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Adenosine Diphosphate Ribosylated Histones

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When rat liver nuclei were incubated with [*adenine*-^{3}H]NAD, besides histone 1, histone 2A and especially histone 2B accepted ^{3}H radioactivity. ^{3}H radioactivity was also found on the non-histone proteins and on the small amounts of histones 1 and 3 released into the supernatant during incubation. [^{14}C]Adenine uptake *in vivo* by liver and thymus nuclei showed radioactivity in histones 1 and 3. After digestion with Pronase and leucine aminopeptidase ^{14}C- or ^{32}P-labelled histone 3 released a serine phosphate-containing nucleotide, which on acid hydrolysis yielded ADP-ribose and serine phosphate. Serine phosphate was also found in the material from the nucleotide peaks from histones 2A and 2B. ADP-ribosylated histones 1 and 3 were more easily released from nuclei than their unmodified forms and showed higher [^{32}P]P$_{i}$ and [^{3}H]lysine uptakes *in vivo* [Ord & Stocken (1975) *FEBS Meet. Proc.* **34**, 113–125].

ADP-ribosylation of nuclear proteins is a post-synthetic modification whose potential has principally been studied by following incubation of nuclei with labelled NAD (see Sugimura, 1973; Stocken, 1977). Histone 1 is an established acceptor both *in vivo* and *in vitro* (Smith & Stocken, 1975; Roberts *et al.*, 1975), and histones 2A, 2B and/or 3 (Smulson & Roberts, 1974; Stocken *et al.*, 1974) are also reported to act as acceptors *in vitro*. This has been confirmed in the present study with nuclei from normal and generating liver, and from *Echinus esculentus* (sea-urchin) eggs during the first cleavage cycle. For studies with rats we chose 13h after partial hepatectomy, as this time is close to the peak of histone phosphorylation preceding DNA synthesis. With *Echinus*, nuclei were isolated in the first telophase following fertilization, which is also a period of active nuclear-protein phosphorylation (M. G. Ord & L. A. Stocken, unpublished work).

Earlier results with adenine uptake (Ord & Stocken, 1966) and experiments using labelled ribose (Ueda *et al.*, 1975) indicated that histones 2 and/or 3 might act as acceptors *in vivo*. Phosphate contents of liver and thymus histones are already known (Ord & Stocken, 1975; Fónagy *et al.*, 1977). These, together with ribose contents, have been considered with results of adenine uptake *in vivo*, by using newer techniques for protein separation to determine which histones may be ADP-ribosylated physiologically.

Materials and Methods

Animals

Male rats were kept under controlled lighting conditions (10h dark, 14h light) with food available only in the dark. Sham operations and partial hepatectomies were performed in the first hour of light on male rats of 180–200g body wt. Four groups of rats were used: for the experiments on [^{3}H]NAD uptakes by liver nuclei *in vitro* seven sham-operated and 18 partially hepatectomized rats were needed. With [^{14}C]adenine and [^{32}P]P$_{i}$ uptakes *in vivo* groups of 12 normal animals were required for each experiment. ADP-ribose-binding sites on histones 2A and 2B were determined with material from the first two groups of rats, and the binding sites on histone 3 with proteins from the other two groups. In experiments where the thymus gland was used the rats were of 120–150g body wt.

Eggs from *Echinus esculentus* were obtained and fertilized as described elsewhere (Ord & Stocken, 1973).

Isotope incorporation in vivo

[^{32}P]P$_{i}$ (100μCi/100g body wt.) or [8-^{14}C]adenine sulphate (30μCi/100g body wt.) were injected intramuscularly 16 and/or 1h before death. The animals were killed by cervical fracture. When the specific radioactivity of plasma P$_{i}$ in control and partially hepatectomized rats was to be compared, blood was collected at death.

Isolation of nuclei and extraction of nuclear proteins

Nuclei were isolated from rat liver and thymus, and from fertilized *Echinus* eggs in telophase, and in all the experiments the nuclear proteins were separated by the same techniques. Purification was by exclusion and ion-exchange chromatography (Ord & Stocken, 1975; Fónagy *et al.*, 1977). The purity and composition of the histones was confirmed by

their homogeneity on acid/urea/polyacrylamide-gel electrophoresis (Panyim & Chalkley, 1969*a*,*b*) and by their amino acid analyses. In experiments with [^{14}C]adenine uptakes *in vivo* negligible ^{14}C radioactivity was detected in histones 2A and 4 after final purification through Bio-Gel P10 columns.

Uptake of [^{3}*H*]*NAD*

Liver nuclei were suspended in 5ml of 0.1M-Tris/HCl, pH 8.0, 40mM-KCl, 10mM-$MgCl_2$, 2mM-dithiothreitol and incubated with 25μCi of [^{3}H]NAD for 20min at 37°C. The concentration of the nuclei was estimated turbidometrically so that equal concentrations could be used from sham-operated and partially hepatectomized rats. DNA (4–5mg/ml) was present in the nuclear suspension (Burton, 1956). At the end of the incubation the nuclei were brought to 0°C, centrifuged at 35000*g* for 15min, and the supernatant proteins and those subsequently extracted from the residue fractionated separately (Fónagy *et al.*, 1977).

Radiochemicals and determination of radioactivity

[^{32}P]P_i (3.47Ci/mmol) and [8-^{14}C]adenine sulphate (specific radioactivity 28.9mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. [*adenine*-2,8-^{3}H]NAD (3.28Ci/mmol) was from New England Nuclear (Boston, MA 02118, U.S.A.), and used within 1 week of receipt. Radioactivity was determined in a Wallac 1210 Ultrobeta liquid-scintillation counter. Approximate efficiencies were 90% for ^{32}P and ^{14}C, and 40% for ^{3}H.

