THE ROLE OF HISTONE AND NON-HISTONE PROTEINS IN NUCLEAR FUNCTION

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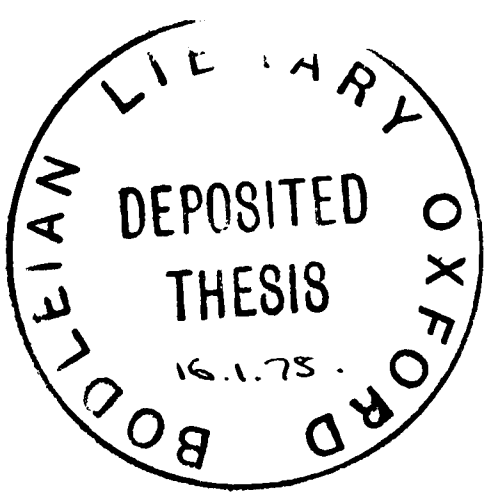
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ABSTRACT

In eukaryotic cells, DNA, which contains the genetic information of the organism, is complexed with histones, a group of small, basic proteins of distinctive amino acid composition. Since the DNA of eukaryotes is mostly repressed, it was suggested by Stedman and Stedman, (1950, 1951) that the histones function as gene inhibitors. Unfortunately, the limited heterogeneity and comparative lack of tissue and species specificity (Panyim et al, 1971) of the histones is inadequate to explain the relative restriction of DNA which is found. Two observations have suggested answers to the problem of selectivity. On the one hand, non-histone proteins are extremely heterogeneous (e.g. Yeoman et al, 1973) and show tissue and species specificity (Dravid and Burdman, 1968; Platz et al, 1970). They are present in high concentration in the chromatin of metabolically active tissues, and there is some evidence that they are responsible for specificity of transcription from chromatin (Paul and Gilmour, 1968). The other relevant observation is that histones are subject to post-transcriptional chemical micromodifications in vivo. The main modifications are methylation (Murray, 1963), acetylation (Allfrey et al,. 1964), thiolation (Ord and Stocken, 1966) and phosphorylation (Ord and Stocken, 1966). Acetylation of histones correlates in general with RNA synthesis, while phosphorylation, and perhaps thiolation, correlate with DNA synthesis and cell division. Current opinion is that modifications of histones are concerned with general changes in the state of the nucleus, while the non-histones include specific genetic repressors.

it is known also that many non-histone proteins are subject to micromodification *in vivo*.

In order to study nuclear proteins, the first requirement is to separate single species of protein. In spite of the distinctive characteristics of the histones, they frequently contain non-histone contaminants (Johns, 1964; Murray, 1965). Although the degree of contamination is not high, it is likely to be significant, particularly in studies of phosphorylation, because of the high phosphorus content of non-histone proteins (Langan, 1968). Since the greatest amount of phosphorylation is observed in the histone F1 fraction (Ord and Stocken, 1966) the first objective of this study was to obtain a pure sample of histone F1, and to characterize its non-histone contaminants. Consequently, non-histone proteins which coextracted with histone F1 were separated from the histone on DEAE Cellulose, and the major component, P1, was purified. It was shown by SDS gel electrophoresis to be a small protein, of molecular weight 20,000 daltons, and to have an N-terminal glycine residue. It contains a high proportion of lysine, and acidic residues. It has a cysteine residue which allows it to form dimers through a disulphide bond. In the oxidised form, it forms complexes with histone F1. Both F1 and P1 have covalently bound phosphate *in vivo*, and both are substrates for cytoplasmic protein kinase *in vitro*, although P1 is less effective than F1 in this respect. Both F1 and P1 are partially extracted from nuclei with 0.35M salt, P1 being more loosely bound.

The comparatively loose binding of both F1 and P1 to chromatin is such as to allow these proteins to be involved in control of chromatin state (see Comings and Tack, 1973). The redox-dependent complex formation between P1 and F1 is such that P1 can 'relay' the redox-dependent complex formation between P1 and F1 is such that P1 can 'relay' the redox state of the cell to produce an effect on the histone.

Now that a pure preparation of histone F1 is available, it is possible to examine the nature of the bound phosphate. In addition to the previously described phosphoserine (Ord and Stocken, 1966), covalently bound ADPribose is also found. The bound ADPribose is stable to neutral hydroxylamine, RNAse and alkaline phosphatase, but is liberated by alkali and venom phosphodiesterase. Enzymic hydrolysis of the protein yields ADPribose monomer bound to the phosphate group of phosphoserine as the major nucleotide product. This product gives phosphoserine and ADPribose on mild acid hydrolysis, with a ratio for serine : adenine : ribose : phosphate of 1 : 1 : 2 : 3. It is proposed that the structure of the product is 1-"(O-serylphosphoryl)-ADPribose. Peptides bearing the nucleotide, isolated from a tryptic digest of histone F1, contained a number of acidic amino acid residues. In resting rat liver, more than 90% of the phosphate bound to histone F1 was present as nucleotide.

The presence of ADPribose bound to nuclear protein after incubation of nuclei <u>in vitro</u> with labelled NAD^+ has been noted previously (Nishizuka et al, 1968). In this study, ADPribose was found bound to non-histone proteins in saline, acid and

urea-saline nuclear extracts. In each case the acceptor protein appears to be a formally acidic non-histone protein with a high serine and glycine content, which forms aggregates readily. The ADPribose is attached by two types of bond, one of which is labile to neutral hydroxylamine.

A peptide of molecular weight about 3,000 daltons is also an acceptor of ADPribose. This compound is most actively synthesised in growing tissues. The nature of its peptide-nucleotide bond is not known.

In perchloric acid extracts from nuclei of resting liver, a large proportion of the histone F1 is aggregated around the ADPribose derivative of the histone. When the cell passes from Go to G1, after partial hepatectomy, F1 accepts phosphate as phosphoserine and the proportion of aggregated material is rapidly reduced.

It is proposed that the aggregation observed in vitro reflects the function of the ADPribosyl derivative of the histone in vivo, namely to maintain Go chromatin in a condensed form.

Increased phosphate contents of histones F2a1, F2a2 and F2b are found at 4 and 13 hours after partial hepatectomy, at times corresponding to the peaks of cAMP levels in liver. The phosphoserine contents of histone F1 and protein P1 are raised to a considerable degree during the period of DNA synthesis. Histone F3 is not phosphorylated prior to mitosis. Both the phosphoserine and ADPribose levels of the non-histone proteins are higher in S phase nuclei than in Go. An increase in synthesis of the small ADPribose - accepting peptide is

observed at early times after partial hepatectomy, which continues until mid S phase.

These results are in keeping with a model in which histones F2 and F3 maintain the secondary and tertiary structure of chromatin, and histone F1 is concerned with higher order structure. Major alterations of chromatin structure are brought about by micromodifications of histones, particularly by phosphorylation. The exact manner in which various non-histone proteins control the function of the nucleus and the details of chromatin structure remain to be elucidated. ADPribosyl derivatives of nuclear proteins would appear to be concerned with broad aspects of nuclear function.

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CHAPTER 1

INTRODUCTION

Ideally, nuclear proteins would be classfied according to their function, but this is not possible in the majority of cases, as their functions are not generally known. Alternatively, it would be helpful to identify in which region of the nucleus they are found; whether in the chromatin, nucleolus, nuclear membrane, or nucleoplasm. However, it is not always clear which part of the nucleus proteins belong to, since the conditions of isolation appear to be critical in this respect. For practical reasons, the differential solubility in various aqueous solvents is a much used method of classification of proteins. However, when a protein has no demonstrable function, it is not always clear whether apparently similar proteins in different nuclear extracts are the same. The only class of nuclear proteins that has received thorough study is that of the histones.

a) Histones:- Characterisation

Histones may be distinguished from other proteins of the nucleus on several counts. They are small (less than 25,000 daltons) basic (with an isoelectric point greater than pH 9) proteins, closely associated with the DNA in chromatin and in the nucleolus. They can be dissociated from the DNA by high concentrations of salt (Phillips, 1961, Busch, 1965, Murray, 1965) or by dilute acids (Davison and Butler, 1954, Daly and Mirsky, 1955, Johns and Butler, 1962). Since the histones were known to be heterogeneous proteins (Kossel, 1928), it was suggested that they might be genetic repressors

Table 1:1

Amino Acid Analyses of Histones from Calf Thymus

	Total Histone	F1	F2a1	F2a2	F2b	F3
Asp	4.9	2.0	5.0	5.5	4.9	4.4
Thr	5.3	5.4	6.6	4.9	6.2	6.5
Ser	5.0	6.7	2.5	5.0	10.9	3.8
Glu	8.4	3.4	6.2	8.7	7.6	10.2
Pro	4.8	10.1	1.3	4.1	4.8	4.4
Gly	8.7	6.9	15.9	9.0	5.6	5.8
Ala	13.6	25.1	7.5	13.2	10.2	13.5
Cys	Trace	0.0	0.0	0.0	0.0	0.3
Val	6.2	4.1	7.8	6.0	7.1	4.6
Met	0.9	0.0	1.0	0.3	1.6	1.5
Ile	4.4	0.8	5.6	4.2	4.9	4.8
Leu	7.7	4.1	8.0	10.1	4.9	9.0
Tyr	2.3	0.5	3.5	2.3	3.8	2.1
Phe	1.7	0.5	2.2	1.0	1.6	2.0
His	2.3	0.0	1.9	2.8	2.3	2.4
Lys	14.9	28.7	9.8	12.5	16.7	10.1
ε-N MethylLys	-	0.0	1.1	0.7	0.0	0.6
Arg	8.9	1.7	13.9	9.3	6.4	13.6

Total histone from Johns, 1971.

Others from Wilhelm et al, 1971.

(Stedman and Stedman, 1950, 1951). In fact, however, the histones divide chemically, chromatographically, or electrophoretically into five major fractions (Johns, 1964, Luck et al, 1958, Panyim and Chalkley, 1969) in most vertebrate tissues (Panyim et al, 1971). Of the five fractions, F1 has been found to consist of four or five subfractions, while the other four are homogeneous as regards primary structure. Table 1:1 shows the amino acid analyses of the five fractions for calf thymus, while Table 1:2 shows the sequences as far as they are known. The sequence of F2a1 is highly conserved, showing only 2 conservative amino acid substitutions between the calf thymus and pea embryo. It may be that the conservation displayed by F2a1 of calf and pea is slightly fortuitous; Cysteine is found in F2a1 in sea urchins (Strickland et al, 1974) indicating that a rather radical difference is found in this species. F3 is also highly conserved across tissues (Fambrough and Bonner, 1968), although it is interesting that some species possess one cysteine residue per molecule, while others have two. F2a2 and F2b are less highly conserved, while F1 shows considerable tissue and species specificity. With the failure to find the degree of heterogeneity in the histones which would be required if they were to be responsible for specific genetic repression, the role of histones has been seen as structural.

Table 1:2

Sequences of Histone from Calf Thymus

a) F1

ac-ser-glu-ala-pro-ala-glu-thr-ala-ala-pro-
ala-pro-ala-pro-lys-ser-pro-ala-lys-thr-
pro-val-lys-ala-ala-lys-lys-lys-lys-pro-
ala-gly-ala-arg-arg-lys-ala-ser-gly-pro-
pro-val-ser-glu-leu-ile-thr-lys-ala-val-
ala-ala-ser-lys-glu-arg-ser-gly-val-ser-
leu-ala-ala-leu-lys-lys-ala-leu-ala-ala-
ala-gly-tyr-(asp-val-glu-lys-asn-asn-ser-
arg-ile-lys-leu-gly-leu-lys-ser-leu-val-
ser-lys-gly-thr-leu-val-glx-thr-lys-gly-
thr-gly-ala-ser-gly-ser-phe-lys-).

From Rall and Cole, 1971 (residues 1-73).
From Jones et al, 1974 (residues 74-108,
from rabbit thymus).

b) F2a1

ac-ser-gly-arg-gly-lys-gly-gly-lys-gly-leu-
gly-lys-gly-gly-ala-lys(ac)-arg-his-arg-lys(me)-
val-leu-arg-asp-asn-ile-glu-gly-ile-thr-
lys-pro-ala-ile-arg-arg-leu-ala-arg-arg-
gly-gly-val-lys-arg-ile-ser-gly-leu-ile-
tyr-glu-glu-thr-arg-gly-val-leu-lys-val-
phe-leu-glu-asn-val-ile-arg-asp-ala-val-
thr-tyr-thr-glu-his-ala-lys-arg-lys-thr-
val-thr-ala-met-asp-val-val-tyr-ala-leu-
lys-arg-glu-gly-arg-thr-leu-tyr-gly-phe-
gly-gly-COOH.

From Ogawa et al, 1969.

c) F2a2

ac-ser-gly-arg-gly-lys-gln-gly-gly-lys-ala-
arg-ala-lys-ala-lys-thr-arg-ser-ser-arg-
ala-gly-leu-gln-phe-pro-val-gly-arg-val-
his-arg-leu-leu-arg-lys-gly-asn-tyr-ala-
glu-arg-val-gly-ala-gly-ala-pro-val-tyr-
leu-ala-ala-val-leu-glu-tyr-leu-thr-ala-
glu-ile-leu-glu-leu-ala-gly-asn-ala-ala-
arg-asp-asn-lys-lys-thr-arg-ile-ile-pro-
arg-his-leu-gln-leu-ala-ile-arg-asn-asp-
glu-glu-leu-asn-lys-leu-leu-gly-lys-val-
thr-ile-ala-gln-gly-gly-val-leu-pro-asn-
ile-gln-ala-val-leu-leu-pro-lys-lys-thr-
glu-ser-his-his-lys-ala-lys-gly-lys-COOH.

From Yeoman et al, 1972.

d) F2b

Hpro-gln-pro-ala-lys-ser-ala-pro-ala-pro-
lys-lys-gly-ser-lys-ala-val-thr-lys-lys-
ala-gln-lys-lys-asp-gly-lys-lys-arg-lys-
arg-ser-ala-lys-glu-ser-tyr-ser-val-tyr-
val-tyr-lys-val-leu-lys-gln-val-his-pro-
asp-thr-gly-ile-ser-ser-lys-ala-met-gly-
ile-met-asn-ser-phe-val-asn-asp-ile-phe-
glu-arg-ile-ala-gly-glu-ala-ser-arg-leu-
ala-his-tyr-asn-lys-arg-ser-thr-ile-thr-
ser-arg-glu-ile-gln-thr-ala-val-arg-leu-
leu-leu-pro-gly-glu-leu-ala-lys-his-ala-
val-ser-glu-gly-thr-lys-ala-val-thr-lys-
tyr-thr-ser-ser-lys-OH.

From Iwai et al, 1970.

e) F3

H_2N-ala-arg-thr-lys-gln-thr-ala-arg-lys(me)-ser-
ala-pro-ala-thr-gly-gly-val-lys-lys-pro-
his-arg-tyr-arg-pro-gly-thr-val-ala-leu-
arg-glu-ile-arg-arg-tyr-gln-lys-ser-thr-
glu-leu-leu-ile-arg-lys-leu-pro-phe-gln-
arg-leu-val-arg-glu-ile-ala-gln-asp-phe-
lys-thr-asp-leu-arg-phe-gln-ser-ser-ala-
val-met-ala-leu-gln-glu-ala-cys-glu-ala-
tyr-leu-val-gly-leu-phe-glu-asp-thr-asn-
leu-cys-ala-ile-his-ala-lys-arg-val-thr-
ile-met-pro-lys-asp-ile-gln-leu-ala-arg-
arg-ile-arg-gly-glu-arg-ala-COOH.

