Summarizing this study, alkyl sulfate type surfactant repressed the hydrolysis of quaternary ammonium salt with ester linkage, and the longer the alkyl chain of surfactant or the acyl chain of the hydrolyzate, the more the degree of repression was enhanced.

The authors express their thanks to Dainippon Pharmaceutical Co., Ltd. for the supply of methantheline bromide and to Nikko Co., Ltd. for the supply of anionic surfactants, and also to Miss Hamada for her technical assistance.

Summary

1) The decomposition of methantheline bromide was repressed by the addition of alkyl sulfate (anionic surfactant). The longer the alkyl chain length of alkyl sulfate, the greater the effect was.

2) The addition of surfactant enhanced the stability of cholinesters, and the effect increased with the acyl chain length.

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202. Tsutomu Unemoto: Studies on polyamines. II.*1 Metabolism of Spermidine and spermine by Amine Oxidase in Beef Serum.

(*Institute of Food Microbiology, Chiba University**2)

Amine oxidase which attacks spermidine and spermine was first described by Hirsch[1] and it was purified from beef plasma some 150 to 200-fold by Tabor, *et al.*[2] Recently this oxidase has been crystallized by Yamada and Yasunobu.[3] In spite of such successful purification of this enzyme, the stoichiometry of the degradation of these polyamines by the oxidase still remaines unsolved. In connection with the finding of Hirsch[1] that some of the oxidation products of these polyamines inhibit the growth of tubercle bacilli, an attempt has been made to clarify the reaction mechanism of this enzyme.

Quantitative studies on the uptake of oxygen and the liberation of ammonia from spermidine and spermine by the action of oxidase have already been reported by other workers. Using unfractionated sheep serum as an enzyme preparation, Hirsch[1] demonstrated that the oxidation of one mole each of spermidine and spermine resulted in the consumption of 2 and 4 atoms of oxygen, respectively. By contrast, our experiments showed that when an unfractionated beef or goat serum was used as an enzyme preparation, the values of oxygen consumption fluctuated from one experiment to the other, say from 1.4 to 1.8 and from 2.6 to 3.4 atoms of oxygen per mole of spermidine and spermine, respectively. Such a fluctuation seemed to be caused by the contamination

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*1 Part I: This Bulletin, **11**, 148 (1963).

*2 Okubo, Narashino, Chiba-ken (畝本 力).

1) J. G. Hirsch: J. Exptl. Med., **97**, 323 (1953).

2) C. W. Tabor, H. Tabor, S. M. Rosenthal: J. Biol. Chem., **208**, 645 (1954).

3) H. Yamada, K. T. Yasunobu: J. Biol. Chem., **237**, 1511 (1962).

of catalase, since when a partially purified enzyme containing no catalase was used, the amount of oxygen uptake settled (Fig. 1) and coincided well with the data of Tabor, *et al.*[2]

The data hitherto reported by several workers on the liberation of ammonia from these polyamines by the said enzyme are somewhat incompatible. Tabor, *et al.*[2] pointed out the possibility of the presence in the reaction products of unknown substance, possibly an unstable amino aldehyde, which behaved like ammonia in the estimation of ammonia. Accordingly the bis-pyrazolone reagent[4] was employed for the estimation of ammonia after diffusion in Conway vessels. This reagent is the most specific for ammonia now available. Using both unfractionated and fractionated beef or goat serum, the liberation of ammonia ranging from 0.8 to 1.0 and from 1.7 to 2.0 moles per mole of spermidine and spermine, respectively, were observed. One of the results is presented in Fig. 1. These values seem to be rather lower than those obtained by Hirsch.[1] This may be accounted for the facts that Hirsch used excess serum (50% of sheep serum) with prolonged incubation (6 hr.); under these conditions exhaustive degradation of oxidation products might occur resulting in the formation of a high amount of ammonia. Fig. 1 also indicates that oxygen uptake and ammonia formation proceeded simultaneously without showing any appreciable lag period.

