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Studies on Unknown Methylated Compounds of Non-Histone Nuclear Protein

Hyang Woo Lee*, Sung-Youl Hong, Sangduk Kim and Woon Ki Paik

Department of Biochemistry, School of Pharmacy, Sung Kyun Kwan University, Suwon 170, Korea

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Abstract □ The HCl hydrolyzate of the non-histone protein fractionated from the rat liver nuclei which have been incubated in the presence of S-adenosyl-L-[*methyl*-^{14}C]-methionine shows at least four unidentified radioactive peaks on a basic amino acid analysis chromatogram. One of these unknown compounds (designated as compound 3) is also formed by the rat liver homogenate with the exogenous addition of an appropriate protein substrate. Since boiled rat liver homogenate or fresh homogenate in the absence of an exogenous protein substrate failed to form compound 3, its formation can be considered to be enzyme-catalyzed. The enzyme which yields compound 3 shows a preference of protein substrate in the order of reductively methylated hemoglobin >native >histone type II-A. The rat enzyme is nuclear in location associated with chromatin, and exhibits the highest activity in the liver among various rat organs. A compound 3-forming enzyme is also present in *Neurospora crassa*, since endogenous formation of the compound 3 can be demonstrated with the crude extract of this mold. The chemical identity of compound 3 is not yet known. However, it resisted to the following treatments; 6 N HCl and 1.0 N NaOH hydrolysis at 110°C, or L-amino acid oxidase.

Keywords □ Unknown methylated compounds of non-histone nuclear protein, S-adenosyl-L-[*methyl*-^{14}C]-methionine, *Neurospora crassa*, Protein-specific methyltransferases.

* Present address: *Fels Research Institute, Temple University School of Medicine, Philadelphia, PA 19140, USA.*

The presence of methylated lysine and arginines in nuclear proteins, and the existence of S-adenosyl-L-methionine:protein methyltransferases forming these methylated residues are well established facts[1]. In particular, the *in vitro* incubation of rat liver nuclei with S-adenosyl-L-[*methyl*-^{14}C]methionine, and the subsequent isolation of tthe histone and non-histone nuclear protein, showed that the histone fraction contains most of the *in vitro* methylated lysines while the non-histone nuclear protein contains most of the methylated arginines[2]. Furthermore, we reported the observation of the presence of at least four unidentified protein methylation-derived products in the non-histone nuclear protein. Recently, we identified one of the products (unknown compound #1) as N-methylamine[3]. The present communication deals principally with one of these unknown compounds, designated as compound 3. In regarding this compound, we have accomplished the followings: (a) The examination of some of the chemical properties of compound 3, (b) the study of the enzyme(s) involved in protein-bound compound 3 formation, and their existence, subcellular and tissue location, and protein substrate preference.

EXPERIMENTAL METHODS

Materials

S-Adenosyl-L-[*methyl*-^{14}C]methionine (specific

activity, 55 mCi/mmol; 80 cpm/pmol) was purchased from Amersham Corp., Arlington Heights, IL., and all the proteins used in Table IV were obtained from Sigma Chemical Co., St. Louis, MO. The remainder of the reagents were obtained from various local sources and were of the highest grade available. The *Neurospora crassa* used in these experiments was a peach mutant strain.

Preparation of modified hemoglobin

Chemically methylated bovine hemoglobin was prepared reductively with formaldehyde and sodium borohydride according to the methods previously published[4]. Heme-free hemoglobin was prepared by the method of Yu and Gunsalus[5].

Methylation of protein in vitro

In order to methylate *Neurospora crassa* protein *in vitro*, the fungus was first grown under sterile conditions in 2,800 ml Fernbach flasks containing 700 ml of Vogel's medium[6] at 30°C with constant shaking for approximately 5 days. The mycelia was then harvested on a Büchner funnel and washed with 500 ml of cold deionized water. 4.5 g of packed mycelia was homogenized in 12 ml of water with a tight fitting glass homogenizer, and the homogenate was passed through a double layer of cheese cloth. Three ml of this homogenate (20 mg of protein), 1.5 ml of 0.5 M Tris-HCl buffer, pH 9.0, 1.5 ml of S-adenosyl-L-[*methyl*-^{14}C]methionine (74.7 nmoles; 6.6×10^6 cpm) and 1.5 ml of water were incubated at 37°C for 2 hours. After terminating the reaction with 7.5 ml of 30% trichloroacetic acid, the nucleic acids and phospholipids were removed according to the published procedure[7]. A portion of this preparation was then hydrolyzed in 6 N HCl *in vacuo* at 110°C for 48 hours, and the hydrolyzate was analyzed on a Perkin-Elmer automatic amino acid analyzer equipped with a flow cell for constant monitoring of radioactivity using a Packard Tri-Carb liquid scintillation spectrometer with a counting efficiency of approximately 60%.