From DeLange et al, 1972.

b) Chromatin Structure:- DNA

X-ray diffraction studies on isolated DNA, have shown that in low ionic strength aqueous solutions, it exists mostly in what is termed the B form, i.e. as an undistorted double helix, with the base pairs perpendicular to the axis of the helix. In fibres drawn from chromatin gels, X-ray diffraction shows an arc at 3.4A, rings at 8 and 12A, and a diffuse 30A equatorial spacing (at 98% relative humidity) (Garrett, 1971, Richards and Pardon, 1970, Wilkins et al, 1959). The similarity of these reflections to those produced by DNA fibres at the same relative humidity indicate that the DNA is in the B form in chromatin (Bram, 1971). If chromatin is examined in the presence of divalent cations, a series of rings at 22, 27, 35 and 55A is observed, together with a meridional reflection at 105A. These are taken as evidence of supercoiling (Garrett, 1971, Richards and Pardon, 1970), and are abolished by stretching the fibres. The supercoils return on an all or none basis for a particular region of chromatin when the stretching is relaxed, as the reflections return in their original position. Supercoil reflections can be obtained from reconstituted nucleohistone (Richards and Pardon, 1970). The best fit of these reflections is for a chromatin fibre of diameter 100A, with the supercoil pitch of 120A.

Electron microscopy has also been used to study fibres of chromatin. In whole nuclei, the fibres generally have a diameter of 80A (Dusenbury and Uretz, 1972, Kaye and McMaster-Kaye, 1966, Stevens and Swift, 1966, Richards and Pardon, 1970) compared with 20A for DNA fibres. On proteolytic

digestion, a network of fibres similar to DNA is produced (Johns and Hoare, 1970, Ris, 1967). Studies using surface-spreading as the method of preparation have shown a species dependent variation in fibre diameter from 80-180A (Zirkin and Wolfe, 1972). On the other hand considerable variation can be produced by the conditions of preparation (Solari, 1971) indicating that the technique is liable to the production of artefacts.

The shortening of chromatin relative to DNA, which is the product of supercoiling, has also been observed by means of light scattering (Zubay and Doty, 1959), viscosity (Henson and Walker, 1971, Wilhelm et al, 1970) and flow birefringence techniques (Ohba, 1966, Zubay and Doty, 1959).

c) Chromatin Structure:- Conformation of Proteins

The study of the conformation of proteins in chromatin is more difficult. It is possible to detect the presence of protein α helix by the peptide carbonyl stretching band in the infra-red, or by optical rotary dichroism, yielding a figure of 50-65% α helix (Zubay and Doty, 1959), or by circular dichroism which gives a figure of 27% (Permogorov et al, 1970), or 35-40% (Simpson and Sober, 1970) although calculations in the latter case are obscured by DNA ellipticity bands. Hydrogen-deuterium exchange experiments show that although histones have no α helix in dilute aqueous solution, some is present in chromatin (Bradbury et al, 1967). Nuclear Magnetic Resonance studies of histones in saline solutions, both alone, and with DNA, have shown that the centre of the molecules contain helical sections (Boublik et al, 1970, 1971).

In particular, the centre of F1 is released from chromatin at concentrations of salt of 0.1 to 0.3M, and the free centres line up in an antiparallel manner, and thereby hang together. The ends of the molecules are freed by 0.6M saline (Bradbury et al, 1973a).

d) Chromatin Structure:- Distribution of Protein

Considerable discussion has been generated by the question of how the proteins are disposed on the DNA, in particular of whether any DNA remains 'free' and if so, how much. Dye binding studies, using Toluidine Blue and Azure A give results of 30-50% phosphate groups remaining accessible in chromatin (Klein and Szirman, 1963, Kurashina et al, 1970, Muira and Ohba, 1967) while poly lysine also binds with 44-50% efficiency (Itzhaki, 1970, Dahmus and McConnell, 1969, Itzhaki and Cooper, 1973). Over 40% of the DNA is also available to DNase (Itzhaki and Cooper, 1973). On the other hand antigen binding to DNA is reduced to 1-5% in chromatin (Stollar, 1970), while about 10% of chromatin is found to melt as if it were free, as indicated from a peak in a differential melting curve (Ansevin and Brown, 1971, Ansevin et al, 1971, Li and Bonner, 1971). These observations are perhaps resolved by the discovery that model proteins will fit in the major groove of the DNA helix (Zubay and Doty, 1959), and that the minor-groove-binding phyleomycin is not affected by the presence of the proteins (Pietsch, 1969). Hewish and Burgoyne, (1973) have shown that chromatin may be broken down into regular subunits by an endonuclease. That the evidence for free regions of DNA may be correct is suggested by

reconstitution experiments which show that the histones bind co-operatively to DNA, forming knobs of protein joined by free DNA, as observed by the electron microscope (Paul and More, 1973). On adding non-histone protein as well, the chromatin is restored to normal as judged by its appearance under the electron microscope and by its template activity. The 45% DNA phosphate which remains free to dye binding is not covered by adding excess histone. Ziccardi and Schumaker, (1973) have also shown co-operative binding of T7 DNA with F2a1 in hydrodynamic studies. Shearing gives rise to DNA histone complexes, together with free DNA.

As far as the proteins are concerned, 20% of the lysine and arginine amino groups are titratable (Walker, 1965) while 25% can be acetylated chemically without altering the chromatin structure (Simpson, 1971). Antisera to histone fractions, to non-histones and to DNA, will all bind to chromatin (Bustin, 1973).

e) Chromatin Structure:- Histone-DNA and Histone-Histone Relations

In order to discover which of the histones is responsible for maintaining chromatin structure, use has been made of selectively deproteinised chromatin. The most straightforward method has been to remove the histones by increasing the salt concentration. Histone F1 is removed first, at a salt concentration of 0.4 to 0.8M, the rest coming off above 0.8M with a variable degree of selectivity (Giannoni and Peacocke, 1963, Ohlenbusch et al, 1967, Garrett, 1971, Richards and Pardon, 1970, Wilhelm and Champagne, 1969). Removal of F1 alters only

the degree of condensation, and the melting curve (Henson and Walker, 1970a, Ohlenbusch et al, 1967, Mirsky et al, 1968) and the chromatin without the F1 is soluble in 0.15M salt. The X-ray reflections at low angles are sharpened somewhat (Bradbury et al, 1972). However, the supercoil rings are not lost until 0.9 - 1.1M (Garrett, 1971) or above 1.1M salt (Richards and Pardon, 1970). Changes in the viscosity and sedimentation properties correlate with removal of F2b, F2a2 and F3 (Henson and Walker, 1970b, 1971), or perhaps with F2a1 which is the last histone to be removed (Ohba, 1966, Fredericq and Houssier, 1967, Wilhelm et al, 1970). Skidmore et al, (1973) found that the supercoil reflections obtained by X-ray diffraction could be maintained by F2a1 alone.

Dissociation of histones from chromatin by sodium deoxycholate takes place in the order F2a2 and F2b > F3 and F2a1 > F1 (Smart and Bonner, 1971a,b), and results using deoxycholate treated chromatin suggest that the flow dichroism and sedimentation properties studied are dependent on the overall protein content rather than a specific histone fraction. Unfortunately, there is evidence that proteins are free to rearrange before they can be seen to dissociate: thus 40% of protein will exchange with exogenous DNA even at 0.6M salt concentration (Clark and Felsenfield, 1971), so it is not safe to assume that proteins remaining are still at their original location. Trypsin attacks the histones in the order F1 > F2a2, F2b and F3 > F2a1 (Toczko and Jazwinski, 1971, Dabebov and Rebentish, 1971). This lends support to the view that F1 is responsible for cross linking the chromatin strands, while the eventual loss of the supercoil agrees with the view that the

centre of the histone molecules are unbound. Simpson, (1972) found that the tryptic digest of chromatin changed the CD spectrum to that of DNA, while the melting temperature remained unchanged. The view that the histones are held together with hydrophobic bonds is also supported by the observation that the viscosity and CD properties of chromatin become like those of DNA in the presence of 4-6M urea, although no proteins are removed by this treatment (Bartley and Chalkley, 1968, Georgiev et al, 1970, Henson and Walker, 1970a,b).

f) Specific Models

Besides the general model of chromatin held together by protein bridges, formed by the interactions of hydrophobic regions of histones, a small number of specific proposals have been made. Sung and Dixon, (1970) showed that if the first 18 amino acid residues of histone F2a1 are arranged in an α helix, and fit into the major groove of DNA, five basic side chains make easy contact with consecutive DNA phosphate groups, and a sixth will meet a phosphate on the other chain. When fitted in this way, the acetylation and phosphorylation sites of the histone are left exposed. A similar model has been put forward for residues 1-23 and 35-45 by Shih and Bonner, (1970). Dixon and his co-workers have studied in detail the replacement of histones by protamines during spermatogenesis in the trout. The protamines are bound in the phosphorylated state, and later dephosphorylated (Louie and Dixon, 1972), the latter condition giving rise to condensation of chromatin in the sperm. Similarly, a high degree of phosphorylation of histone found in trout (Louie et al, 1973) is

correlated with an extended state of chromatin, and it is proposed that phosphorylation and dephosphorylation allows correct binding to take place, and that orientation is performed by serial acetylation (Louie and Dixon, 1973, Dixon et al, 1973). In the trout system, acetylation and phosphorylation follow closely on synthesis (Louie et al, 1973). It is interesting that for trout histone F1, the possibility of four sites being simultaneously phosphorylated is advocated, on the basis of running characteristics of this histone on starch gels (Louie and Dixon, 1973). Bradbury et al, (1974) working with Physarum polycephalum have found that histone F1 is phosphorylated at an exponential rate, reaching a maximum at mitosis, and propose that phosphorylation of this histone frees the interacting centre section from the DNA, and, by allowing the previously postulated antiparallel interactions (Bradbury et al, 1973) to take place, causes chromatin condensation. The weaker binding of proteins to DNA during mitosis has been noticed by Sadgopal and Bonner, (1970b) and Lake et al, (1972).

g) Micromodifications of Histones

The assignment of a structural role to histones does not leave them passive and unable to participate in vital chromosomal processes. A flexibility is allowed them by considerable experimental evidence that their binding to chromatin may be modified by chemical alterations to the histones themselves. These well attested micromodifications are methylation, acetylation, thiolation and phosphorylation. Some of these micromodifications occur at a readily detectable level in

resting tissue, and are included in the sequence data in Table 1:2; others are found in significant amounts only at specific parts of the cell cycle.

(i) Methylation

ε-N-methyllysine was found by Murray, (1963) in calf thymus histones F2a1, F2a2 and F3. The methyl group is derived from methionine (Murray, 1964, Allfrey et al, 1964) via S adenosyl methionine (Kim and Paik, 1965). Since then, ε-N-dimethyllysine has been found in calf thymus histone (Paik and Kim, 1967) ε-N-trimethyllysine in chick erythrocyte histone (Hempel and Lange, 1968), ω-N-methlarginine in rat liver and calf thymus (Paik and Kim, 1970a,b) and traces of 3 methyl histidine in immature avian erythrocytes (Gershey et al, 1969). Apart from Sekeris et al, (1967) who reported methylation of F1 and F2b, it is generally found that only the arginine-rich histones are methylated in vivo (Murray, 1965, DeLange et al, 1969a, 1970, Quagliarrotti et al, 1969, Patterson and Davies, 1969, Borun et al, 1972). During regeneration of rat liver after partial hepatectomy, increased levels of methylation are observed, that of arginine and histidine peaking during S, and that of lysine during G2 (Borun et al, 1972), although Hardin et al, (1971) reported that methylkinase levels remained constant during the cell cycle in synchronised cell culture. Possibly the increase of methylation is due to increased availability of substrate, or the report of Burdon and Pearce, (1971) that histone methylation by chromatin preparation is stimulated by AMP may have relevance here. Histone methylase activities in

embryonic and neoplastic rat liver were found to be higher than in normal adult tissue (Turner and Hancock, 1970). Lee and Paik, (1972) found that the maximum content of methyllysine in regenerating rat liver precedes the time at which incorporation of radioactive methyl groups from methionine is maximal.

(ii) Acetylation

Phillips, (1963) found that the N terminal amino groups of histones F1, F2a1 and F2a2 were blocked by acetate. Allfrey et al, (1964a) and Allfrey, (1965) showed that the in vitro inhibition of RNA synthesis by histones could be prevented by acetylation. For calf thymus, F3 has three internally acetylated peptides, while F2a1 has one (DeLange et al, 1970, 1969a,b). Acetate was shown to turn over on histones without concomitant turnover of amino acids (Pogo et al, 1966, 1967), and is derived from acetyl co-enzyme A (Nohara et al, 1966). It is present as N-acetyl serine on F2a1 and F2a2, and as N- and O-acetyl serine in F3 (Gershey et al, 1968, Pogo et al, 1968, 1969) and as ε-N-acetyllysine in those histones (Marzluff and McCarty, 1970). In synchronised CHO cells, all histones were acetylated last in S, except for F1 which reached a peak at mid-S phase (Shepherd et al, 1971c). The turnover rate is highest for F1 and F2b, a feature apparently restricted to CHO cells (Wilhelm and McCarty, 1970, Marzluff and McCarty, 1970). On the other hand, in exponential CHO cultures, double labelling experiments using acetate and arginine have shown that the arginine/acetate incorporation ratio is constant with time for each histone fraction, and that the label is not chased out over

a 30 hour period, implying that the acetate does not turn over (Shepherd et al, 1972). Shepherd, (1973) has also provided evidence that F2a1 is acetylated internally during the same cycle as it is synthesised, which rules out the possibility that it might be a trigger for DNA synthesis. During regeneration of rat liver, following partial hepatectomy, there is an early rise in acetylation from 1-3 hours, or a little later involving histones F2a1, F2a2 and F3 and corresponding with a rise in RNA synthesis, and a late acetylation period during S, when F1 is also acetylated (Pogo et al, 1968, 1969, Jones and Irvin, 1972). Cortisol inhibits DNA synthesis and late F2a2 acetylation, while reducing the synthesis of F2a2 and F2b (Jones and Irvin, 1972).

(iii) Thiolation

The presence of cysteine in F3, alone of the histones, was reported by Phillips, (1965) and later confirmed (Phillips, 1966, Jellum, 1966, Ord and Stocken, 1967). Deakin et al, (1963) calculated that 71% of nuclear protein thiol groups were associated with F3 histone. Fambrough and Bonner, (1968) found only one oligomeric form of F3 for pea bud histone F3, but many for calf thymus F3, suggesting that they had one and two molecules of cysteine respectively per monomer histone. The ratio of thiol to disulphide was higher in diffuse than dense chromatin (Ord and Stocken, 1966) and the disulphide containing chromatin was less efficient as template for *in vitro* RNA synthesis (Hilton and Stocken, 1966), although no difference was found with respect to *in vitro* DNA synthesis (Ord and Stocken, 1968). The thiol content is raised at S

and at mitosis in sea urchin embryos (Ord and Stocken, 1969, 1970) and at S in PHA stimulated lymphocytes (Cross and Ord, 1970). Sadgopal and Bonner, (1970a,b) on the contrary, using synchronised HeLa cells, found a four-fold increase in the disulphide form of F3 in metaphase (condensed) chromatin over interphase chromatin.