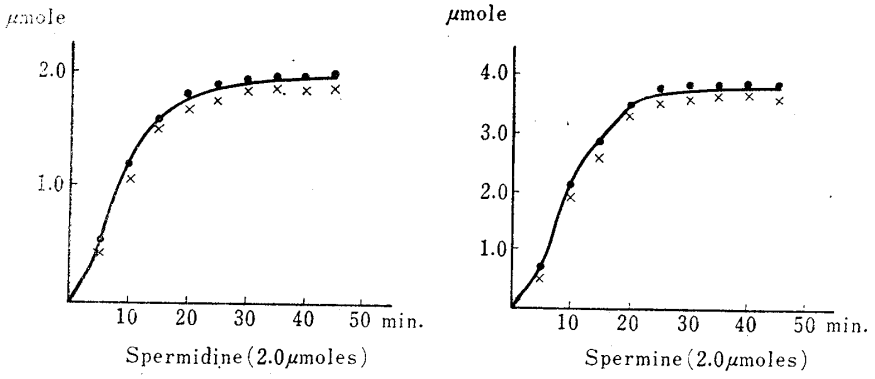

Fig. 1. Relationship between the Oxygen Uptake and Ammonia Liberation during the Course of Oxidation

•—• Oxygen uptake ×—× Ammonia liberation

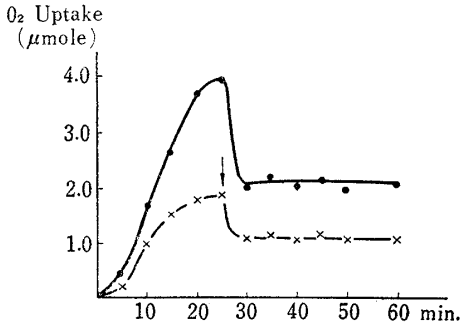

Fig. 2. Formation of Hydrogen Peroxide from Spermidine and Spermine by Enzymatic Oxidation

At the end of oxidation of the substrates, catalase (prepared from beef blood) was added to each flask as indicated by arrows.

×—× Spermidine (2.0 μ moles)
•—• Spermine (2.0 μ moles)

The formation of hydrogen peroxide from spermidine and spermine by this enzyme was examined and the results are indicated in Fig. 2. These data seem to be in quite agreement with those of Tabor, *et al.*[2] From the present observations, concerning the oxygen consumption and the formations of ammonia and hydrogen peroxide, the following equations may be formulated :

4) T. Unemoto, Y. Tsuda, M. Hayashi : Yakugaku Zasshi, **80**, 1089 (1960).

$$\text{Spermidine} + O_2 = NH_3 + H_2O_2 + ?$$

$$\text{Spermine} + 2O_2 = 2NH_3 + 2H_2O_2 + ?$$

Fig. 3 indicates the relationship between the oxygen uptake and the decrease in amino groups of the polyamines during oxidation. At the time when the oxygen uptake reached its maximum, amino groups being present initially in amounts of 3 and 4 μ moles equivalent for spermidine and spermine, respectively, were reduced to about 2.0 μ moles equivalent per μ mole of each substrate; *i.e.*, spermidine lost one amino group and spermine, two amino groups. These observations agree well with the amount of ammonia liberated. Dubin[5] reported recently that the molecular ratio of primary and secondary amino groups could be estimated by the ratio of absorbancies at 350 and 390 mμ of 2,4-dinitrophenyl derivatives of an amine. Fig. 3 also indicates the change in the ratio during the oxidation of spermidine and spermine. The fact that the value of the ratio at the end of the reaction became ca. 2.0 in the cases of both polyamines showed that solely the primary amino groups existed at the end of oxidation. In case of spermine, increase in the ratio, however, was much less marked as compared with the case of spermidine.

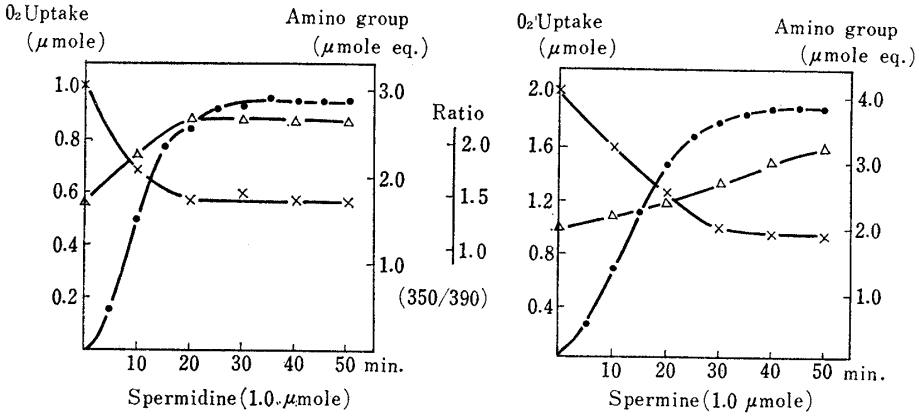

Fig. 3. Decrease of Amino Groups of Spermidine and Spermine during Oxidation

•—• Oxygen uptake
×—× Amino groups
△—△ Ratio of absorbancies at 350 and 390 mμ for 2,4-dinitrophenyl derivatives