The *in vitro* methylation of rat liver nuclei with S-adenosyl-L-[*methyl*-^{14}C]methionine and subsequent fractionation of the protein into histone and acid-insoluble protein ("non-histone nuclear protein") were carried out according to the method described previously[2]. An analysis of the radioactivity incorporated into various amino acids was performed as described above for the *N. crassa* protein.

Enzyme assay

The reaction mixture for the routine enzyme assay contained 0.1 ml of enzyme preparation (protein concentration ranged 0.03~0.10 mg), 0.1 ml of S-adenosyl-L-[*methyl*-^{14}C]methionine (4.98 nmoles; 80 cpm/pmole), 0.1 ml of 0.5 M phosphate buffer, pH 8.6, 0.1 ml of chemically methylated bovine serum hemoglobin (20 mg) and 0.1 ml of water. The reaction was carried out for 20 minutes at 37 °C. The reaction was terminated by the addition of 0.5 ml of 30% trichloroacetic acid, the mixture was heated at 90 °C for 15 minutes, followed by washing with 15% trichloroacetic acid three times. These procedures remove nucleic acids as well as unreacted S-adenosyl-L-[*methyl*-^{14}C]methionine. The above reaction mixture was now treated successively with hot ethanol and once with a mixture of ether:ethanol:chloroform (2 : 2 : 1) to remove phospholipids. When counted for radioactivity at this stage, the substrate (hemoglobin) had an extremely strong quenching effect due to its color. Therefore, 0.3 ml of 30% H_2O_2 was added into the above mixture and heated at about 70 °C for 10 minutes. This process bleached the red color. Finally, the contents were

.nsferred to a scintillation vials which contai-
d 10 ml of scintillation liquid (Formula 963
New England Nuclear Co.). Enzyme activity
expressed as picomoles of S-adenosyl-L-[*me-*
yl-^{14}C]methionine used/min/mg of enzyme
ɔtein.

Subcellular fractionation of rat liver was per-
·med according to the method of Schneider[8],
d the protein concentration was estimated by
: method of Lowry *et al*[9].