(iv) Phosphorylation

The incorporation of ^{32}P radioactivity into histones by rat liver *in vivo*, or thymus nuclei *in vitro* was reported by Ord and Stocken, (1966) and Kleinsmith et al, (1966). The phosphate was bound via an ester link to serine (Ord and Stocken, 1966, Kleinsmith et al, 1966, Langan, 1968a), and to a lesser extent threonine (Ord and Stocken, 1966, Kleinsmith et al, 1966), mainly to histone F1. The phosphate content of F1 was shown to increase during S in regenerating rat liver (Ord and Stocken, 1968, 1969), while cAMP increased incorporation *in vitro* and in perfused liver (Langan, 1969b). *In vitro*, ^{32}P was incorporated mainly into the serine residue which was at that time thought to be residue 28, but which is now relocated at position 37 (in the rabbit thymus sequence, or 38 for calf thymus (Langan, 1968a,b, 1969b, Langan, 1971a, Rall and Cole, 1970, Rall and Cole, 1971)). Langan, (1971b) has found two kinases from different tissues that phosphorylate preferentially different sites. Smith et al, (1973) have recently claimed to have isolated two new kinases that transfer phosphate to histidine on F1 and F2a1. Phosphohistidine, being acid labile would be lost during acid extraction of histones. Apart from the surprising pH optima

of these enzymes (pH 6.5 and 9.5) it should be noted that F1 is believed not to contain histidine (e.g. Wilhelm et al, 1971).

The changes in phosphorylation of histones through the cell cycle have been followed in a number of systems. In CHO cells, F2a2 is phosphorylated throughout the cell cycle, while F1 is phosphorylated in early S, parallel to its synthesis, and again, on a separate fraction, during mitosis. F3 is phosphorylated only at mitosis (Gurley et al, 1974). In G1 arrested cells, turnover is reported of phosphate on F2a2, but not F1 (Gurley et al, 1973). Lake and Salzman, (1972), and Lake et al, (1972) report that in these cells F1 is phosphorylated at mitosis rather than during S. In HeLa cells, F1 is phosphorylated early in S, and continues to incorporate phosphate until the end of mitosis. The turnover of phosphate in F1 is reduced during S. In the other histones, phosphate is incorporated during S, parallel to the synthesis of the histones, such that the overall phosphate content remains constant (Marks et al, 1973). In PHA stimulated lymphocytes, F1 is phosphorylated early, an increase in histone kinase activity being detectable within 30 minutes, while the other histones are only phosphorylated later (Cross and Ord, 1970). In regenerating rat liver, F1 phosphorylation is high during S, and again at mitosis (Balhorn et al, 1971), although an early 2-6 hour increase in F1 phosphorylation has been reported (Gutierrez-Cernosek and Hnilica, 1971). Again, the other histones are phosphorylated at a rate parallel to synthesis (Gutierrez-Cernosek and Hnilica, 1971). In Physarum polycephalum,

F1 phosphorylation is found to take place parallel to synthesis during S, but to rise steeply (and exponentially) until mitosis, which it is believed to initiate in this species (Bradbury et al, 1973b, 1974).

(v) Modifications during Spermatogenesis

One system in which the modifications to histones have been studied in depth is that of spermatogenesis in trout (Dixon et al, 1973). As a result of complete sequencing of all the histones except F1, the following modifications have been recognised:

F2a1 phosphorylation of N terminal acetylserine, acetylation of lysines 5, 8, 12, 16.

F2a2 phosphorylation of N terminal acetylserine, acetylation of lysine 5.

F2b phosphorylation of serine 6, acetylation of lysines 5, 10, 13, 19.

F3 acetylation of lysines 9, 14, 18, 23, phospho-peptides not yet identified, but are to serines 10 and 27 in calf thymus (Marzluff and McCarty, 1972).

F1 phosphorylation of serine 157, predominantly, no acetylation.

In both F2a1 and F2a2, the phosphorylated peptide is N acetyl-O-phospho-seryl-glycyl-arginine. The interpretation of phosphorylation and the serial acetylation of F2a1 has already been referred to, but it must remain questionable to what extent the terminal differentiation of trout testis can

be used as a model for other systems of cell division.

h) Non-Histone Proteins

In spite of an early interest in the non-histone proteins of the cell nucleus (Stedman and Stedman, 1943, 1944), they have not proved easy to work with, because of their strong tendency to form aggregates, and because of their extreme heterogeneity, which means that any one protein is present only in minute quantities. Non-histone proteins have been separated from the DNA and histones using phenol (Teng et al, 1971) detergent (Elgin and Bonner, 1970) or salt (Benjamin and Gelhorn, 1968), or by dissociating the chromatin with salt, and allowing the DNA and histone to reconstitute at low ionic strength (Langan, 1967). Richter and Sekeris, (1972) have succeeded in keeping virtually all of the non-histone proteins in solution at low ionic strength, in the presence of urea, by dint of thoroughly removing the DNA, and by separating the histones on Biorex 70. Preliminary fractionaticns of non-histone proteins have been performed by chromatography on QAE Sephadex (Richter and Sekeris, 1972) on hydroxyapatite (Rickwood et al, 1972) and on SE Sephadex Elgin and Bonner, 1972). An acid soluble nucleolar non-histone protein has been isolated by means of polyacrylamide gel electrophoresis (Knecht and Busch, 1971) in sufficient quantity to allow amino acid analysis and N terminal acid (serine) to be determined. A good separation of nuclear membrane proteins has been achieved by Bornens and Kasper, (1973) using a Sephadex G150 column, in the presence of SDS. Most of the membrane proteins had a molecular weight of 60-75,000 daltons.

Since separation of non-histone nuclear proteins has proved so troublesome, most of the information available about them has been obtained by using heterogeneous mixtures, which can then be analysed by polyacrylamide gel electrophoresis. The heterogeneity of these proteins, originally inferred from N terminal amino acid determinations (Steele and Busch, 1963, 1964) has been confirmed by polyacrylamide gel electrophoresis in the presence of SDS (Elgin and Bonner, 1970, Graziano and Huang, 1971), which showed a range in molecular weight from 5,000 to 200,000 daltons, and by two-dimensional polyacrylamide gel electrophoresis (Yeoman et al, 1973a,b, MacGillivray and Rickwood, 1974). The 80 or more proteins distinguished in this way is undoubtedly a minimum estimate of those present.

Tissue specificity has been shown for the gel densitometer profiles of these proteins, or by direct observation of protein bands on gels (Dravid and Burdman, 1968, Platz et al, 1970, Wang, 1971, Teng et al, 1971). Differences between the two-dimensional gel patterns of non-histone proteins of liver and ascites hepatoma cells have also been noted (Yeoman et al, 1973a,b). The tissue specific acetylation of non-histone proteins has been reported (Suria and Liew, 1974), both N acetyl serine and N acetyl lysine being found. Many nuclear phosphoproteins are known (Langan, 1967, Langan and Smith, 1967, Kleinsmith et al, 1966a,b, Kleinsmith and Allfrey, 1969a,b, Gershey and Kleinsmith, 1969a,b), and phosphate incorporation is also tissue specific (Teng et al, 1971). A specific phosphorylated non-histone protein is

known from the early stages of urchin development (Platz and Hnilica, 1973). Non-histone phosphokinases have been isolated on phosphocellulose columns (Takeda et al, 1971, Kish and Kleinsmith, 1974) which are present in different concentrations in different tissues. Varying effects of cAMP on these kinases are noted, and some are subject to inhibition by histones. Some nuclear acidic proteins have been observed to bind to DNA-cellulose columns, binding specifically to homologous DNA (Kleinsmith et al, 1970, Kleinsmith, 1973), although Rowekamp and Sekeris, (1974) have found that only nuclear membrane proteins bind in this manner, and that nuclei prepared by methods using non-ionic detergents, and therefore lack the outer membrane, have no binding proteins.

Non-histone proteins are present in high concentrations in the chromatin of metabolically active tissues (Dingman and Sporn, 1964, Paoletti and Huang, 1969) and are preferentially localised in chromatin regions most active in RNA synthesis (Frenster, 1965). There is evidence that specificity of transcription in reconstituted chromatin preparations resides in the non-histones (Gilmour and Paul, 1970, Paul and Gilmour, 1968, Stein and Farber, 1972).

Besides the membrane proteins, and those which may control gene activation a number of enzyme activities are known from the nucleus. They include a NAD nucleosidase (Bock et al, 1968), a neutral protease (Furlan and Jericijo, 1967a,b, Bartley and Chalkley, 1970), protein phosphokinases for both histone (Siebert et al, 1971) and non-histone proteins (Kish and Kleinsmith, 1974), histone methylases (Comb et al, 1966)

acetylases (Gallwitz, 1970, 1971), poly (ADPribose) polymerase, poly (ADPribose) glycohydrolase (Sugimura et al, 1973), glutamate dehydrogenase, lactate dehydrogenase, malate dehydrogenase, ATPase (Wang, 1967) and various enzymes concerned with RNA and DNA metabolism (Howk and Wang, 1969a b, 1970a,b, O'Connor, 1969, Liao et al, 1968, Sekeris et al, 1967). Thymus nuclei also contain a number of other enzymes, including those required for glycolysis (Siebert and Humphrey, 1965). Siebert and Humphrey add the caution, however, that thymus nuclei are atypical, and results obtained with them should not be applied to nuclei in general.

Another function which some of the non-histone proteins fill is that of transporting dRNA away from the nucleus; the protein particles responsible have been termed informofers (Samarina et al, 1966, 1968, Lukanidin et al, 1972). The two main proteins involved in transporting presumed mRNA are of low molecular weight (32-40,000 daltons) and they, together with the associated heterogeneous fraction of proteins which are also isolated on these RNP particles are capable of being phosphorylated in vivo (Bryan and Hayashi, 1973, Gallinaro-Matringe and Jacob, 1973).

The non-histone proteins are selectively affected by hormones. Shelton and Allfrey, (1970) found that cortisol enhanced the synthesis of a single non-histone protein of molecular weight 41,000 daltons. Pederson, (1974) observed that the increased radioactivity in protein, when following the incorporation of a labelled amino acid in the cortisol stimulated rat liver, was associated solely with the RNP particles of the nucleus, and proposed that cortisol acts by

a post trans criptional regulatory mechanism. An acidic nuclear protein fraction which provides a tissue specific binding site for a cytosol protein-progesterone complex has been isolated from chick oviduct chromatin (O'Malley et al, 1972, Spelsberg et al, 1972). Changes in the acidic proteins have been observed as a result of adminstering diethyl stilbesterol to the immature chick oviduct (Spelsberg et al, 1973). Courtois et al, (1974) has reported the enhanced synthesis of a single non-histone protein in response to lysine starvation of cultured cells, and another selectively labelled one on stimulating the cells with 30% serum.

From the foregoing, it is apparent that the non-histone proteins of the nucleus have a variety of functions, and can only be properly studied as their function becomes known. In some respects, however, they do seem to behave as a whole. During the cell cycle in CHO and HeLa cells, for instance, a general synthesis of non-histone proteins takes place during late G1 and early S (Gerner and Humphrey, 1973, Karn et al, 1974). An increase in synthesis of non-histone nuclear proteins is observed in rat liver, 3 hours after partial hepatectomy (Pogo et al, 1968), as a result of which the template activity of the chromatin for RNA synthesis is enhanced two-fold (Kostraba and Wang, 1973). The rate of turnover of nuclear non-histone proteins also appears to be fairly general (Dice and Schimke, 1973), save that the larger proteins are degraded slightly more rapidly. These co-ordinate properties are probably those that are required to preserve the balance of components of the nucleus as a whole, and do not imply any common function.

i) Poly ADPribose

Before leaving this introduction to the proteins of the nucleus, it is relevant to consider what is known about a nuclear polyribonucleotide:- poly (ADPribose), which is closely bound in with nuclear proteins in chromatin. Poly (ADPribose) was discovered by Chambon et al, (1963), who noted that the incorporation of radioactivity from [^{14}C]Adenine ATP into acid insoluble material in hen liver nuclei was stimulated 1,000-fold by NMN. The product was not digested by NaOH, as would be expected of poly A, an observation which prompted the identification of the product as poly (ADPribose) (Chambon et al, 1966). Poly (ADPribose) is digested by venom phosphodiesterase (from Crotalus adamanteus - the major activity being a pyrophosphatase) to give phosphoribosyl AMP, together with a smaller amount of AMP (Chambon et al, 1966, Hasegawa et al, 1967, Reeder et al, 1967). After phosphodiesterase hydrolysis, all phosphate groups are labile to alkaline phosphatase (Chambon et al, 1966, Shima et al, 1968). Acid hydrolysis yields adenine and ribose-5-phosphate only (Reeder et al, 1967). An average chain length of 25 units is indicated by the ratio of AMP to phosphoribosyl AMP in the phosphodiesterase digest (Chambon et al, 1966, Hasegawa et al, 1967, Reeder et al, 1967). The polymer is presumed to have some tertiary structure by reason of its 20% hypochromicity (Sugimura and Shimizu, 1968, Shima et al, 1970).

The polymer is synthesised from NMN and ATP, with NAD as a compulsory intermediate. NADH, NADP, and ADPribose do not function as intermediates (Chambon et al, 1966,

Fujimura et al, 1967). Polymerase activity of nuclei appears to correlate with the DNA content rather than the growth rate of the tissue (Hilz and Kittler, 1971, Leiber et al, 1973), while on separation of nuclei on the zonal rotor, the polymerase activity associates with those nuclei which are engaging in RNA synthesis (Haines et al, 1969). Lehmann et al, (1974), however find an increase in poly (ADPribose) polymerase activity in PHA stimulated lymphocytes. The activity during S is nearly 3 times the control level. The polymerase itself is tightly chromatin bound (Ueda et al, 1968) but may be solubilised by DNase treatment (Yamada et al, 1971), and is found to have a requirement for histone, DNA and magnesium ions. Synthesis of the polymer is inhibited by nicotinamide (Fujimura et al, 1967, Clark et al, 1971).

The significance of poly (ADPribose) to the nucleus is not understood, although it is reported that incubation of nuclei with NAD suppresses DNA synthesis (Burzio and Koide, 1970, Ueda, 1971) but not RNA synthesis (Burzio and Koide, 1971). Novikoff hepatoma nuclei are not suppressed in this way (Burzio and Koide, 1972). Incubation of nuclei with NAD has been shown to release DNA polymerase into the medium from the chromatin, and this is believed to be the mechanism by which DNA synthesis is suppressed (Yoshihara and Koide, 1973). NMN is not as effective as NAD in this respect; the only difference observed is in ADPribosylation of proteins, in the 'run-off' peak of acid-extractable proteins from Amberlite CG-50 (Koide and Burzio, 1972).

j) ADPribosylation of Proteins

Poly (ADPribose) is also found bound to histones (Nishizuka et al, 1968, Otake et al, 1969, Nishizuka et al, 1971, Ueda et al, 1972, Koide and Burzio, 1972, Dietrich et al, 1973) and/or non-histone nuclear proteins (Koide and Burzio, 1972, Dietrich and Siebert, 1974), depending on the method of extraction of the proteins (Dietrich and Siebert, 1974). The nucleotide appears to be covalently bound to the protein, remaining attached in the presence of 2% SDS, 8M urea, 2M guanidinium chloride, 0.01M EDTA, DNase, RNase, acid phosphatase, or alkaline phosphatase (Nishizuka et al, 1968). The protein-nucleotide bond is, however, labile to alkali, or neutral hydroxylamine (Nishizuka et al, 1971, Ueda et al, 1972, Dietrich et al, 1973, Dietrich and Siebert, 1973), while its molecular sieving properties are altered by trypsin (Ueda et al, 1972). Proteins bearing ADPribose appear to aggregate in the presence of other, not necessarily related, nuclear proteins (Adamietz and Hilz, 1972), so the fact that histones separate from the aggregate on digestion with phosphodiesterase and alkaline phosphatase (Ueda et al, 1972) does not provide evidence that the ADPribose is bound to these histones. Equally, the separation of ADPribose from the bulk of the histones by selective extraction or chromatography (Ueda et al, 1972, Koide and Burzio, 1972, Dietrich and Siebert, 1974) does not imply that the nucleotide is not bonded to histone. Unfortunately histone F1 has been lost in some studies, either by washing nuclei with 3% PCA which extracts this histone (Dietrich and Siebert, 1973, Dietrich et al, 1973) or by

considering only 5% TCA precipitable material (Ueda et al, 1972). The protein acceptor has proved difficult to identify, presumably because the nucleotide contributes a sizeable negative charge to the molecule, while the properties of aggregation will alter the apparent molecular weight, so that the substituted protein molecule will behave quite differently from the parent, and may separate from it early in the extraction procedure.

k) Protein-Nucleotide Bonds

The protein-bound poly (ADPribose is of importance because the whole structure of chromatin revolves around protein-nucleic acid interactions. Besides the ionic- and hydrogen-bonded interactions of DNA and histones, a number of covalent linkages have been claimed.