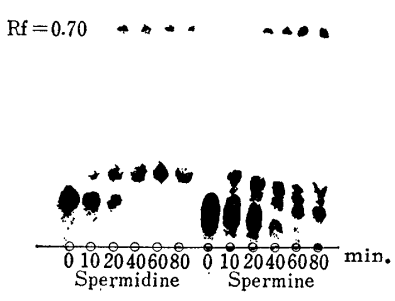

Fig. 4. Paper Chromatogram of the Reaction Mixture taken at Time Intervals during Oxidation

After removal of protein with TCA, the acid was extracted with Et_2O and 0.01 ml. aliquot of the reaction mixture was applied on to the paper (Toyo Roshi No. 51) and developed with a solvent system of BuOH-AcOH-H_2O(4:1:2). Spots were detected with 0.1% Ninhydrin in BuOH containing 10% of pyridine.

5) D. T. Dubin : J. Biol. Chem., **235**, 783 (1960).

The paper chromatogram of the reaction mixture taken at time intervals during oxidation is shown in Fig. 4. The original spots of spermidine and spermine disappeared when the oxygen uptake became maximum (after 40 and 60 minutes, respectively). The upper spot (Rf being 0.70) appeared in both cases showed aldehyde reaction. This spot seems to correspond to that of amino aldehyde described by Tabor, *et al.*[2]

This compound was found to react with resorcinol or orcinol to give a fluorescence. This finding was already found to be applicable for the specific estimation of these polyamines.[6] When the oxidation of spermidine by the enzyme was performed in the presence of resorcinol, the same fluorescent spot appeared on a paper chromatogram showing the Rf value of 0.74, slightly higher than that of an unknown amino aldehyde (Rf=0.70). It is noteworthy that when the reaction mixture containing resorcinol was heated at 60° for 20 minutes, when the intensity of fluorescence became maximum, the amino aldehyde spot of Rf is 0.70 completely disappeared (Fig. 5). This spot does not fade away under the same heating conditions in the absence of resorcinol.

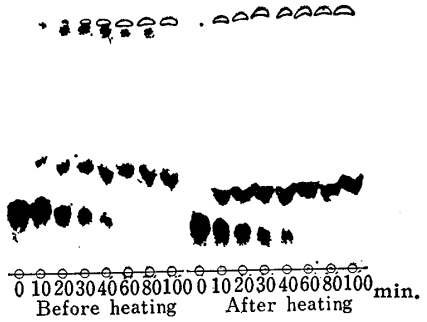

Fig. 5. Paper Chromatogram of the Oxidation Mixture of Spermidine in the Presence of Resorcinol before and after Heating

The reaction mixture was collected at time intervals indicated above and divided into two portions and one of them was heated at 60° for 20 min. and the both portions were treated in the same manner as in Fig. 4. The circles on the paper indicate the spots of fluorescent compound.

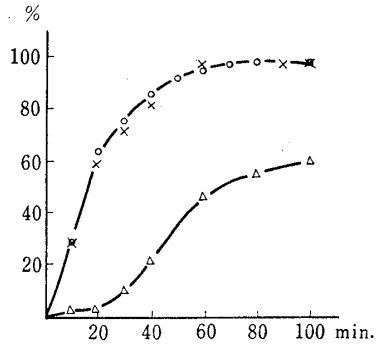

Fig. 6. Relationship between the Oxygen Uptake and the Increase in the Intensity of Fluorescence during Oxidation

Two aliquots of reaction mixture containing 0.01*M* resorcinol were taken at intervals. One of them was heated at 60° for 20 min. and the intensity of fluorescence was measured together with the other aliquot which had not been heat treated.

o—o Oxygen uptake
×—× Fluorescence intensity (heat treated)
△—△ Fluorescence intensity (untreated)

The amount of fluorescent compoud formed after heating in the presence of resorcinol seemed to increase during the course of oxidative degradation of the polyamines. Aliquots of the reaction mixture containing resorcinol were taken at intervals, heated at 60° for 20 minutes and the intensty of fluorescence was measured as indicated in Fig. 6. Thus it increased in parallel with the consumption of oxygen as reaction proceeded. Although the chemical nature of the fluorescent compound obtained either from spermidine or spermine has not been identified yet, the substance derived from either polyamines seemed to be the same judging from the results of paper chromatography, paper electrophoresis and ultraviolet absorption spectra in acid and alkaline (Fig. 7). These results suggest that at least one of the oxidation products by the enzyme is common to spermidine and spermine used as substrates.

6) T. Unemoto, K. Ikeda, M. Hayashi, K. Miyaki: This Bulletin, **11**, 148 (1963).

