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PREPARATION AND PROPERTIES OF N-(RING)-RIBOSYLHISTAMINE DERIVATIVES

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Abstract—The preparation, purification and characterization of histamine adenine dinucleotide, histamine mononucleotide and histamine nucleoside are described. All of these compounds show no histamine-like activity as proved by tests on the contraction of guinea pig ileum, the fall in blood pressure of the cat and on the local accumulation of circulating dye in guinea pig skin. Histamine nucleoside is easily cleaved by nucleoside hydrolase from *Lactobacillus delbrueckii*. However, the phosphorolytic cleavage of nucleoside and the reverse reaction are not demonstrable with the mammalian nucleoside phosphorylase.

THERE is some evidence which suggests that nucleotide metabolism involves in the detoxification of histamine. In earlier experiments, imidazoleacetic acid riboside was isolated as a major product from urine of rats injected with ^{14}C-labelled histamine by Tabor and Hayaishi,[1] and Karjala.[2] Alivisatos *et al.*[3] reported that histamine adenine dinucleotide (HRPPRA) is formed from histamine and nicotinamide adenine dinucleotide (NAD) with the use of mammalian NADase. Subsequently, we succeeded in synthesizing histamine mononucleotide (HRP)[4] from the dinucleotide by means of snake venom phosphodiesterase and histamine nucleoside (HR)[5] from the resulting nucleotide using human prostatic acid phosphatase. Furthermore, Abdel-Latif and Alivisatos[6,7] reported an alternative pathway of formation of HRP and HR by the direct interaction of histamine on the nucleotide or nucleoside of nicotinamide.

Since the preliminary evidence showed that the nucleotide pathway does not include the process of phosphorolytic cleavage of HR and the reverse reaction, and that even *in vivo* the histamine-ribose bond of ribosylhistamine derivatives is difficult to split, we proposed that the final product(s) of this pathway must be HR and/or its derivatives.[5] This hypothesis was recently substantiated from the *in vivo* experiments by Alivisatos *et al.*[8]

Present report deals with the enzymic synthesis and isolation procedures of HRPPRA, HRP and HR, as well as the chemical, enzymic and pharmacological properties of these compounds. Preliminary reports of this work have been published.[4,5]

MATERIALS AND METHODS

Preparation of enzymes and substrates

NADase from beef spleen and pig brain was prepared by the method of Kaplan.[9] Phosphodiesterase[10] from snake venom (*Agkistrodon halys blomhoffi* Boie) and 5′-nucleotidase[10] from snake venom and bull semen were generously supplied by Dr. S. Iwanaga, Institute for Protein Research, Osaka University. Human prostatic acid

phosphatase was prepared by the procedure of Davidson *et al.*[11] Nucleotide phosphorylase was purified from rat liver by the method of Kalckar.[12] Nicotinamide ribosidase was partially purified from the acetone powder of beef liver by ammonium sulfate fractionation as described by Rowen and Kornberg.[13] Milk xanthine oxidase was kindly supplied by Dr. M. Yoneda, Takeda Research Laboratory, Osaka, who prepared the enzyme by Ball's method.[14] Nucleoside hydrolase was partially purified from *Lactobacillus delbrueckii* by ammonium sulphate fractionation as described by Takagi and Horecker.[15] Diamine oxidase was extracted from the acetone powder of hog kidney.[16]

NAD used was the preparation from baker's yeast by the procedure of Kornberg.[17] ^{14}C-labelled histamine was a commercial product from the Radiochemical Centre, Amersham, England.

Isolation procedures and methods for chemical analysis

Ion exchange procedures for the isolation of HRPPRA and HRP employed the chloride form of Dowex-1 (X 10, 200–400 mesh). For the isolation of HR, the column of cotton acid succinate was employed. This exchanger was prepared by McIntire's method.[18]

Phosphate was determined by the method of Nakamura,[19] while ribose by the periodate consumption[20] and orcinol reaction.[21] In the latter method, ribose was estimated by referring to 5′-adenylic acid as a standard. The Pauly diazo reaction was carried out according to Macpherson.[22] Florimetry of N-ribosylhistamine derivatives was attempted by the method of Shore *et al.*[23] using the Farrand spectrofluorometer.