(i) Bound Chromosomal Nucleotides

Nucleotide material associated with histones has been known for a long time as material adsorbing at 260nm (Hnilica and Bess, 1965) or [^{14}C]Adenine counts (Ord and Stocken, 1967), attached mainly to F3. These may represent early observations of protein-bound ADPribose. Such material has also been regarded as pure contaminant (Shepherd et al, 1970, Balhorn et al, 1971, Greenaway and Murray, 1973), or as covalently bound chromosomal RNA (Huang and Bonner, 1965, Jacobson and Bonner, 1968, Bonner et al, 1968, Huang and Huang, 1969, Bekhor, 1973), although the experimental methods and their interpretation has been questioned (Heyden and Zachau, 1971, Artman and Roth, 1971, Szeszak and Pihl, 1971). Patel and Holoubek, (1973) found both free and apparently bound RNA

in saline nuclear extracts. Bekhor, (1973) considers that protein-bound chromosomal RNA is essential in order to reconstitute chromatin correctly.

(ii) Residual DNA-Bound Protein

Possible peptide 'linkers' to join together DNA lengths into chromosomes have been postulated, partly because it has proved impossible to completely separate peptide material from eukaryotic DNA (Bendich and Rosenkranz, 1963, Champagne et al, 1964, Balis et al, 1964). While this material is reported to have a fairly constant amino acid composition, that is not evidence that it is covalently bound to the DNA. Hilgartner, (1968) however, claims to have isolated material which he terms 'junction points', consisting of nucleotide peptide material not digested by nuclease or protease.

(iii) Small Nucleotide-Peptides

A number of 'small' molecules have been reported to be oligopeptides with covalently bound nucleotide. The two which are specifically nuclear in location were isolated from a 5% TCA extract of nuclei from ascites hepatoma cells (Islam and Kay, 1972). They were separated by polyacrylamide gel electrophoresis, and adenine was found to be the only base in each. The main amino acids identified were alanine, aspartate, glutamate, lysine, proline, serine and threonine. they incorporate formate and glutamate radioactivity rapidly in a puromycin insensitive synthesis. The cells accumulated added peptides in the nucleus, inhibiting DNA and protein synthesis. The peptides precipitate DNA *in vitro*. Other small nucleotide-peptides have been isolated from 5% TCA extracts of liver, and separated by chromatography on

Dowex-chloride (Szafranski et al, 1959, Wilken and Hansen, 1961, Binkley, 1960, Ondarza, 1962, Veinova et al, 1966). Veinova et al, (1966) investigated the nature of the peptide-nucleotide bond, and found that it was labile to hydroxylamine and alkali. A bond between a peptide carboxyl and a ribosyl-OH was postulated. Adenine was the main base present, with some cytosine on two fractions.

(iv) Amino Acyl tRNA - A Well Characterised Peptide-Nucleotide Bond

Probably the best characterised bond between an amino acid and a nucleotide is that of amino-acyl transfer RNA (Hoagland et al, 1957). The bond is acid stable, but labile to alkali and hydroxylamine, and is an ester between the carboxyl group of the amino acid, and the 3'OH of the terminal adenosine of transfer RNA (Sonnenbichler et al, 1963). 90% of the hydroxylamine lability is lost when the molecule is ribosome-bound (Arlinghaus et al, 1968).

(v) ADPribosylation of Amino Acyl Transferase II

Another instance is the inhibition of amino acyl transferase II by diphtheria toxin, in which the enzyme accepts one residue of ADPribose (Honjo et al, 1968, 1969). The bond is stable to mild treatment with acid, alkali, or hydroxylamine.

CHAPTER 2

INTRODUCTION

Histone F1 is not a homogeneous protein fraction. It has long been known (Luck et al, 1958) that more than one fraction could be obtained by using an Amberlite IRC 50 column, and if a sufficiently shallow gradient of Guanidinium chloride is used, four or five peaks may be resolved reproducibly, whose content is species and tissue specific (Kinkade and Cole, 1966a,b, Bustin and Cole, 1969, Stellwagen and Cole, 1969). Bustin and Stollar, (1972, 1973) showed immunological specificity in the F1 subfractions of rat liver and thymus, and demonstrated the close similarity of the subfractions of these tissues. Hekman and Sluyser, (1973), while finding more cross-reaction across species, showed that the antigenic determinants of histone F1 are in the C terminal half of the molecule. In addition to these fractions, standard preparations of 'histone F1' were shown to contain at least two non-histone protein components, which are separable on CM cellulose (Johns, 1964).

At least three of the five components of histone F1 resolved by preparative polyacrylamide gel electrophoresis have been shown to carry phosphate (Buckingham and Stocken, 1970b) while more than 20% of ^{32}P radioactivity incorporated into the F1 fraction in resting liver *in vivo* was found to be associated with the non-histone components (Buckingham and Stocken, 1970a). Stevely and Stocken, (1968) obtained phosphate-rich and phosphate-poor fractions of histone F1 by DEAE Cellulose chromatography. Langan, (1968a,b, 1969) isolated a tryptic peptide from histone F1, which carried a considerable proportion of

the ^{32}P radioactivity incorporated *in vivo* by rat liver, stimulated by glucagon or insulin, and found that this peptide was heavily labelled when histone F1 was incubated with a histone kinase preparation and [^{32}P] ATP *in vitro*. However, isotope dilution studies showed that only 25% of the phosphate in histone F1 was due to that histone kinase. Langan, (1968a) pointed out that since the non-histone phosphoproteins of the nucleus contain more than 200 times the amount of phosphorus found in histone F1, a comparatively small contamination with these proteins could lead to a large increase in the apparent phosphate content of the histone.

The standard preparation of histone F1, then, contains at least seven protein components, five histone, two non-histone, of which at least three of the former, and one of the latter carry phosphate. The crude F1 preparation, however, is often dealt with as a whole (Takeda and Ohga, 1973, Johnson and Allfrey, 1972, Shepherd et al, 1972, Gottesfeld et al, 1972) and it is not clear how far the histone itself is responsible for the properties reported. The non-histone components of the fraction have not been characterised, and it is not known whether they represent casual contamination, or are associated with histone F1 *in vivo*.

Results

The non-histone components were separated from the histone on a column of DEAE Cellulose, (Buckingham and Stocken, 1970a). The crude preparation was applied to the column in 50mM Tris-HCl buffer, pH 7.4. The non-histone protein was retained on the column, and, after washing with Tris-HCl buffer, was eluted with 0.5M NaCl, 50mM Tris-HCl buffer, pH 7.4, (Fig. 2:1). On washing the column with 0.1N NaOH, the third peak of Buckingham and Stocken, (1970a) was not found. In the three tissues investigated:- sheep thymus, rat liver and ascites hepatoma cells, the non-histone components accounted for 5 to 8% of the protein, although this represented 25-35% of the OD at 280nm, or Lowry equivalent.

Analysis of the protein fractions by electrophoresis on polyacrylamide gels containing acetic acid and 2.5M urea (Fig. 2:3) showed the histone to be substantially free of impurities, with one major, and one minor band (the latter being F1a (Panyim and Chalkley, 1969c)). No other bands were seen, even with loads of up to 1mg protein, indicating the absence of protein impurities more than 0.1% of the histone. However, the histone is still heterogeneous on the gel system of Reisfield et al, (1962), showing the characteristic five bands, together with a minor band that only just enters the gel. The non-histone fraction contained two major bands, whose mobilities relative to that of histone F1 were 0.92 and 0.15 (or 0.86 and 0.33 respectively when the urea concentration in the gel was increased to 6.25M). The latter band was not observed if βmercaptoethanol was used to reduce the protein

Fig. 2:1

Separation of non-histone components from histone F1

On DEAE Cellulose (Whatman DE52) (20cm x 3cm^2).

Flow rate: 16mls/hr.

The histone (approx. 60mgs) was derived from livers of 12 rats (approx. 250g) injected with 250 µCi [^{32}P] ortho-phosphate 90 minutes before being killed.

The protein was monitored as OD_{280}.

Arrow indicates addition of salt-containing buffer.

Fig 2:1

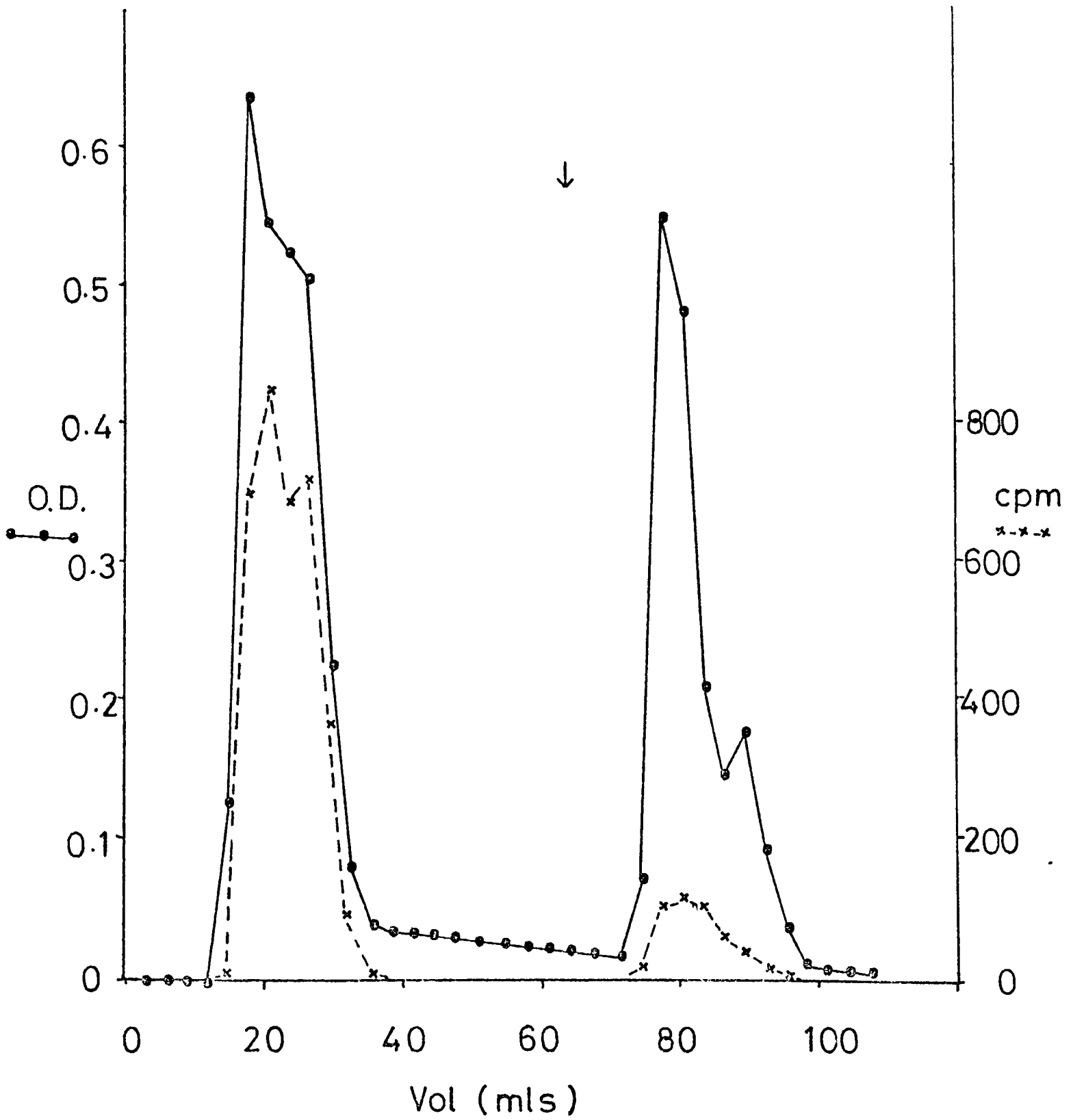


Fig. 2:2

Purification of major component of the non-histone proteins co-extracted with histone F1

On CM Sephadex (Pharmacia CM 50) (20cm x 3cm^2).

Flow rate: 16mls/hr.

The non-histone protein (approx. 15mgs) was derived from 200g sheep thymus.

Arrows indicate changes of buffer.

