Metabolic and Chemical Properties of Basic Proteins Isolated from Nuclei of Rat Liver and Thymus Gland

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1. The effects of alkylating agents and disulphides on the thiol-containing proteins of nuclei from rat thymus and liver were studied. Three protein fractions were examined: histones extracted with 50mM- and 250mM-hydrochloric acid and the residual protein. None of the reagents selectively reacted with any one of the protein fractions. 2. Amino acid uptake *in vitro* into the histones of nuclei from rat thymus was analysed by preparative electrophoresis of the proteins extracted with 50mM- and 250mM-hydrochloric acid. After 1 hr. at 37° the greater incorporation was into the proteins extracted with 50mM-hydrochloric acid. 3. Preparative electrophoresis was used to study the relative thiol contents of the proteins of the 50mM-hydrochloric acid extract from thymus nuclei by labelling the histones *in vitro* with ¹⁴C-labelled *N*-ethylmaleimide. 4. The capacity of the proteins extracted from rat thymus with 50mM- and 250mM-hydrochloric acid, and of the components from these extracts separated by preparative electrophoresis, to combine with DNA and to depress DNA-dependent RNA synthesis was studied. The histones extracted with 50mM-hydrochloric acid were more lysine-rich than those extracted with 250mM-hydrochloric acid. Wide variations were found in the abilities of the separated components to depress RNA synthesis.

The detection of thiol groups in proteins of nuclei from rat liver and thymus (Deakin, Ord & Stocken, 1963; Jellum & Eldjarn, 1964) prompted an investigation of any function these groups might have in nuclear metabolism. Three aspects of nuclear function have been considered: the change in oxidation-reduction state of thiol groups in nuclear proteins believed to occur during mitosis (Rapkin, 1931; Mazia, 1961), the effect of histones in modifying information transfer from DNA (Stedman & Stedman, 1951; Huang & Bonner, 1962; Allfrey, Littau & Mirsky, 1963) and the inhibition produced by alkylating or oxidizing agents on precursor uptake into nuclear proteins and RNA (Srinivasan, Brunfaut & Errera, 1964; Smit & Stocken, 1964).

About 50% of nuclear thiol groups in liver and thymus is associated with histones that can be extracted with 50mM- and 250mM-hydrochloric acid (Marsh, Ord & Stocken, 1964). Any attempt to study variations in the ratio of thiol to disulphide groups in nuclei that might occur under physiological conditions demands a means by which the initial content of thiol groups can be determined, or, alternatively, corrections be made for the changes

taking place during the experimental procedures. Because of the practical difficulties involved in isolating nuclei under anaerobic conditions, the effects of different thiol reagents on nuclei were examined to see if it was possible to label the thiol groups initially present, and to determine what effect such labelling had on the behaviour of the histones in the RNA-polymerase (EC 2.7.7.6) system.

We have also studied the separation of the histones by disk electrophoresis on polyacrylamide gels. The method was then employed on a preparative scale to investigate into which of the histones of thymus nuclei amino acids were incorporated after incubation of the nuclei *in vitro* and to test the effects of the separated histone fractions on DNA-dependent RNA synthesis.

METHODS

Animals. This Laboratory strain of Wistar rats was used. With nuclei prepared from thymus gland, animals of 100–140 g. body wt. were used; with nuclei from liver the animals were of 400–450 g. body wt.

Nuclei. Nuclei were isolated from liver as described by Chaveau, Moulé & Rouiller (1956) by using 2.2M-sucrose–5mM-CaCl₂–5mM-tris-HCl buffer, pH 7.2. Nuclei from thymus gland were isolated in 0.25M-sucrose–5mM-CaCl₂–5mM-tris-HCl, pH 7.2 (Creasey & Stocken, 1959).

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Acid extraction of nuclei. Extraction with 50 mM- and 250 mM-HCl was performed as described by Marsh *et al.* (1964). The material remaining after the second extraction with 250 mM-HCl was resuspended in water and used as the residual protein fraction. The extracts were analysed electrophoretically without dialysis.

Reaction of thiol groups with alkylating agents and disulphides. Nuclei from liver and thymus gland were incubated with a number of alkylating agents and disulphides. The reagent (0.3 ml.) was added to 10–30 ml. of the experimental sample of nuclei and the same volume of water to the control. With *p*-chloromercuribenzenesulphonate, *p*-chloromercuribenzoate and Ellman's (1959) reagent [bis-(3-carboxy-4-nitrophenyl) disulphide] the nuclei were kept for 5 min. at 2° after the addition of the reagent. With the other inhibitors the nuclei were kept with the reagent for 30 min. at 20°; with *N*-ethylmaleimide the nuclei were kept for 60 min. at 20°. All reactions were carried out at pH 7.2. After incubation the preparations were centrifuged for 1 min. at 3500 g at 2° and the excess of reagent was removed by twice washing and resuspending the nuclei in 0.25 M-sucrose medium. Control nuclei were treated similarly.