Chemicals

ADP-ribose was from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. Its composition and structure was confirmed by nuclear-magnetic-resonance spectroscopy by Dr. C. H. Reynolds and Dr. P. J. Seeley (Department of Biochemistry, University of Oxford, Oxford, U.K.).

Other analytical methods

These were as previously reported (Fónagy *et al.*, 1977).

Results

[*Adenine*-^{3}*H*]*NAD uptake by liver nuclei in vitro*

Experiments with thymus nuclei (Fónagy *et al.*, 1977) indicated that non-histone proteins and phosphorylated histones were preferentially released into the supernatant during incubation in a slightly hypo-osmotic medium. Liver nuclei from normal and partially hepatectomized rats were isolated 1h after the animals had received an injection of [^{32}P]P_i. After incubation with [^{3}H]NAD under standard conditions the histones released into the supernatant had, as expected, higher ^{32}P radioactivity/mg of protein than the histones subsequently extracted from the

Table 1. [^{3}*H*]*NAD uptake by nuclear proteins from nuclei of normal and regenerating rat liver*

Partially hepatectomized animals were given [^{32}P]P_i 12h after operation, 1h before death. Sham-operated rats were treated similarly. Nuclei were isolated and incubated with [^{3}H]NAD as described in the text; after 20min at 37°C the suspensions were centrifuged for 15min at 35000*g*. The supernatant was removed and the residual nuclei extracted with $HClO_4$ and 250mM-HCl as described in the Materials and Methods section. Proteins in the $HClO_4$ extract were separated as described in the text. Radioactivity of the proteins is expressed as c.p.m./μg of protein; for ^{32}P this is corrected for the difference in specific radioactivity of liver P_i pools. There were six rats in the control group and twenty rats in the experimental group.

	Specific radioactivities (c.p.m./μg of protein)			
	Normal liver nuclei		After partial hepatectomy	
Supernatant proteins	^{3}H	^{32}P	^{3}H	^{32}P
Total	3.6	2.7	14.2	10.3
$HClO_4$ (5%, w/v) soluble	30.6	2.25	15.2	2.4
HCl (250mM) soluble	9.0	2.3	5.9	2.3
'Bound' proteins				
$HClO_4$ (5%, w/v) soluble	39.8	0.8	46.9	1.1
HCl (250mM) soluble	10.9	1.4	20.0	1.8
Histone 1a	161	7.7	315	6.0
Histone 1b	14.9	0.2	4.1	1.1
Non-histone protein P1	17.6	1.3	37.4	1.9
Histones 2A, 3, 4	12.2	1.0	13.9	1.3
Histone 2B	85.8	1.7	107	1.3

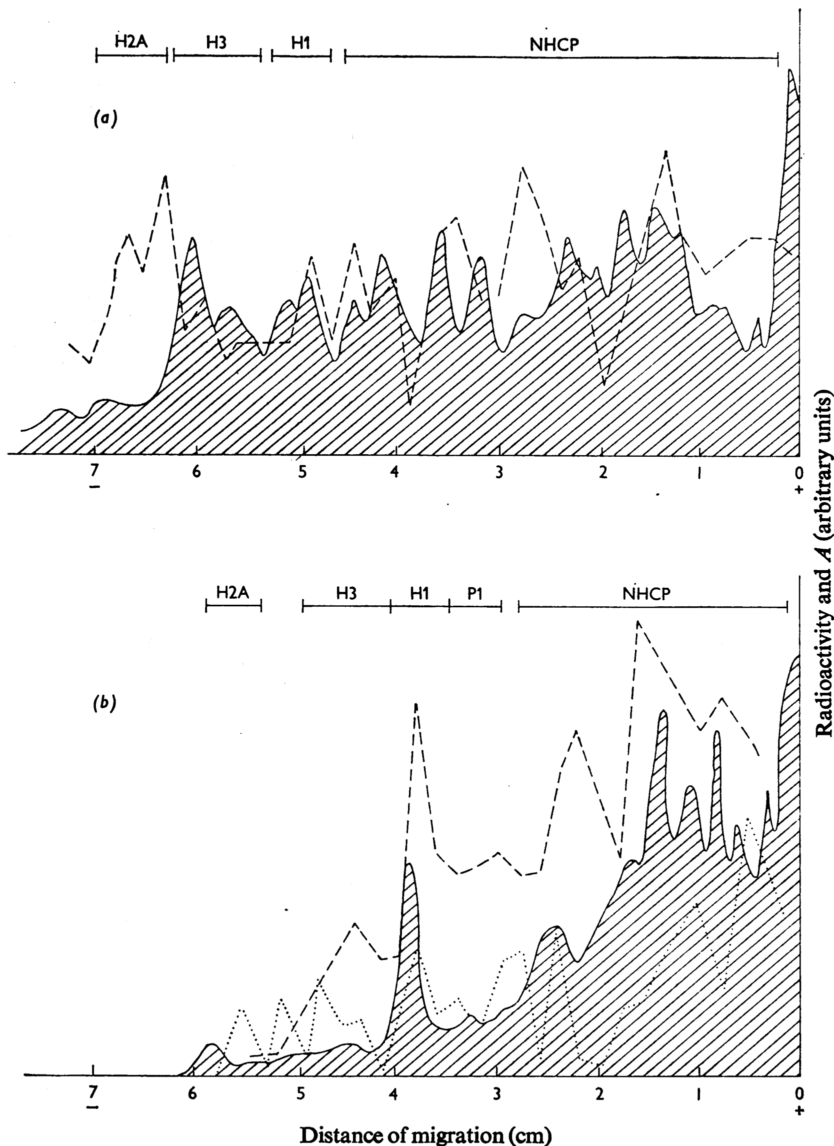


Fig. 1. *Acid/urea/polyacrylamide-gel electrophoresis of proteins from liver and Echinus nuclei, that had been incubated with [adenine-^{3}H]NAD*