With respect to the spots of low Rf's other than the substrate polyamines shown in Fig. 4, it was revealed that Rf values were largely affected by the presence of salts which were contained in the reaction mixture. Therefore, the identification of these reaction products was performed by an ion-exchanger chromatography referring to the

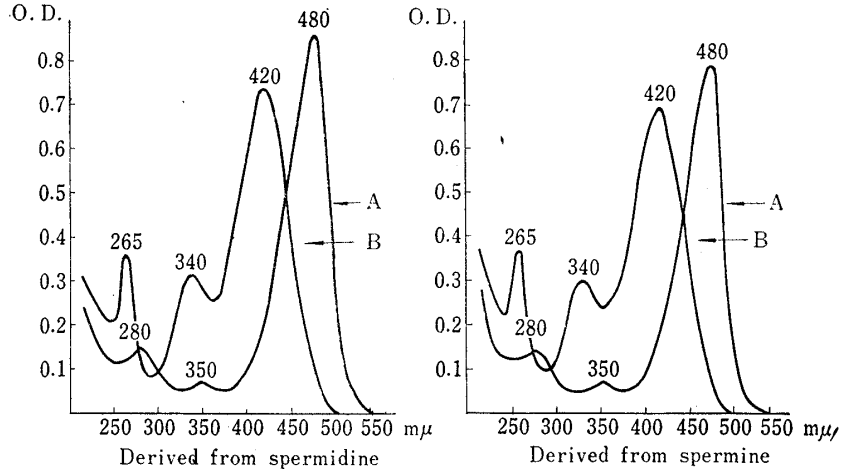

Fig. 7. Absorption Spectra of the Fluorescent Compounds obtained from Spermidine and Spermine

One hundred μ moles of each substrate were oxidized with the enzyme in the presence of resorcinol (10^{-2} *M*) for 90 min. and heated at 60° for 20 min. After removal of protein with TCA, the reaction mixture was treated with Dowex-1 (OH^- form) to remove TCA. The supernatant (adjusted to pH 5.0) was passed through the column of Amberlite XE-64 (H^+ form, 2×10 cm.) and the fluorescent compound was adsorbed on the column. The column was then washed with 500 ml. of 0.05*N* AcOH, then the fluorescent compound was eluted with 0.05*N* HCl. The eluate was concentrated *in vacuo* under the N_2 flow and dissolved in H_2O. An aliquot was used for the measurement of absorption spectra.

A : in 0.05*N* NaOH B : in 0.05*N* HCl

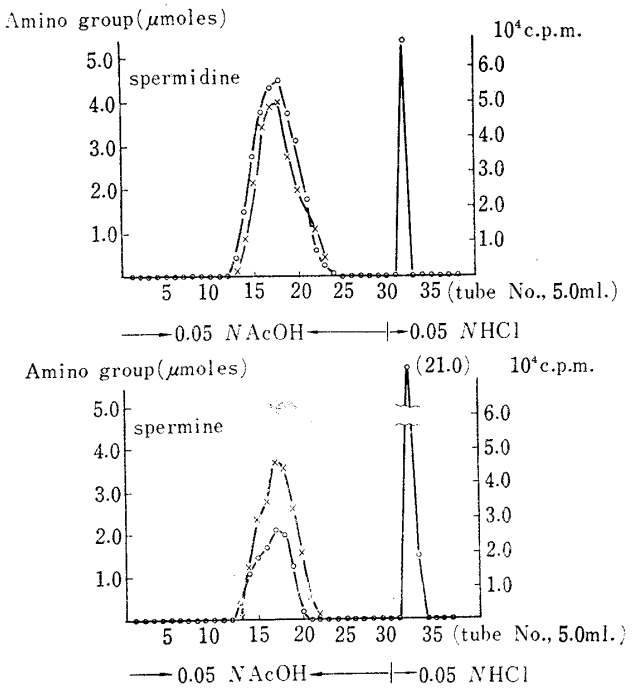

Fig. 8. Column Chromatogram (Amberlite XE-64) of Oxidation Products of Spermidine and Spermine

An aliquot of the reaction mixture containing initially 20 μ moles of each substrate was supplemented with 0.60 μ mole of putrescine-1,4-^{14}C (2.9×10^5 c.p.m.) at the end of oxidation, and the mixture was applied on the column (1.2×13 cm., adjusted to pH 5.0 prior to use). Each fraction of the eluate was estimated for the amount of amino groups and for radio activity.

o—o Amino groups
×—× Counts of radio activity

previous reports on the isolation of putrescine[7] and spermidine[2] from the oxidation products of spermine.