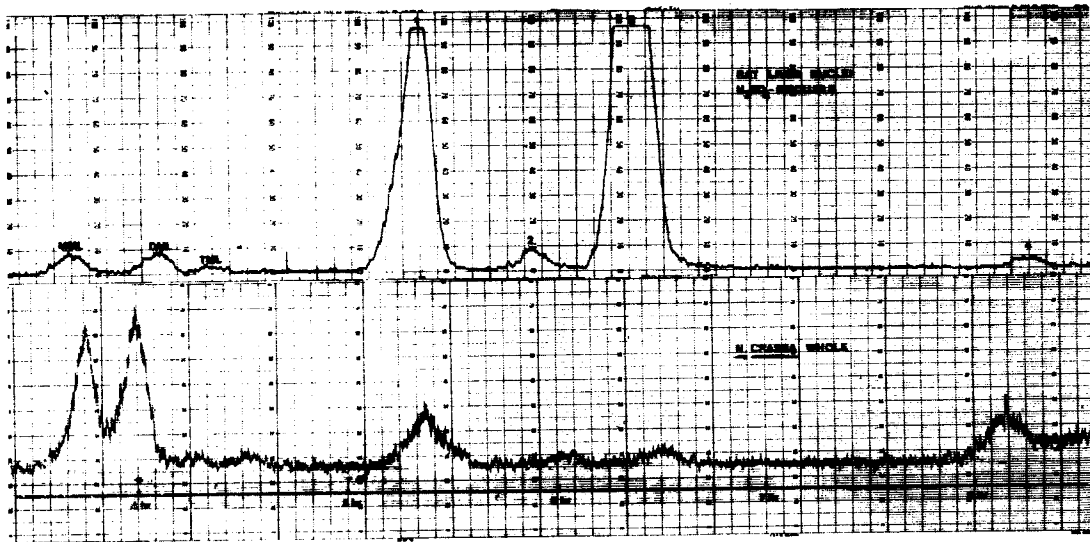
RESULTS

ɛthyl-^{14}C] incorporation pattern of non-histone
clear protein of rat liver

The top panel of Fig. 1 illustrates the [*me-*
yl-^{14}C] distribution pattern in the hydrolyzate
non-histone nuclear protein of rat liver nuclei
methylated *in vitro* with S-adenosyl-L-[*methyl*-^{14}C]methionine. It is seen that, in addition to the three well-characterized lysines (ε-mono, ε-N-di and ε-N-trimethyllysine), there are also four radioactivity peaks (compound # 1 has since been ideyntified as N-methylamine, and three methlated arginine derivatives elute much later on the chromatogram). Previously, it was found that these unknown radioactivity peaks were not present in histone hydrolyzate radiogram[2]. These uncharacterized peaks may be ubiquitous in their occurrence in nature since, as shown in the lower panel of the figure, they also are seen when crude extracts of *N. crassa* are incubated with S-adenosyl-L-[*methyl*-^{14}C]methionine.

Evidence of an enzyme

We attempted to identify a possible enzyme(s)



.g. 1. *Radiogram of HCl-hydrolyzate of non-histone proteins of rat liver nuclei and N. crassa crude extract methylated in in vitro.*
The top panel represents the amino acid analysis radiogram of the HCl-hydrolyzate of non-histone nuclear protein of rat liver nuclei methylated *in vitro* with S-adenosyl-L-[*methyl*-^{14}C]methionine. The lower panel is the hydrolyzate radiogram for the *N. crassa*. MML, DML, and TML represent ε-N-mono-, ε-N-di and ε-N-trimethyllysine, respectively. Detailed experimental procedures are described under Methods.