Amino acid uptake into isolated nuclei from thymus gland. Six rats were used for the isolation of nuclei. The final concentration of the nuclear suspension was the equivalent of two thymus glands/ml. of medium. The incubation mixture contained: nuclear suspension (3.0 ml.), 100 μ C of ¹⁴C-labelled algal-protein hydrolysate (specific activity 100 μ C/0.77 mg.), glucose (220 μ moles) and NaCl (220 μ moles) in 0.25 M-sucrose–5 mM-CaCl₂–5 mM-tris-HCl buffer, pH 7.2 (0.3 ml.). The incubation was carried out aerobically with shaking at 37° for 60 min. The nuclei were centrifuged (3000 g for 3 min.) and the nuclear precipitate was washed six times with sucrose medium containing 1% (w/v) of casein hydrolysate.

Preparative electrophoresis of labelled histones on polyacrylamide gel. The procedure followed was a modification of that described by Racusen & Calvanico (1964). The gel container consisted of two flanged glass tubes, respective lengths 5.0 and 11.5 cm. (internal diam. 2.5 cm.), separated by a polythene chamber, total capacity 1.22 ml. (0.25 cm. thick), into which two stainless-steel hypodermic syringe needles (B.W.G. 21) were inserted to provide an inlet and outlet for the buffer solution used as eluent. The flanged end of the long tube was tightly covered with Parafilm, placed flanged-end down and then partially filled with the acrylamide monomer solution (McAllister, Wan & Irwin, 1963) (25 ml.) to give a column of gel (5 cm. high). The acrylamide solution was overlaid with distilled water (0.5 cm. depth), care being taken not to disturb the interface of the solutions, and then left to polymerize.

The distilled water and unchanged monomer were then drained off, the Parafilm seal was removed and the flanged ends of each tube were lightly greased with petroleum jelly. The polythene chamber, which contained a recessed stainless-steel wire grid (20 mesh, 26 gauge, diam. 2.8 cm.) on the top to support the polyacrylamide gel and Visking dialysis membrane (diam. 3.5 cm.) on the bottom, was then bolted between the two flanged tubes in a watertight assembly.

A 2 ml. portion of histone solution, containing about 5 mg. of protein, was mixed with an equal volume of 2 M-sucrose–74 mM-glycine-acetic acid buffer, pH 4.0, and

placed on the gel column; this was then gently covered with 37 mM-glycine-acetic acid buffer, pH 4.0, without disturbing the interface.

Electrophoresis was performed in a vertical position so that the histones moved down the column. The buffer vessel at either end of the tube contained approx. 500 ml. of 37 mM-glycine-acetic acid buffer, pH 4.0. Because the runs sometimes lasted for up to 5 days additional buffer was continuously added to each reservoir at a rate of 25 ml./hr. Each buffer compartment was drained and refilled every 12 hr. of running time.

A continuous-infusion apparatus (distributors: B. Braun, Melsungen, Germany) was connected to one side arm of the eluting chamber. The buffer used for elution (37 mM-glycine-acetic acid buffer, pH 4.0) was run into the chamber at 7.5 ml./hr. Air bubbles were carefully flushed out of the tubing and chamber and the apparatus was checked for watertightness before use. The outlet side arm was led to a fraction collector and 2.5 ml. fractions were collected.

Electrophoresis was carried out at 360 v (72 v/cm. of gel), 12 mA, which led to little heat development (approx. 2–3°) in the gel (ambient temp. 20°).

Analytical electrophoresis. This was performed on polyacrylamide gel in 37 mM-glycine-acetic acid buffer, pH 4.0 (McAllister *et al.* 1963; Marsh *et al.* 1964).

Determination of thiol groups. Masked and free thiol groups were determined as described by Marsh *et al.* (1964) by using the method of Ellman (1959).

Determination of DNA. DNA was measured by the diphenylamine method of Burton (1956) and expressed as DNA phosphorus. For assays with the RNA-polymerase system and in the melting profiles DNA was determined spectrophotometrically by using E_{260} (50 μ g./ml.) = 1.0.

Determination of protein. The protein content of 50 mM-HCl and 250 mM-HCl extracts and of fractions eluted in preparative electrophoresis was determined either spectrophotometrically or by the method of Lowry, Rosebrough, Farr & Randall (1951). The procedures were standardized by using dialysed HCl extracts that had been dried *in vacuo* over P₂O₅.

RNA polymerase. The enzyme was prepared from *E. coli* (Chamberlin & Berg, 1962) up to the step of fraction 3. Incubation was carried out for 8 min. as described by Huang, Bonner & Murray (1964), except that the precipitates were collected on B-6 membrane filters (Schleicher & Schuell, Dassel, Germany), washed and counted directly (J. M. Bonner, personal communication). The incubation mixture contained (final vol. 0.26 ml.): tris-HCl buffer, pH 8.0, 10 μ moles; MgCl₂, 1 μ mole; MnCl₂, 0.25 μ mole; GTP, CTP and UTP, each 0.1 μ mole; β -mercaptoethanol, 3 μ moles; [8-¹⁴C]ATP (specific activity 1 μ C/ μ mole), 0.05 μ mole; DNA, 5–10 μ g.; histones, 0–30 μ g., as stated in the text; RNA polymerase, 0.01 ml.

Soluble reconstituted rat-thymus nucleohistones. These were prepared by gradient dialysis as described by Huang *et al.* (1964). The ratio of extinction at 230 m μ to that at 260 m μ in the reconstituted nucleohistones was 0.54–0.58:1, compared with 0.37:1 in calf-thymus DNA (Sigma Chemical Co., St Louis, Mo., U.S.A.).

