HIGHLY SELECTIVE IN VIVO ETHYLATION OF RAT LIVER NUCLEAR PROTEIN BY ETHIONINE*

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SUMMARY

Ethionine ethylates rat liver nuclear proteins in a highly selective manner. The proteins most ethylated are those insoluble in 20-40% $(NH_4)_2SO_4$ in the saline soluble fraction. With L-ethionine-ethyl-1-^{14}C, only one peak of radioactivity was found. This peak has been tentatively identified as an ethyl analogue of methyl-guanidino arginine, recently reported to occur in nuclear proteins. No radioactive ethionine was found in the proteins after giving ethyl-labeled or ^{35}S-labeled ethionine. The pattern of labeling with methionine-methyl-^{14}C is more complex and is presented.

Ethionine, the ethyl analogue of methionine (1) and a carcinogen for the rat (cf.2), has several metabolic effects including the rapid induction of an hepatic ATP deficiency and the ethylation of various cellular components (cf. 2-4). Among the latter, transfer RNA (5-7) and some proteins of the nucleus (7) are particularly active as acceptors. The chemical nature of the ethylated components in tRNA has been clarified to a considerable extent by Rosen (8). However, the nature of the protein components labeled by ethionine in liver nuclei is not well understood and is the subject of this study. A non-histone saline soluble fraction of nuclear proteins is much more heavily ethylated than is any other fraction. This fraction contains only one labeled component, a basic amino acid which is probably the ethyl analogue of a guanidino-methylated arginine, recently reported by Paik and Kim (9, 10) to occur in mammalian cell nuclei.

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MATERIALS AND METHODS

Male white Wistar rats, weighing from 260 - 400 grams were used. The L-ethionine-ethyl-1-^{14}C (3.95 mc/mmole), L-methionine-methyl-^{14}C (14.75 mc/mmole), and D, L-arginine-guanidino ^{14}C (2.9 mc/mmole) were purchased from New England Nuclear, glycine-2-^{14}C (6.75 mc/mmole) from Nuclear Chicago Corp., and D, L-arginine-5-^{14}C (4.5 mc/mmole) from Schwarz BioResearch. Rats treated with ethionine-ethyl-1-^{14}C, methionine-methyl-^{14}C or arginine-guanidino-^{14}C were injected intraperitoneally once a day for 4 days with 14.3 μc. These animals were killed on the morning of the fifth day.

Animals were maintained on Wayne Lab Blox ad libitum. Food was removed 16 hours before the animals were killed by decapitation. The livers were quickly removed, rinsed in ice cold physiological saline and weighed. Liver cell nuclei were isolated as described previously (7). The nuclear pellet was then extracted 3 times with 15 ml of ice cold 0.14 M NaCl, followed by centrifugation for 20 min at 10,000 rpm in a Spinco type 30 rotor. The protein in the supernatant is designated saline soluble protein. The final pellet was then extracted with 15 ml of 0.25 N HCl at 4° C for 1 hour to yield a histone fraction. This extract was centrifuged for 10 min at 20,000 rpm in a Spinco 30 rotor. The supernatant fluid of this centrifugation yielded histones while the pellet represents acid insoluble protein. Ammonium sulfate fractionations of the saline soluble protein were performed at 0° C followed by centrifugation at 20,000 rpm for 10 min in a Spinco type 30 rotor.

Proteins were prepared from all fractions, except histones, by TCA precipitation as described previously (7). Proteins from the histone fraction were precipitated in 25% TCA and washed in the following manner: once with 25% TCA, once with absolute ethanol containing 10% potassium acetate, once with