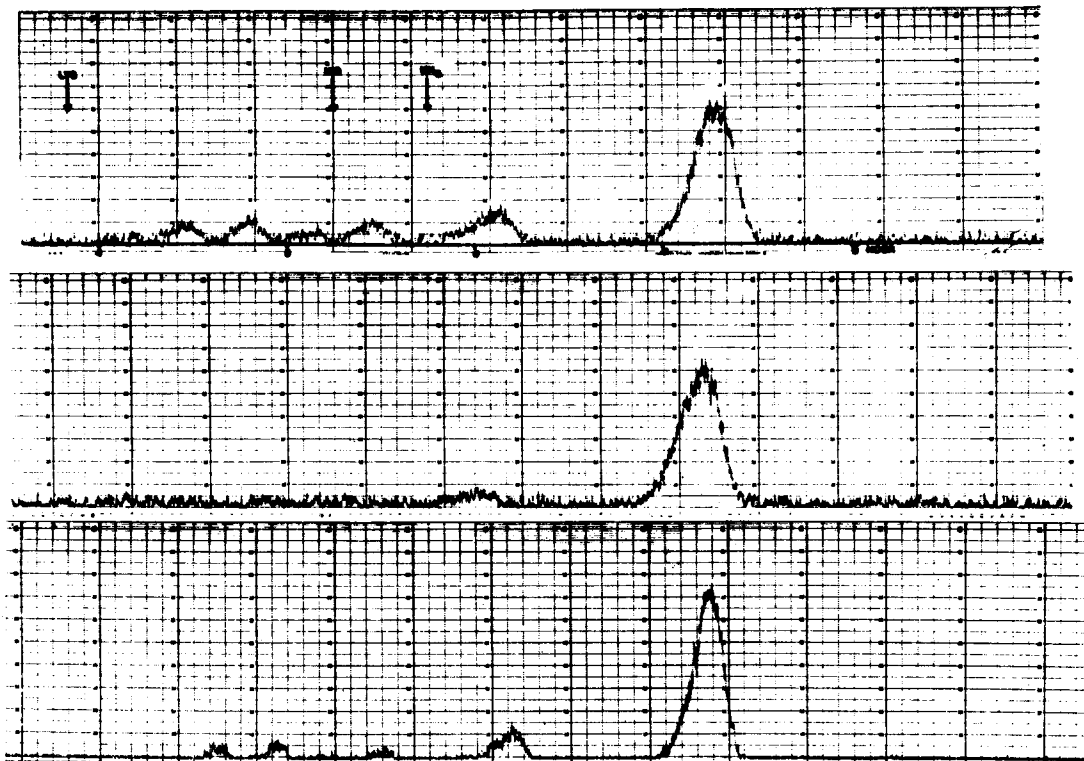


Fig. 2. ***Radiogram of HCl-hydrolyzate of bovine hemoglobin methylated by rat liver homogenate.***
Top panel: Purified rat liver nuclei were methylated *in vitro* with S-adenosyl-L-[*methyl*-^{14}C]methioni: in the absence of exogenously added protein substrate, and non-histone nuclear protein was isolated aı hydrolyzed.
Middle panel: Rat liver whole homogenate was incubated with S-adenosyl-L-[*methyl*-^{14}C]methionine the presence of bovine hemoglobin, and the total protein after removing unreacted S-adenosyl-L-[*m thyl*-^{14}C]methionine, nucleic acids and phospholipids was hydrolyzed.
Lower panel: An equal amounts of both *Top* and *Middle* hydrolyzates were analyzed together. Tl rest of the experimental procedures are described under Methods.

responsible for the synthesis of these compounds. The results depicted in Fig. 2 demonstrate that the formation of compound 3 seen in Fig. 1 is enzymatically catalyzed. The top panel of Fig. 2 is the [*methyl*-^{14}C] distribution of acidic nuclear protein similarly shown in the top panel of Fig. 1, in a much reduced amount in order to accentuate the peak of compound 3. When rat liver homogenate was reacted with either bovine hemoglobin of histone in the presence of S-adenosyl-L-[*methyl*-^{14}C]methionine an the assay mixture treated as described in Mє thods, the subsequent HCl-hydrolyzate analysi by radiochromatography showed a single radioac tivtiy peak which is seen in the middle panє of Fig. 2. Neither endogenous (in the absenc of added substrate protein) nor boiled homogє nate produced this peak. Furthermore, whe analyzed together with the HCl-hydrolyzate c acidic nuclear protein (sample in the top panel)

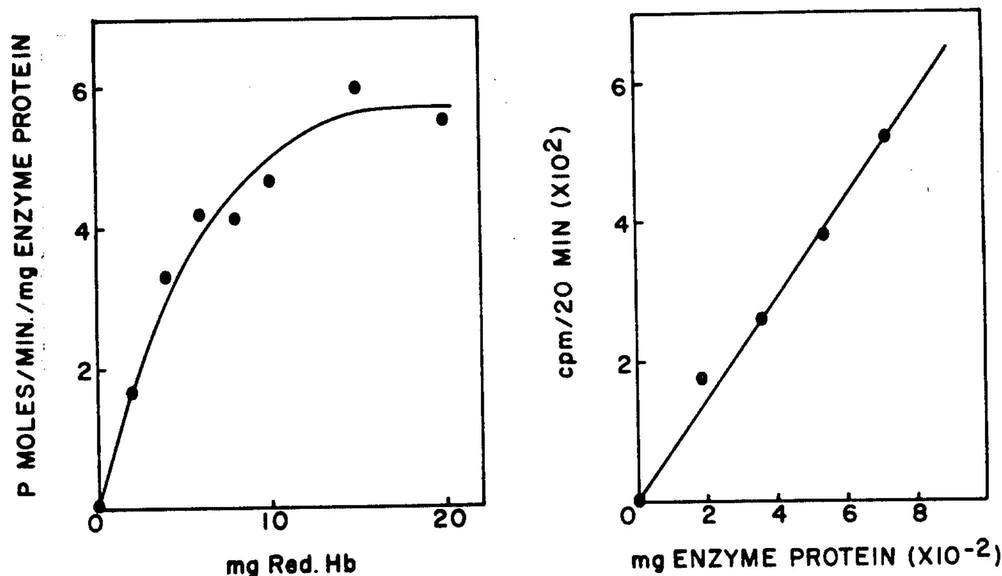


Fig. 3. *Effect of substrate protein concentration and the enzyme on the enzyme activity*
Left panel illustrates the effect of hemoglobin concentration on the enzyme activity, and the right panel shows the effect of enzyme protein concentration. Detailed experimental procedures are described under Methods.

the radioactivity co-migrated with the compound 3 peak (the lower panel).