Melting profiles. Melting profiles of DNA and of nucleohistones (3 ml., containing approx. 75 μ g. of DNA) in 15 mM-NaCl–1.5 mM-sodium citrate were determined in the temperature-regulated cuvette holder of a Beckman DK-2A spectrophotometer. The extinction was read at

257 $m\mu$ every 2° above 50° until there was no further increase in absorption; the time taken was about 20 min. and good reproducibility was obtained.

Determination of ^{14}C activity. This was determined either by scintillation counting (Isotope Electronics Ltd. Tritomat) or by gas-flow counting (Nuclear-Chicago Corp.). A 0.1 ml. portion of the protein solution eluted from the columns was added to 2.0 ml. of scintillator [1,4-bis-(5-phenyloxazol-2-yl)benzene; Thorn Electronics Ltd.] or 0.5 ml. was evaporated on aluminium planchets for gas-flow counting. The efficiency of the scintillation counter was 34%; that of the gas-flow counter was about 2%. Sufficient counts were recorded to give an error of less than 2%.

Amino acid analysis. This was performed by the procedure of Crestfield, Moore & Stein (1963). The dialysed proteins were freeze-dried, and a 2 mg. sample was hydro-

lysed with 2 ml. of constant-boiling HCl (twice-redistilled) for 17 hr. *in vacuo*, the tubes being sealed as described by Crestfield *et al.* (1963). The hydrolysates were dried *in vacuo* over NaOH pellets and anhydrous $CaCl_2$.

RESULTS

Effect of alkylating agents and disulphides on nuclear thiol groups. The distribution of thiol groups in control nuclei was similar to that reported by Marsh *et al.* (1964), except that the content of apparent (immediately reacting) thiol groups was rather higher, probably because of the additional washings and resuspensions required for these experiments (Table 1).

Table 1. *Thiol contents of protein fractions of normal rat-liver and rat-thymus nuclei*

The apparent thiol content was the portion of the total thiol content that reacted directly with the Ellman reagent; the total thiol content was the amount that reacted after the nuclei had stood for 5 min. in 4 M-urea. The total thiol content is given as μ moles of SH/mg. of DNA phosphorus. The thiol content of each fraction is given as a percentage of the total thiol content. The protein content of these fractions was estimated from the extinction at 280 $m\mu$ and is given as mg. of protein/mg. of DNA phosphorus of the original nuclei. The results are given as means $\pm$ S.E.M. (or means and ranges), with the numbers of experiments in parentheses.

Fraction	Liver nuclei	Thymus nuclei
Whole nuclei		
Total thiol content	1.36 $\pm$ 0.25 (6)	0.84 $\pm$ 0.16 (8)
Apparent thiol content (% of total)	56.7 $\pm$ 13.4 (8)	55.6 $\pm$ 12.0 (8)
50 mM-HCl extract		
Thiol content (% of total)	47.6 $\pm$ 18.7 (8)	30.7 $\pm$ 10.6 (8)
Protein content	17.3 $\pm$ 4.51 (7)	9.9 $\pm$ 3.32 (6)
250 mM-HCl extract		
Thiol content (% of total)	8.7 $\pm$ 4.4 (7)	14.6 $\pm$ 4.9 (7)
Protein content	5.6 $\pm$ 1.14 (7)	8.1 $\pm$ 1.25 (7)
Residual protein		
Thiol content (% of total)	34.0 (range 20.2–46.3) (4)	39.3 (range 32.5–48.5) (5)

Table 2. *Effect of alkylating agents and disulphides on thiol groups of liver nuclei*

The reagents were incubated with liver nuclei as described in the text. At the end of the incubation period the control and treated nuclei were centrifuged and washed twice before final resuspension. Portions of the washed nuclear suspension were taken for determination of the apparent (immediately reacting) and total thiol contents, and the remainder was used for the acid extraction. The masked thiol content is the increment in thiol content found in the presence of 4 M-urea.

Reagent	Concn. of reagent (mM)	Inhibitor/total nuclear SH molar ratio	Inhibition (percentage decrease in thiol content)				
			Apparent thiol content	Masked thiol content	Histones		Residual protein
					50 mM-HCl extract	250 mM-HCl extract	
Bis-(3-carboxy-4-nitrophenyl) disulphide	0.2	3.6	100	0	46	42	31
Dithiodiglycol	11.65	186	44	30	—	—	—
Iodoacetate	0.14	2.7	0	0	—	—	—
N-Ethylmaleimide	0.13	3.2	27	31	32	13	42
p-Chloromercuribenzenesulphonate	0.20	4.6	—	—	76	61	23
	0.14	9.5	70	29	85	—	—
	0.14	3.1	68	—	72	—	—
	0.02	0.4	37	4	24	0	26
	0.02	0.5	—	—	8	7	0

Table 3. *Effect of alkylating agents and disulphides on thiol groups of thymus nuclei*

The reagents were incubated with thymus nuclei as described in the text. At the end of the incubation period the control and treated nuclei were centrifuged and washed twice with 0.25 M-sucrose medium before the acid-extraction procedure.