Fig 2:2

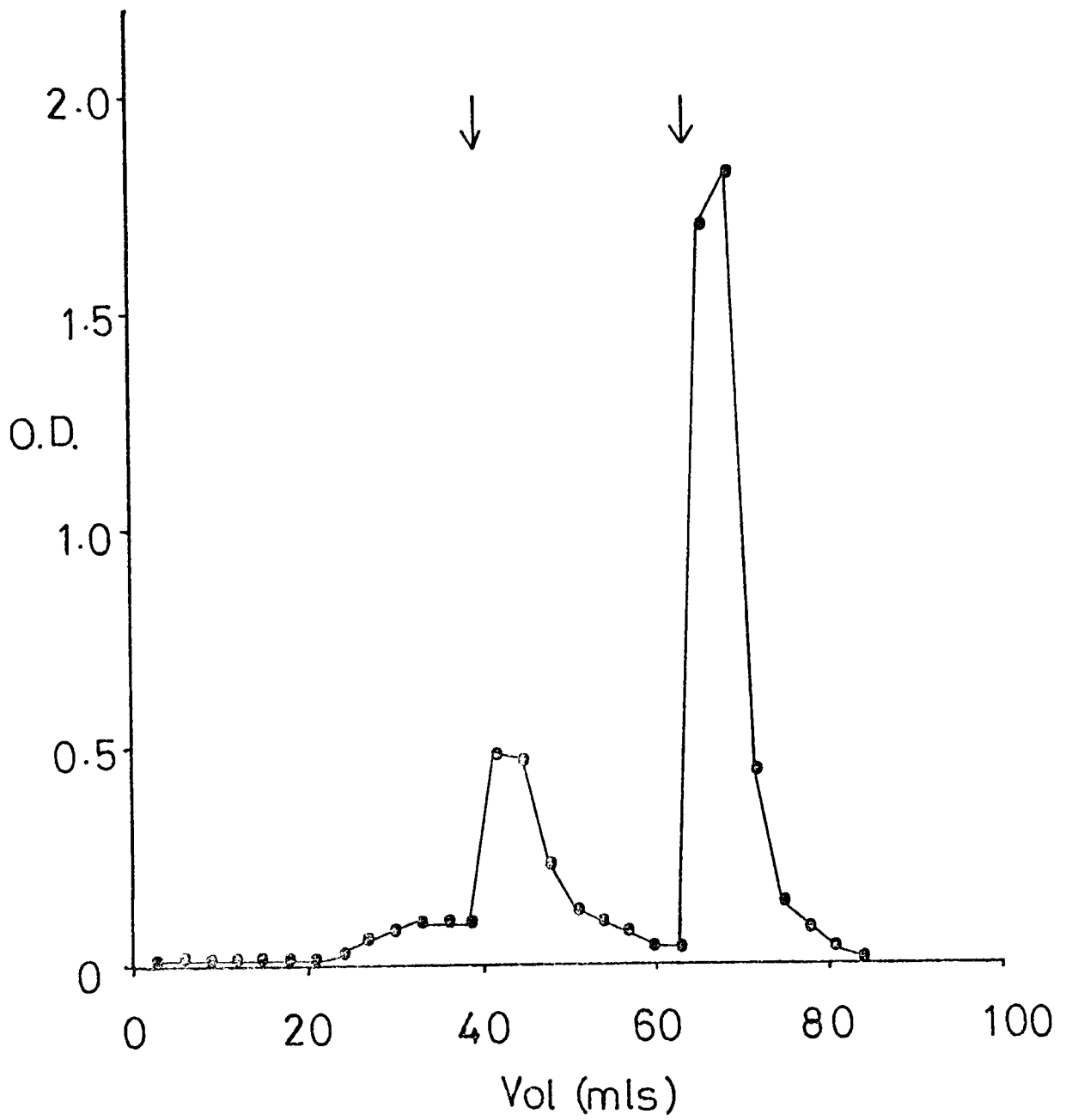


Fig. 2:3

Analysis of protein fractions by polyacrylamide gel electrophoresis in the presence of acetic acid - 2.5M urea

a) sheep thymus 'histone F1'.

b) non-histone proteins separated from sheep thymus histone F1.

c) major non-histone component of sheep thymus histone F1.

d) component P1 (after reduction with βmercaptoethanol).

Approx. 50µg protein was loaded.

The gel top (origin) is to the right.

Fig 2:3

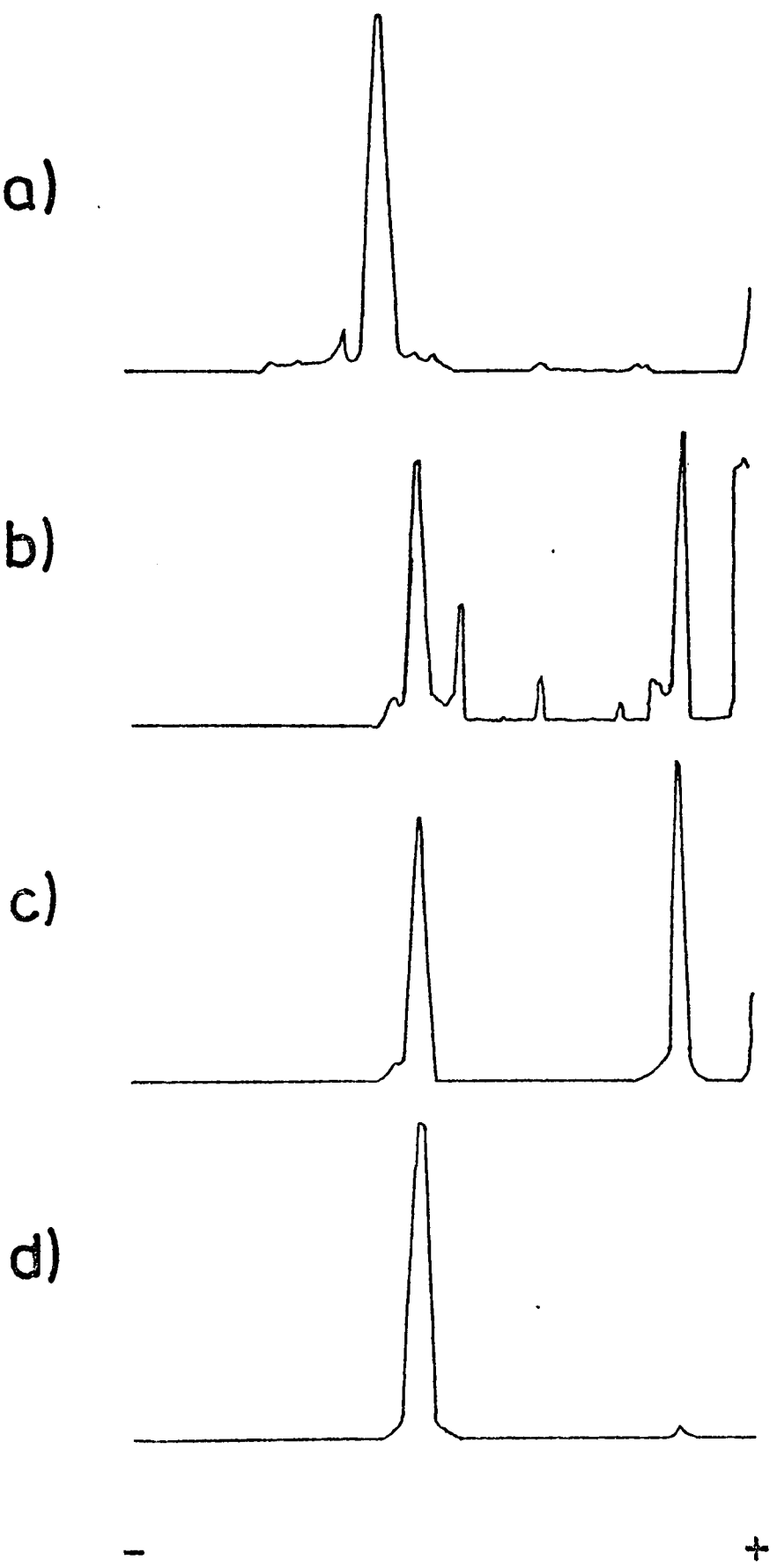


Table 2:1

Amino Acid Analyses of Non-Histone Proteins Co-Extracted with Histone P1

	P1	* Others	** Fraction 1 (Johns, 1964)	** Fraction 2	HMG 1 (Goodwin et al, 1973)	Slow-running band from Reisfield gel
Asx	11.7	7.6	11.4	30.9++	10.8	3.3
Thr	2.6	4.2	2.9	2.3	2.2	5.5
Ser	5.9	11.3	5.6	7.6	5.9	7.3
Glx	17.8	10.8	20.1	++	18.3	5.0
Pro	4.1	3.9	5.8	7.2	5.9	8.2
Gly	4.7	13.6	5.6	6.5	5.6	7.9
Ala	8.3	21.4	10.1	8.3	8.9	20.5
Cys	0.3	0.8	ND	ND	1.1	0
Val	2.3	4.8	2.4	2.5	2.1	4.5
Met	1.8	0.8	ND	ND	0.9	0
Ile	1.9	2.6	5.6	4.2	2.0	1.4
Leu	2.2	4.1			2.1	4.9
Tyr	2.9	0.0	2.4	3.0	3.0	0.7
Phe	3.9	1.7	3.2	3.5	4.3	1.9
His	1.1	4.1	1.3	0.9	1.8	0
Lys	23.9	4.1+	19.4	19.0	19.9	25.9
Arg	4.0	2.2	4.2	4.2	4.3	3.0

+ Unidentified peak of 1.9 moles % eluting between His and Lys.

++ Asx + Glx.

* First peak from CM Sephadex.

** Unadsorbed, and slightly adsorbed fractions from CM cellulose.

ND indicates amino acid not determined.

All values are in moles %.

solution, prior to application (Fig. 2:3d). A number of slow running minor bands were also present, most of them remaining near the top of the gel.

The major component (designated P1) of the non-histone fraction was purified by adsorption to a column of CM Sephadex (CM 50). The protein was applied in 0.3M NaCl, 50mM Tris-HCl buffer, pH 7.4, the column washed with 6M urea, 0.3M NaCl, 50mM Tris-HCl buffer, pH 7.4, and the main part of the protein eluted in 6M urea, 1M NaCl, 50mM Tris-HCl buffer, pH 7.4 (Fig. 2:2). When this eluate was dialysed against water it generally became cloudy. It was clarified by making the solution 5% (w/v) with respect to perchloric acid, and centrifuging at 10,000g for 20 minutes. The supernatant remained clear on dialysis against water.

Analysis of the final supernatant by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (and βmercaptoethanol) (Fig. 2:4c) reveals a single band, while on urea-acetic acid gels, it may be resolved as a close doublet. From its position on the SDS gels, a molecular weight of approximately 20,000 daltons is inferred. The amino acid analysis of component P1 (Table 2:1) shows that it is formally slightly acidic. However, since it runs so closely to histone F1 on urea-acetic acid polyacrylamide gel electrophoresis, it is probable that many of the acidic residues are present as amides.

Besides the high percentage of charged residues, the amino acid analysis of P1 shows cysteine to be present to the extent of approximately one residue per molecule of 20,000 daltons.

Fig. 2:4

Analysis of protein fractions by polyacrylamide gel electrophoresis in the presence of SDS (pH 7.0)

a) sheep thymus 'histone F1'.

b) sheep thymus component P1.

c) non-histone proteins extracted from rat liver nuclei by 0.6M NaCl.

Approx. 50µg protein was loaded (a + b) or 100µg (c).
The gel top (origin) is to the right.

Fig 2:4

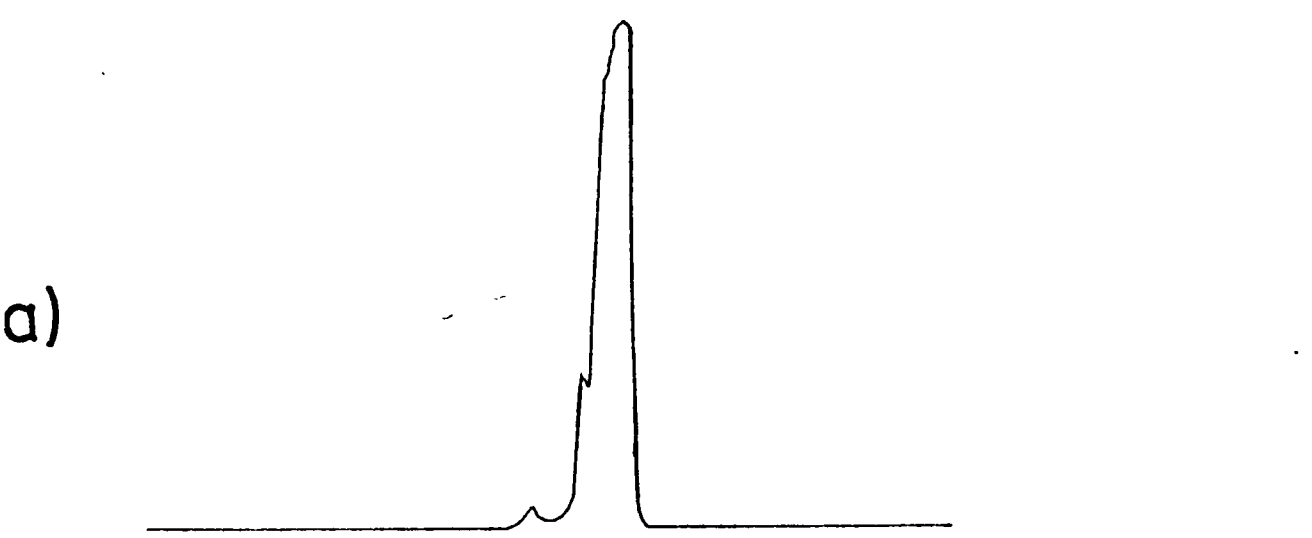


Table 2:2

Amino Acid Analyses of Histone and Non-histone Proteins

Extracted with 5% PCA

	Rat Liver		Ascites Hepatoma		Sheep Thymus	
	Histone	Non-Histone	Histone	Non-Histone	Histone	Non-Histone
Asx	2.5	10.8	4.0	10.8	3.5	11.6
Thr	6.1	4.9	4.1	2.9	6.1	3.1
Ser	7.4	8.5	12.1	7.3	7.6	5.4
Glx	4.0	13.8	5.5	15.0	4.8	15.8
Pro	8.6	6.6	9.0	5.0	8.3	4.7
Gly	6.8	7.2	7.4	6.2	5.8	5.2
Ala	24.0	10.5	21.0	9.8	20.7	9.2
Cys	0	0.2	0	0.8	0	0.9
Val	4.8	5.1	5.0	2.0	5.2	4.0
Met	0.2	1.3	0.4	7.5	1.2	4.6
Ile	1.5	3.4	1.5	2.3	1.3	2.1
Leu	4.2	5.3	4.2	3.5	4.6	2.6
Tyr	0.6	1.4	0.6	1.1	0.6	3.8
Phe	0.7	2.8	0.8	2.6	0.8	
His	0	1.3	0.3	1.2	0.3	1.3
Lys	26.5	13.2	32.5	16.7	27.0	20.1
Arg	2.8	4.0	3.5	4.1	2.4	4.7
Phosphate	15.4	50.9	13.5	140	2.4	81
	23.5*	146.1*				

* 21hr regenerating liver.

Amino acid values expressed as moles %.

Phosphate in nmoles/mg protein.

Table 2:3

Efficiency as Substrate for 'Histone Kinase'	
Substrate	cpm incorporated/ 100µg protein/10 mins
F1	2,664* 2,760 2,625
P1	610* 460 660
F1 : P1 (2 : 1)	2,055*
Ratio of efficiency F1 : P1 4.8 (± 0.9 S.D.).	

All figures are averages of at least two estimations.

*TCA precipitable counts as described in materials and methods.

In order to check that the counts transferred were covalently bound to the substrate protein, rather than simply co-precipitated, or incorporated into kinase associated proteins, the second and third figures for each substrate were obtained from acid/urea and SDS polyacrylamide gels respectively.

The formation of dimers by disulphide linkages, which are reducible with βmercaptoethanol, explains the presence of the slower running band on polyacrylamide gels under oxidising conditions. From its amino acid analysis, N terminal glycine and molecular weight, P1 appears to be identical with the protein HMG 1 isolated from saline extracts of thymus nuclei by Goodwin et al, (1973).

Histones are retarded in mobility on Sephadex and on polyacrylamide gels at pH 2.4, considering their molecular weight (Phillips and Clarke, 1970) but are only slightly retarded on polyacrylamide gels in the presence of SDS (Panyim and Chalkley, 1971). Component P1 shows a retardation similar to the polycationic histones. It has glycine as N terminal amino acid, in contrast to F1 whose N terminal is acetylated (Phillips, 1963) and consequently gives no dansyl derivative. The remaining non-histone proteins are considerably more acidic (Table 2:1), having a low lysine content. They have high contents of glycine and alanine. Amino acid analysis of non-histone components of liver and ascites F1 preparations (Table 2:2) show that P1 is less predominant among the non-histone components of 5% PCA extracts of these tissues than is the case for thymus. This may simply reflect the low content of non-histone nuclear protein in sheep thymus.

The non-histone components carry a significant proportion of the phosphate of the crude F1 preparation (Table 2:2) being in each tissue much more heavily phosphorylated than the histone itself. Component P1 acts as a substrate for the cytoplasmic 'histone kinase' <u>in vitro</u> (Table 2:3). However, it is considerably less effective than the histone itself.

