level to the control in the initial stage, was dropped after 6 weeks from the start. Considering that the LDH activity did not decrease in the group of rats fed cupric acetate with the dyes, a role of copper might be to recover the LDH activity that was reduced by the administration of DAB.

Isozyme Pattern of LDH

The main component of LDH isozyme in the rat liver was LDH_5 fraction. When 2% homogenate was applied to the electrophoretic separation, only LDH_5 component was detected on the plate. If 10% homogenate was subjected to the separation, LDH_4 fraction, as well as LDH_5 fraction, was appeared. The administration of cupric acetate with and without DAB did not change the isozyme pattern observed in the control, while the administration of DAB alone was appeared to affect the isozyme pattern. As shown in Fig. 2, LDH_3 fraction was newly found in group(i) after 11 weeks from the start, and this pattern seemed to resemble that of the primary tumor induced by DAB. From the above facts, it was found that the administration of cupric acetate repressed the change of the LDH isozyme pattern in the course of carcinogenesis in the liver of the rat fed DAB.

Discussion

The administration of DAB, though reduced the total activity of LDH, gave rise to the appearance of a new LDH isozyme, LDH_3 fraction, which was never detected in the homogenate of the control. The fact indicates that the administration of DAB might mainly repress LDH_5 fraction of LDH isozyme, and that thereby the total activity is reduced. The appearance of aerobic fraction, LDH_3 might be related to the induction of hepatic carcinoma by feeding DAB, because another aerobic fraction, LDH_2, was appeared in primary carcinoma. Thus, the effect of feeding DAB might be related to the repression of anaerobic fraction of LDH, and the feeding of copper might recover the activity and isozyme pattern of this enzyme.

The above facts were added to support of the results of the previous report which indicated that the administration of basic cupric acetate stimulates remarkably of azo-reduction and protects the hepatic carcinogenesis in the rat fed DAB.

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Synthesis of Adenosine 2′-, and 3′-Phosphate 5′-Pyrophosphates and Their Esters[1)]

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Adenosine 2′- (IVa) and 3′-phosphate 5′-pyrophosphate (Va) are the known compounds, of which the former was obtained from the enzymatic hydrolyzate by Kaplan, *et al.*[3)] and the

1) This report represents Part XXV of "Investigations on Pantothenic Acid and Its Related Compounds"–Chemical Studies (12); *a*) Part XXIV: M. Shimizu, O. Nagase, Y. Hosokawa, H. Tagawa, and Y. Yotsui, *Chem. Pharm. Bull.* (Tokyo), **18**, 838 (1970).
2) Location: *Minamifunabori-cho, Edogawa-ku, Tokyo, 132, Japan.*
3) N.O. Kaplan, S.P. Colowick, E.F. Neufeld, and M.M. Ciotti, *J. Biol. Chem.*, **205**, 17 (1953).