Reagent	Concn. of reagent (mm)	Inhibitor/total nuclear SH molar ratio	Inhibition (percentage decrease in thiol content)		
			Histones		Residual protein
			50 mM-HCl extract	250 mM-HCl extract	
Bis-(3-carboxy-4-nitrophenyl) disulphide	0.48	5.1	56	33	33
N-Ethylmaleimide	0.23	5.4	39	0	27
p-Chloromercuribenzenesulphonate	0.23	2.4	77	49	25
	0.23	2.4	75	53	52
p-Chloromercuribenzoate	0.23	2.4	72	43	54
	0.23	1.7	64	49	—

Fig. 1. Preparative electrophoresis on polyacrylamide gel of 50 mM-HCl extract from rat-thymus nuclei that had been incubated for 60 min. at 37° with ^{14}C -labelled amino acids. The incubation mixture, preparation of the acid extract and electrophoretic procedure were as described in the text. A 3.7 mg. sample of protein was applied to the column and electrophoresis carried out in 37 mM-glycine-acetic acid buffer, pH 4.0. Fractions (2.5 ml.) were collected at a flow rate of 7.5 ml./hr. Protein (---) was determined by the method of Lowry *et al.* (1951), and the ^{14}C activity (—) by gas-flow counting. The peak numbers correspond to those referred to in Tables 4–6.

The thiol reagents that received most attention were bis-(3-carboxy-4-nitrophenyl) disulphide, *N*-ethylmaleimide and *p*-chloromercuriphenyl compounds (Tables 2 and 3). Incubating the nuclei with the disulphide for 5 min. enabled an analysis to be

made of the protein fractions making up the apparent and masked thiol groups in normal liver and thymus nuclei. Nuclei from the two tissues reacted very similarly, and it seemed that in the histone fraction extracted with 50 mM-hydrochloric acid

about 50% of the thiol groups reacted immediately with the disulphide and so contributed to the apparent thiol fraction. The residual protein reacted to a slightly smaller extent, but there was little selectivity of reaction between the three protein fractions isolated. *p*-Chloromercuribenzenesulphonate was a more effective and selective inhibitor, preferentially reacting with the histone fraction extracted with 50mM-hydrochloric acid. The extent of the inhibition was increased with increasing molar ratio of inhibitor to thiol groups. *p*-Chloromercuribenzoate gave similar results, and from the extinctions at 255m μ (Boyer, 1954) of the thymus-nuclei histones extracted with 50mM- and 250mM-hydrochloric acid evidence was obtained for the binding of phenyl groups to the protein fractions in excess of their thiol content. Comparable observations with *p*-chloromercuribenzenesulphonate were precluded because the absorption of the mercaptide occurs at shorter wavelengths (Boyer, 1954). Direct comparison of the results obtained by the Ellman (1959) and Boyer (1954)

methods for the thiol content of the histone fraction extracted from thymus nuclei with 50mM-hydrochloric acid confirmed that additional groups were reacting with the alkylating agent, approximately equimolar in amount to the thiol content determined by the Ellman (1959) method.

With *N*-ethylmaleimide the extent of inhibition was very much less than with the *p*-chloromercuriphenyl compounds used in the same, or smaller, molar excess, and the selectivity of inhibitor between the different fractions was not marked. Previous experiments (Marsh *et al.* 1964) had shown that isolated histone fractions gave identical thiol contents by the Ellman (1959) method and by the use of ^{14}C -labelled *N*-ethylmaleimide. Analytical electrophoresis of the material extracted with 50mM- and 250mM-hydrochloric acid from liver and thymus nuclei which had been treated with *p*-chloromercuribenzoate or *N*-ethylmaleimide failed to show any differences in mobility as compared with extracts from control nuclei.

Preparative electrophoresis of histone fractions

Fig. 2. Preparative electrophoresis on polyacrylamide gel of 250mM-HCl extract from rat-thymus nuclei that had been incubated for 60 min. at 37° with ^{14}C -labelled amino acids. The incubation mixture, preparation of the acid extract and electrophoretic procedure were as described in the text. A 3.6mg. sample of protein was applied to the column and electrophoresis carried out in 37mM-glycine-acetic acid buffer, pH4.0. Fractions (2.5ml.) were collected at a flow rate of 7.5ml./hr. Protein (---) was determined by the method of Lowry *et al.* (1951), and the ^{14}C activity (—) by gas flow counting. The peak numbers correspond to those referred to in Tables 4-6.

from thymus nuclei. As the thiol groups of the different histone fractions had failed to react selectively with the various reagents tried, it was decided to investigate the separation of the histones by preparative electrophoresis on polyacrylamide gel. The procedure was used to study any possible differences in metabolic activity of the histone components isolated from thymus nuclei that had been incubated for 60 min. at 37° with ^{14}C -labelled amino acids from an algal-protein hydrolysate. Incorporation of amino acids into the protein of isolated thymus nuclei had been demonstrated by Allfrey, Mirsky & Osawa (1955), and has since been extensively confirmed. Usually incorporation into residual protein is more marked than into histones (Allfrey *et al.* 1955; Zbarskii & Samarina, 1963), but uptake into the latter fractions certainly occurs (Allfrey, Faulkner & Mirsky, 1964; Allfrey, Littau & Mirsky, 1964).