Fig. 2:5

Formation of complexes by P1 with histone F1 and poly L lysine

The assay contains 100µg P1 in 2mls

together with F1 (closed circles).

F1 and βmercaptoethanol (open circles).

Poly L lysine (crosses).

Complex formation is monitored by the O.D. at 450nm.

Fig 2:5

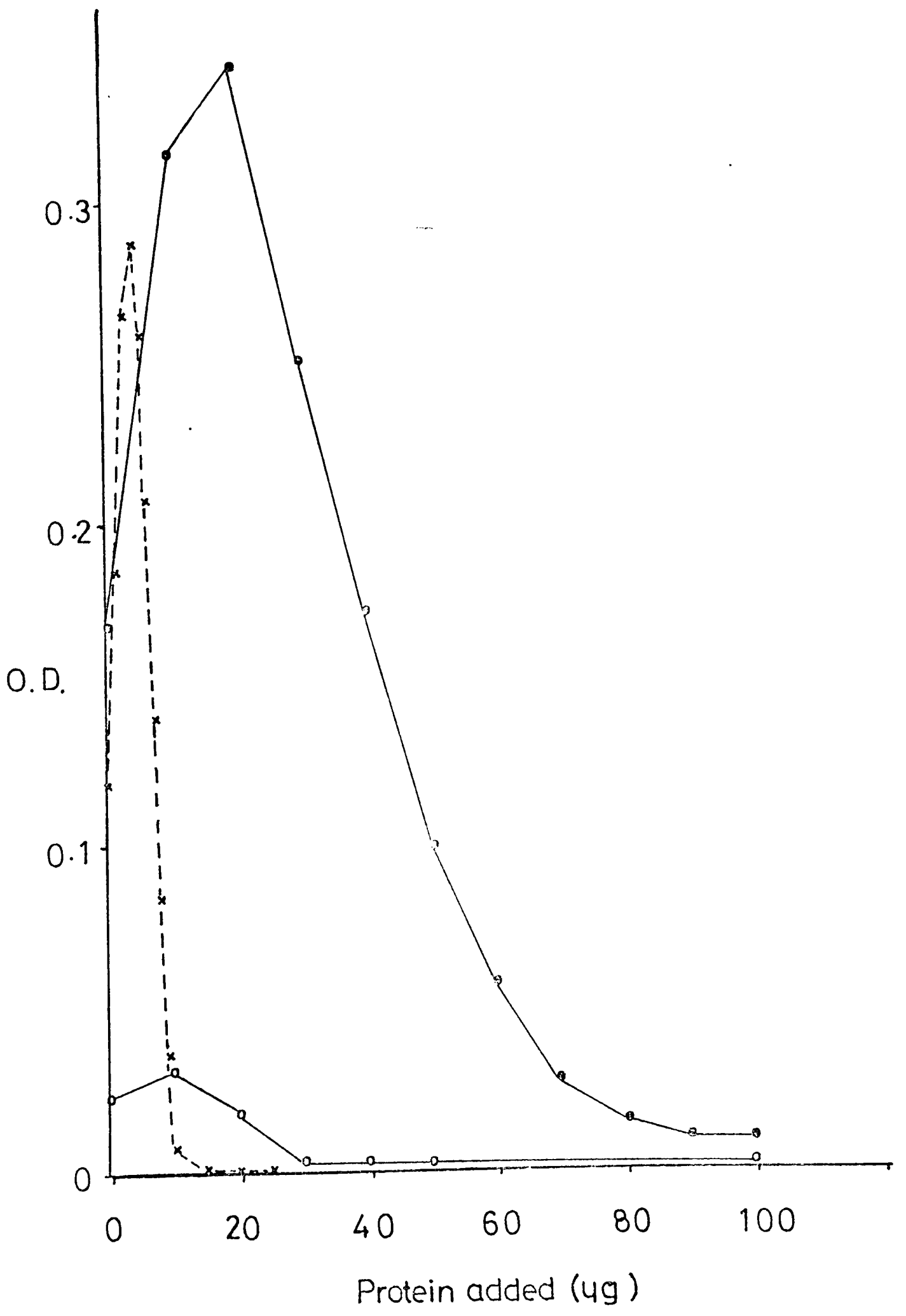


Table 2:4

Effect of P1 on Template Activity of DNA for *E.coli* RNA Polymerase

	cpm
DNA alone	23,511
DNA + 10µg P1	24,693
DNA + 20µg P1	25,226
DNA + 40µg P1	21,041
DNA + 40µg F1	5,656
DNA + 40µg F1 + 10µg P1	5,261
DNA + 40µg F1 + 20µg P1	6,206
DNA + 40µg F1 + 40µg P1	4,971
DNA + 40µg F1 + 60µg P1	6,149
DNA + 40µg F1 + 80µg P1	6,151
DNA + 40µg F1 + 100µg P1	5,559
No DNA	787

cpm are counts precipitable by 5% TCA.

On the basis of its *in vitro* behaviour with this enzyme, it would be expected that only 1% of ^{32}P counts incorporated *in vivo* would be associated with the non-histone fraction if this kinase were solely responsible for phosphorylation. In fact, the figures observed are 6-7% for a 90 minute incubation, increasing to as much as 29% for an incubation of 18 hours. The question of the enzyme responsible for *in vivo* phosphorylation will be discussed later, but it is clear that the population of phosphate molecules associated with the non-histone components is metabolically distinct from that associated with histone F1 itself.

The question remains whether the non-histone components are involved in the function of the histone. It is found that component P1 forms complexes with histone F1, or poly L lysine (Fig. 2:5) under the same conditions as those for which Langan and Smith, (1965) and Wang and Johns, (1968) observed complex formation between histones and acidic nuclear proteins. Complex formation is not found in the presence of βmercaptoethanol (Fig. 2:5).

Component P1 does not reduce the template activity of DNA for Escherichia coli DNA dependent RNA polymerase *in vitro*, nor does it relieve inhibition of this activity by histone F1, (Table 2:4) possibly because the assay mixture contains βmercaptoethanol, which prevents complex formation.

On washing nuclei with various concentrations of saline solution, many nuclear proteins are lost (Johns and Forrester, 1969, Goodwin and Johns, 1972, Comings and Tack, 1973) and extensively washed chromatin may be devoid of non-histone protein (Johns and Forrester, 1969). It is found that the

Table 2:5

Amounts of Non-Histone Protein Associated with Histone F1 Extracted from Rat Liver Nuclei in Various Ways

Method of Extraction	Yield (mgs)		%
	F1	Non-histone	Non-histone
A) Extracted twice with 5% PCA	37.3	1.8	4.8
B) Extracted twice with 5% PCA after two washes with 0.14M NaCl	26.4	0.3	1.2
C) Extracted twice with 5% PCA after two washes with 0.3M NaCl	16.5	0.18	1.1
D) Extracted twice with 5% TCA	28.2	0.6	2.1
E) Extracted twice with 0.25N HCl(5% PCA supernatant)	31.4	0.7	2.2
F) Extracted twice with 0.6M NaCl	41.0	3.6	8.8

Nuclei from 150g rat liver were divided into 6 equal portions for extraction. In each case the protein was precipitated with 25% TCA, and resuspended in water before separation on DEAE Cellulose.

Fraction F contained much non-histone material that did not dissolve in water, and is not included in the Table.

In fractions A-E, the major non-histone component was P1.

amount of non-histone associated with F1 is reduced by washing with salt, and some F1 is also lost (Table 2:5a-c). Component P1 is the predominant non-histone component in all except the 0.6M saline extract, which contains many non-histone proteins (Fig. 2:4c). The non-histone is evidently slightly less tightly bound to the chromatin than histone F1, in agreement with the finding of Lake et al, (1972), who finds a non-histone band which runs close to F1 on polyacrylamide gel electrophoresis, and which is washed from nuclei at slightly lower salt concentration than is F1.

Discussion

Histone F1 preparations from all the tissues examined have a fairly constant content of component P1, together with varying amounts of other non-histone proteins. (Proteins similar in mobility to P1 appear also to be present in histone preparations from urchins (Easton and Chalkley, 1972) HeLa cells (Sadgopal and Bonner, 1970a,b) and in yeast (Wintersberger et al, 1973) in which the contents of both F1 and P1 are reduced). Since component P1 carries a high level of phosphate, it is clear that from a purely practical point of view it needs to be separated from the histone before the estimation of the phosphate of the latter is possible. Similarly, metabolic studies of the histone-phosphate are invalid in the presence of the metabolically distinct P1-phosphate.

The protein itself is nuclear, and while it is partly removed from chromatin by washing with 0.3M NaCl, this is true of most, if not all non-histone nuclear proteins (Fujitani and Holoubek, 1973, Comings and Tack, 1973). It

does not preclude a chromosomal function. Histone F1 itself is washed out gradually at a salt concentration of 0.35M (Lake et al, 1972, Johns and Forrester, 1969) or under the ionic conditions believed by Langendorf et al, (1961) to exist inside the nucleus (Comings and Tack, 1973). While the binding of proteins to 'chromatin', is presumed to be tighter, the higher the ionic strength which is required to extract them, the difference between histones and non-histones would appear to be quantitative rather than qualitative. Thus, washing nuclei twice with 0.3M NaCl has removed 50% F1, while 10% of the 5%-perchloric-acid-soluble non-histone still remains (Table 2:5). Comings and Tack, (1973) point out that a non-histone protein with a function as, say, a gene repressor, might be expected to bind loosely to chromatin, such that its control function could be modified by a small alteration in the ionic enviroment of the nucleus, or by the binding of a small molecule to the protein such that its affinity for the chromatin was altered. A protein with a control function would thus be expected to exist with 'bound' and 'free' populations in equilibrium.

Whether the P1 acts on F1 in vivo is not determined. P1 does form complexes with F1 in vitro, but such complexes are observed between heterogeneous preparations of non-histones and histones (Langan and Smith, 1965, Wang and Johns, 1968) and between different fractions of histones (Kelly, 1973, D'Anna and Isenberg, 1973, Kornberg and Thomas, 1974), while individual histone fractions themselves aggregate (Johns, 1971).

Non-histone proteins which complex with histones, also relieve the inhibition by histones of DNA dependent RNA

polymerase *in vitro* (Wang, 1968, Kamiyama and Wang, 1971, Spelsberg and Hnilica, 1969, 1971a,b, Spelsberg et al, 1971, Teng and Hamilton, 1969, Allfrey et al, 1963). Unfortunately, the validity of results of *in vitro* experiments using *E.coli* RNA polymerase is doubtful, since Johns and Hoare, (1970) claim that the apparent template activity is simply a measure of the degree of precipitation of the DNA, while Keshgegian and Furth, (1972) have shown that *E.coli* RNA polymerase is less specific at transcription than the endogenous polymerase.

If it should be that complex formation of P1 with F1 reflects function *in vivo*, it is noteworthy that the complexes are dependent on the redox state of P1 (Fig. 2:5). It is known that thiolation is related to replication (Ord and Stocken, 1966), so alteration of histone F1 complexes with P1 could provide a further means for the control of nuclear function. A mechanism is available by means of which the histone can respond to a change in the redox state of the nucleus.

Since the phosphate attached to P1 and to F1 are metabolically distinct, it is likely that the kinases involved in phosphorylating these two proteins are different. The best studied of enzymes that can phosphorylate histone F1 is the cAMP stimulated protein kinase of the cytoplasm, which has also been shown to phosphorylate phosphorylase kinase, glycogen synthetase, hormone sensitive lipase, *E.coli* RNA polymerase and brain microtubule protein (Chen and Walsh, 1971). It phosphorylates *in vitro* the same tryptic peptide of F1 which is phosphorylated *in vivo* by the glucagon or

insulin stimulated liver (Langan, 1968a). However, this peptide is one of many phosphorylated slightly in the unstimulated liver, and is not selectively phosphorylated during regeneration. An accurate impression of the current state of this field is given in a recent review by Langan, (1973). Siebert et al, (1971) has shown the presence of a nuclear histone kinase which is not stimulated by cAMP, and whose activity increases sixfold during liver regeneration while the cytoplasmic enzyme remains constant. Kish and Kleinsmith, (1974) have distinguished at least 12 non-histone kinases, by separation on a phosphocellulose column, some of which may phosphorylate histone, and find the distribution of these kinases to be tissue specific. It is evident from this report that much more work will be needed in this field before a clear picture is obtained.

CHAPTER 3

INTRODUCTION

Nucleotides have been reported as contaminants of preparations of histone F1 (Shepherd et al, 1970, Balhorn et al, 1971, Greenaway and Murray, 1973).

They are probably present as contaminants in these preparations because acetone or ethanol are used as precipitants, or the acid extract is freeze-dried to obtain the dry histone. The high proportions of precipitant required ensure that acid or salt soluble oligonucleotides are precipitated with the histone, while small molecules are all retained by freeze drying.

In nuclei incubated with [^{14}C] Adenine labelled NAD, ^{14}C radioactivity is found associated with histone F1 (and other histones) separated on CM Cellulose or Amberlite CG50 columns, presumably in the form of covalently bound ADPribose (Nishizuka et al, 1968, Ueda et al, 1972).

Results

That not all the phosphate associated with histone F1, and other histones, was bound as phosphoserine, was indicated by the difference in behaviour on polyacrylamide gel electrophoresis between material labelled with [^{32}P] phosphate *in vivo* and *in vitro*, (Table 3:1). Thus F1 or P1 phosphorylated *in vitro* with γ [^{32}P] ATP by cytoplasmic 'histone kinase', carried a minimum of 80% of the loaded radioactivity in the protein band, while protein labelled *in vivo* carried only a small percentage of apparently associated radioactivity with it. Some of the missing radioactivity remains at the gel origin; much never enters the gel.

Fig. 3:1

Ultraviolet absorption spectra

of histone F1, 1mg/ml, λ max. 273, OD_{273}:- 0.16

P1, 1mg/ml λ max. 275, OD_{275}:- 0.45

perchloric acid precipitate, 100µg/ml, λ max. 260, OD_{260}:- 0.33.

All proteins were derived from sheep thymus.