Since it was highly probable that the reaction products contained putrescine, the separation of putrescine from the oxidation mixture was attempted by using Amberlite XE-64 (buffered at pH 5.0 prior to use). Under the present condition, putrescine was eluted fom the column by 0.05*N* AcOH (AcOH-fraction), and spermidine and spermine were eluted by 0.05*N* HCl (HCl-fraction); thus the putrescine was successfully separated from the polyamines. Fig. 8 shows elution diagrams of the reaction products obtained by the enzymatic oxidation of spermidine and spermine. Putrescine-1,4-^{14}C added to the reaction mixture was recovered in the acetic acid fraction and the other amines, in the hydrochloric acid fraction. The total amount of amino groups assayed in the two fractions of eluate was found to be from 60 to 80% of that applied to the column. This indicates that the degradation or absorption of some amines in the product had occurred during the column chromatography. As a matter of fact an unknown amino aldehyde appeared at the top of paper chromatography described above could not be found in either fraction. Nevertheless, another substance which emits fluorescence by reacting with resorcinol was found in the hydrochloric acid fraction.

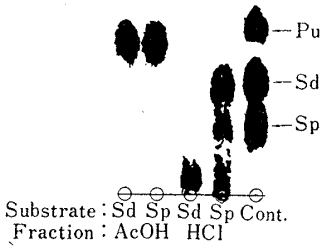

Fig. 9. Paper Chromatogram of the Fraction separated by Amberlite XE-64

The paper was developed with a solvent system of 2-ethoxy-2'-hydroxydiethyl ether-propionic acid-H_2O (70:15:15) saturated with NaCl.[8] Pu, Sd and Sp denote, respectively, putrescine, spermidine and spermine. For the detection of spots see Fig. 4.

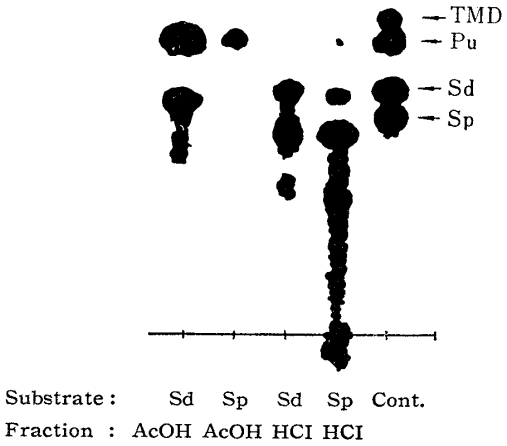

Fig. 10. Paper Electrophoresis of the Fraction separated by Amberlite XE-64

Paper electrophoresis was performed using 0.05*M* sulfosalicylate buffer, pH 3.4, for 40 min. at 20 v./cm. and 10 mA. For Pu, Sd and Sp see Fig. 9. TMD denotes trimethylenediamine used as a reference standard. For the detection of spots see Fig. 4. Figure shows TMD is not present in the reaction products.

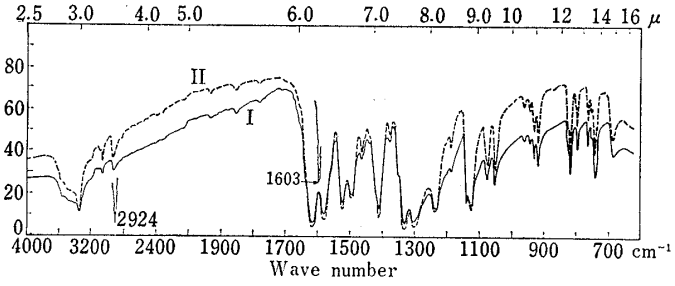

Fig. 11. Infrared Spectra (KBr disc) of 2,4-dinitrophenyl Derivatives of authentic Putrescine (I) and One of the Oxidation Products of Spermidine (II)

7) C. W. Tabor, S. M. Rosenthal : J. Pharm. Exptl. Therap., **116**, 139 (1956).

Figs. 9 and 10, respectively, show the results of paper chromatography[8] and paper electrophoresis[8] of the each fraction. The radioactivity of putrescine-1,4-^{14}C added to the reaction mixture was recovered in the spot of putrescine. Three millimoles of spermidine was then oxidized with a partially purified amine oxidase from beef serum and the reaction mixture was subjected to the column chromatography as indicated above. When the acetic acid fraction thus obtained was reacted with 2,4-dinitrofluorobenzene, the resulting derivative, recrystallized twice from 4-methyl-2-pentanone, melted at 240°. The results of elementary analysis and infrared spectrum (Fig. 11) of this substance agreed well with those of the same derivative of an authentic putrescine.