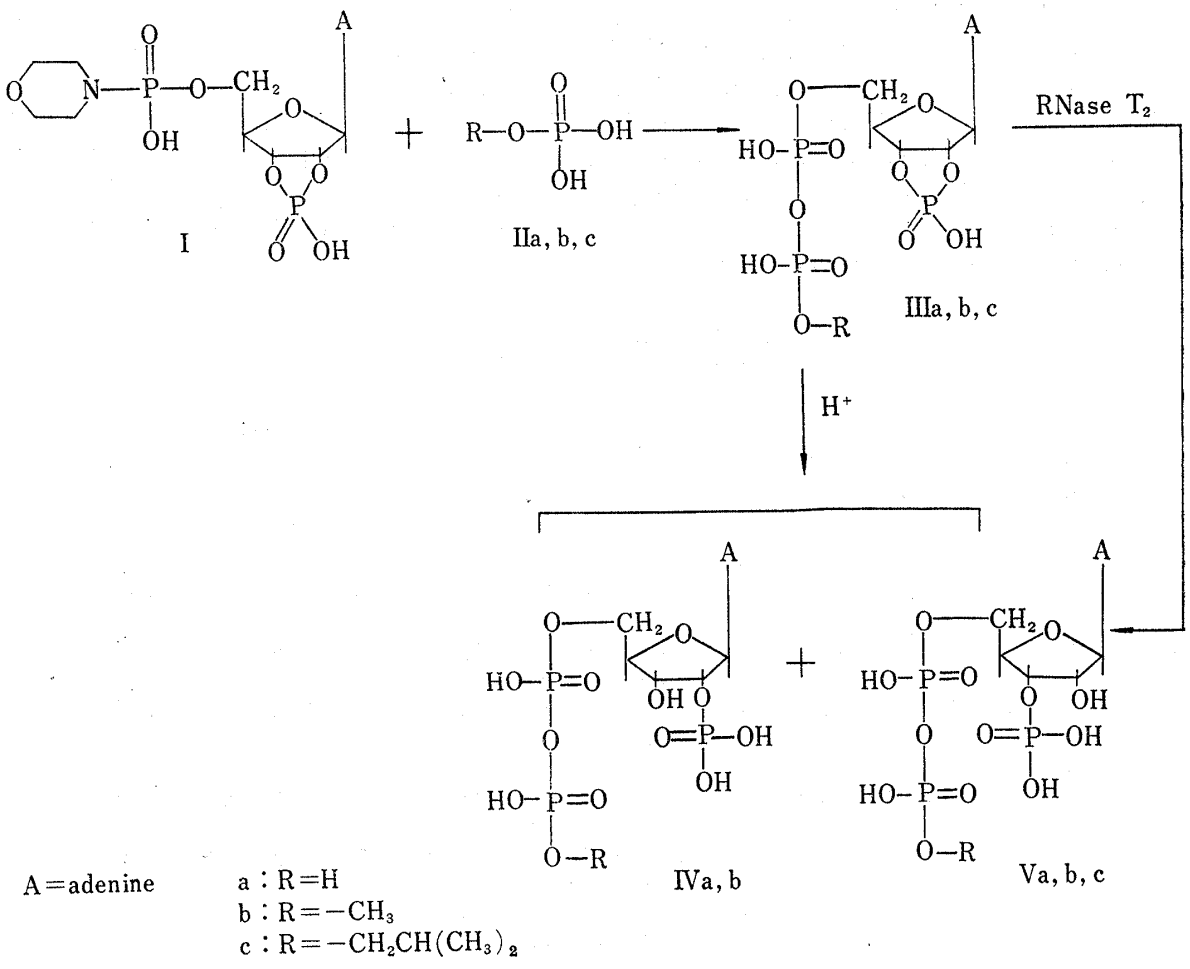

Chart 1

latter prepared synthetically by Michelson.[4] Their descriptions, however, have lacked in detailed data. We necessitated these nucleotides and their unknown alkyl esters during the course of the studies on structural specificity of coenzyme A for phosphotransacetylase. This paper deals with the modified preparation method and the chemical properties of these compounds.

The method employed for forming the pyrophosphate bond was the condensation reaction of adenosine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate (I) with orthophosphoric acid (IIa) or its esters (IIb,c) similarly with the cases of coenzyme A[5] and its analogues[1a,6,7] reported previously. One of the condensation products, adenosine 2′,3′-cyclic phosphate 5′-pyrophosphate (IIIa), has been synthesized by Michelson[4] using his anionic displace-

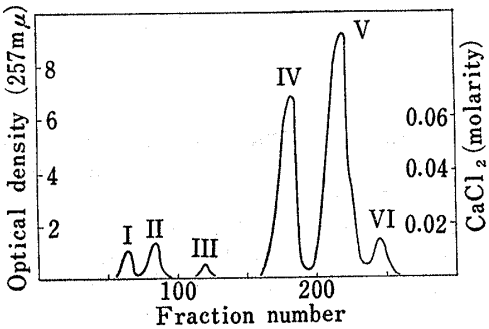

Fig. 1. Chromatography of the Reaction Products in the Preparation of IVb and Vb on a Dowex 1 (Cl^-) Column

peaks I and II: adenosine 2′,5′-and 3′,5′-bisphosphate, respectively, peak IV: adenosine 2′-phosphate 5′-pyrophosphate 5′-methyl ester (IVb, 25%), peak V: the 3′-isomer of IVb (Vb, 43%)