Preparation and extraction of nuclei are described in the text. The rats were given [^{32}P]P_i 1h before death. (*a*) HCl (250mM) extract from chromatin obtained from *Echinus* nuclei 140min after fertilization (telophase); (*b*) supernatant proteins released from normal liver nuclei. The migration was from right to left. The hatched areas show the Vitatron scans. ----, ^{3}H radioactivity; · · · ·, ^{32}P radioactivity. The positions of histones 1 (H1), 3 (H3) and 2A (H2A) are indicated and also that of the non-histone protein P1. NHCP are the non-histone chromosomal proteins.

nuclear residue (Table 1, Fig. 1). ^{3}H radioactivity in the supernatant was associated with four proteins in the non-histone region, including protein P1 (Smith & Stocken, 1973), and with histones 1 and 3. The acceptor proteins were phosphorylated. With histone 3 released into the supernatant during incubation (Fig. 1*b*), two modified forms may have been present, one of which appeared to carry [^{3}H]-adenine radioactivity. This might also be evident for the non-histone protein P1.

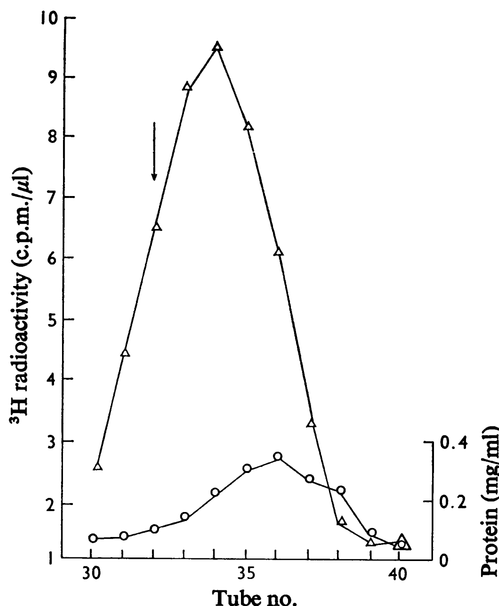


Fig. 2. *Bio-Gel P*-10 *separation of crude histone* 2*B from rat liver nuclei isolated* 13 *h after partial hepatectomy and incubated with* [^{3}*H*]*NAD*

Conditions of nuclear isolation, incubation and histone extraction are as described in the text. Crude histone 2B (4.5mg) was applied to a Bio-Gel P-10 column (400cm × 0.5cm). The protein was eluted with 10mM-HCl/1mM-2-mercaptoethanol with a flow rate of 2ml/h. The volume in each tube was 1ml. △, ^{3}H radioactivity; ○, protein. The arrow indicates the peak in specific activity.

Separation of the bound histones indicated no significant differences in ^{3}H radioactivity for histone 2B, nor for the acid/ethanol-soluble group of proteins, between normal and regenerating liver. Column-chromatographic separation on Bio-Gel P-10 showed (Fig. 2) that ^{3}H radioactivity equivalent to 0.5mol of adenine/mol of protein was associated with histone 2B emerging on the ascending side of the protein peak. Comparison of the u.v. spectra with histone 2B previously isolated from normal rat liver also confirmed the presence of adenine in the material (0.4 mol/mol). For histone 2A the protein emerging on the leading edge of the peak had ^{3}H radioactivity equivalent to 0.35mol of adenine/mol (Fig. 3, tubes 37 and 38). The bulk of the remaining histone 2A carried 0.13mol of adenine/mol (Fig. 3, tubes 40–45). From acid/urea/polyacrylamide-gel electrophoresis the acceptors appear to correspond to histone 2A carrying 2 and 1 mol of phosphate/mol of protein respectively. The Bio-Gel separations also suggested negligible radioactivity had been incorporated into the bound forms of either histones 3 or 4 (Fig. 3).

Fractionation of the $HClO_4$-soluble proteins (Table 1) showed marked [^{3}H]NAD uptake into ADP-ribosylated histone 1 (H1a) (Smith & Stocken, 1975), negligible uptake into the remaining histone 1 (H1b) and some incorporation into the non-histone protein P1. This distinction between the two forms of histone 1 was unchanged 13h after operation, when nuclei from partially hepatectomized rats showed approximately twice the radioactivity in histone 1a and protein P1 than from controls, corresponding to 2 and 0.3 mol of adenine/mol of protein respectively. At this time after partial hepatectomy the phosphate content of histone 1b increases from 0.2 to 1.4mol/mol (Ord & Stocken, 1975). Acid/urea/polyacrylamide-gel electrophoresis indicated that the most mobile subclass of histone 1 was probably not an acceptor of ^{3}H radioactivity from NAD *in vitro*. The other three subclasses separating electrophoretically all showed ^{3}H uptake.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that all the ^{3}H radioactivity was released from all the histones and the soluble non-histone proteins after treatment of the supernatant proteins with 0.1M-NaOH (30min, 20°C); only one protein showed ^{3}H radioactivity, which was partially released by 0.4M-NH_2OH, pH 7.5, under the same conditions. This was the second least mobile in the non-histone region.