The number of major components and their protein content (Figs. 1 and 2) corresponded approximately to the distribution found by electrophoresis on polyacrylamide gel on a small scale (Marsh *et al.* 1964), although some of the most mobile peaks in the material extracted with 50 mM-hydrochloric acid (peaks 1–3 in Table 4) were not always detected on the small-scale runs. Although the recoveries were rather variable (Tables 4 and 5) it appears that differences in metabolic activity of the components occurred (Table 4): the proteins extracted with 50 mM-hydrochloric acid were generally more labelled than those extracted with 250 mM-hydrochloric acid, and in the material extracted with 50 mM-

hydrochloric acid the more mobile peaks were the more active. As had been indicated from small-scale electrophoresis, some overlap between the two extracts may have occurred.

The preparative technique was then used to determine the thiol contents of the different components in the proteins extracted with 50 mM-hydrochloric acid, $1\text{-}^{14}\text{C}$ -labelled *N*-ethylmaleimide being used to label the thiol groups. The distribution of the fractions (Table 5) was similar to that in the preceding experiment, and wide differences in the *N*-ethylmaleimide contents were found. Marsh *et al.* (1964) had shown that the slowest component (4) in the proteins extracted with 50 mM-hydrochloric acid had a higher thiol content than the immediately succeeding peak, and this is confirmed here (peaks 9 and 7+8). The most mobile peaks, which apparently had the highest thiol contents, were not detected regularly on small-scale electrophoresis. For a molecular weight of 20 000 the original material extracted with 50 mM-hydrochloric acid would have a thiol content of about 0.5 mole of SH/mole of histone, and peak 9 about 1.0 mole/mole.

Effect of histones from thymus nuclei on the priming activity of DNA in DNA-dependent RNA synthesis. The possible function of the proteins extracted from rat-thymus nuclei with 50 mM- and 250 mM-hydrochloric acid was investigated by studying the capacity of these proteins to stabilize DNA against thermal denaturation and to inhibit DNA-dependent RNA synthesis. Huang *et al.* (1964) have shown a marked parallelism between these two effects. Soluble nucleohistones were

Table 4. *Preparative electrophoresis of proteins extracted with 50 mM- and 250 mM-hydrochloric acid from thymus nuclei that had been incubated with ^{14}C -labelled amino acids*

Nuclei were suspended in 0.25 M-sucrose medium and incubated with ^{14}C -labelled algal-protein hydrolysate for 60 min. at 37°. After being washed, the nuclei were extracted with 50 mM- and 250 mM-HCl, and the extracts applied to the column and eluted as described in the text. The main peaks are given in order of elution from the columns. A 1.0 ml. sample of each fraction was taken for ^{14}C assays, and 0.5 ml. for protein determinations (Lowry *et al.* 1951). Of the radioactivity applied to the columns 91% was recovered from the 50 mM-HCl extract and 33% from the 250 mM-HCl extract. Specific activities are given as counts/min./ $\mu\text{g.}$ of protein.

Peak no.	50 mM-HCl extract			250 mM-HCl extract		
	Radioactivity (counts/min.)	Protein ($\mu\text{g.}$)	Specific activity	Radioactivity (counts/min.)	Protein ($\mu\text{g.}$)	Specific activity
1	1880	48.0	34.2	800	168.5	4.8
2	2135	68.0	31.4	818	269.5	3.1
3	2240	117.5	19.3	503	142.5	3.6
4	2435	51.0	47.8	43	24.0	1.8
5	1270	27.5	46.3	76	30.5	2.5
6	1015	10.0	101.5	140	4.3	8.3
7	305	8.0	38.2	52	22.5	2.3
8	240	12.0	40.0	69	21.0	3.3
9	305	62.0	4.9			
10	260	30.0	8.7			

Table 5. Preparative electrophoresis of proteins extracted with 50mm-hydrochloric acid from rat-thymus nuclei after the extract had been labelled with 1-¹⁴C-labelled N-ethylmaleimide in vitro

A twofold excess of N-ethylmaleimide (specific activity 8μc/mg.) was allowed to react with the extract for 60 min. at 20° at pH 4.5. The solution was dialysed before being put on the column. A 0.1 ml. sample of each fraction was taken for ¹⁴C assays, and 0.5 ml. for protein determination (Lowry *et al.* 1951). The peaks are numbered to correspond to those shown in Table 4. About 47% of the material was recovered.

Peak no.	Radioactivity (counts/min.)	Protein (μg.)	Specific activity (counts/min./μg. of protein)
1	49	15	3.3
2	126	22.5	5.6
3	86	—	2.9
4	214	270	0.8
5	429	602	0.7
6	72	43	1.7
7+8	68	529	0.1
9	213	255	0.8

Fig. 3. Melting profiles of DNA and of nucleohistones reconstituted from calf-thymus DNA and of histones from rat-thymus nuclei. Nucleohistones were reconstituted from DNA and the 50mm-HCl and 250mm-HCl extracts of rat-thymus nuclei, and the melting profiles obtained as described in the text. ○, Calf-thymus DNA alone; Δ, nucleohistone from calf-thymus DNA and 50mm-HCl extract of rat-thymus nuclei; □, nucleohistone from calf-thymus DNA and 250mm-HCl extract of rat-thymus nuclei.

therefore reconstituted by gradient dialysis from calf-thymus DNA and the histones extracted with 50mm- and 250mm-hydrochloric acid in a weight ratio of 1.0:1.3, and the melting profiles were determined. The half-melting temperature (*T_m*) for both reconstituted nucleohistones was 80°, which is 6° higher than the *T_m* for uncombined calf-thymus DNA. The nucleoprotein reconstituted from the material extracted with 250mm-hydrochloric acid showed a greater heterogeneity in melting, with considerably more material melting above 90° (Fig. 3).