Those spots other than that of putrescine in acetic acid fraction (Fig. 10) seem to be some of the degradation products derived from amino aldehyde or of the intermediates of oxidation products. When spermidine and spermine were oxidized by the enzyme in the presence of resorcinol, these spots disappeared. The separation and identification of these substances has yet been unsuccessful.

The components of the hydrochloric acid fraction have not still been fully understood. There found a trace amount of the original polyamines that were left unoxidized. A spot apparently corresponding to spermidine was also observed when spermine was used as substrate. Other spots in this fraction may be some of the intermediates or decomposed compounds of unstable oxidation products. The fact that the amount of amino groups in the hydrochloric acid fraction was very small when spermine had been oxidized in the presence of resorcinol suggested the presence of polymerized or decomposed amino aldehyde in this fraction.

Quantitative analysis of putrescine formed from spermidine and spermine by the action of the present enzyme was performed employing the isotope dilution method with the addition of putrescine-1,4-^{14}C to the end products of oxidation. Bachrach and Bar-Or,[9] using unfractionated sheep serum as an enzyme preparation, demonstrated by quantitative paper chromatography that all of spermine turned at first into spermidine that accumlated and that subsequently oxidation of the spermidine resulted in its degradation. If all of spermine is converted to spermidine, the amount of resultant putrescine derived originally from spermidine and spermine should be the same. This is not in accord with the present results shown in Table I. This discrepancy could be understood by a consideration that not all of spermine is converted to spermidine at least by the amine oxidase of beef serum. This table also indicates that much more putrescine was recovered in the presence than in the absence of resorcinol or orcinol. Since the presence of orcinol did not alter the amount of oxygen uptake

Table I. Recovery of Putrescine from the Oxidation Products of Spermidine and Spermine as revealed by the Isotope Dilution Method

Exp. No.	Addition of resorcinol derivatives	Putrescine (μ moles)	
		Spermidine substrate (10 μ moles)	Spermine substrate (10 μ moles)
1	none	6.7	3.2
2	〃	6.3	3.1
3	〃	6.0	2.2
4	resorcinol ($5\times10^{-3}M$)	7.2	5.1
5	orcinol (〃)	7.6	5.1

At the end of incubation, putrescine-1,4-^{14}C (5.3×10^5 c.p.m. per μ mole) was added and the putrescine was separated by Amberlite XE-64 column. The AcOH-fraction was rechromatographed through the same resin until impurities disappeared; the purity of putrescine was confirmed by paper electrophoresis.

8) E. J. Herbst, D. L. Keister, R. H. Weaver : Arch. Biochem. Biophys., **75**, 178 (1958).
9) U. Bachrach, R. Bar-Or : Biochim. et Biophys. Acta, **40**, 545 (1960).

and of ammonia formation (Table II), the above results may not be caused by changing the site of oxidation of either spermidine or spermine. It seems likely that resorcinol derivatives trapped unstable compounds produced by oxidation and prevented from unfavorable side reaction otherwise may occur.

Table II. Formation of Ammonia from Spermidine and Spermine in the Presence of Resorcinol or Orcinol

	Ammonia (μ moles per μ mole of substrate)	
	Spermidine substrate	Spermine substrate
Resorcinol ($5\times10^{-3}M$)	1.02	1.98
	(1.06)	(1.80)
Orcinol (〃)	0.88	1.88
	(0.96)	(1.96)

Values in parentheses are those obtained after heating the end products at 60° for 20 min.; by this treatment the formation of fluorescent compound became maximum. Initial concentrations of polyamines were $5\times10^{-4}M$.

Discusion

Since the chemical structure of spermidine is simpler than that of spermine, the degradation pathway of the former is necessarily less complicated. The data on the amount of oxygen uptake and of ammonia formation from spermidine indicate that the spermidine molecule may be oxidized at one site. As shown in the formula of spermidine in Chart 1, there are four possible splitting positions, A, B, C, or D. Assuming that the spermidine is oxidized at D, putrescine will never be formed from any possible oxidation products, irrespective of the accompanying formations of equimolar ammonia and hydrogen peroxide. Thus a possibility of oxidation at D may be excluded. Provided that the spermidine is oxidized at C, 1,3-diaminopropane must be found as a product, but no such compound was so far detected in the reaction mixture. If, then, the spermidine is oxidized at B, the quantitative recovery of putrescine should be observed and in addition, quantitative and non-oxidative formation of ammonia from 3-aminopropionaldehyde should be presumed. Such a situation is also inconsistent with the present results. Consequently the oxidative cleavage at A as shown in Chart 1 seems to be the most probable. The fact that primary amino groups exist in the end products as a whole (Fig. 3) necessitates the non-oxidative cleavage of the compound (I) at the site b or c. The formation of putrescine suggests the site of splitting rather at b than at c.