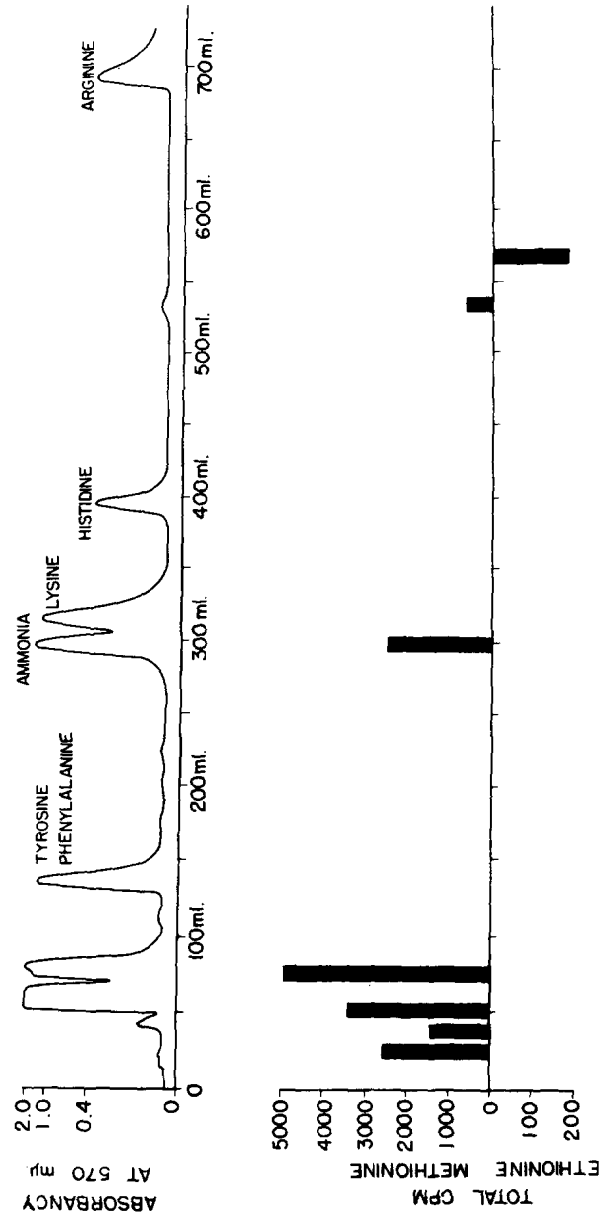

Figure 1:

Amino Acid Pattern of Saline Soluble Nuclear Protein: About 7-8 mg of protein hydrolysate were chromatographed on a 50 cm x 0.9 cm column of a Beckman model 120 B automatic amino acid analyzer. The column temperature was held at 30° C for the first 680 minutes and then held at 50° C for the duration of the analysis. The eluting buffer contained 0.38 M sodium citrate, pH 4.26, and had a flow rate of 0.5 ml per minute. Figure 1A indicates positions of amino acids as determined by ninhydrin reaction, while Figure 1B represents radioactivity from methionine-methyl-^{14}C or as indicated, radioactivity from ethionine-ethyl-^{14}C.

absolute ethanol, twice with ethanol ether (3:1), and twice with ether. After drying, the protein powder was hydrolyzed and counted in the same manner as the other protein powders.

Aliquots of the dried protein used for amino acid analysis were hydrolyzed in 6 N HCl for 16 hours at 110° C. Following flash evaporation of the HCl, the hydrolysates were dissolved in pH 2.2 citrate buffer containing thiodiglycol and Brig-50 (see Figure 1). Aliquots were analyzed on a Beckman 120 amino acid analyzer to which was attached in series an ultraviolet monitor (260 mμ) and a Nuclear Chicago scintillation counter equipped with a flow cell as previously described (4).

RESULTS

Labeling Patterns of Nuclear Proteins. Table 1 records the different labeling patterns for different liver nuclear protein fractions following the administration of L-ethionine-ethyl-1-^{14}C, L-^{35}S-ethionine or L-methionine-methyl-^{14}C. With ethyl-labeled ethionine, the highest specific activity (SA) by far was found in the 20 - 40% $(NH_4)_2 SO_4$ fraction of the saline soluble (SS) proteins. It appeared probable that the bulk of this label is in the form of an ethylated component rather than as ethionine per se, since this fraction was not heavily labeled by ^{35}S-ethionine (Table 1). With methionine, the various fractions showed much less difference, with the exception of the histone fraction which had only one-half or less the SA of the others.