The effect of the proteins extracted with 50mm- and 250mm-hydrochloric acid on DNA-dependent RNA synthesis was examined by using RNA polymerase from *E. coli* (Chamberlin & Berg, 1962; Huang *et al.* 1964). Consistent with the greater

content of high-melting material in the nucleohistone of the material extracted with 250mm-hydrochloric acid, this fraction was also more effective in rendering the DNA inactive as primer for RNA polymerase. The dependency of RNA synthesis on the concentration of DNA provided either alone or as soluble nucleohistone was studied (Fig. 4). With 20μg. of DNA the nucleohistone obtained from the material extracted with 50mm-hydrochloric acid was 43% and that from the material extracted with 250mm-hydrochloric acid 18% as effective as DNA alone.

To demonstrate that these decreases in RNA synthesis were due only to a decrease in the ability of DNA to prime and not to some other form of inhibition, the priming activity of uncombined DNA was examined in the presence of nucleohistone. When 2μg. of either nucleohistone was added to 2μg. of DNA, no decrease in RNA synthesis occurred; in fact, with the nucleohistone from the material extracted with 50mm-hydrochloric acid slightly more synthesis was found. If an amount of DNA (10μg.) that saturates the polymerase is used together with an equal amount as nucleohistone, then the rate of RNA synthesis is intermediate between the rates when either DNA or nucleohistone is used. This suggests that some competition exists for enzyme between DNA and partially inhibited primer.

Huang *et al.* (1964) have shown that the addition of histones to the RNA-polymerase system provides a direct and sensitive assay of the ability of the histone to inhibit the priming activity of DNA. The addition of the histone to DNA in a solution of low ionic strength causes an insoluble nucleohistone to be formed that is nevertheless capable of priming DNA-dependent RNA synthesis. The effects of the proteins extracted with 50mm- and 250mm-hydrochloric acid on RNA synthesis were therefore assayed by this method. Since the

Fig. 4. Dependency of RNA synthesis on the concentration of DNA and nucleohistone. The RNA-polymerase system was as described in the text, as was the procedure for reconstituting soluble nucleohistones from calf-thymus DNA and either the 50 mM-HCl or the 250 mM-HCl extract from rat-thymus nuclei. $\circ$, Calf-thymus DNA alone; Δ , nucleohistone reconstituted from calf-thymus DNA and 50 mM-HCl extract from rat-thymus nuclei; $\square$, nucleohistone reconstituted from calf-thymus DNA and 250 mM-HCl extract from rat-thymus nuclei. A, 2 μ g. of DNA + nucleohistone reconstituted from 2 μ g. of DNA and histone extracted with 50 mM-HCl; B, as A, but with 250 mM-HCl extract; C, as A, but with 10 μ g. instead of 2 μ g.; D, as B, but with 10 μ g. instead of 2 μ g.

material extracted with 50 mM-hydrochloric acid itself tends to precipitate after storage at -15° under nitrogen or after adjusting the pH above 3.5, the proteins extracted with 50 mM-hydrochloric acid were always centrifuged (at 4000g for 5 min.) to remove insoluble material before use in this assay, and the protein content of the supernatants was checked spectrophotometrically.

The material extracted with 250 mM-hydrochloric acid was much more effective than that extracted with 50 mM-hydrochloric acid in preventing RNA synthesis (Fig. 5). This agrees with the results obtained with soluble nucleohistones.

Histone fractions obtained by preparative electrophoresis from the material extracted with 50 mM- and 250 mM-hydrochloric acid were added to either 5 μ g. or 10 μ g. of DNA, and the ability of the resulting insoluble nucleohistones to support RNA synthesis was determined. Although insufficient material was available to test all of the peaks, assay of several separated components (Table 6) showed that they differed widely in their

Fig. 5. Capacity of the 50 mM-HCl and 250 mM-HCl extracts from thymus nuclei to repress DNA-dependent RNA synthesis. The preparation of the extracts and the polymerase system were as described in the text. A 10 μ g. portion of calf-thymus DNA was used in the presence of 0–30 μ g. of histone. The priming activity is expressed as a percentage of the activity obtained with DNA alone. The results given are the means of experiments with three batches of nuclei. $\circ$, 50 mM-HCl extract; Δ , 250 mM-HCl extract.

ability to inhibit, the more mobile proteins being the more effective in both the extracts.

The effect of blocking the thiol groups of the crude fractions by *N*-ethylmaleimide on their ability to inhibit was also studied. To minimize attack on groups other than thiol a molar concentration of maleimide equal to the thiol content in the extracts was used at pH 3.5 (Smyth, Blumenfeld & Konigsberg, 1964). Four times the amount of maleimide equivalent to the thiol groups of the histones had no effect on the RNA-polymerase system, which contains a 1000-fold excess of β -mercaptoethanol (12 mM). *N*-Ethylmaleimide

Table 6. *Effect on RNA synthesis of histone fractions obtained by preparative electrophoresis of material extracted from thymus nuclei with 50mm- and 250mm-hydrochloric acid*

The RNA-polymerase system is described in the text. The concentration of the histone fractions was determined by the method of Lowry *et al.* (1951). The activity of each fraction is given as the percentage of the [^{14}C]ATP incorporation obtained in the presence of DNA. With components from the 50mm-HCl extract 10 μg . of DNA was used; with the 250mm-HCl extract 5 μg . of DNA was used. The peaks are numbered to correspond to those shown in Table 4.

