Comparison of the Acetylation of Proteins and Nucleic Acids

BY WOON KI PAIK AND SANGDUK KIM

*Fels Research Institute and Department of Biochemistry, Temple University School of Medicine,
Philadelphia, Pa. 19140, U.S.A.*

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The possibility of acetylation of nucleic acids was examined. Although protein is actively acetylated with [$1\text{-}^{14}\text{C}$]acetic acid in rat liver systems *in vivo* and *in vitro* and in a frog liver system *in vivo*, nucleic acids are not acetylated under these conditions; nucleic acids purified from these sources are without radioactivity. Requirements for acetylation *in vitro* of protein in rat liver are different from those in frog liver; GSH has no effect in the rat liver system and is inhibitory in the frog liver system. Among various acetylated proteins, proteins insoluble in 0.1M-sulphuric acid have the highest radioactivity.

It is well known that nucleic acids are methylated at the macromolecular level, and several review articles have been presented (Borek, 1963; Srinivasan & Borek, 1964, 1966; Borek & Srinivasan, 1966). Enzymic methylation of nucleic acids has been suggested to play a role in neoplasia or differentiation (Borek, 1963). Since proteins such as histones are acetylated (Phillips, 1963) as well as methylated (Murray, 1964; Paik & Kim, 1967*a*) and acetylation of nucleic acids might have profound biological effects, the question arises whether nucleic acids might also be acetylated. We have investigated the problem and have concluded that acetylation of nucleic acids does not occur under conditions in which protein is actively acetylated.

MATERIALS AND METHODS

Sodium [$1\text{-}^{14}\text{C}$]acetate (sp. radioactivity, 19.61mCi/mmol) was purchased from Tracerlab, Waltham, Mass., U.S.A., [$1\text{-}^{14}\text{C}$]acetic acid (sp. radioactivity, 57mCi/mmol; 86c.p.m./pmol) from Amersham/Searle Corp., Des Plaines, Ill., U.S.A., and Bio-Gel from Bio-Rad Laboratories, Richmond, Calif., U.S.A. ATP, CoA, GSH, once-crystallized pancreatic deoxyribonuclease, 5 $\times$ recrystallized pancreatic ribonuclease and trypsin were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Giant bullfrogs (*Rana catesbeiana*) were purchased from Connecticut Valley Biological Supply Co., Southampton, Mass., U.S.A. All other reagents were obtained from local sources.

Acetylation of rat liver homogenate. Rat liver was homogenized in 4 vol. of water and the homogenate was passed through a double layer of cheese-cloth. Acetylation of the homogenate was carried out by the method described by Nohara, Takahashi & Ogata (1968) on a larger scale; 20ml of the homogenate, 2.5mmol of tris-HCl buffer, pH 7.4, 0.25mmol of MgCl_2 , 0.25mmol of ATP, 0.30mmol of GSH, 2.5mmol of KF, 1.0mg of CoA and

10 μC of sodium [$1\text{-}^{14}\text{C}$]acetate in a total volume of 50ml were incubated at 37°C for 2h.

There are three methods available for isolating nucleic acids from tissues: (1) the procedure of Schneider (1945), in which tissues are treated with trichloroacetic acid or perchloric acid at 90°C; (2) NaCl extraction (Davidson & Smellie, 1952); (3) phenol extraction (Kirby, 1956). Since extraction of nucleic acids with trichloroacetic acids or perchloric acid at 90°C degrades a considerable amount of nucleic acids, we adopted the last two methods.