[*adenine*-^{3}*H*]*NAD uptake by Echinus nuclei in vitro*

HCl (250mM) extracts of chromatin from nuclei isolated from *Echinus* eggs 140min after fertilization (in telophase) (M. G. Ord & L. A. Stocken, unpublished work) showed similar patterns of ^{3}H radioactivity on acid/urea/polyacrylamide-gel electrophoresis to those shown by liver nuclei (Fig. 1*a*); five proteins in the non-histone region appeared to be labelled as well as histones 1, 3 and 2A/2B. Modified forms of histones 1 and 3 were seen. Separation of the $HClO_4$- and acid/ethanol-soluble fractions confirmed ^{3}H uptake in histone 1, and histones 3 and 2A. Histone 4 was probably not significantly radioactive. Histone 2B was not easily separated from other acid-soluble proteins, and was not further examined.

Adenine uptake in vivo by liver and thymus histones

Phosphate contents of histones from liver (Ord & Stocken, 1975) and thymus (Fónagy *et al.*, 1977) indicated that ADP-ribosylation of histones 2A and 2B must be very low *in vivo*. To investigate this further [^{14}C]adenine was given to rats 16h before death, and liver and thymus nuclei isolated. They were incubated with 2mM-ATP for 20min at 37°C to promote release of phosphorylated histones and non-histone proteins (Fónagy *et al.*, 1977). The proteins

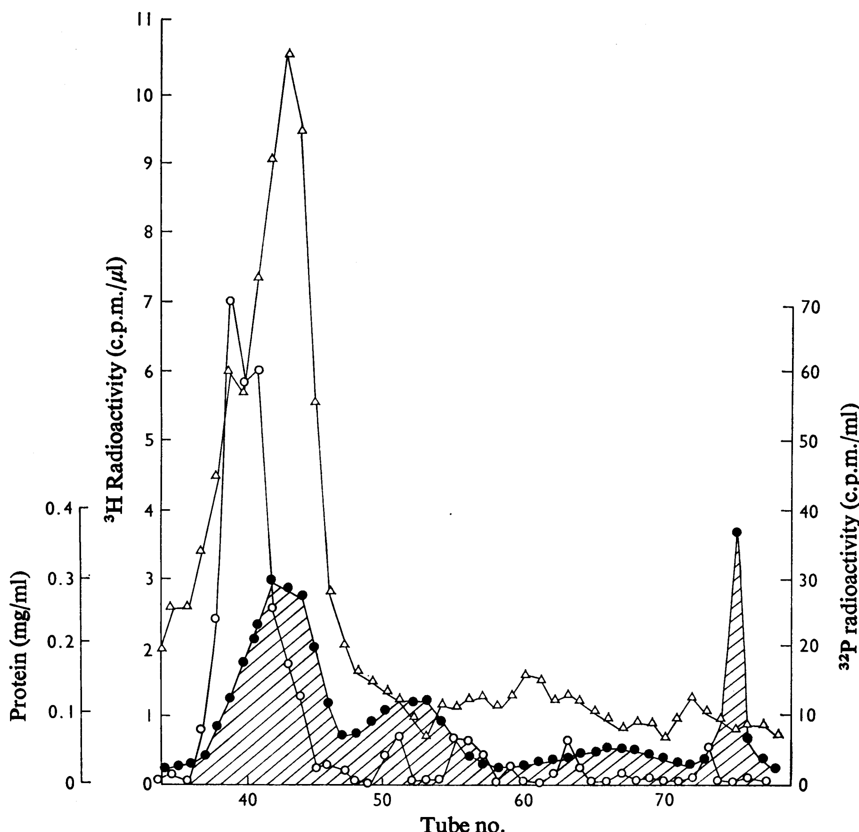


Fig. 3. *Bio-Gel P-10 separation of crude histones 2A, 3 and 4 from rat liver nuclei isolated 13h after partial hepatectomy and incubated with [^{3}H]NAD*

Conditions of nuclear isolation, incubation and histone extraction are as described in the text; 4.6mg of protein was applied to the column. Otherwise the conditions were as in Fig. 2. ●, Protein (hatched); △, ^{3}H radioactivity; ○, ^{32}P radioactivity.

so released into the supernatant and those subsequently extracted with acid were fractionated in the normal way. Similar results were obtained with both types of nuclei.