Patterns of Radioactivity in Hydrolysis Products. Chromatographic analysis of the amino acids from the 20 - 40% $(NH_4)_2 SO_4$ fraction labeled with ethionine-ethyl-1-^{14}C showed no radioactivity in any of the acidic or neutral amino acids including ethionine, which in this system elutes with leucine. All the radioactivity appeared as a single peak in the region of elution of basic amino acids

TABLE 1

SPECIFIC ACTIVITY OF RAT LIVER PROTEIN FRACTIONS FOLLOWING ADMINISTRATION OF RADIOACTIVE ETHIONINE OR METHIONINE

Nuclear Protein Fraction	Specific Activity of Protein Fraction after Administration of# L-Ethionine-Ethyl-1-^{14}C	L-^{35}S-Ethionine	L-Methyl-^{14}C-Methionine
		cpm/mg	
Acid Insoluble Protein	140	143	2300
Histones	110	38	855
Ammonium Sulfate Fractions of Saline Soluble Protein			
0% - 20%	319	744	1791
20% - 40%	600	166	2241
40% - 60%	335	-	2451
60% - 80%	151	-	-
80% -	107	-	-
40% -	-	97	-
60 -	-	-	1582

Each rat received daily injections for four days of either 14.3 μc of L-ethyl-1-^{14}C ethionine, (4 rats), 14.3 μc of L-methyl-^{14}C-methionine, (4 rats), or 60 μc of L-^{35}S-ethionine, (4 rats). Animals were sacrificed and various protein fractions isolated as described in the text.

Each value is the specific activity in cpm/mg protein of the corresponding pooled sample.

between histidine and arginine (Figure 1B). This peak was consistently preceded by a small ninhydrin-positive peak (peak VI in Figure 1A) which was quite heavily labeled with methionine-methyl-^{14}C but not by ^{35}S-ethionine, even though the latter significantly labeled both cystine and methionine. No peaks of radioactivity were observed at positions corresponding to ϵ-N-monoethyl- or ϵ-N-diethyl-lysine[1]. With ^{35}S-ethionine, the only radioactive peaks were methionine and cystine. As with ethionine-ethyl-^{14}C, the ethionine peak was not labeled with ^{35}S-ethionine. Thus, with two forms of labeled ethionine, no evidence of incorporation of ethionine into nuclear proteins could be found. This finding complicates the interpretation of the biochemical significance of different ratios of labeling protein with these two radioactive forms of ethionine (11).

With methionine-methyl-^{14}C, six radioactive peaks were consistently found. In addition to the methionine peak, which had the highest SA, 3 other peaks were present in the neutral and acidic amino acid fractions. These no doubt represented methionine sulfoxide or sulfone, serine and glycine. In the basic amino acid range, one peak preceded lysine and the other was the pre-arginine peak (peak VI, Figure 1A). The pre-lysine peak is most probably ϵ-N-monomethyl- or ϵ-N-dimethyl-lysine.

The pattern of labeling with methionine or ethionine-ethyl-^{14}C was reproducibly different with different protein fractions. For example, with the whole histone fraction, the only radioactive peak found with ethionine-ethyl-^{14}C was the pre-arginine one but the level of radioactivity was much less than in the SS proteins. With methionine, the same fraction had 5 radioactive peaks with 3 in the basic amino acid range. The positions of the first two peaks in this

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[1] These compounds were kindly sent to us by Dr. Paik.

range coincided with N-monomethyl- and di-methyl- lysines while the third one was the pre-arginine peak coinciding with a small ninhydrin-positive one just preceding the peak labeled by ethionine. The height of this ninhydrin-positive peak varied consistently in different nuclear protein fractions. Also, this peak was not detectable in hydrolysates of whole cytoplasmic or ribosomal protein (14 mg of protein).

DISCUSSION

It is evident that the labeling patterns of some nuclear protein fractions are quite different with each of the three labeled compounds. The new findings are the unusually high activity of a pre-arginine peak with either ethionine-ethyl-^{14}C or methionine-methyl-^{14}C without any evidence of incorporation of ethionine per se. With methionine, the pre-arginine peak coincides with a ninhydrin-positive peak which appears to be neither methyl lysines nor methyl histidines. This peak also labels to a small degree with guanidino-^{14}C-arginine or arginine-5-^{14}C but not with labeled lysine or histidine[2]. Thus, the available data point to the radioactivity in the pre-arginine peaks from methionine or ethionine as being most likely methylated or ethylated arginine respectively, probably alkylated in the guanidino group (10). This offers new support for the conclusion of Paik and Kim (9, 10) concerning the presence of methylated guanidines in nuclear proteins and suggests that at least one is a very active site for both methylation and ethylation.

The highly selective nature of the ethylation of the nuclear protein fractions could conceivably have a relationship to carcinogenesis (7). In addition, when highly purified, these proteins may prove to be useful in further

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[2] Friedman and Farber, unpublished data.

studies on the possible role of selectively alkylated proteins in nuclear and DNA function.

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