4) A.M. Michelson, *Biochim. Biophys. Acta*, **114**, 460 (1966).
5) M. Shimizu, O. Nagase, S. Okada, Y. Hosokawa, H. Tagawa, Y. Abiko, and T. Suzuki, *Chem. Pharm. Bull.* (Tokyo), **13**, 1142 (1965); **15**, 655 (1967).
6) M. Shimizu, O. Nagase, Y. Hosokawa, and H. Tagawa, *Tetrahedron*, **24**, 5241 (1968).
7) M. Shimizu, O. Nagase, S. Okada, and Y. Hosokawa, *Chem. Pharm. Bull.* (Tokyo), **18**, 313 (1970).

ment method. Acidic fission of IIIa or its ester (IIIb) gave a mixture of 2′-phosphate (IVa, b) and 3′-phosphate (Va, b), respectively, as in the synthesis of coenzyme A.[8] The separation of two isomers has been most efficiently effected by the column chromatography on the Dowex 1 (Cl^-) using linear gradient elution with calcium chloride and hydrochloric acid. For the exclusive preparation of 3′-phosphate (Va,b,c) was very suitable the enzymatic fission of IIIa, b,c with ribonuclease T_2 according to Michelson.[4] The structure of the products thus obtained was confirmed by paper chromatography of the alkaline hydrolyzate. The above reaction sequences are shown in Chart 1.

Table I. Adenosine 2′- (IVa), and 3′-Phosphate 5′-Pyrophosphate (Va) and their Esters (IVb, Vb, Vc)

Compd.	Method[a]	Yield (%)	*Rf*[b] I	*Rf*[b] II	*Rf*[b] III	Formula	Analysis (%) Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
IVa	B	22.0	0.08	0.32	0.52	$C_{10}H_{11}O_{13}N_5Ca_{2.5}P_3\cdot 8H_2O$	16.09	3.65	9.38	15.97	3.47	7.92
IVb	B	23.2	0.16	0.32	0.48	$C_{11}H_{14}O_{13}N_5Ca_2P_3\cdot 9H_2O$	17.39	4.25	9.22	17.75	4.40	8.64
Va	A	39.6	0.08	0.32	0.44	$C_{10}H_{13}O_{13}N_5Li_3P_3\cdot 5H_2O$	19.53	3.77	11.39	19.94	3.96	11.02
	B	16.4										
Vb	A	55.7	0.16	0.32	0.38	$C_{11}H_{15}O_{13}N_5Li_3P_3\cdot 3H_2O$	22.28	3.57	11.81	22.59	4.20	11.04
	B	40.3				$C_{11}H_{14}O_{13}N_5Ca_2P_3\cdot 9H_2O$	17.39	4.25	9.22	17.20	4.09	8.77
Vc	A	49.5	0.33	0.55	0.35	$C_{14}H_{21}O_{13}N_5Li_3P_3\cdot 4H_2O$	25.74	4.48	10.72	25.46	4.85	10.23

a) A: enzymatic hydrolysis B: acid hydrolysis
b) Paper chromatography was performed on Toyo Roshi No. 51A by the ascending technique. solvent system: I, EtOH–0.5N $AcONH_4$ buffer (pH 3.8)(5:2); II, isobutyric acid–1N NH_4OH–0.1N EDTA·2Na (100:60:1.6); III, satd. $(NH_4)_2SO_4$–0.1M $AcONH_4$ (pH 6)–iso-PrOH (79:19:2)

Experimental

Adenosine 2′-Phosphate 5′-Pyrophosphate (IVa) and its Methyl Ester (IVb)——The mono-tributylammonium salt of orthophosphoric acid (IIa, 1 mmole) was rendered anhydrous by several evaporations with added pyridine. The barium salt of methyl phosphate (IIb, 1 mmole) was converted to the pyridinium salt by passage through a column of Amberlite IR 120 (H^+) and evaporating the effluent with pyridine to dryness *in vacuo*, and then rendered anhydrous as described above. Bis(4-morpholine-N,N′-dicyclohexylcarboxamidinium) salt of adenosine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate[8] (I, 565 mg, 0.5 mmole) was also dried and mixed with II in anhydrous pyridine (30 ml). The mixture was allowed to stand at room temperature overnight. After evaporation of the solvent *in vacuo*, the residue was dissolved in 0.1N HCl (30 ml) and left at room temperature for 1 hr. HCl was removed by several evaporations *in vacuo* with water. The residue was dissolved in water, adjusted to pH 6 with dilute NH_4OH, and applied to a column (1.9×60 cm) of Dowex 1 X 4 (Cl^-). The column was washed with water and then eluted with a linear gradient using 0.03M $CaCl_2$ in 0.0045N HCl (3 liters) in a mixing chamber and 0.06M $CaCl_2$ in 0.006N HCl (3 liters) in a reservoir.[9] Fractions of 20 ml were collected every 10 min. The elution pattern of methyl ester is shown in Fig. 1. Peak IV containing the compound IV was adjusted to pH 7 with 2% $Ca(OH)_2$ suspension and evaporated to dryness *in vacuo*. The residue was triturated with EtOH (100 ml), centrifuged, washed with EtOH, and dried over P_2O_5 *in vacuo* to give the trilithium salt of IVa or IVb as a white powder.