Isolation of nucleic acids by the NaCl extraction method. The incubation mixture described above was cooled in ice and 50ml of 30% (w/v) trichloroacetic acid was added to the mixture. The reaction mixture was centrifuged at 39100*g* for 10min. The precipitate was washed with 2 $\times$ 50ml of cold 15% (w/v) trichloroacetic acid. The precipitate was then washed with 50ml of 1M-ammonium acetate. This process has two advantages: since the pH of ammonium acetate is neutral, treatment of the trichloroacetic acid-treated sample with ammonium acetate gives a neutral pH, which is essential for the next step, and secondly, the unlabelled acetate dilutes out unchanged [^{14}C]acetate. To the residue, 25ml of 2M-NaCl was added and the mixture was incubated at 100°C for 30min. This step to extract nucleic acids was repeated once more with 25ml of 2M-NaCl. The extracts were combined and dialysed overnight against running water. The material remaining in the bag was freeze-dried. The preparation contained DNA, RNA and protein in the proportions 16:47:38, by wt. In a separate experiment in which the extraction was performed with smaller volumes, it was found that the radioactivity recovered in this NaCl extract was approximately 20% of that found in residual protein in rat liver, and about 60% of that in tadpole liver. 'Residual protein' represents the protein fraction that remained after NaCl treatment.

Preparation of RNA by the phenol extraction method. The method employed is essentially that described by Kirby (1956); 40ml of the above incubation mixture or whole homogenate was added to an equal volume of 90% (w/v) phenol and the mixture was stirred magnetically for 1h.

The mixture was centrifuged at 3000g for 1h and the upper layer was pipetted off. The mixture was washed once with 25ml of water and after centrifugation at 3000g for 45 min the aqueous layer was again kept. Potassium acetate was added to the combined water extracts to a concentration of 2% (w/v). RNA was precipitated by the addition of 2vol. of alcohol. After standing overnight the precipitate was collected by centrifugation and washed once with alcohol. The insoluble pellet was dissolved in 20ml of water. Any remaining alcohol was evaporated at 23°C under reduced pressure. The aqueous solution was mixed with an equal volume of 2.5M-K₂HPO₄, 0.05vol. of aq. 33.3% (w/v) H₃PO₄ and 1vol. of 2-methoxyethanol. The mixture was centrifuged and the upper aqueous layer was taken and dialysed against 4l of water overnight at 3°C. The dialysed sample was now made 2% with respect to potassium acetate and RNA was precipitated by addition of 2vol. of alcohol. The precipitate after centrifugation was washed once with alcohol and dissolved in 10ml of water.

Preparation of DNA. The method used is essentially that described by Marmur (1961) for preparation of DNA from micro-organisms; 40ml of the above incubation mixture or whole homogenate was centrifuged at 1000g for 10min and the residue was suspended in 20ml of 0.15M-NaCl-0.1M-EDTA, pH 8.0. To the suspension, 1.6ml of 25% (w/v) sodium lauryl sulphate solution was added and the mixture was incubated at 60°C for 10min. The mixture was cooled, sodium perchlorate was added (final concn. 1M) to the lysed sample, and the whole mixture was shaken with an equal volume of chloroform-3-methylbutan-1-ol (24:1, v/v). The emulsion was centrifuged at 8000g for 5min and the upper layer was carefully pipetted off into a 100ml measuring cylinder. The nucleic acids were precipitated by gentle layering of 2vol. of alcohol on top of the water layer and were separated by centrifugation. In the experiments *in vivo* in which homogenates of rat or frog had been processed, DNA could be 'spooled' on a rod as a thread-like precipitate and was collected in this manner. The precipitate was dissolved in 15ml of 15mM-NaCl-1.5mM-trisodium citrate buffer, pH 7.0. The solution was adjusted to 0.15M-NaCl-0.015M-trisodium citrate by addition of 1.5ml of 1.5M-NaCl-0.15M-trisodium citrate. The mixture was shaken with an equal volume of chloroform-3-methylbutan-1-ol (24:1, v/v) for 15min and centrifuged, and the supernatant was collected. This deproteinization process was repeated five times. The supernatant obtained after the last deproteinization was precipitated with 2vol. of alcohol and the precipitate was dissolved in 20ml of 15mM-NaCl-1.5mM-trisodium citrate. Pancreatic ribonuclease A (1mg) was added to the above suspension and was incubated at 37°C for 30min. After digestion of contaminating RNA, the digest was deproteinized three times with chloroform-3-methylbutan-1-ol (24:1, v/v). Finally, DNA was precipitated with 2vol. of alcohol and was dissolved in 10ml of 15mM-NaCl-1.5mM-trisodium citrate.