Pharmacological effect

The contraction of the guinea pig ileum was measured on a piece of ileum suspended in a 10 ml Tyrode bath containing 0·5 μg of atropine sulphate. The identification of histamine was carried out using the antagonistic action of the antihistamine Mepyramine. The effect of histamine and its ribosyl derivatives on the arterial pressure was determined by the use of a pentobarbitone-anaesthetized cat. The blueing test[24] was conducted by the observation on the local blueing of the skin by intracutaneous injection in a guinea pig previously administered pontamine sky blue by cardiac puncture.

RESULTS

Enzymic formation and isolation of histamine adenine dinucleotide, histamine mononucleotide and histamine nucleoside

HRPPRA was synthesized from ^{14}C-histamine and NAD as substrate, and with the use of insoluble, partially purified NADase from beef spleen or pig brain by the procedure of Alivisatos *et al.*[3], which had been slightly modified. The isolation of HRPPRA from the incubation mixture was carried out by the column of Dowex-1 (x 10, chloride form). HRPPRA, which has the radioactivity due to ^{14}C-histamine and exhibits the absorption at 260 mμ, was eluted by 0·002 N hydrochloric acid and was recrystallized from acetone–water mixture after lyophilization. Yield 20–30 per cent.

HRP was synthesized with the use of snake venom phosphodiesterase. In a typical experiment, 15 ml of reaction mixture containing 1 g of ^{14}C-HRPPRA, 12 mg of phosphodiesterase and 2000 μmoles of tris buffer (pH 8·4) were incubated for 5 hr at 37°. After diluting to 500 ml, HRP was isolated by means of the column chromatography of Dowex-1 (x 10, chloride form). The fraction eluted by 0·001 N hydrochloric acid (pH 3·1) showed the radioactivity of ^{14}C-histamine and the absorption of imidazole portion at 220 mμ. This was collected, lyophilized and recrystallized from acetone–water mixture to 250 mg (yield 58 per cent) of crystals, m.p. 176–180° (decomp.). At the same time, a substance having absorption at 260 mμ was obtained by elution with 0·004 N hydrochloric acid (pH 2·4) and this was identified as 5′-adenylic acid

The degradation of HRP proceeded with the use of partially purified human prostatic acid phosphatase. Reaction mixture containing 50 mg of HRP, 4 ml of 0·01 M $MgCl_2$ and 12 ml of 0·2 M acetate buffer (pH 5·0) was incubated for 3 hr at 37°. For the isolation of the nucleoside from the incubation mixture, the purification method for tissue histamine, involving butanol extraction at pH 12·5 and column chromatography by cotton acid succinate, was applied. The fraction having absorption at 220 mμ was obtained by elution with 0·004 N hydrochloric acid and was recrystallized from alcohol–ether (1 : 1) after lyophilization. The crystalline preparation of the nucleoside was so highly hygroscopic at room temperature that accurate measurements of the weight and melting point were impossible.

TABLE 1. ANALYTICAL DATA FOR HISTAMINE ADENINE DINUCLEOTIDE AND HISTAMINE MONONUCLEOTIDE

	Dinucleotide (HRPPRA)	Mononucleotide (HRP)
Formula	$C_{20}H_{29}N_8O_{13}P_2Cl{\cdot}3H_2O$	$C_{10}H_{18}N_3O_7P{\cdot}HCl$
Molecular weight	740·5*	359·5
mp (°C)	———	176-180 (decomp.)
Specific activity (cpm/μmole)	1419	1466
Ribose (μmoles)		
orcinol reaction	0·86 (1·00)†	0 (0)
periodate consumption	1·80 (2·00)	0·96 (1·00)
λmax (mμ)	215 258	215
Rf values (0·1 M HAc-ethanol)	0·45	0·58
Color reaction in paperchromatograms		
diazo	orange	yellow
ninhydrin	violet	violet
phosphorous (Wade)	positive	positive
dark spot (UV)	positive	positive

* Alivisatos *et al.*[3]
† The values in parentheses indicate the calculated values.