General properties of the enzyme

The left panel of Fig. 3 illustrates the effect of substrate protein concentration (non-enzymatically methylated bovine hemoglobin) on the enzyme activity. It is obvious from the figure that more than 20 mg of hemoglobin per assay is required to saturate the enzyme. However, due to its low solubility, 20 mg per assay was employed routinely in the following experiments. The right panel of Fig. 3 shows the relationship between the amount of enzyme (rat liver homogenate) and the enzyme activity. Here, it is seen that a linear relationship exists with up to 0.08 mg of enzyme protein.

Fig. 4 illustrates the effect of the time of incubation (left panel) and pH (right panel) on the enzyme activity. The enzyme activity is

Table I: Distribution of the Enzyme Among Various Rat Organs.

Organs	Enzyme activity (p mols methyl used/min/mg enzyme protein)
Liver	4.53
Thymus	1.11
Testis	0.83
Spleen	0.73
Kidneys	0.57
Brain	0.48
Heart	0.33
Muscle	0.27
Lungs	0.17
Pancreas	0.02

A male rat weighing approximately 220 g was used. The whole homogenate was prepared in 9 volumes of cold water by electrically driven teflon-glass homogenizer and the whole homogenates were passed through a double layer of cheese cloth. One-tenth ml of whole homogenate (protein concentration ranges between 0.03~0.1 mg), 0.1 ml of S-adenosyl-L-[*methyl*-^{14}C] methionine (4.98 nmoles; 80 cpm/pmol), 0.1 ml of 0.5 M phosphate buffer at pH 8.6, 0.1 ml of non-enzymatically methylated bovine hemoglobin (20 mg) and 0.1 ml of water were incubated at 37 °C for 20 minutes. The rests of the experimental procedures are described under Methods.

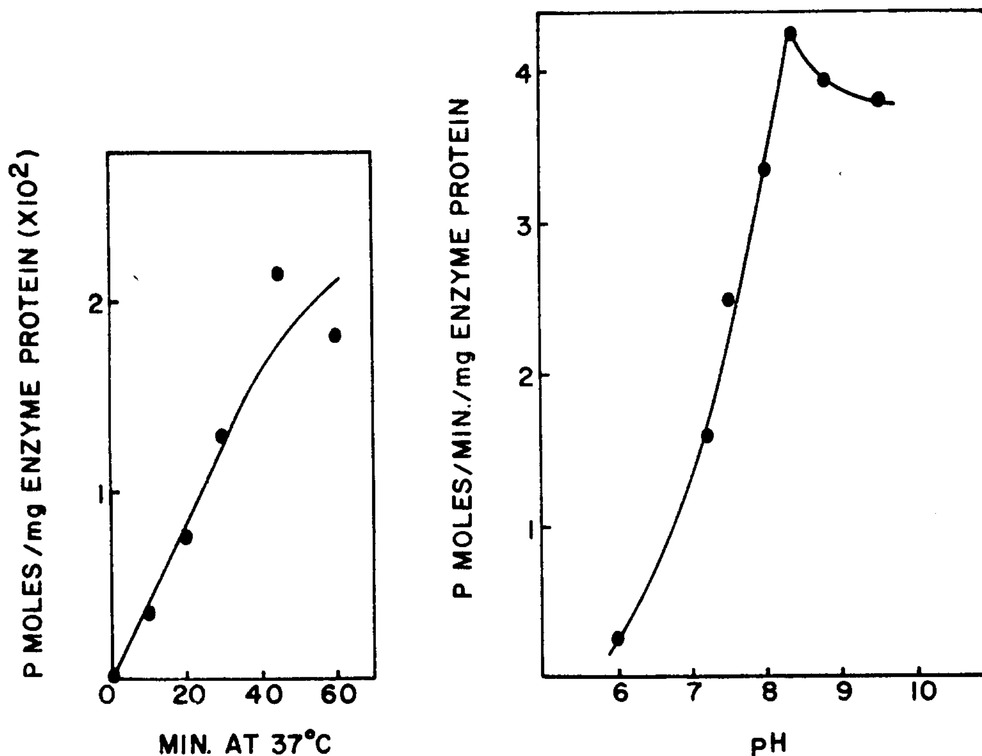


Fig. 4. *Effect of incubation period and pH on the enzyme activity*
Left panel represents the effect of incubation time, and the right panel shows the effect of pH on the enzyme activity. Rat liver homogenate was used as the enzyme. In the pH study, phosphate buffer was used for pH 6~9, and Tris-HCl for pH 9.5. More detailed experimental procedures are described under Methods.