Fractionation of acetylated protein into 0.1M-H₂SO₄-insoluble, 0.1M-H₂SO₄-soluble, ethanol-soluble and water-insoluble fractions was carried out by the method described by Paik & Kim (1967b). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), RNA by the method of Dische & Schwartz (1937)

and DNA by the method of Burton (1956). Finally, radioactivity was determined in a scintillation spectrometer with 10ml of Bray's (1960) solution.

RESULTS AND DISCUSSION

Effect of deoxyribonuclease, ribonuclease and trypsin treatment on the sodium chloride extract. Fig. 1 illustrates the elution pattern of the sodium chloride-extracted nucleic acid fraction on Bio-Gel P-100 in 0.01M-potassium phosphate buffer, pH 7.2. Since the exclusion limit of this resin is 100 000 daltons, over 65% of the radioactivity was shown to be associated with molecules with a molecular weight of over 100 000. [1-¹⁴C]Acetate, therefore, must have been incorporated either into nucleic acids or into proteins that are free or conjugated with nucleic acids during dialysis. If the radioactivity is associated with nucleic acids,

Fig. 1. Elution pattern of NaCl extract and effect of deoxyribonuclease, ribonuclease and trypsin treatment. Freeze-dried NaCl extract was dissolved in 0.01M-phosphate buffer, pH 7.2, giving sp. radioactivity 1650 c.p.m./0.5ml. Of this, 0.5ml and 0.5ml of the buffer were loaded on a column (0.8 cm × 60 cm) of Bio-Gel P-100 that had been previously equilibrated with the above buffer. Elution was carried out with the same buffer. Fractions (1ml) were collected at room temperature. Radioactivity was measured by introducing the whole sample into 10ml of Bray's (1960) solution and counting in a scintillation counter. To digest DNA with pancreatic deoxyribonuclease, 0.5ml of the NaCl extract, 0.3ml of 0.2M-phosphate buffer, pH 6.5, 0.2ml of 0.1M-MgCl₂ and 1.0mg of the enzyme were incubated at 37°C for 4h and the incubation mixture was loaded on the column as described. For the action of pancreatic ribonuclease, 0.5ml of the NaCl extract, 0.5ml of 0.1M-phosphate buffer, pH 7.3, and 1.0mg of the enzyme were incubated, and for trypsin, 0.5ml of the NaCl extract, 0.5ml of 0.1M-phosphate buffer, pH 7.3, and 1.0mg of trypsin were incubated. The rest of the procedures were the same as before. Recovery of the radioactivity on the column was practically complete. ○, Control; ●, deoxyribonuclease-treated; △, ribonuclease-treated; ▲, trypsin-treated.

treatment of the sample with nucleases should diminish the size of the molecule, the radioactivity appearing as a result in later fractions of the column. As shown in Fig. 1, treatment of the sample with pancreatic deoxyribonuclease or ribonuclease decreased the amount of radioactivity appearing at the void volume and increased the radioactivity in the region corresponding to a smaller molecule. However, unexpectedly, trypsin had the most profound effect on the elution pattern of the radioactivity. Since trypsin is unable to hydrolyse acetamide bonds, as in *N*-acetyl-L-leucine (Dixon & Webb, 1964), it is highly unlikely that the enzyme deacetylated nucleic acids that might have been acetylated. The results in Fig. 1 therefore strongly suggest that [^{14}C]acetate is associated with protein but not with nucleic acids. Since it has been reported that histone is one of the major acetylated proteins (Nohara *et al.* 1968; Pogo, Pogo, Allfrey & Mirsky, 1968), and the molecular weight of histone is about 21 000 (Teller, Kinkada & Cole, 1965), the effect of deoxyribonuclease or ribonuclease is most likely due to the dissolution of a histone-nucleic acid complex (nucleohistone) by the degradation of the nucleic acids.