In case of spermine, there are six possible sites to be attacked by the oxidase (see the formula of spermine in Chart 1). Therefore, a large number of combinations of oxidative cleavages can be considered probable. Among those one of the most probable pathways of degradation of spermine referring to the pressent results is tentatively indicated in Chart 1. First, oxidative splitting at position A yields an intermediate compound (II) with the formation of two moles each of ammonia and hydrogen peroxide. It will further be hydrolysed non-oxidatively at the site b or c. The hydrolysis at b may give a compound (I), which is considered to be formed in the case of spermidine. Thus the compound (I) may further be converted to putrescine. By contrast, the hydrolysis of the compound (II) at c may result in the formation of 3-aminopropanal and compound (III), the latter may further be degraded into smaller fragments without form ing putrescine. These considerations are as yet only conjecture until more data are available.

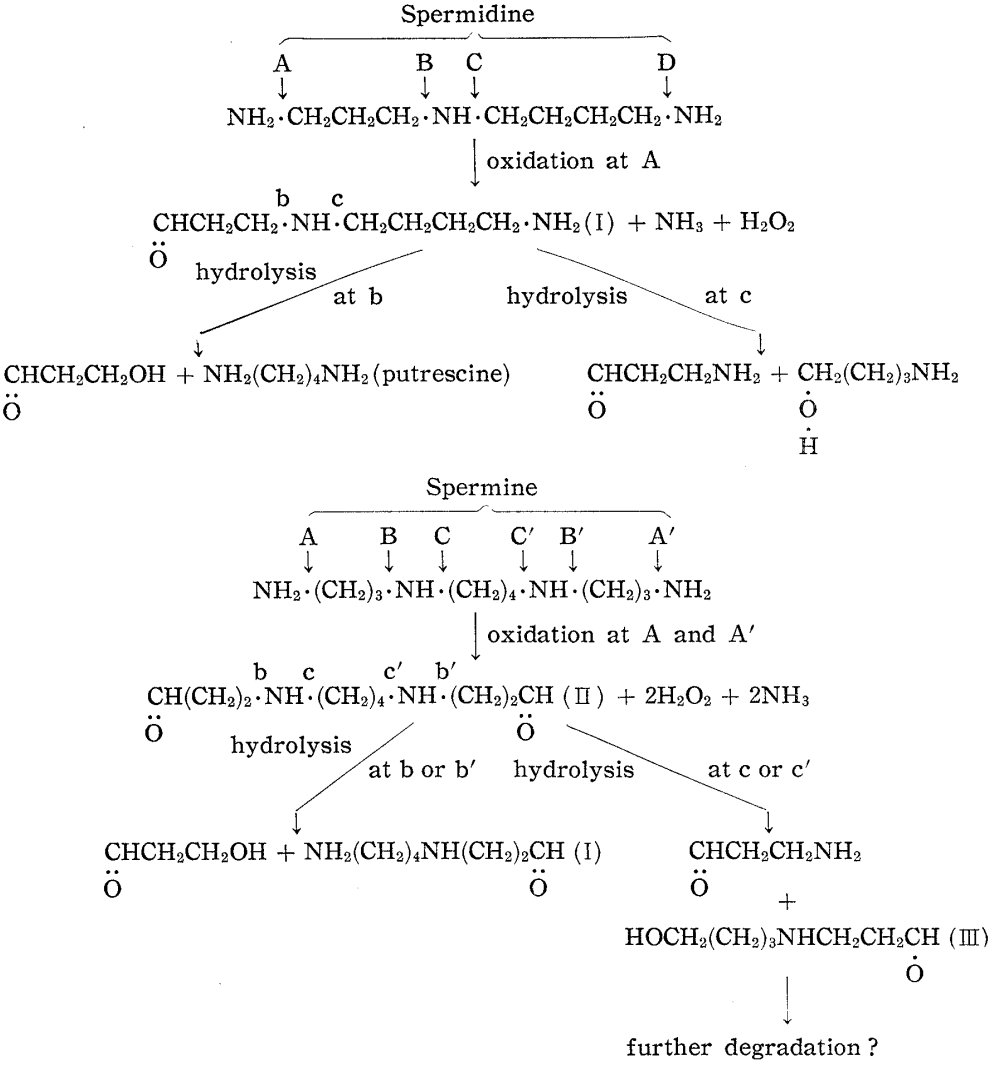

Chart 1. Possible Pathway of Degradation of Spermidine and Spermine

Experimental

Materials——Fresh beef blood was collected at the slaughter house in Funabashi City. Putrescine-1,4-^{14}C was purchased from Daiichi Pure Chemicals Co. and spermidine and spermine hydrochloride were obtained from Nutritional Biochemicals Corp. Spermine phosphate synthesized in this laboratory was also used.

