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Incorporation *in vivo* of Methionine and Ethionine into and the Methylation and Ethylation of Rat Liver Nuclear Proteins

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Modification of nuclear proteins can be expected to change their interaction with nucleic acids, and this in turn could have profound effects on the synthetic activity of the nucleus. Methylation of histones has been shown to occur on residues already in polypeptide chains (Allfrey, Faulkner & Mirsky, 1964). Methylation *in vitro* of calf thymus nuclear proteins (Paik & Kim, 1967) and of isolated rat liver nuclei (Sekeris, Sekeri & Gallwitz, 1967) has been reported with *S*-adenosyl[*Me*-^{14}C]methionine as the source of methyl groups. In the thymus study all of the nuclear protein fractions isolated were methylated. Ethylation of liver nuclear proteins has also been shown to occur when [*Et*-^{14}C]ethionine was injected into rats (Farber *et al.* 1967). However, there was a higher relative degree of methylation than ethylation.

In the present work *in vivo* we found methionine to be incorporated more readily than ethionine into all the nuclear proteins studied. Lysine-rich histones incorporated more methionine and ethionine than did arginine-rich histones. Chromatin-associated acid protein also exceeded residual acid protein in this capacity. In contrast with amino acid incorporation, alkyl group transfer (ethylation and methylation of residues) occurred more to the arginine-rich histones, especially fraction F3, than to the lysine-rich histones. Chromatin-associated acid protein accepted few ethyl or methyl groups; however, methylation of residual acid protein was considerable.

L-[^{35}S]Methionine (175mc/m-mole), L-[*Me*-^{3}H]-methionine (2·92c/m-mole), L-[*Me*-^{14}C]methionine (52mc/m-mole), L-[^{35}S]ethionine (3·1mc/m-mole), L-[*Et*-1-^{3}H]ethionine (41·3mc/m-mole) and L-[*Et*-1-^{14}C]ethionine (3·67mc/m-mole) were purchased from New England Nuclear Corp. (Boston, Mass., U.S.A.) or Nuclear–Chicago Corp. (Des Plaines, Ill., U.S.A.). After 12hr. starvation, groups of five or six male 180–220g. Nelson Wistar rats were injected intraperitoneally with either 100μc of ^{14}C- or ^{35}S-labelled compound or 200μc of ^{3}H-labelled compound dissolved in 0·14M-NaCl. At 4hr. after injection they were killed by decapitation. The livers were homogenized in 0·25M-sucrose–2mM-$CaCl_2$–2mM-$MgCl_2$–5mM-tris–HCl buffer, pH7·2 (Hnilica, Kappler & Hnilica, 1965), and filtered through two layers of cheesecloth. The crude nuclei were sedimented by centrifugation at 600*g* for 10min. The nuclei were then purified by centrifugation for 1hr. at 44000*g* in 2·2M-sucrose–5mM - $MgCl_2$ – 5mM - tris – HCl buffer, pH7·2 (Chauveau, Moulé & Rouiller, 1956). The nuclei were resuspended in and sedimented from 0·25M-sucrose–5mM-$MgCl_2$–5mM-tris–HCl buffer, pH7·2. The nuclei were then extracted twice by stirring for 20min. with 0·14M-NaCl followed by 0·5mM-$MgCl_2$–10mM-tris–HCl buffer, pH7·6. Chromatin was extracted with 1M-NaCl (Wang, 1967). The insoluble material was extracted with 50mM-NaOH and is referred to below as residual acid protein. Nucleohistone was precipitated from the chromatin extract by dialysing it against 6vol. of water (Wang, 1967). The soluble material constitutes the chromatin-associated acid protein. The nucleohistone was washed with ethanol. Fractionation of histone into lysine-rich histone fractions F1 and F2b and into arginine-rich histone fractions F2a and F3 was essentially performed as described by Johns (1964) (method 2).