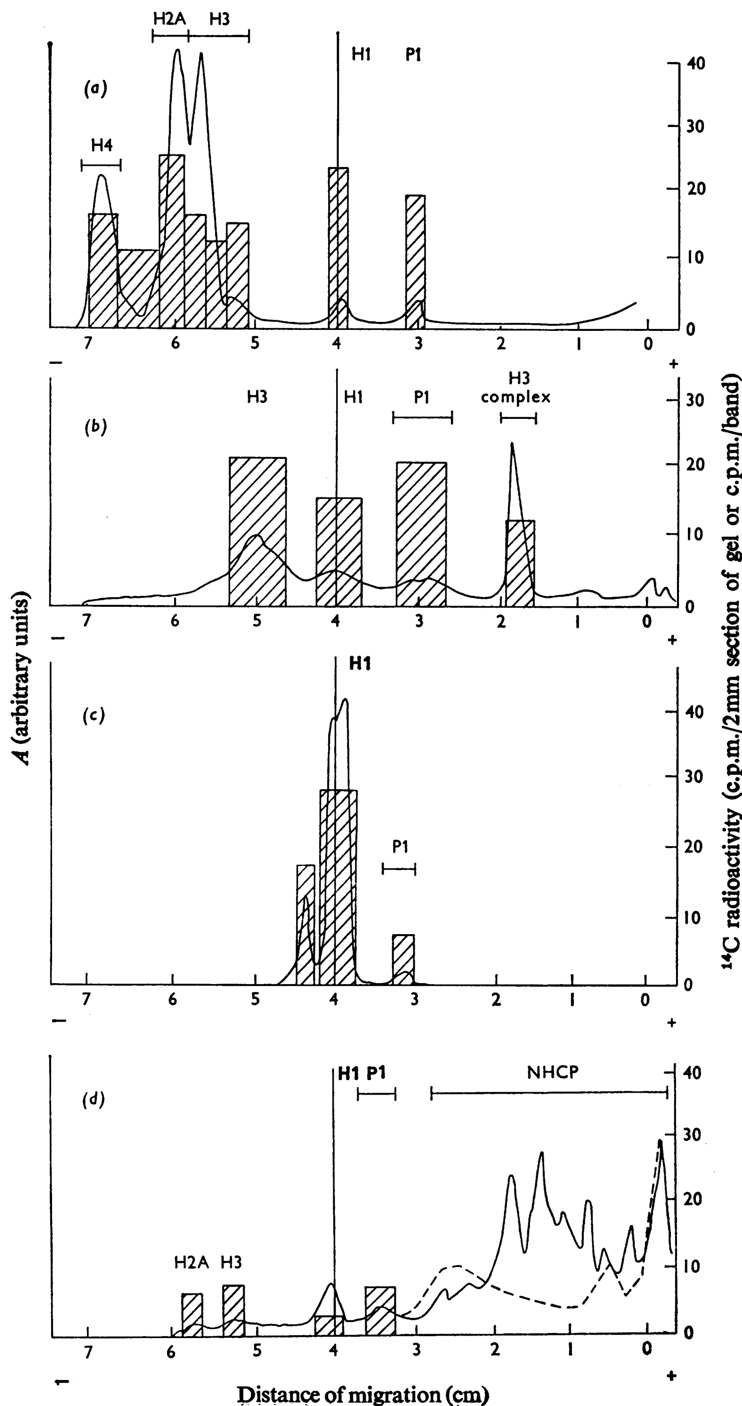
On acid/urea/polyacrylamide-gel electrophoresis, ^{14}C radioactivity was principally associated with four acid-soluble proteins in the non-histone region in thymus and with three in liver, including protein P1 in both cases (Figs. 4*d* and 5*c*). Histone 1 (Figs. 4*c* and 5*b*) and that fraction of histone 3 which, with liver nuclei, was released into the supernatant during incubation, also showed [^{14}C]adenine uptake (Fig. 4*d*); this was especially evident when the acid/ethanol-soluble histones in the supernatant were separated and examined (Fig. 4*b*).

It was also observed that the acid/ethanol-soluble proteins appeared to contain traces of histone 1 and non-histone P1, which had high [^{14}C]adenine uptakes (Figs. 4*a* and 4*b*, and 5*a*). It was assumed that these ribosylated compounds were more easily aggregated with other proteins during $HClO_4$ precipitation and were not subsequently removed in 5% (w/v) $HClO_4$.

Bound histones 2A, 3 and 4 showed radioactivity (Figs. 4*a* and 5*a*), but that on histones 2A and 4 was directly proportional to the protein concentration and was assumed to be non-specific binding. This was confirmed when these acid/ethanol-soluble proteins from thymus were separated on Bio-Gel P-10 after removing possible polyphosphorylated compounds on DEAE-cellulose at pH 7.4. Only histone 3 then showed ^{14}C radioactivity on the ascending side of the protein peak.

Acid/urea/polyacrylamide-gels of the acid/ethanol-soluble proteins extracted from the supernatant proteins released from liver nuclei (Fig. 4*b*) and the residual fraction from thymus nuclei (Fig. 5*a*) showed the presence of a slowly migrating compound, which

had high [^{14}C]adenine radioactivity and was removed by passage through DEAE-cellulose at pH 7.4. This component migrated on the acid/urea/polyacrylamide-gels in an identical position to a ribonucleotide-associated complex of histone 3, which had been found earlier in liver and thymus histones (Ord & Stocken, 1975; Fónagy *et al.*, 1977) and which contained 16–20 mol of P/mol of histone.



This protein in normal liver amounted to about 5% of the total histone 3, contained ribose and had a high ^{32}P uptake *in vivo*. The greater part of the histone 3 in liver contained 0.24 ± 0.06 mol of phosphate/mol of protein and 0.15 ± 0.06 mol/mol of ribose (six determinations): it did not show [^{32}P]P_i uptake *in vivo* after a pulse of 1 h.

These results from the incorporation studies *in vivo* and *in vitro* and from the phosphate and ribose analyses obtained earlier (Fónagy *et al.*, 1977) suggested that in addition to ADP-ribosylated histone 1, a small amount of histone 3 carried polymeric ADP-ribose *in vivo*. This fraction of histone 3, which was more easily lost from nuclei during incubations, was not in rapid equilibrium with the bulk of histone 3 assumed to be part of the chromatin core.

Phosphate and ribose analyses of the non-histone protein P1 also suggested its ADP-ribosylation *in vivo*. In normal liver the protein carries 2.3 ± 0.6 mol of phosphate/mol and has a ribose : phosphate ratio of 1:1.7. By 13 h after partial hepatectomy, ADP-ribosylation of this protein may increase, since phosphate contents of 8.4 mol/mol have been reported (Ord & Stocken, 1975).