Peak no.	Histone (μg .)	Activity (%)
Original 50mm-HCl extract	20	78
	30	70
1	20	2
3	20	8
	25	5
4+5	20	7
	30	9
7+8	20	12
Original 250mm-HCl extract	5	68
	10	14
*	5	21
†	5	13
4	5	20
	10	19
5	5	22
6	5	91
7	5	62
‡	5	66

* Peak preceding peak 1.

† Peak between peaks 3 and 4.

‡ Peak following peak 8.

treatment did not alter the action of the proteins extracted with 250mm-hydrochloric acid, but it caused a slight but reproducible decrease in the ability of the proteins extracted with 50mm-hydrochloric acid extract to decrease RNA synthesis (11% decrease with both 20 and 30 μg . of histone).

DISCUSSION

The effects on nuclei of the alkylating agents and disulphides used showed that none of the inhibitors examined was ideal for the selective labelling of either the immediately reacting or the masked thiol groups initially present in the nuclei. The *p*-chloromercuriphenyl compounds were the most effective but they appeared to be bound on groups additional to thiols (see also Boyer, 1959), and the selectivity of low concentrations of the alkylating agents for histones extractable with 50mm-hydro-

chloric acid was very dependent on the concentration of thiol originally present. *N*-Ethylmaleimide, which had been used for earlier radioautographic experiments with thymus nuclei (Deakin *et al.* 1963), reacted with only about 30% of the total thiol groups after 60 min. at 20°, and iodoacetate at pH 7 was ineffective. Of the disulphides used, Ellman's (1959) reagent [bis-(3-carboxy-4-nitrophenyl) disulphide] reacted more with the histone fraction extracted with 50mm-hydrochloric acid than with the residual protein, which indicated that the histone thiol contributed a large proportion of the immediately reacting thiols in the initial nuclear suspensions. Residual protein did not react with an inhibitor to more than 54% of its total thiol groups, suggesting that 40–50% of the thiol groups of residual protein in the intact nuclei might be unavailable to the reagents used and only become reactive when the tertiary structure had been disorganized with acid or 4M-urea.

Since the inhibitors did not react selectively with any broad fraction of nuclear thiols, preparative electrophoresis was used as a means of separating the different histones. At present this technique has only been satisfactory with about 5mg. of protein. Increasing the load on the column or the size of the column has not so far led to adequate recoveries. We have therefore not been able to obtain amino acid analyses on the separated histones. From the three parameters used, amino acid uptake, labelling with *N*-ethylmaleimide and repression of RNA synthesis, there appears to be good evidence that some separation of the components of the crude material obtained from thymus nuclei by extraction with 50mm- and 250mm-hydrochloric acid has been achieved. Very good agreement was found between the different tests with fractions which separated as 'paired peaks'.

The quantitative interpretation of the results is based on a method of protein determination (Lowry *et al.* 1951) that is weighted for tyrosine. The specific activities of these components after amino acid uptake, and especially after maleimide labelling, may therefore reflect in part a low tyrosine content in these proteins. A low tyrosine content in components 1–3 of the material extracted with 50mm-hydrochloric acid would also explain the marked repression produced by these fractions on RNA synthesis, where again the amount of protein to be assayed was based on the Lowry method.

The average amino acid composition of the two extracts from thymus (Table 7) confirmed that the proteins extracted with 50mm-hydrochloric acid were more rich in lysine than those extracted with 250mm-hydrochloric acid. The proteins extracted with 50mm-hydrochloric acid also had more proline and less tyrosine than those extracted with 250mm-

Table 7. *Amino acid composition of acid extracts from liver and thymus nuclei*

Experimental details are given in the text. The results for the acid extract of liver nuclei are taken from Marsh *et al.* (1964).

	Amino acid content (moles/100 moles)		
	Liver nuclei 50 mM-HCl extract	Thymus nuclei	
		50 mM-HCl extract	250 mM-HCl extract
Lysine	12.6	16.4	11.4
Histidine	2.38	1.90	2.16
Arginine	7.87	5.76	11.5
Aspartic acid	6.72	6.27	5.49
Threonine	5.67	5.40	6.44
Serine	6.41	7.17	4.50
Glutamic acid	10.19	8.83	9.37
Proline	5.22	6.76	3.61
Glycine	8.84	8.36	9.86
Alanine	10.8	12.6	10.1
Cystine (half)	Trace	Trace	Trace
Valine	4.63	6.13	6.15
Methionine	1.52	1.12	1.28
Isoleucine	3.76	3.10	4.59
Leucine	7.32	6.42	8.16
Tyrosine	2.55	2.00	2.93
Phenylalanine	2.40	1.81	2.41
Ammonia	—	7.52	6.81
Lysine/arginine ratio	1.60	2.85	0.99

hydrochloric acid (cf. the lysine-rich histones obtained by Johns, Phillips, Simson & Butler, 1960; Rasmussen, Murray & Luck, 1962). Previous results with analytical electrophoresis (Marsh *et al.* 1964) had shown that the proteins extracted from liver nuclei with 50 mM-hydrochloric acid did not contain as much of the more mobile components as did the corresponding extract from thymus nuclei. This suggests that some of the more mobile components (peaks 1–3 in Table 4) were lysine-rich (cf. Reid & Cole, 1964) and also probably contained the least tyrosine and the most proline.