Acid protein fractions were brought to 10% (w/v) trichloroacetic acid and stored at 4° overnight. The resulting precipitate was treated with 10% trichloroacetic acid for 15min. at 90° followed by 5% trichloroacetic acid for 10min. at 95° to solubilize contaminating nucleic acids. The insoluble acid protein was washed with ether to remove excess of trichloroacetic acid and dissolved in 50mM-NaOH. After determination of the protein content by the method of Lowry, Rosebrough, Farr & Randall (1951) and the RNA content by the orcinol method (Dische & Schwartz, 1937), 0·5ml. of protein sample was transferred to a counting vial and evaporated to dryness in an oven at 50°. It was wetted with a minimal amount of water, dissolved in Nuclear–Chicago Solubilizer and counted on a three-channel scintillation counter with 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene. The efficiency was determined by the channels-ratio method, with an external barium standard, and the results are presented in distintegrations/min./mg. of protein.

Whereas the incorporation of the ^{35}S label was due primarily to the amino acid alone (Gaitonde &

Table 1. *Incorporation of label from ethionine and methionine into nuclear proteins*

The Table shows the degree of labelling of nuclear proteins extracted from the livers of male rats (180–220g.) injected 4hr. previously with 100μc of ^{14}C or ^{35}S-labelled compound or 200μc of ^{3}H-labelled compound. Incorporatiion is expressed in disintegrations/min./mg. and $\mu\mu$moles of labelled amino acid after correction to equal amounts of amino acid injected. The final column in each set expresses in the form of a ratio the incorporation of the ethyl (methyl)-labelled compound as compared with the ^{35}S-labelled compound.

	L-Ethionine				
	Et-1-$^{3}H(^{14}C)$-labelled		^{35}S-labelled		
	(disintegrations/min./mg.)	($\mu\mu$moles/mg.)	(disintegrations/min./mg.)	($\mu\mu$moles/mg.)	$^{3}H(^{14}C)/^{35}S$ ratio
Chromatin-associated acid protein	1944	210	1096	190	1·1
Residual acid protein	1231	120	763	133	0·9
Arginine-rich histone	426	46	72	12	3·8
Lysine-rich histone	343	37	89	15	2·5
Lysine-rich histone fractions					
Fraction F1	361	39	90	15	2·6
Fraction F2b	306	33	79	14	2·4
Arginine-rich histone fractions					
Fraction F2a	333	36	61	10	3·6
Fraction F3	618	66	75	13	5·1
	L-Methionine				
	Me-$^{3}H(^{14}C)$-labelled		^{35}S-labelled		
	(disintegrations/min./mg.)	($\mu\mu$moles/mg.)	(disintegrations/min./mg.)	($\mu\mu$moles/mg.)	$^{3}H(^{14}C)/^{35}S$ ratio
Chromatin-associated acid protein	16180	3700	17670	4700	0·8
Residual acid protein	33400	7600	9510	2500	3·0
Arginine-rich histone	3640	830	1020	270	3·1
Lysine-rich histone	2790	640	1330	350	1·8
Lysine-rich histone fractions					
Fraction F1	2440	560	1360	360	1·6
Fraction F2b	2940	670	1350	360	1·9
Arginine-rich histone fractions					
Fraction F2a	3060	700	980	260	2·7
Fraction F3	4520	1030	1010	270	3·8

Gaull, 1967), when the label was on the alkyl group the radioactivities were due not only to the incorporation of the amino acid but also to transferred ethyl group (ethylation) and transferred methyl group (methylation). The differences in ^{35}S-, ^{14}C- and ^{3}H-labelling are shown in Table 1. The specific radioactivity of chromatin-associated acid protein exceeded that of residual acid protein for both of the ^{35}S-labelled compounds. This relationship was also maintained when the label was on the ethyl group of ethionine. However, when methyl-labelled methionine was used the high degree of methylation of residual acid protein caused a reversal in the ratios of the specific radioactivities. Lysine-rich histones incorporated more [^{35}S]ethionine and [^{35}S]methionine than did arginine-rich histones. On the other hand the degree of both ethylation and methylation of arginine-rich histones exceeded that of lysine-rich histones by enough to cause a reversal in the relationship of their specific radioactivities. The specific radioactivities of the subfractions (lower part of Table 1) reflect these changes. In particular it is the high degree of ethylation and methylation of the arginine-rich histone fraction F3 that stands out.