Fig 3:1

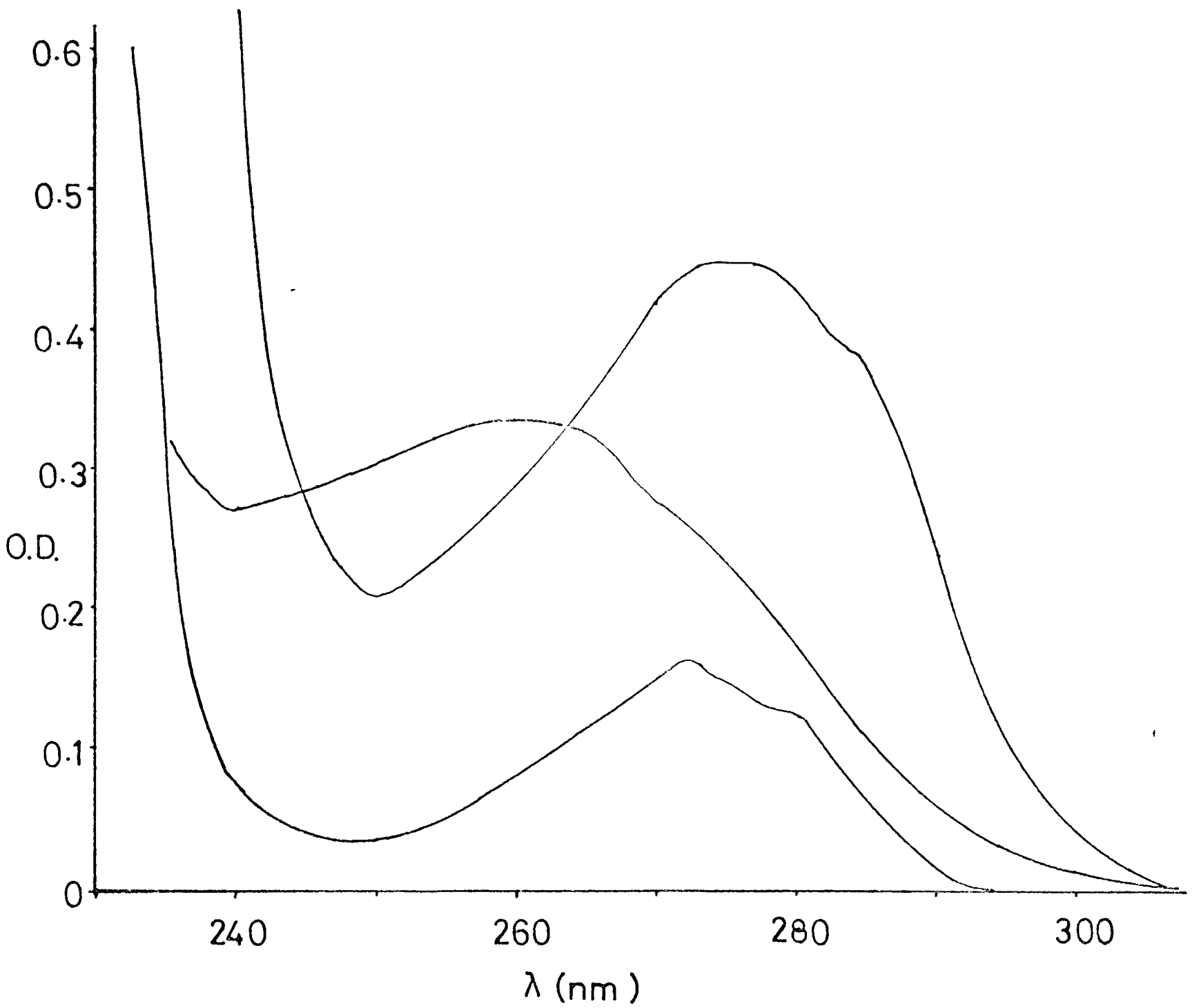


Fig. 3:2

Dowex-1-formate (AG-1-X4) chromatography of alkaline digest of histone F1

The ascites hepatoma histone F1, labelled with ^{32}P for 47hrs *in vivo* was digested with 0.5N KOH for 2hrs at 37°C. After neutralisation the digest was applied to the Dowex-1-formate column (20cm x 3cm^2, flow rate 20ml/hr). The formic acid gradient was formed by running first 0.5N formic acid, and then 6N formic acid into a 300ml reservoir of water. The arrows at the top of the Fig. indicate the start of the 0.5N and 6N formic acid gradients respectively. The lower arrows mark the points of elution of AMP and ADP respectively.

cpm = counts per minute per (5ml) fraction.
15,000cpm loaded.

Fig 3:2

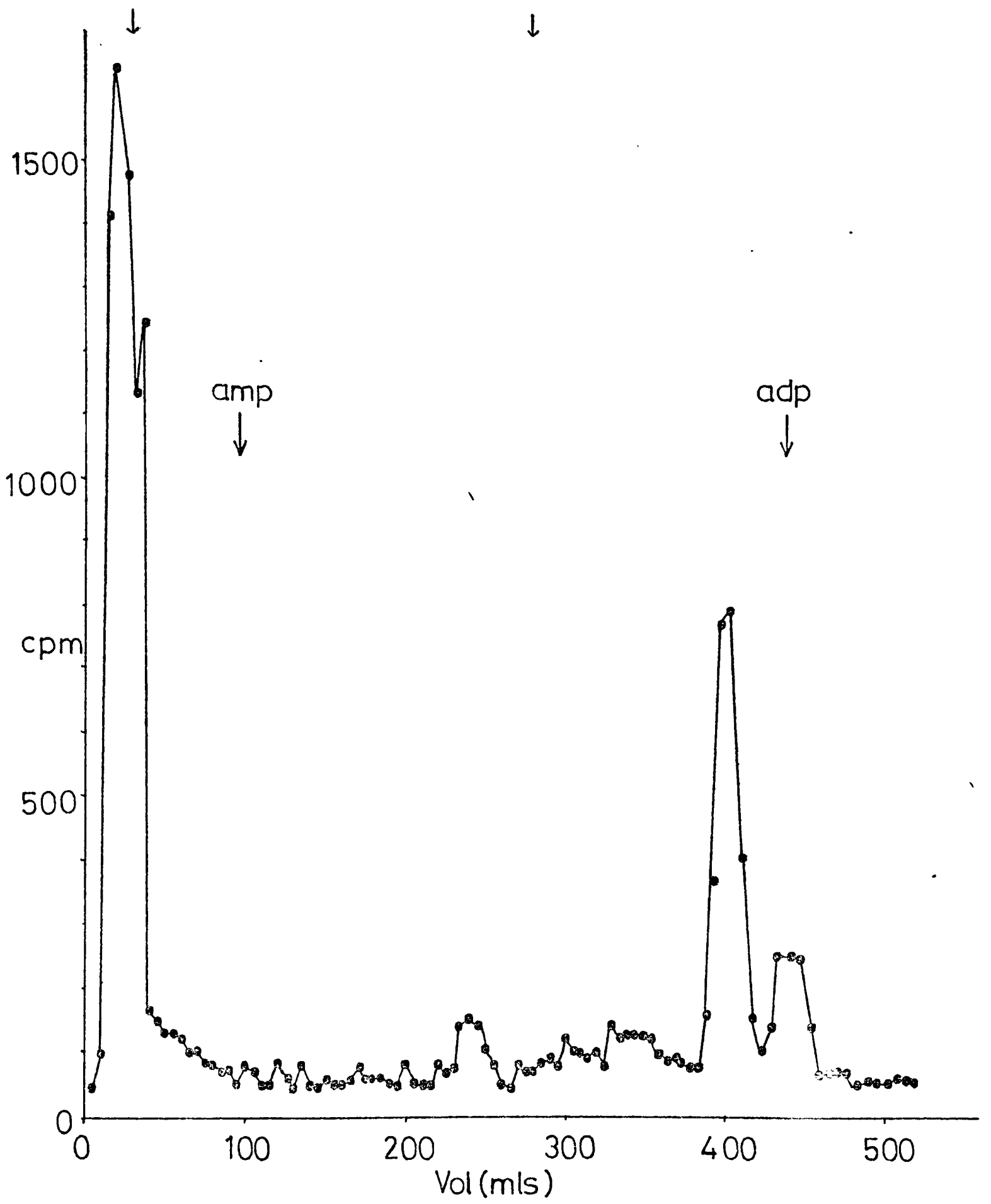


Table 3:1

^{32}P Radioactivity Recovered from Polyacrylamide Gel Electrophoresis						
Tissue	Protein	Method of Labelling	Gel	Load cpm	Recovered with protein cpm	Recovered with protein %
Sheep Thymus	F1	'histone kinase' in vitro	A	15,000	13,800	92
	P1			1,500	1,200	80
	F1		S	14,000	12,600	90
	P1			3,000	2,700	90
Rat Liver	F1	90 min i.p	A	56	10	18
	TH-F1			700	69	10
	F1		S	56	10	18
	TH-F1			700	100	14
21hr regenerating rat liver	F1	90 min i.p	A	456	174	38
	TH-F1			1,350	106	8
	F1		S	456	177	39
	TH-F1			1,350	160	12
Rat Liver	F1	18hr i.p	S	300	0	0
	TH-F1			300	0	0
Ascites Hepatoma	F1	48hr i.p	A	1,800	90	5
	TH-F1			3,400	10	0.3

A = acetic acid/urea gel system.

S = sodium dodecyl sulphate gel system pH 7.0.

i.p = intraperitoneal injection of [^{32}P] orthophosphate administered in 0.3mls 0.14M saline, given the stated time before death.

It was also noticed that purified F1 and P1 prepared from sheep thymus contain limited amounts of phosphate, i.e. F1 - 2.4nmoles phosphate/mg protein, P1 - 14.7nmoles/mg. However, the material which was precipitated by 5% (w/v) perchloric acid during the final step of purification of P1, had a phosphate content of 184nM/mg protein; this material, which consisted of about 5% of the P1 protein could be re-suspended in water to give a clear solution. It gave an ultraviolet absorption spectrum with a peak at 260nm, as opposed to the normal protein peak at 275nm which is found for pure F1 and P1 (Fig. 3:1). Since a peak at 260nm is characteristic of nucleotides it was necessary to check for nucleotide contamination.

Ascites hepatoma cells were labelled with [^{32}P] ortho-phosphate in vivo. (Ascites cells were chosen as they readily incorporate label into F1, and because the label incorporated separates from the protein band on polyacrylamide gel electrophoresis (Table 3:1). Histone F1 was prepared and purified, and incubated with 0.5N KOH for 2 hours at 37^{o}C to hydrolyse any RNA present. After neutralising the solution with perchloric acid, and centrifuging out the $KClO_4$ formed, the solution was loaded on to a Dowex-1-formate column, and eluted with a gradient of formic acid (Nishizuka et al, 1967). Apart from the radioactivity which was not retained by the column, and which contained inorganic phosphate derived from phosphoserine, only 2 peaks of any significance were observed. One of these was at the position where UMP and ADPribose elute, the other at the position of ADP (Fig. 3:2). When a sample of

Fig. 3:3

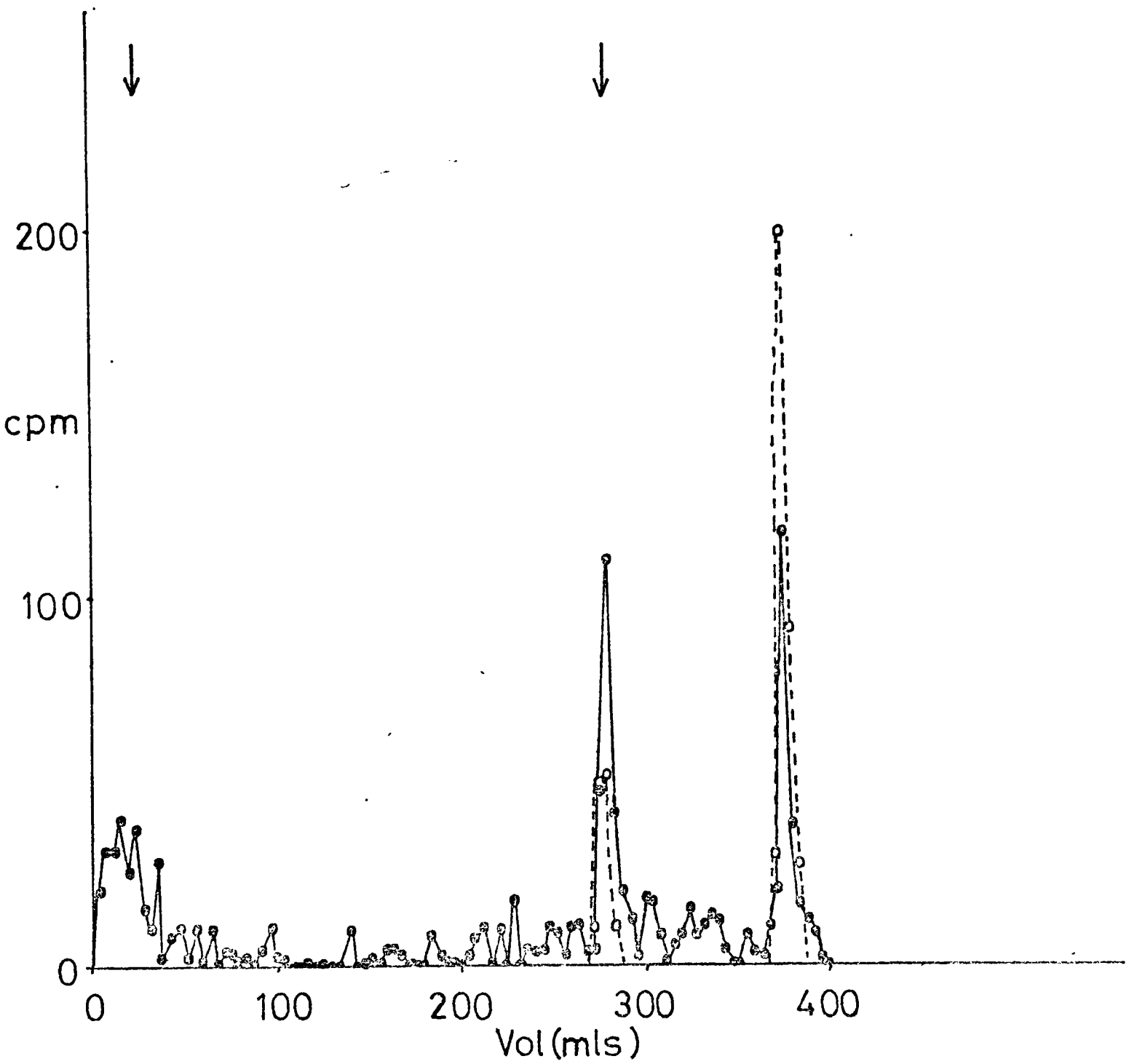
Dowex-1-formate chromatography of phosphodiesterase digest of histone F1 and component P1

The rat liver histone F1, or P1 labelled with ^{32}P for 18hrs *in vivo* was digested with venom phosphodiesterase for 4hrs, and chromatographed under the same conditions as in Fig. 3:2. 4ml fractions were collected. 1,000cpm loaded in each case.

Closed circles - F1

Open circles - P1

Fig 3:3



ascites histone F1 was digested with 60% perchloric acid at 100°C for 60 minutes, the digest neutralised with KOH, and the supernatant chromatographed according to Lane, (1963), adenine was the only base observed. These results were taken to indicate that RNA was not a serious contaminant of histone F1, and that nucleotide material present in the preparation was probably in the form of (poly) ADPribose.

Preparations of F1 and P1, from rat liver, also labelled in vivo with ^{32}P were digested with venom phosphodiesterase (from Crotalus atrox, containing pyrophosphatase and phosphodiesterase activities, together with a low activity of phosphomonoesterase). The digests were applied to a Dowex-1-formate column, and eluted as above. Again, there is an unretained peak, which in this case is presumably protein bound phosphoserine, a peak near the UMP elution position, which could be phospho-ribosyl-AMP, and a third peak eluting at the ribose-5-phosphate position, which also behaves as ribose-5-phosphate when chromatographed on paper with isobutyric acid/NH_4OH (Nishizuka et al, 1967). AMP was not observed. (Fig. 3:3).