The melting profiles and effects in the RNA-polymerase system of the unfractionated extracts from thymus are self-consistent, higher-melting material being found in the material extracted with 250 mM-hydrochloric acid (Fig. 3), which was apparently more active in depressing RNA synthesis than the material extracted with 50 mM-hydrochloric acid. The separated components of the material extracted with 250 mM-hydrochloric acid showed fairly wide differences in their capacity to depress RNA synthesis, the most mobile components being the most effective. Unfortunately, insufficient material was available to examine all the components of the material extracted with 50 mM-hydrochloric acid, but again the most mobile proteins were the most effective inhibitors. The possibility that more protein was used in these

assays with RNA polymerase than with the unfractionated material extracted with 50 mM-hydrochloric acid has already been discussed. Previous data linking the decrease of DNA-dependent RNA synthesis with histone composition are still conflicting (Huang *et al.* 1964; Allfrey *et al.* 1963; Hindley, 1963) and incomplete, and the relation of this property of the histones to DNA structure is virtually unexplored. These data are urgently required if the inhibition of DNA-dependent RNA synthesis by histones is to be equated with repression of genetic function. The present results confirm that very wide variations are found in the ability of different histones to repress; there was no clear correlation between their capacity to repress and the rate of amino acid uptake into the components.

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REFERENCES

- Allfrey, V. G., Faulkner, R. & Mirsky, A. E. (1964). *Proc. nat. Acad. Sci., Wash.*, **51**, 786.

- Allfrey, V. G., Littau, V. C. & Mirsky, A. E. (1963). *Proc. nat. Acad. Sci., Wash.*, **49**, 414.
- Allfrey, V. G., Littau, V. C. & Mirsky, A. E. (1964). *J. Cell Biol.* **21**, 213.
- Allfrey, V. G., Mirsky, A. E. & Osawa, S. (1955). *Nature, Lond.*, **176**, 1042.
- Boyer, P. D. (1954). *J. Amer. chem. Soc.* **76**, 4331.
- Boyer, P. D., (1959). In *The Enzymes*, 2nd ed., vol. 1, p. 511. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York: Academic Press Inc.
- Burton, K. (1956). *Biochem. J.* **62**, 315.
- Chamberlin, M. & Berg, P. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 81.
- Chaveau, J., Moulé, Y. & Rouiller, Ch. (1956). *Exp. Cell Res.* **11**, 317.
- Creasey, W. A. & Stocken, L. A. (1959). *Biochem. J.* **72**, 519.
- Crestfield, A. M., Moore, S. & Stein, W. H. (1963). *J. biol. Chem.* **225**, 363.
- Deakin, H., Ord, M. G. & Stocken, L. A. (1963). *Biochem. J.* **89**, 296.
- Ellman, C. L. (1959). *Arch. Biochem. Biophys.* **82**, 70.
- Hindley, J. (1963). *Biochem. biophys. Res. Commun.* **12**, 175.
- Huang, R. C. C. & Bonner, J. M. (1962). *Proc. nat. Acad. Sci., Wash.*, **49**, 1216.
- Huang, R. C. C., Bonner, J. M. & Murray, K. (1964). *J. molec. Biol.* **8**, 54.
- Jellum, E. & Eldjarn, L. (1964). *Biochem. J.* **92**, 7P.
- Johns, E. W., Phillips, D. M. P., Simson, P. & Butler, J. A. V. (1960). *Biochem. J.* **77**, 631.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- McAllister, M. C., Wan, Y. C. & Irwin, J. L. (1963). *Analyt. Biochem.* **5**, 321.
- Marsh, W. H., Ord, M. G. & Stocken, L. A. (1964). *Biochem. J.* **93**, 539.
- Mazia, D. (1961). In *The Cell*, vol. 3, p. 77. Ed. by Brachet, J. & Mirsky, A. E. New York: Academic Press Inc.
- Racusen, D. & Calvanico, N. (1964). *Analyt. Biochem.* **7**, 62.
- Rapkin, L. (1931). *Ann. Physiol. Physicochim. biol.* **7**, 382.
- Rasmussen, P. S., Murray, K. & Luck, J. M. (1962). *Biochemistry*, **1**, 79.
- Reid, D. R. & Cole, R. D. (1964). *Proc. nat. Acad. Sci., Wash.*, **51**, 1044.
- Smit, J. A. & Stocken, L. A. (1964). *Biochem. J.* **91**, 155.
- Smyth, D. G., Blumenfeld, O. O. & Konigsberg, W. (1964). *Biochem. J.* **91**, 589.
- Srinivasan, P. R., Brunfaut, M. & Errera, M. (1964). *Exp. Cell Res.* **34**, 61.
- Stedman, E. & Stedman, E. (1951). *Phil. Trans. B*, **235**, 565.
- Zbarskii, I. B. & Samarina, O. P. (1963). *Biokhimiya*, **27**, 471.