Instruments——A Hitachi Spectrophotometer, EPU-2 and a Nihon Bunko, IR-S were used for spectrophotometry. A Toyo Roshi Power Supply, Model No. 3BA8 was used for paper electrophoresis. For the measurement of radioactivity, a window-less gas flow counter, Rikagaku Kenkyusho, Model-100, was employed under a condition which ignored self-absorption.

Preparation of enzyme——Fresh beef serum was fractionated by $(NH_4)_2SO_4$, the fraction that precipitates between 30 and 50% of saturation of the salt was collected and dialysed against 0.05M phosphate buffer, pH 7.0, for 16 hr. An excess of ammonium salt in the preparation was completely removed by gel filtration using Sephadex G-25 (medium) and the concentration of protein was adjusted to 50 mg. per ml. with 0.1M phosphate buffer, pH 7.0. The enzyme activity which was measured manometrically under the conditions that the reaction mixture contained $10^{-3}M$ spermidine, 0.1M phosphate buffer and 10% of the enzyme preparation in a total volume of 2.0 ml. was from 1.6 to 2.4 μl. per 10 min. per mg. of protein. This partially purified enzyme which contained no catalase was used as an enzyme preparation throughout the experiments.

Oxidation of Spermidine and Spermine——Following procedures were employed as a standard method unless otherwise noted. A reaction mixture consisted of $10^{-3}M$ substrate (spermidine or spermine), 0.1M phosphate buffer (pH 7.0) and 10% of the enzyme preparation. The reaction mixture was incubated

at 37° and oxygen uptake was followed by the conventional Warburg apparatus. At the end of incubation, protein was removed by centrifugation after the addition of trichloroacetic acid (TCA, final concentration being 5%) and the supernatant was extracted with ether or treated with an anion exchanger (Dowex-1 or Amberlite 400, OH^- form) to remove TCA. The supernatant, when treated with the resin, was neutralized with AcOH to pH 5.0 and subjected to subsequent analyses.

Determination of Ammonia——A saturated solution (0.5 ml.) of glycin was added to 1.0 ml. of the reaction mixture contained in the outer well of Conway No. 1 unit to prevent ammonia from being trapped by aldehyde compounds that is considered to exist in the reaction mixture. After diffusion in Conway unit for 2 hr. at 20°, with 1.0 ml. of the saturated solution of K_2CO_3 for alkalization, an aliquot of 0.02*N* H_2SO_4 solution in the center well was analysed with the bis-pyrazolone reagent.[4] When resorcinol or orcinol was added to the reaction mixture, addition of glycine solution was omitted.

Determination of Amino Groups——After removal of protein with TCA, followed by Et_2O extraction of TCA, 0.2 ml. of aliquot was analysed by the method of Dubin.[5] Under this condition, Beer's law was followed for 0.1 and 0.4 μ equivalent of amino groups at 370 mμ.

Identification of Putrescine——Three millimoles of spermidine was oxidized under the standard by the anion exchanger column. The supernatant was fractionated by Amberlite XE-64 and the AcOH-fraction was rechromatographed in the similar manner, then the fraction eluted by AcOH was reacted with 2,4-dinitrofluorobenzene. The resultant precipitate was washed with H_2O (three times), hot EtOH (two times), and recrystallized twice from 4-methyl-2-pentanone. It melted at 240°. *Anal.* Calcd. for $C_{16}H_{16}O_8N_6$: C, 45.72; H, 3.84; N, 20.00. Found : C, 44.93; H, 3.96; N, 19.94.

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Summary

The oxidation of spermidine and spermine by the partially purified amine oxidase of beef serum was examined. Under the present conditions, when spermidine was used as substrate, 1 mole each of ammonia and hydrogen peroxide were formed consuming 1 mole of oxygen. By contrast, in the case of spermine 2 moles each of ammonia and hydrogen peroxide were formed consuming 2 moles of oxygen. At the end of reaction, amino groups assayed in the reaction products of the two polyamines were found to be 2 moles equivalent of primary amino groups per 1 mole of the substrates. Another finding was that putrescine was identified and determined in the end products. The presence of an unidentified amino aldehyde in the end products was suggested using the resorcinol reagent which yield a fluorescent compound with the amino aldehyde. From these observations, possible pathways of the oxidative degradation of spermidine and spermine by the said enzyme were discussed.

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