Table 1. *Effect of deoxyribonuclease or ribonuclease on solubilization of the NaCl extract in 15% trichloroacetic acid*

The NaCl extract (0.5 ml) contained 3400 c.p.m. The conditions for incubation of the NaCl extract with the nuclease are described in the legend of Fig. 1.

Incubated with	Amount of DNA or RNA in acid-insoluble fraction (μg)		Radioactivity in acid-soluble fraction (c.p.m.)	
	Control	Treated	Control	Treated
Deoxyribonuclease	820	95	1200	1230
Ribonuclease	2280	1205	830	840

The above contention was further confirmed by the following experiments. The NaCl extract was treated with either deoxyribonuclease or ribonuclease and the radioactivity and the amounts of nucleic acids solubilized in 15% trichloroacetic acid were compared. As shown in Table 1, even though DNA and RNA were extensively hydrolysed by the appropriate enzymes, no significant increase in radioactivity was detected in the 15% trichloroacetic acid-soluble fraction. Further, exogenous DNA and RNA were found to be incapable of stimulating the incorporation of radioactivity either into the NaCl extract or into the residual protein. Rather, an adverse effect on the incorporation was found.

Purified RNA or DNA acetylated in vitro. Table 2 lists the relationship between the amount of radioactivity incorporated and the degree of purification of the nucleic acids. Since acid-soluble material and phospholipid were removed by treatment of the sample with trichloroacetic acid and alcohol respectively, the amount of radioactivity listed in Table 2 indicates the amount incorporated into protein or possibly into nucleic acids. The crude incubation mixture of RNA, DNA and protein was shown to contain a considerable amount of radioactivity (620 c.p.m.). However, highly purified RNA or DNA preparations contained no radioactivity.

Purified RNA and DNA acetylated in vivo. To confirm further the results obtained with acetylation *in vitro*, rats and giant bullfrogs were injected intraperitoneally with [^{14}C]acetic acid. Although protein isolated from livers treated with [^{14}C]acetic acid *in vivo* showed a large amount of radioactivity, purified RNA and DNA from these sources showed no significant incorporation of radioactivity (Table 3). The results in Table 3 are additionally noteworthy, since protein acetylation in rat and frog liver are quite different in terms of their requirements, as is shown below.

Fractionation of acetylated protein. When whole homogenates acetylated *in vivo* were fractionated,

Table 2. *Purified RNA and DNA from rat liver homogenate incubated with [^{14}C]acetic acid in vitro*

Samples, consisting of RNA, DNA and protein as listed in the table, were washed three times with cold 15% trichloroacetic acid and twice with hot alcohol to remove the acid-soluble material and phospholipid respectively (Allfrey, Mirsky & Osawa, 1957). For radioactivity counting, the washed sample was suspended in 0.2 ml of 1.5 M- NH_3 and quantitatively transferred into 10 ml of Bray's (1960) solution.

Preparation	Amount in sample				Radioactivity (c.p.m.)
	RNA (mg)	DNA (mg)	Protein (mg)	Ratio of protein/(RNA+DNA)	
Incubation mixture	1.50	0.53	16.2	7.98	620
RNA	4.86	0.45	0.07	0.01	0
DNA	0.03	0.61	0.03	0.05	0

Table 3. *Purified RNA and DNA from rat and frog livers acetylated in vivo*

Rats (weighing about 350 g) and frogs (*Rana catesbeiana*, weighing about 300 g) were injected intraperitoneally with 125 μ Ci and 100 μ Ci of [1-¹⁴C]acetic acid respectively. The animals were killed after 2 h and the livers were homogenized in water to give a 10% (w/v) homogenate. The homogenates were passed through a double layer of cheese-cloth. RNA and DNA were purified from 40 ml samples as described in the Materials and Methods section. Radioactivity was measured as described in Table 2.