Identification of ADP-ribose-acceptor sites in histones 2A, 2B and 3

The [*adenine*-^{3}H]NAD-labelled histones 2A and 2B, ^{32}P-labelled histone 3 complex (Ord & Stocken, 1975) from rat liver nuclei from rats given [^{32}P]P_i 1 and 16 h before death, and histone 3 from thymus nuclei from the rats, which had been labelled overnight with [^{14}C]adenine, were digested with Pronase and leucine aminopeptidase (Balhorn *et al.*, 1971; Smith & Stocken, 1975). The digests were brought to pH 3 and applied to Dowex 50 columns (Fig. 6).

[^{3}H]Adenine-labelled material from histones 2A and 2B migrated in an identical position to the [^{14}C]-adenine radioactivity from histone 3 (Fig. 6*d*). Histone 2A had been obtained from rats which had received [^{32}P]P_i 1 h before death. Under these circumstances ^{32}P is found on serines 1 and 19 (Ord & Stocken, 1975; Shlyapnikov *et al.*, 1975). The ^{3}H-containing material from histone 2A also contained ^{32}P, implicating serine phosphate at positions 1 or 19 in the binding site.

When the material from the radioactive nucleotide peaks from histones 2A, 2B and 3 were hydrolysed in 1 M-HCl for 30 min at 37°C (Spackman *et al.*, 1958) and amino acid analyses performed, only serine and serine phosphate were detectable from histones 2A and 2B (Table 2). Amounts of adenine present could be calculated from the ^{3}H radioactivity in histones 2A and 2B, assuming no dilution had occurred from endogenous NAD in the unwashed nuclei (Table 2). With histone 3, which had been labelled *in vivo*, no such calculation was possible. With this protein, threonine and glutamic acid were also present with serine and serine phosphate in the nucleotide-containing peak.

Pronase/leucine aminopeptidase digests of histone 3 complex gave one main peak of radioactivity off the Dowex 50 (Fig. 6*e*). Considerable ^{32}P radioactivity was present in the run-off material. This was hydrolysed in 1 M-HCl for 30 min at 37°C; ^{32}P radioactivity appeared where ADP-ribose and serine phosphate would be eluted (Fig. 6*d*). AMP, which might have been present as a contaminant, is eluted after serine phosphate, and at a pH considerably higher than 1.5. The release of the serine phosphate suggested its involvement in an acid-labile linkage with ADP-ribose. Similar behaviour was shown by that fraction of the histone 3 complex eluting in 3–8 ml of 50 mM-HCl (Fig. 6*c*).

When the material from the nucleotide peaks from histone 2B and the [^{14}C]adenine-labelled histone 3 were hydrolysed in 1 M-HCl as above, and rechromatographed on Dowex 50, together with authentic ADP-ribose, the ^{3}H or ^{14}C radioactivities now co-migrated with the ADP-ribose (Fig. 6*a*). With histone 2A, acid hydrolysis decreased the ^{32}P radioactivity, but the position at which the ^{3}H radioactivity eluted was unchanged, retarded with respect to ADP-ribose.

Discussion

Much work on ADP-ribosylation of nuclear proteins has been concerned with the functions of poly-

Fig. 4. *Acid/urea/polyacrylamide-gel electrophoresis of liver nuclear proteins isolated from rats* 16*h after exposure to* [^{14}C]*adenine*

Conditions of nuclear isolation and protein extraction are given in the text. The nuclei were incubated for 20 min at 37°C with 2 mM-ATP. They were then cooled to 0°C and centrifuged for 15 min at 35000 g. The proteins released into the supernatant were subsequently fractionated with $HClO_4$ and acid/ethanol, as were the residual nuclei. Proteins obtained from the latter are described as 'bound'. Vitatron scans are from right to left. Positions of the principal components are indicated. Non-histone protein P1 migrated about 3.2 cm and histone 1 4 cm. NHCP are the other non-histone chromosomal proteins and H3 complex is the poly(ADP-ribosyl)ated form of histone 3 discussed in the text. The other histones are labelled as H1, H2A etc. ----, Radioactivity (or shown as a hatched bar for the total in that region). The radioactivity in the interband regions was indistinguishable from the background value. (*a*) Acid/ethanol-soluble proteins from residual nuclei; (*b*) acid/ethanol-soluble proteins from supernatant (*d*); (*c*) histone 1 from residual nuclei; (*d*) supernatant proteins; ——, *A*.