To compare the incorporation, considering that the specific radioactivity of the injected material varied greatly, the amount incorporated into each of these fractions has also been expressed (in Table 1) as $\mu\mu$moles of amino acid incorporated/mg. of protein after correction to equal amounts of amino acid injected. The final column in each set gives the ratio of incorporation of the sum of the alkyl

group plus its corresponding amino acid as compared with the amino acid alone. Anything greater than 1 is presumably due to alkylation. There was no apparent ethylation of residual acid protein or of chromatin-associated acid protein. Although there was no apparent methylation of chromatin-associated acid protein, the residual acid protein was methylated to a high degree. There was both ethylation and methylation of all histone fractions studied, with fraction F3 showing the highest degree of incorporation of label.

It should also be noted that the ratios of the specific radioactivities of nuclear acid proteins to histones in these experiments ranged from 5·5 to 7·1 for ethyl-labelled ethionine or methyl-labelled methionine, whereas it ranged from 12 to 14 for the ^{35}S-labelled amino acids. This would further point to the higher relative degree of ethylation and methylation of histones as compared with nuclear acid proteins.

When one compares the relative degrees of incorporation [^{35}S]methionine was 19–26 times more readily incorporated into the nuclear proteins than [^{35}S]ethionine.

The possibility of contamination of all nuclear protein fractions by highly methylated or ethylated RNA presents a real problem. This was especially true for ethylation, where the specific radioactivity of RNA was as high as four times that of the total nuclear protein. Ultraviolet-spectral analysis revealed the reproducibility of these fractions and did not reveal any appreciable absorption at 260mμ. The orcinol test showed 0·1–0·5% contamination for histones and 1·3% contamination for acid proteins after trichloroacetic acid treatment. Disc electrophoresis, as well as revealing the reproducibility of the fractions, showed no non-migrating material, no RNA-like staining material and no highly labelled non-protein material on the gels. One presumes that RNA contamination has not significantly influenced these results.

Preliminary ion-exchange column studies (by the method of Paik & Kim, 1967) of the basic amino acids from hydrolysates of acid protein from [*Me*-^{14}C]methionine-labelled material corroborate certain of these findings. For the highly methylated residual acid protein, of the recovered radioactivity 74% was found in the area that corresponded to methylated lysine and 24% was eluted with the neutral and acidic amino acids. It was also found that over 85% of the radioactivity of the sparsely methylated chromatin-associated acid protein was not eluted with the basic amino acids.

Contrary to the finding presented here, Paik & Kim (1967) found for methylation *in vitro* of calf thymus nuclei with *S*-adenosylmethionine that lysine-rich histones had the highest percentage of the total radioactivity of all the histone fractions. Tidwell, Allfrey & Mirsky (1968) found for regenerating rat liver that only the arginine-rich histones are methylated. Using *S*-adenosylmethionine with isolated rat liver nuclei, Sekeris *et al.* (1967) found *in vitro*, as we did with methionine *in vivo*, that of the four histone subfractions the arginine-rich fraction F3 had the highest specific radioactivity.

All histone fractions are methylated and ethylated in somewhat the same general pattern; however, the absolute extents of these two processes are different. The identity of pattern is absent with the acid protein fractions, where the patterns of ethylation and methylation of especially residual acid protein are strikingly different. This would point to the presence of a number of different enzymes or enzyme systems with different efficiencies of handling the ethyl group and methyl group.

As is the case *in vivo* with methylation of RNA, the methylation of protein is a complex phenomenon. Both occur on the macromolecules at particular positions and to specific degrees for each molecule. Methylation of histone probably occurs at specific time in the cell cycle (Paik & Kim, 1967; Srinivasan & Borek, 1966; Tidwell *et al.* 1968). The role that alkylation of protein and RNA plays is far from clear, though some RNA function seems to be correlated with its state of methylation (Ortwerth, Del Monte, Rosen & Novelli, 1968).

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