Chemical properties of ribosylhistamine derivatives

The summarized chemical properties of HRPPRA, HRP and HR are shown in Tables 1 and 2.

The ribosylhistamine compounds were orcinol reaction negative for ribose, while close to the theoretical value was obtained by the periodate reaction. It was also

demonstrated that the absorption spectrum of diazo reaction with these compounds lacks the peak at 500 mμ characteristic of free histamine but one peak at around 380 mμ is noted (Fig. 1).

The ultra-violet spectrum of the dinucleotide exhibited absorption maxima at around 215 mμ and 258 mμ, and that of nucleotide at around 215 mμ (Fig. 2). Attempted formation of a fluorescent product by condensation with *o*-

TABLE 2. ANALYTICAL DATA FOR HISTAMINE NUCLEOSIDE

	Value per μmole histamine* Calculated	Found
Ribose		
periodate consumption	1·00	0·99
orcinol reaction	0·00	0·00
Bound histamine†		
biologically‡	1·00	0·93
fluorometrically	1·00	0·96
Free histamine		
biologically	0·00	0·00
fluorometrically	0·00	0·00
Total phosphorous†	0·00	0·00
λmax (mμ)		210
Rf value (0·1 M acetic acid–ethanol)		0·79
Color reaction in paperchromatograms		
diazo		yellow
ninhydrin		violet

* Value with respect to histamine determined by the absorption at 220 mμ ($\epsilon_{220m\mu} = 3{\cdot}36 \times 10^3$ at pH 7·0).
† Data from acid hydrolysate (2·5 N HCl, 170°C, 5 hr).
‡ With the use of guinea pig ileum (see text).

phthalaldehyde, observed by Shore *et al.*[23] with histamine, failed in these three compounds.

It was observed that the N-ribosyl bond of these compounds was very resistant to acid hydrolysis and a high concentration of hydrochloric acid could not decompose them under the normal pressure. The negative values of ribose by the orcinol reaction are also considered to be due to the stability of the N–C bond between their imidazole and ribose. However, they were easily hydrolysed when heated with dilute acid under a high pressure. For example, the liberation of 93–96 per cent of free histamine from the nucleoside was demonstrated with the use of 2·5 N hydrochloric acid in a sealed tube as shown in Table 2.

Enzymic properties of ribosylhistamine derivatives

(a) *Action of prostatic acid phosphatase and 5′-nucleotidase upon histamine mononucleotide.* As mentioned, HRP was decomposed by human prostatic acid phosphatase resulting in the formation of HR and inorganic phosphate. The rate of the liberation of inorganic phosphate from HRP appeared to be somewhat slower than that with 5′- adenylic acid (Fig. 3). 5′-Nucleotidase from snake venom or bull semen failed to decompose HRP, since these enzyme preparations, in an amount sufficient to completely decompose 5′-adenylic acid in 5 min, failed to give evidence for the presence of inorganic phosphate in 60 min which should be liberated from HR.