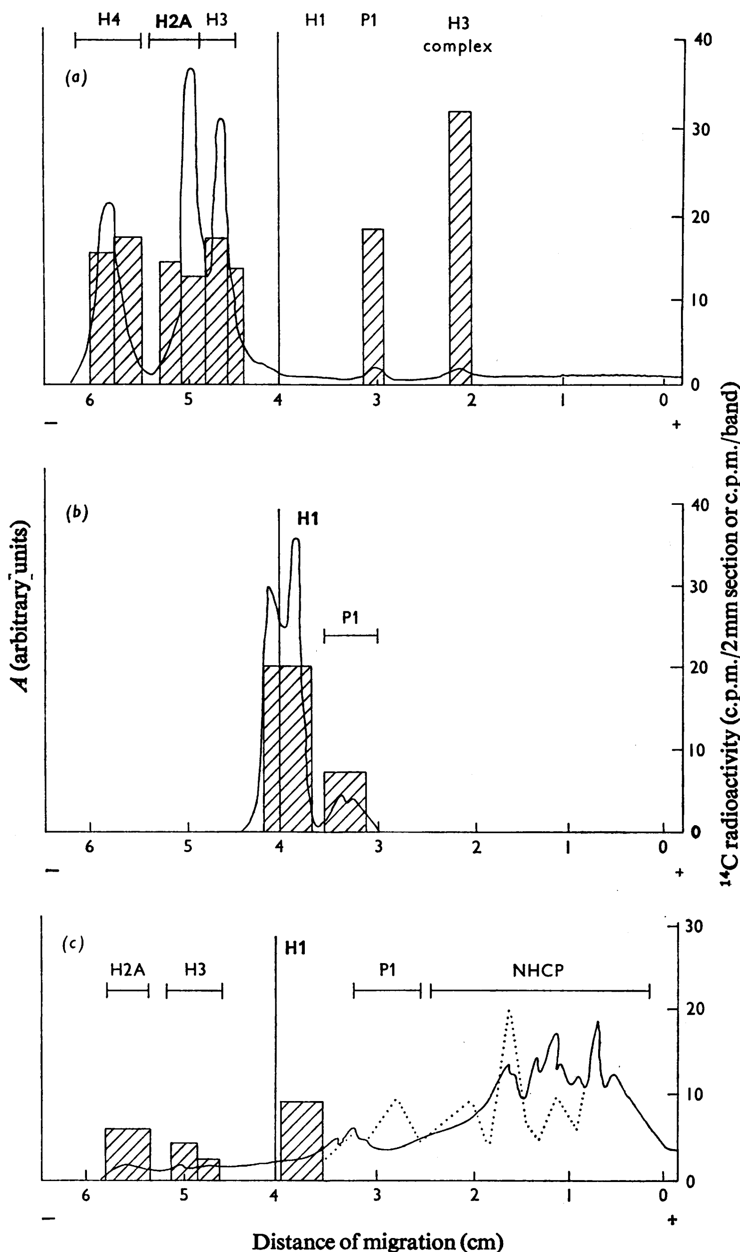


Fig. 5. *Acid/urea/polyacrylamide-gel electrophoresis of thymus nuclear proteins isolated from rats 16h after exposure to [^{14}C]adenine*

Conditions of nuclear isolation and protein extraction are given in the text. The nuclei were incubated for 20min at 37°C with 2mM-ATP. They were then cooled to 0°C and centrifuged for 15min at 35000*g*. The proteins released into the supernatant were subsequently fractionated with $HClO_4$ and acid/ethanol, as were the residual nuclei. Proteins obtained from the latter are described as 'bound'. Vitatron scans are from right to left. Positions of the principal components are indicated as in Fig. 4. NHCP are the non-histone chromosomal proteins and H3 complex is the poly-(ADP-ribosyl)ated form of histone 3 discussed in the text. ----, Radioactivity (or shown as a hatched bar for the total in that region). The radioactivity in the interband regions was indistinguishable from the background value. (*a*) Acid/ethanol-soluble proteins from residual nuclei; (*b*) histone 1 from residual nuclei; (*c*) supernatant proteins.

(ADP-ribose). Our interest here was with the changes ADP-ribosylation may make to the behaviour of histone acceptors, although these may account for only a small proportion of the total ADP-ribosylated proteins in nuclei (Adamietz & Hilz, 1976). Two histones appear to be acceptors *in vivo*: histone 1, where in adult liver the substituent is predominantly in the monomeric form (Smith & Stocken, 1975), and histone 3, of which in liver about 5% might be poly(ADP-ribosyl)ated with 8–10 units/molecule, and another part of which appears to carry the monomeric substituent. ADP-ribosylation of histone 3 has also been reported by Caplan during differentiation in chick mesenchymal cells (see Smulson & Shall, 1976).

Table 2. *Analyses of nucleotide peaks from histone digests separated on Dowex 50 columns*

Histones 2A and 2B had been obtained from rat liver nuclei incubated with [*adenine*-^{3}H]NAD; [^{14}C]adenine-labelled histone 3 was obtained from rat thymus (see the text). The material from the nucleotide peaks was hydrolysed in 1M-HCl for 30min at 37°C and their amino acid contents determined. ^{3}H radioactivity of histones 2A and 2B were used to determine their adenine contents (see the text).

	Composition (nmol)		
Histone ...	2A	2B	3
Serine phosphate	1.8	2.4	1.6
Threonine	—	—	1.7
Serine	1.7	1.7	1.6
Glutamic acid	—	—	1.9
Adenine	1.7	2.9	—