Table II: Subcellular Distribution of the Enzyme in Rat Liver

Subcellular fractions	Specific activity	Total enzyme activity	Per cent of total enzyme activity
	(pmoles of methyl/min/mg enzyme protein)	(pmoles of methyl/min)	(%)
Whole homogenate	4.20	719.5	100.0
Nuclear	5.47	547.9	76.2
Mitochondrial	13.50	29.8	4.1
Microsomal	6.98	15.7	2.2
Cytosol	0.62	39.8	5.5

directly proportional to the period of incubation up to 60 minutes, and the pH optimum was found to lie in the range of 8~10. Thus, in subsequent experiments, the pH of 8.6 and 20 minute incubation period were used.

Distribution of the enzyme

Table I lists the specific activity of the enzyme among various rat organs. Liver showed the highest enzyme activity followed by thymus and testis. The results on the enzyme's subce-

Table III: Localization of the Enzyme in the Chromatin of Rat Liver

	Enzyme activity (pmoles of methyl used/min/mg enzyme protein)
Whole homogenate	3.78
"Pure" nuclei	2.03
Chromatin	3.42

Ten g of rat liver was homogenized in 90 ml of 0.25 M sucrose solution containing 6×10^{-3} M $CaCl_2$, passed through a double layer of cheese cloth, and then centrifuged at 700 g for 10 minutes. Crude nuclei pellet was washed twice with the above sucrose solution, resuspended in 60 ml of 2.4 M sucrose solution, and centrifuged at 63,000 g for 1 hour. One-fifth of the pellet was resuspended in an appropriate amount of 0.01 M Tris-HCI buffer at pH 8.0. This is designated as "pure" nuclei. The remaining portion of the above pellet nuclei was homogenized in 35 ml of 0.01 M Tris-HCl buffer at pH 8.0 and then centrifuged at 39,000 g for 10 minutes. The pellet was washed once with 35 ml of Tris-HCl buffer, resuspended in 30 ml of 1.7 M sucrose solution, and then centrifuged at 63,000 g (in swinging bucket head) for 3 hours. The pellet was resuspended in 4 ml of the above Tris-HCl buffer. The final preparation is designated as chromatin.

llular and suborganelle location are summarized in Table II and III respectively. It is apparent that the enzyme is mostly nuclear-bound, and further study specifically showed that this enzyme is bound to chromatin.

Substrate specificity

A list of various proteins as substrate for the enzyme is shown in Table IV. Bovine hemoglobin appears to be the best substrate followed by histone. It is of interest to note that reductive methylation of the hemoglobin with formaldehyde and sodium borohydride. increased the substrate-capacity of this protein.

All attempts at purifying the enzyme from rat liver were unsuccessful. These attempts included ammonium sulfate precipitation, calcium phosphate gel treatment, and DEAE-Sephadex column chromatography.

Table IV: Substrate Specificity of the Enzyme

Protein substrate	Enzyme activity (pmoles of methyl used/min/mg enzyme protein)
Hemoglobin (bovine)	
"Native"	2.39
Reductively methylated	4.73
Reductively methylated and heme-free*	0.50
Reduced with 2-mercaptoethanol*	0.33
Histone type II-A (Sigma Chemical Co.)	1.45
γ-Globulin	0.49
Ribonuclease (pancreatic)	0
Albumin (bovine serum)	0

One-tenth ml of 0.5 M phosphate buffer at pH 8.6, 0.1 ml of S-adenosyl-L-[*methyl*-^{14}C]methionine, 0.1 or 0.2 ml of protein substrate solution (suspended as 100 mg/ml in water). and 0.1 ml of rat liver whole homogenate (0.031 mg protein) in a total 0.5 ml of incubation mixture were incubated at 37 °C for 20 minutes. The rest of the experimental procedures are described under Methods.

Further characterization of the enzyme product

It was found that compound 3 seen in Fig. 3 is resistant to 1.0 N NaOH hydrolysis at 110°C for 48 hours. This indicates that the compound is not a methylated derivative of serine, threonine, cysteine or arginine.[10,11] When reacted with L-amino acid oxidase from snake venom [L-Amino acid:0_2 oxidoreductase; EC 1.4.3..2] at both pH 7.2 and 8.6,[12] the compound remained unaltered. This result indicates that either the α-NH_2 group is blocked, or that the mother compound itself has a nature of insensitivity towards the L-amino acid oxidase.[12] The radioactivity peak still appeared on the radiochromatogram even after an exhaustive extraction of nucleic acids by repeated treatment of the sample at 90 °C in 15% trichloroacetic acid, thus excluding the possibility that the radioactive compound resulted from the nucleic acids.