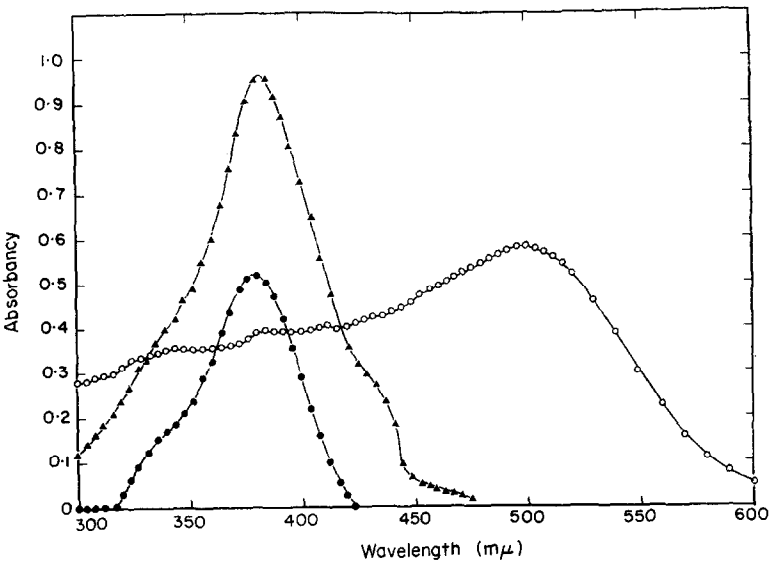

FIG. 1. Absorption spectra of azo-dyes produced by reaction of diazotized sulfanilic acid with histamine, histamine dinucleotide and histamine mononucleotide. ○———○, $2{\cdot}0 \times 10^{-7}$ M of histamine; ●———●, $8{\cdot}7 \times 10^{-7}$ M of histamine mononucleotide; ▲———▲, $1{\cdot}0 \times 10^{-6}$M of histamine dinucleotide.

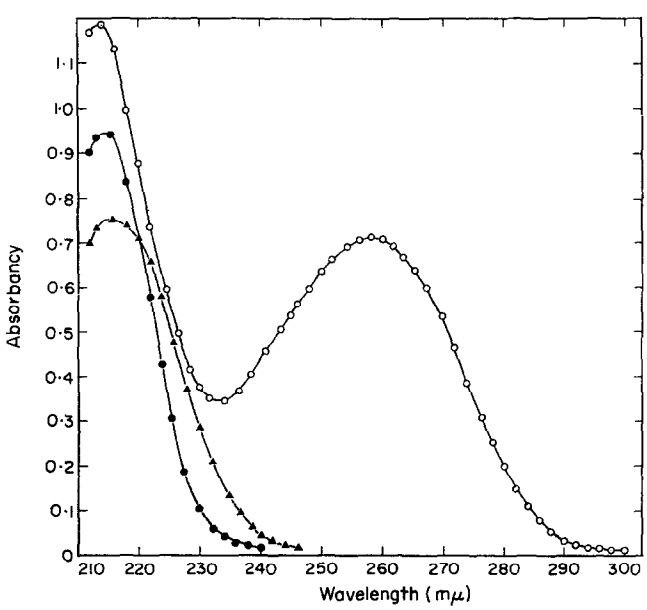

FIG. 2. Ultra-violet absorption spectra of histamine dinucleotide and histamine mononucleotide at pH 2·4. ●———●, $3{\cdot}33 \times 10^{-4}$ M of histamine; ○———⊙, $6{\cdot}66 \times 10^{-5}$ M of histamine dinucleotide; ▲———▲, $1{\cdot}45 \times 10^{-4}$ M of histamine mononucleotide.

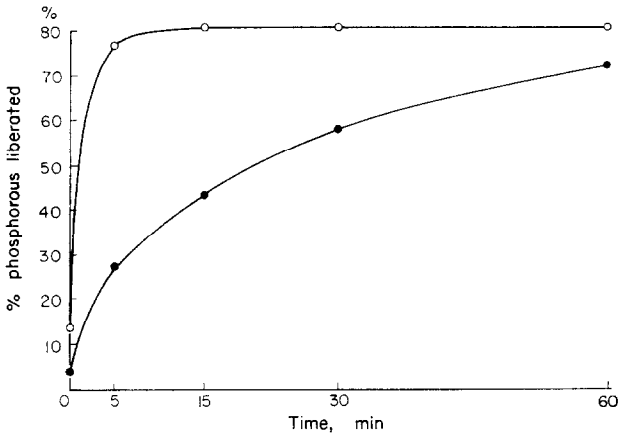

FIG. 3. Dephosphorylation of histamine mononucleotide and adenine mononucleotide with human prostatic acid phosphatase. Reaction mixture contained 2μmoles of histamine mononucleotide or adenine mononucleotide, 0·2 ml of 0·2 M acetate buffer, 0·1 ml of 0·01 M $MgCl_2$ and 0·2 ml of enzyme preparation. ●——●, histamine mononucleotide; ○——○, adenine mononucleotide