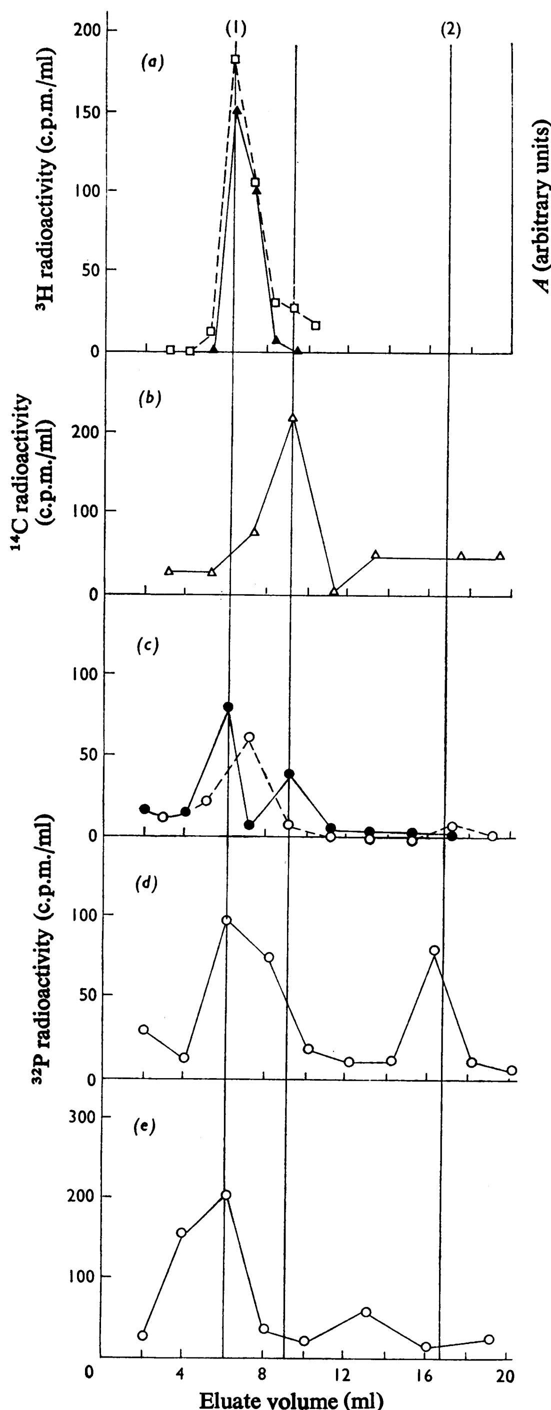


Fig. 6. *Dowex 50 separations of histones 3 and 2B digests after Pronase and leucine aminopeptidase treatment*

Histone 3 was prepared from liver and thymus nuclei from rats that had been given [^{32}P]P_i 1 and 16h before death or [^{14}C]adenine 16h before death. Histone 3 complex from liver and histone 3 monomer from thymus were separated on Bio-Gel P-10, digested (Balhorn *et al.*, 1971) and applied to Dowex 50 columns. Histone 2B was prepared from liver nuclei that had been incubated with [^{3}H]NAD. It was separated on Bio-Gel P-10, digested and analysed as for histone 3. Elution was with 50mM-HCl. (*a*) [^{3}H]Adenine-labelled histone 2B after chromatography on Dowex 50, hydrolysis was done in 1M-HCl as for (*c*) and rechromatography with added ADP-ribose; (*b*) [^{14}C]adenine-labelled histone 3 monomer from thymus; (*c*) the fraction of histone 3 complex from (*e*) separating in 4ml, re-run with (○) and without (●) hydrolysis in 1M-HCl for 30min at 37°C; (*d*) the run-off fraction from (*e*), hydrolysed for 30min at 37°C and re-applied to a Dowex 50 column; (*e*) ^{32}P-labelled liver histone 3 complex. The positions of authentic ADP-ribose (1) and serine phosphate (2) are also shown. (*a*) ▲, ^{3}H radioactivity; □, A_{260}.

The methods of extraction and purification of the histones used here would not allow the isolation of acid-labile ADP-ribosylated derivatives with linkages through *N*-phosphate or acyl groups. The ADP-ribosylated histones we have analysed contain serine phosphate in the acceptor sites, and in contrast with other histone–ADP-ribose derivatives, which are labile in 0.4 M-NH_2OH at pH 7.5 (see Smulson & Shall, 1976), the linkages here were stable to neutral NH_2OH. The lability of the linkages in histones 1 and 3 to 1 M-HCl at 37°C, and consequent release of serine phosphate and ADP-ribose, reported here and elsewhere (Smith & Stocken, 1975), is consistent with ADP-ribosylation on to serine phosphate. With histone 3, the presence of significant amounts of threonine and glutamic acid in the nucleotide peak raises the possibility of alternative or additional ester or ribosidic, acid-labile, linkages for the ADP-ribose. The tripeptide serylthreonylglutamate could have been derived from positions 57–59 in histone 3.

From its ^{32}P radioactivity the substituent on histone 2A must have been attached at either serines 1 or 19. We are unable at present to explain the failure of acid hydrolysis to alter the position at which the ^{3}H-labelled nucleotide from this histone eluted on Dowex 50. In histone 2B, phosphorylation *in vitro* has been reported on serine residues at positions 14 and 36 (Shlyapnikov *et al.*, 1975) or 32 and 36 (Hashimoto *et al.*, 1975). Histone 2B is probably only phosphorylated in the cell cycle at the G_1-phase–S-phase transition, and its phosphate content in resting liver is negligible. The size of the [^{3}H]NAD uptake into this protein *in vitro* was quite high, and it is possible that the histone was phosphorylated during the incubation, since some ATP is likely to have been retained on the unwashed nuclei, which have an extremely active kinase phosphorylating histone 2B (Fónagy *et al.*, 1977).

ADP-ribosylation markedly affects the behaviour of the proteins. When nuclei in a slightly hypo-osmotic medium were incubated with [γ-^{32}P]ATP, only ADP-ribosylated histone 1 (H1a) was a major phosphate-accepting histone unless chromatin dissociation had been promoted (Fónagy *et al.*, 1977). ADP-ribosylated non-histone protein P1 was phosphorylated under the same conditions. The ADP-ribosylated form of histone 3 was also preferentially released into the incubation medium.

Studies on [^{3}H]lysine uptake *in vivo* (Ord & Stocken, 1975) showed additionally that 'turnover' of histones in resting liver was only detectable in ADP-ribosylated histones 1 and 3. When histone biosynthesis commenced in rat livers 13 h after partial hepatectomy, [^{3}H]lysine uptake into poly(ADP-ribosyl)ated histone 3 was again much more pronounced than into the remainder of the histone.

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