DISCUSSION

In this communication, we have shown that compound 3, one of the as yet unidentified protein methylation products, is formed by an enzymatic process. This assertion is substantiated by the fact that boiled rat liver homogenate in the presence of substrate as well as native rat liver homogenate in the absence of added substrate both failed to show compound 3 formation. The PH optimum of the compound 3-forming enzyme(s) is about 8.6 which is rather close to the PH optimum of the well-characterized protein methylase III [S-Adenosyl-L-methionine: protein-lysine N-methyltransferase; EC 2.1.1.43]. However, due to the unknown nature of the methylated product, this enzyme (s) can not be one of the protein-specific methyltransferases previously characterized[1] Thus, the present findings indicate the discovery of a new enzyme(s).

The subcellular location of most of the rat liver enzymatic activity is in the nucleus with an indication from Table III that the enzyme (s) is chromatin-bound. This subcellular and suborganelle location of the enzyme(s) is consistent with the observed methylation of non-histone nuclear protein in the intact rat liver nuclei.[2] However, an interesting controversy is raised in the fact that no compound 3 formation is seen in the histone fraction of the *in vitro* methylated nuclei;[2] while exogenously added histone to the rat liver homogenate demonstrates that it is a good substrate for the enzyme (Table IV). This apparent inconsistency can probably explained by reasoning that a steric hindrance or protein conformation effect prevents histone, when in the intact nuclei, from being methylated by the compound 3-forming enzyme(s). Thus, the addition of exogenous free histone to the rat liver homogenate shows that no such steric hindrance exists when the histone is in the free form. Another instance where protein conformation seems to play a crucial role in determining the substrate-capacity of a protein towards this enzyme(s) is shown in the substrate-capacity of the variously treated hemoglobins (Table IV). Here, it is seen that reductively methylated hemoglobin serves as the the best substrate.

Another interesting observation is the fact that compound 3 formation is seen in the *N. crassa* crude homogenate. Although it is a matter of pure speculation as to the nature of the respective protein substrate(s) in *N. crassa*, it is still interesting that the compound 3 forming enzyme occurs in such diverse organisms as *N. crassa* and rat. An object of further study would be to ascertain whether the respective compound 3 forming enzyme in both systems have functionally similar roles.

The radioactive compound 3 has the following properties; the compound is resistant towards treatment with 6 N HCl, 1.0 N NaOH and the action of L-amino acid oxidase. These properties exclude the possibility that the compound is a derivative of serine, threonine, cysteine, arginine or tryptophan. Furthermore, the α-NH_2 group may be blocked by methyl substitution. In addition, the following possibilities are also ruled out due to their respective elution positions on the amino acid analyzer: O-Methylserine, O-methylthreonine, O-methyltyrosine, S-methylcysteine, 1-N-methylhistidine, 3-N-methylhistidine, 1,3-dimethylhistidine, ε-N-mono-, ε-N-di- and ε-N-trimethyllysine, N^G-mono-, N^G,N^G-di- and N^G,N'^G-dimethylarginine. Recently, various α-N-methyl substituted amino acids have been reported; such as α-N-trimethylalanine in *E.*

oli ribosomal protein[13] or N-dimethylproline in ytochrome *c*.[14] It is quite possible that the ompound 3 might be α-N-methyl substituted asic amino acid such as lysine or histidine. Iowever, a definite conclusion can only come rom further investigation.

The biological significance of the present nding is not clear at present. Recently, after njecting [*methyl*-^{14}C]methionine into rat, Boffa *t al.* observed an unknown radioactive peak on he amino acid radiochromatogram of the HCl-ydrolyzate of liver nuclear 40s HnRNP[15]. The nknown radioactivity peak appeared between mmonia and N^G,N^G-dimethylarginine, which losely corresponds to the elution position of ur unknown compound 3. Since nuclear acidic rotein[16] or nuclear 40s HnRNP[17] may play n important role in genetic expression or trans-ription, methylation of these proteins by a hromatin-bound enzyme could be profoundly mportant in differentiation and development.

ACKNOWLEDGEMENTS

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