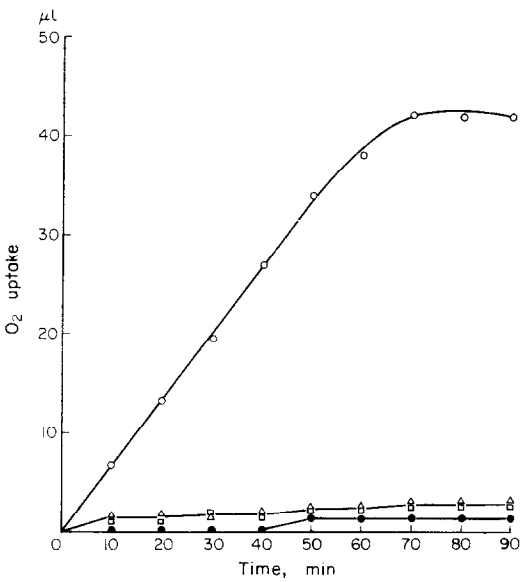

FIG. 4. Effect of ribosylhistamine derivatives on oxygen uptake in the presence of diamine oxidase from hog kidney. 3·2 ml of reaction mixture containing 2 μmoles of histamine or its ribosyl derivatives, 0·5 ml of diamine oxidase solution, and 2·5 ml of 0·2 M potassium phosphate buffer (pH 7·2) was incubated at 37°C. ○——○, histamine; ●——●, histamine dinucleotide; △——△, histamine mononucleotide; □——□, histamine nucleoside.

(b) *Action of diamine oxidase upon ribosylhistamine derivatives.* As shown in Fig. 4, in the presence of diamine oxidase partially purified from hog kidney, the oxygen uptake proved to be 38·4 μl by histamine, 1·8 μl by HR, 2·4 μl by HRP and 1·9 μl by HRPPRA in 2 μmoles each for 1 hr. Therefore, this enzyme can be said to act almost specifically on free histamine only.

(c) *Action of nucleoside phosphorylase and nucleoside hydrolase upon histamine nucleoside.* The liberation of free histamine from HR was investigated taking as its criterion the contraction of the guinea pig ileum suspended in Tyrode bath kept at 37°C in the presence of HR and enzyme preparation. Nucleoside hydrolase, prepared from *Lactobacillus delbrueckii*, did cause a contraction of ileum incubated with 50 mμmoles of HR, and this contraction was relaxed by the antihistamine (Mepyramine) or by washing. This means that free histamine is liberated from HR by nucleoside hydrolase. However, nucleoside phosphorylase from rat liver, nicotinamide ribosidases from beef liver, hemolysed human erythrocytes and crude snake venom (*Agkistrodon halys blomhoffi* Boie) did not cause the liberation of histamine under the same condition.

The formation of ribose-1-phosphate or ribose from HR was estimated by the orcinol reaction, since the imidazole-bound ribose was negative for the orcinol reaction. As shown in Fig. 5, the degradation of the nucleoside did not occur by adding a large amount of purified nucleoside phosphorylase (Fig. 5, curve b), though

