure——To 2.0 cc. of the above neutral solution containing 0.2 to 0.6 μmole of spermine or 0.4 to 1.0μmole of spermidine, are added 0.5 cc. of the phosphate buffer, 0.3 cc. of resorcinol solution and 0.2 cc. of the enzyme preparation succesively. The mixture is incubated at 37° for 1 hr. and then at 60° for 15 min. After the end of incubation period, 1.5 cc. of acetic acid solution is added and the fluorescence intensity is measured at 520 mμ. A standard curve is prepared for each of these amines and the concentration of unknown amines is calculated by interpolation.

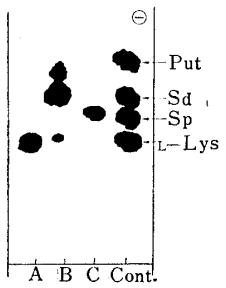

Fig. 3. Diagram of Paper Electrophoresis Conditions; 15 v/cm., 10 mA., 2~5°, 45 min. Solvent; pyridine 40 cc., glacial acetic acid 20 cc., citric acid 20 g. and water 940 cc. (Solvent C, reported by Fischer *et al.*[1])
A : Basic Amino Acids(fraction number 12~18)
B : Sd fraction (fraction number 22~30)
C : Sp fraction (fraction number 37~38)
Put : 1,4-diaminobutane. L-Lys. : L-Lysine.

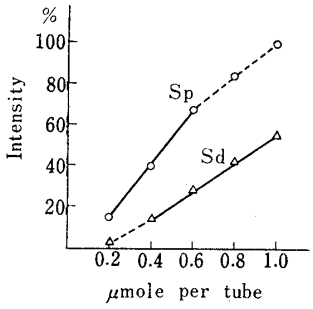

Fig. 4. Standard Curves

Standard Curves——As shown in Fig. 4, the fluorescence intensity is linear with the concentration of spermine or spermidine up to about 0.6 μmole and 1.0 μmole, respectively. And the intensity obtained with spermine is about twice as great as that with spermidine. In practice, a standard curve is plotted by adjusting the readings of the most intense fluorescence to 100 per cent in each case.

Notes on the Method——The effect of the amount of resorcinol, the time of addition of resorcinol and the heating time to the final intensity of fluorescence are shown in Fig. 5, Table III and Fig. 6, respectively. These figures were obtained by using 0.5 μmole of spermine and similar results were

Table III. Relationship between the Time of Addition of Resorcinol and the Fluorescence Intensity

	Fluorescence intensity	
	spermine (0.5 μmole)	spermidine (1.0μmole)
at the start of enzymatic reaction	100	100
after 20 min.	69	78
after 40 min.	56	62
at the end of enzymatic reaction	43	52

Table IV. Effect of Diamines and Basic Amino Acid

Substance	μmole	Relative intensity	
		spermine (0.5 μmole)	spermidine (1.0 μmole)
none	—	100	100
$NH_2(CH_2)_3NH_2$	1.0	102	99
	5.0	103	102
$NH_2(CH_2)_4NH_2$	1.0	106	107
	5.0	109	108
$NH_2(CH_2)_5NH_2$	1.0	105	101
	5.0	105	81
$NH_2(CH_2)_6NH_2$	1.0	91	61
	5.0	55	13
L-histidine	5.0	104	98
L-arginine	5.0	105	102
L-lysine	5.0	100	101

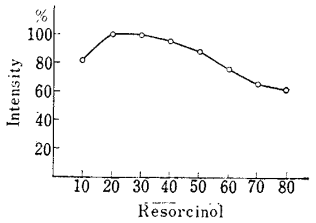

Fig. 5. Effect of the Amount of Resorcinol (μmole per tube)

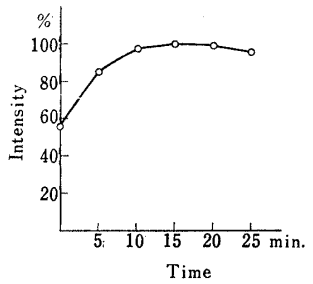

Fig. 6. Effect of Heating Time at 60°

obtained in the case of spermidine. The mixture was heated at 60° to intensify the fluorescence. Since at higher temperatures, proteins in the mixture were denatured and became insoluble, it was neceseary to remove them before measurement. Under the present conditions, however, the presence of proteins in the mixture did not affect the intensity of fluorescence and measurement was possible without removing the proteins.

Specificity of the Method——2-Aminoethanol, 2-aminopropanol, 3-aminopropanol, 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane and β-alanine are all negative by the present method.

Interfering Materials——Diamines and basic amino acids are likely to contaminate both fractions during separation procedure and their effect on the present method was examined as illustrated in Table IV. It is seen that in general, the effect was small and the contamination of 1,6-diaminohexane might cause inhibition of the enzymatic reaction.

Application of the Method to the Analysis of Rat Liver——a) Preparation of rat liver extract: fresh rat liver is homogenized with 4 parts of 5% trichloroacetic acid and the proteins are removed by centrifugation. The supernatant solution is extracted with Et_2O to remove the acid until its pH becomes above 4.0.

b) Separation of spermine and spermidine: 1.5 cc. of Amberlite XE-64 (Na-form) is placed in a small glass tube of 0.5 cm. in diameter. The liver extract thus prepared and containing no more than 1.0 g. of tissue is passed through the column and the column is washed first with the same amount of H_2O, then with appoximately 40 cc. of 0.05*N* AcOH to remove basic amino acids. After the pH of the effluent goes down below 7.0, the spermidine fraction is collected by eluting the column with 35 cc. of the same fluid. The spermine fraction is then eluted with 5.0 cc. of 0.1*N* HCl. Each fraction is concentrated *in vacuo* and dried in a desiccator containing NaOH and P_2O_5 to remove excess acids. The dried sample is dissolved in 2.0 cc. of distilled H_2O and applied to the assay.

Summary

A new fluorometric method for the estimation of spermine and spermidine was devised on the basis of the findings that one of the oxidation products of spermine and spermidine by beef plasma amine oxidase reacts with resorcinol to produce a fluorescent compound. It was also attempted to estimate spermidine in biological materials by the combination of a simple separation procedure with the specific assay method presented in this investigation.

(Received February 21, 1962)