Preparation	Amount in sample				
	RNA (mg)	DNA (mg)	Protein (mg)	Ratio of protein/ (RNA + DNA)	Radioactivity (c.p.m.)
Rat					
Whole homogenate	1.20	0.304	18.72	12.4	10650
RNA	1.43	0	0.004	0.003	0
DNA	0.12	0.289	0.005	0.012	3
Frog					
Whole homogenate	1.07	0.296	11.68	8.57	10080
RNA	0.42	0.030	0.04	0.09	6
DNA	0.05	0.517	0.04	0.07	0

Table 4. *Fractionation of proteins of rat and frog liver acetylated in vivo*

Whole homogenates (5 ml each) from liver of rat and frog that had been injected with [1-¹⁴C]acetic acid (sample identical with that used in Table 3) were fractionated by the method described by Paik & Kim (1967b). Procedures for removing the acid-soluble material and phospholipids and counting the incorporated radioactivity are described in Table 2.

Fractions	Sp. radioactivity (c.p.m./mg of protein)		Total radioactivity (c.p.m.)		Percentage of total radioactivity in fraction	
	Rat	Frog	Rat	Frog	Rat	Frog
0.1M-H ₂ SO ₄ -insoluble	1029	1270	32 200	21 700	60.5	53.9
0.1M-H ₂ SO ₄ -soluble	288	614	2780	4190	5.2	10.4
Ethanol-soluble	121	631	228	1730	0.4	4.3
Water-insoluble	573	652	18 050	12 650	33.9	31.4

most of the radioactivity was found in 0.1M-sulphuric acid-insoluble protein (Table 4). Further, this fraction had the highest specific radioactivity (c.p.m./mg of protein). These general patterns are common to both rat and frog livers. Water-insoluble protein comes next to the sulphuric acid-insoluble protein in the total amount of radioactivity and specific radioactivity.

Requirements for incorporation of acetyl groups. Table 5 lists the requirements for protein acetylation in both rat and frog liver, and shows that the rate of acetylation of protein in rat liver is almost eight times that of frog liver. Mg²⁺, ATP, fluoride and CoA are requirements for both systems. However, GSH does not seem to be required for protein acetylation in rat liver and when added to the frog liver system is actually found to be inhibitory.

When protein acetylated *in vitro* in rat or frog homogenates was hydrolysed under reflux in 2M-H₃PO₄ at 110°C for 20 h and the hydrolysates were distilled, approx. 95% of the total radioactivity

Table 5. *Requirements for incorporation of [1-¹⁴C]-acetyl groups into endogenous proteins*

Tissues were homogenized in water to give a 20% (w/v) homogenate and were passed through a double layer of cheese-cloth. The complete system contained the following: 0.1 ml of 0.5M-tris-HCl buffer, pH 7.4, containing 0.05M-MgCl₂, 0.1 ml of 0.05M-ATP (pH adjusted to 7.0), 0.1 ml of 0.06M-GSH, 0.1 ml of 0.5M-KF, 0.1 ml (40 μ g) of CoA, 0.2 ml of homogenate, 0.1 ml of [1-¹⁴C]acetic acid (47.2 nmol; 86 c.p.m./pmol) in a total volume of 1.0 ml were incubated at 37°C for 30 min. The rest of the procedures are described in Table 2.

System	Acetyl groups incorporated (pmol/30 min/mg of protein)	
	Rat	Frog
Complete	55.8	6.93
Minus Mg ²⁺	26.6	0.66
Minus ATP	0.8	0.01
Minus GSH	53.2	16.47
Minus KF	23.4	0.84
Minus CoA	45.9	2.59

incorporated was distilled over (samples in Tables 2 and 5). On the other hand, hydrolysates of proteins acetylated *in vivo* (samples in Tables 3 and 4) lost only about 30% of the incorporated radioactivity during distillation. The remaining radioactivity is probably due to the radioactivity of amino acids synthesized from [$1\text{-}^{14}\text{C}$]acetic acid *in vivo*.

The question of possible acetylation of nucleic acids has never been raised before. The above results therefore led us to the conclusion that nucleic acids were not acetylated in the rat liver under conditions in which protein was actively acetylated. Since identical results were obtained with frog liver, this conclusion may be generally applicable.

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