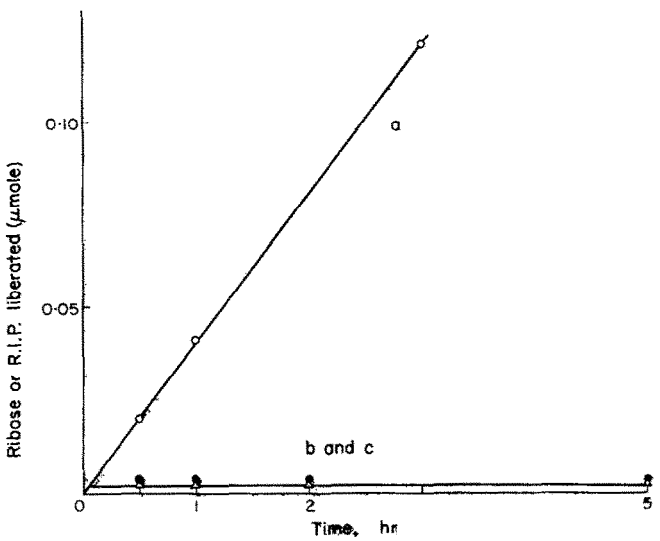

FIG. 5. Effect of nucleoside phosphorylase and nucleoside hydrolase on the liberation of ribose or ribose-1-phosphate from histamine nucleoside. 0·8 ml of reaction mixture containing 0·2 μmole of histamine nucleoside, 0·2 ml of phosphate buffer (pH 6·5 or 7·4) and enzyme solution was incubated at 37°. Curve a, 0·7 mg of nucleoside hydrolase from *Lactobacillus delbrueckii* (pH 6·5); curve b, 320 units of nucleoside phosphorylase from rat liver (pH 7·4); curve c, the same as curve b, but with diamine oxidase from hog kidney.

nucleoside hydrolase from *Lactobacillus delbrueckii* did cleave the nucleoside quantitatively (Fig. 5, curve a). In view of the fact that the phosphorolytic cleavage of inosine was coupled with the xanthine oxidase reaction[25] and that diamine oxidase could act almost specifically on free histamine only, an excess amount of diamine oxidase was added to the reaction system just mentioned, but an acceleration of the reaction failed to take place (Fig. 5, curve c).

(d) *Failure of the formation of histamine nucleoside by mammalian nucleoside phosphorylase.* It was thought that the following reaction might have occured in the presence of purine nucleoside phosphorylase:

inosine + inorganic phosphate ⟶ hypoxanthine + ribose-1-phosphate (1)

ribose-1-phosphate + histamine ⟶ histamine nucleoside + inorganic phosphate (2)

For the removal of hypoxanthine and increased formation of ribose-1-phosphate, xanthine oxidase was added in the reaction mixture. But the formation of HR was not demonstrated because free histamine determined by the diazo reaction at 530 mμ did not decrease in the medium (Fig. 6). Unexpectedly, however, a large amount of uric

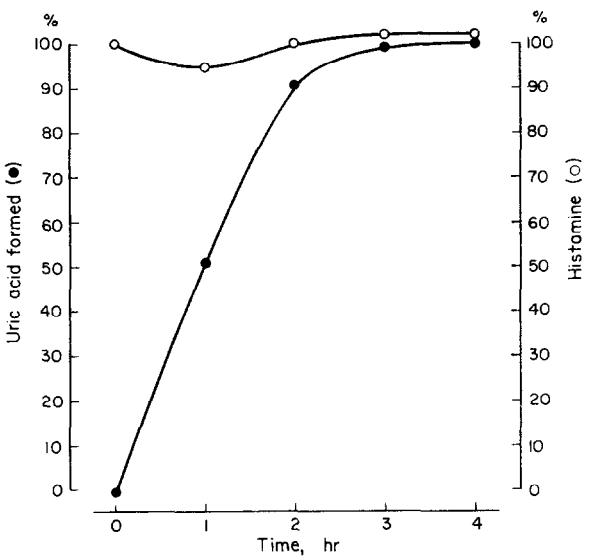

FIG. 6. Failure of the formation of histamine nucleoside from histamine and inosine in the presence of nucleoside phosphorylase and xanthine oxidase. 3 ml of reaction mixture containing 1·5 μmoles of inosine, 1·5 μmoles of histamine, 50 units of nucleoside phosphorylase, 400 units of xanthine oxidase and 0·1 M potassium phosphate buffer (pH 7·4) was incubated at 37°C. ●————●, absorbancy at 290 mμ; ○————○, absorbancy at 530 mμ (diazo reaction).