Adenosine 3′-Phosphate 5′-Pyrophosphate (Va) and its Esters (Vb,c)——1) The intermediate compound (III) obtained from I (0.3 mmole) and II (0.6 mmole) as described above was dissolved in 0.02M

8) J.G. Moffatt and H.G. Khorana, *J. Am. Chem. Soc.*, **83**, 663 (1961).
9) The similar system had been used for the separation of adenosine 2′,5′- and 3′,5′-bisphosphate from each other (J. Baddiley, J.G. Buchanan, and R. Letters, *J. Chem. Soc.*, **1958**, 1000).

acetate buffer (pH 4.5, 2 ml) and partially purified RNase T_2 (1500 units)[5] was added. The mixture was incubated at 25° for 2 hr and then chromatographed on a column (1.55×25 cm) of DEAE-Sephadex A-25 (Cl^-), which was eluted with a linear gradient using 0.003N HCl and 0.15M LiCl in 0.003N HCl (each 1.5 liters). The main peak containing V was adjusted to pH 4.5 with LiOH and evaporated to dryness *in vacuo*. LiCl was removed by dissolving the residue in MeOH and precipitating with acetone repeatedly. Drying over P_2O_5 *in vacuo* gave the trilithium salt of Va(b,c).

2) The peak V obtained by chromatography of acid hydrolyzate described above was worked-up in the same manner as described for Peak IV to give the calicium salt of Va or IVb. Yields and analytical data are summarized in Table I.

Alkaline hydrolysis (1N NaOH, 100°, 90 min) of IV and V thus obtained gave adenosine 2′,5′- and 3′,5′-bisphosphate, respectively, which were detected by paper chromatography using solvent III.

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Selective and Sensitive Method for Determination of Vanillylmandelic Acid (VMA) in Urine

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The major metabolite, vanillylmandelic acid (VMA), of norepinephrine and epinephrine in human urine has been measured by colorimetric[2,3] and gas chromatographic[4–6] methods. Those methods have some faults as follows: 1) The colorimetric methods are not selective for VMA on account of the obstruction by other urinary phenolic compounds, such as *p*-hydroxymandelic acid,[2] *etc.*,[3] and sensitivity is low. 2) The gas chromatography by ionization detector[4,5] is not sensitive enough for measurement of VMA in urine of normal person. 3) Wilk, *et al.* converted VMA to vanillin and analysed it as trifluoroacetyl derivative by gas chromatography equipped with electron capture detector,[6] however, the accuracy of their methods is low because of the absence of an internal standard in gas chromatographic analysis and the recovery is about fifty percent. 4) The common separation procedure of those methods depends on extraction by organic solvent such as ethyl acetate or ether from acidified urine and it is the reason why the prepared sample is contaminated with other substances to lower the selectivity of them.

Now we investigated the separation with adsorbents and gas chromatographic analysis with electron capture detector without conversion of VMA to vanillin. The established method consists of the separation of VMA by adsorption on Amberlite XAD-2 from urine at pH 1 and gas chromatography of VMA after methylation in ethanol in a short period and trifluoroacetylation.[7]

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1) Location: *Hongo-7-3-1, Bunkyo-ku, Tokyo.*

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3) S.E. Gitlow, L. Ornstein, M. Mendlowitz, S. Khassis and E. Kruk, *AM. J. Med*, **28**, 921 (1960).
4) C.M. Williams and M. Greer, *Clin. Chim. Acta*, **7**, 880 (1962).
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6) S. Wilk, S.E. Gitlow, M. Mendlowitz, M.J. Franklin, H.E. Carr and D.D. Clarke, *Anal. Biochem.*, **13**, 544 (1965).
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