acid was formed in the above reaction only in the presence of histamine. Subsequent experiments showed that an increased formation of uric acid was not due to the activation of nucleoside phosphorylase but simply due to the activation of xanthine oxidase by histamine.[26–28]

Pharmacological effect of ribosylhistamine derivatives

The ileum of a guinea pig sensitive to 5 mμmoles/10 ml of histamine was not affected by HRPPRA, HRP and HR at the same concentration with histamine. However, 100–300 mμmoles of HRP and HR caused a slow increase of tone, but this action was apparently different from that of histamine in that the contraction occurred gradually and was not inhibited by antihistamine.

The arterial pressure of a cat, which had shown a characteristic fall with 1·35 mμmoles of histamine, was negligibly affected by intravenous injection of 178 mμmoles of the nucleoside or 500 mμmoles of the nucleotide. Only a larger amount of the dinucleotide such as 500–1000 mμmoles exhibited a very slight fall, somewhat slower than that of histamine. This might be due to the action of adenylic portion of the dinucleotide.

The local blueing test was negative in all these three ribosylhistamine derivatives even at 500 mμmoles, while histamine was positive at 1 mμmole. These results indicate that histamine containing nucleotides or nucleoside all show no histamine-like effect. It seems that the histamine-ribose bond of these compounds is difficult to split in both *in vitro* and *in vivo* and the structure-activity relationship is highly strict.

DISCUSSION

In this experiment it was successfully demonstrated that HRPPRA could be synthesized from histamine and NAD with the use of partially purified beef spleen or pig brain NADase, HRP from the resulting dinucleotide with the phosphodiesterase of snake venom, and HR from HRP with human prostatic acid phosphatase. Among these ribosylhistamine derivatives, HRPPRA and HRP, both being acidic, could be isolated in a chemically pure form by the anion exchanger and HR, because of its basic property, by means of the cation exchanger. It was also demonstrated that neither decomposition of HR by phosphorolysis nor the formation of HR from histamine and ribose-1-phosphate occurred. The results of the pharmacological study indicate that even *in vivo* the histamine–ribose bond of N-ribosylhistamine is not easy to split. This is in good agreement with the results of enzymic studies *in vitro*.

Chemical studies have also demonstrated that the N–C bond of histamine–ribose is difficult to split and every one of ribosylhistamine derivatives does not undergo hydrolysis in the presence of concentrated mineral acid under normal pressure. It seems that each of these compounds proves to be orcinol negative for the same reason.

The fact that N-ribosylhistamines do not show a histamine-like activity in the contraction of guinea pig ileum and in the fall of the blood pressure of the cat is interesting from the viewpoint of correlations between chemical constitution and histamine activity. Previously Lee *et al.*[29] reported that 3-methylhistamine, having a fragment of $=N-\overset{\|}{C}-CH_2CH_2NH_2$ in its structure, definitely did have histamine activity in the above two biological systems but 1-methylhistamine, having no such fragment, had no activity. Since N-ribosylhistamine derivatives have no histamine-like activity, the ribosyl group may bind to the N atom at 1 position of imidazole as in the case of 1-methylhistamine.

In the known catabolic products of histamine, imidazoleacetic acid riboside is closely related to the compounds in the present study. This substance was isolated from urine of the rats injected with ^{14}C-labelled histamine and considered to be one of the major metabolites of histamine in various animals.[1, 2, 30] However, the present experiments have failed to show the oxidative transformation of N-ribosylhistamine derivatives to N-ribosylimidazoleacetic acid derivatives, since the oxygen uptake by histamine nucleotides or by nucleoside was negligible in the presence of diamine oxidase preparation from hog kidney.

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