In order to establish whether the nucleotide was free or protein bound, rat liver histone F1 labelled with ^{32}P in vivo was digested with pronase, and leucineaminopeptidase, the digest made 50mM in HCl, and applied to a Dowex 50 column. On eluting with 50mM HCl, nucleotide material was eluted immediately, phosphoserine eluted at approximately 3 column volumes, while other amino acids were retained on the column (Pizer, 1963, Balhorn et al, 1971). The nucleotide containing fraction was applied to a Dowex-1-formate column, washed with 0.3N formic

Table 3:2 (a)

Analysis of Material from Histone F1 Retained on Dowex Formate after Separation of Serine Phosphate

Serine	0.31
Ribose	1.73
Phosphate	1.83
Adenine	0.89

(b)

Analysis of Material from Histone F1 Eluting from DEAE Sephadex in the Monomer Position

	Expt I	Expt II
Serine	1.89	1.77 (1.80 ser P)
Ribose	4.40	3.33
Phosphate	6.01	5.37
Adenine	1.86	1.74

All values are in nM/mg protein

Ribose and phosphate were determined directly on the eluate. Serine was determined by the method of Spackman et al, (1958) on a sample of the eluate which was lyophilised and incubated in 0.5N KOH for 30 minutes at 37°C, or for 21hrs in 6N HCl at 110°C. Adenine was determined by its optical density at 260nm. It was isolated from the eluate by paper chromatography after hydrolysis in 2M HCl for 1hr at 100°C. Serine phosphate was determined by the method of Spackman et al, (1958) on a sample of the eluate after hydrolysis in M HCl for 30 minutes at 37°C. ADPribose was identified by paper chromatography of a sample of the eluate, hydrolysed with 0.3N HCl for 5 minutes at 30°C, against an authentic specimen, in isobutyric acid/NH_4OH.

Fig 3:4

Proposed Structure for O-phosphoseryl(ADPribose)$_n$

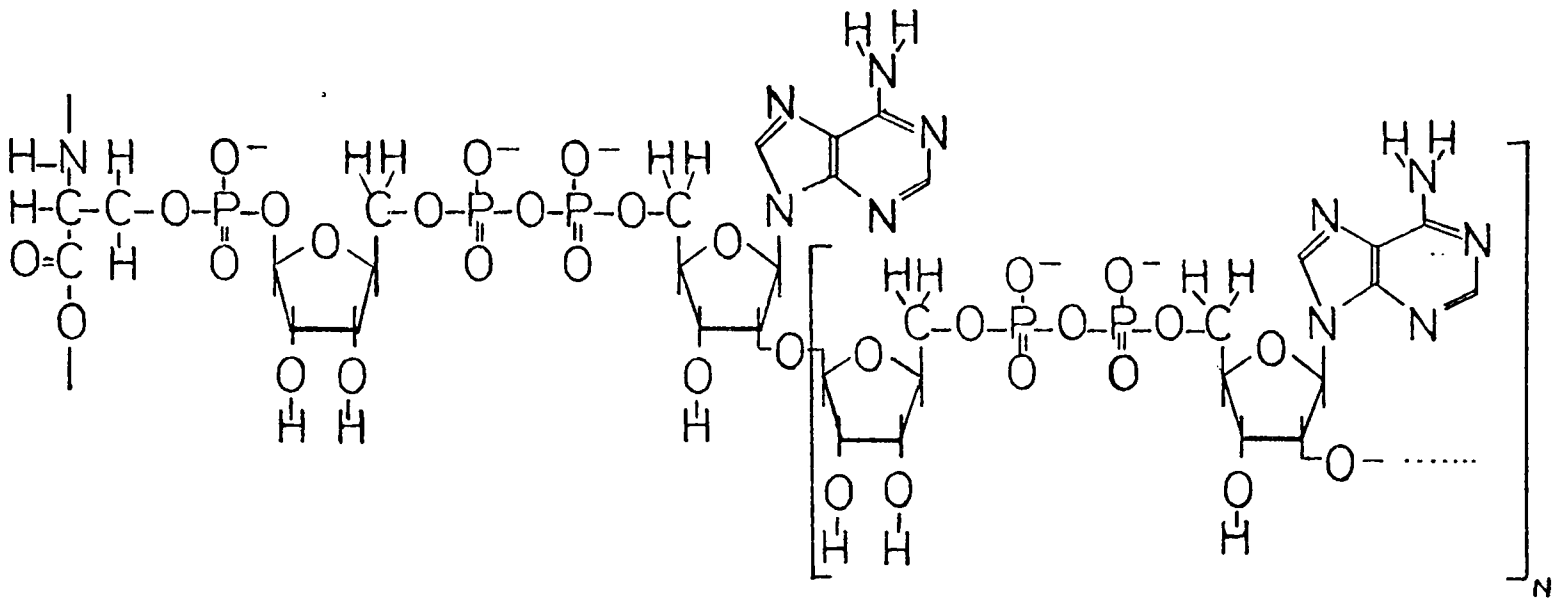


Fig. 3:5

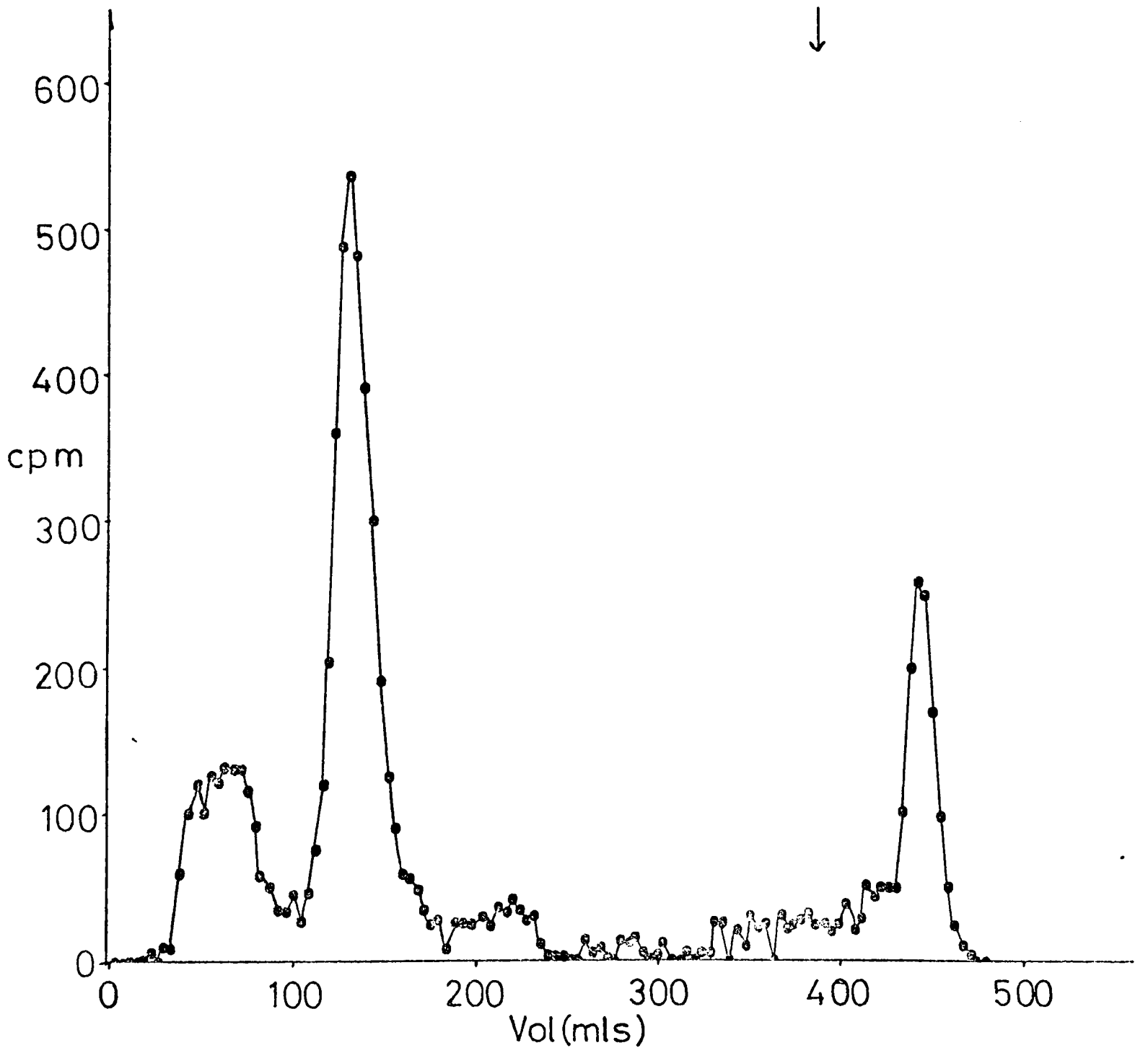
DEAE Sephadex chromatography of pronase-leucineaminopeptidase digest of histone F1

The rat liver histone F1, labelled *in vivo* with ^{32}P for 18hrs was digested with pronase for 17hrs, then leucineamino-peptidase for 96hrs. It was applied to a column (20cm x $3cm^2$, flow rate 16mls/hr) of DEAE Sephadex (A25), and eluted with a salt gradient formed by running 7M urea, 0.7M NaCl, 20mM Tris-HCl buffer, pH 7.4 into a 400ml reservoir of 7M urea, 0.14M NaCl, 20mM Tris-HCl buffer, pH 7.4. The arrow at the top indicates where the column was washed with the 0.7M NaCl buffer.

Load: 8,000cpm.

Yield: 1,300cpm phosphoserine.
3,480cpm phosphoseryl ADPribose.
435cpm phosphoseryl $[ADP\ ribose]_2$
1,200cpm phosphoseryl $[ADP\ ribose]_n$

Fig 3:5



acid, and eluted in 3N formic acid. The analysis of the eluate is shown in Table 3:2a. Phosphoserine was identified in a sample of the eluate after hydrolysis in 2M HCl for 1 hour at 100°C, by comparison of its elution position on the amino acid analyser with that of a specimen of authentic O-phospho-L-serine. Since the phosphoserine from the histone digest had already been separated from the nucleotide on Dowex 50, the phosphoserine identified in the nucleotide eluate must have been covalently bound to the nucleotide. A structure may now be proposed for (poly)ADPribose, bound to histone F1 via phosphoserine as shown in Fig. 3:4.

The analysis presented in Table 3:2a is compatible with the presence of F1 - bound (poly)ADPribose with an average chain length (ser: ade ratio) of three ADPribose units, but does not indicate the distribution of lengths. A pronase, leucineaminopeptidase digest of *in vivo* ^{32}P labelled rat liver histone F1 was made 7M in urea and 0.14M in NaCl and applied to a column of DEAE Sephadex pre-equilibrated with the same solvent buffered with 20mM Tris-HCl, pH 7.4, and eluted with a salt gradient to determine the chain length (Fig.3:5) (Seno et al, 1968). After the elution of the 'run-off' peak, containing the bulk of the amino acids, including phosphoserine, the majority of the counts are found in the peak expected to contain the phosphoserine-bound monomer. Many of the remaining counts are not recovered until the column is washed with 7M urea, 0.7M NaCl, 20mM Tris-HCl, pH 7.4, and represent a polymer of more than 6 units of ADPribose. The analysis of the peak containing the monomer is given in Table 3:2b, and

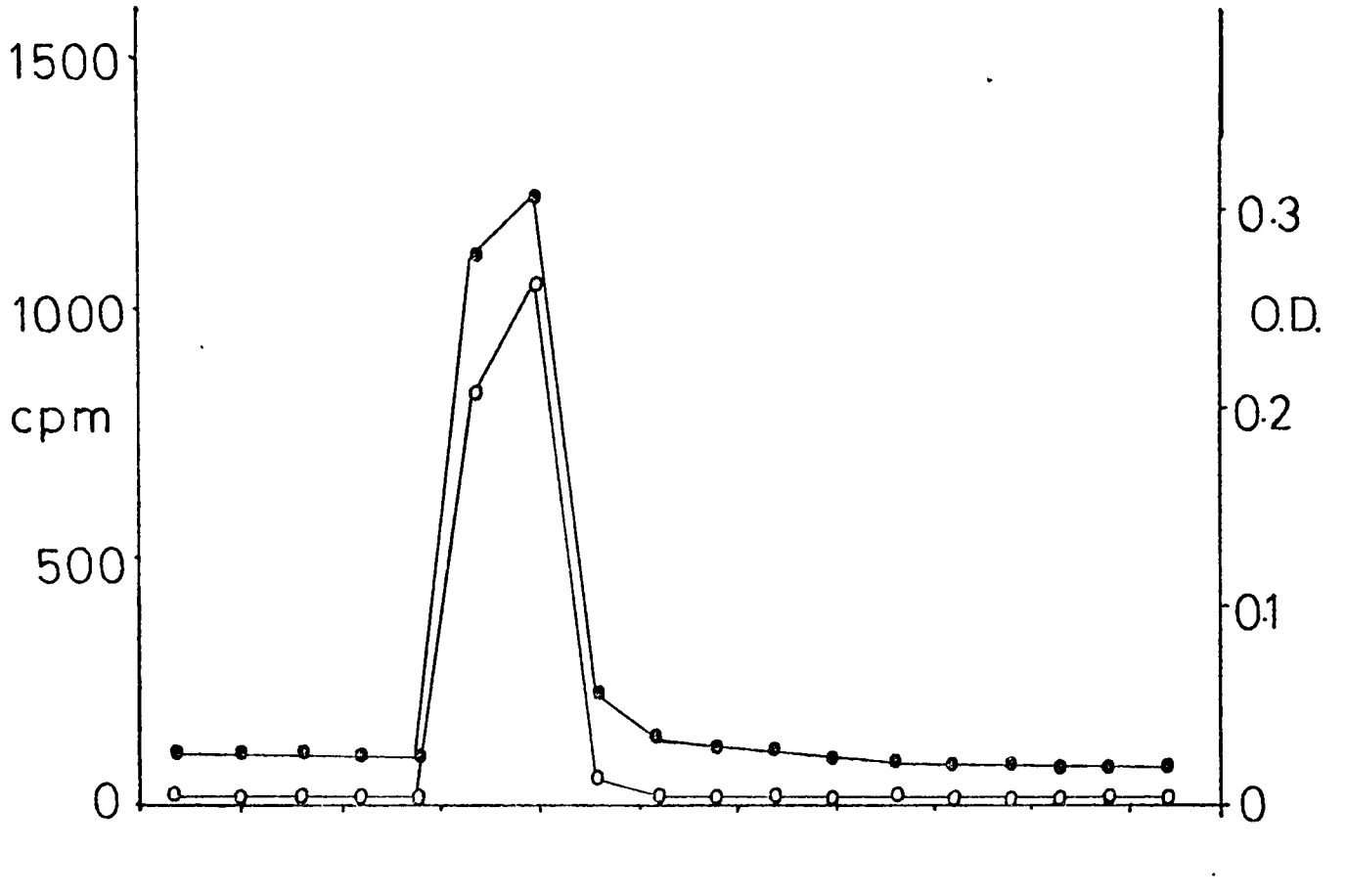
Fig. 3:6

Sephadex G75 exclusion chromatography of histone F1

a) Rat liver histone F1 incubated for 18hrs with ^{32}P in vivo was chromatographed on a Sephadex G75 column (20cm x 3cm^2, flow rate 20mls/hr) in 50mM Tris-HCl pH 7.4.
Closed circles = cpm/fraction.
Open circles = OD at 280nm.
Load: 2,500cpm, 3.3mgs.
Yield: 2,200cpm, 3.2mgs.
Identical results were obtained with 7M urea, 2% SDS, or 5M urea, 2M NaCl as solvent.

b) As above, but with phosphoseryl-ADPribose added.
Load: 920cpm F1, 480cpm phosphoseryl-ADPribose.
Yield: 880cpm on F1, 450cpm free.

Fig 3:6


a)
1500
1000
cpm
500
0
0·3
O.D.
0·2
0·1
0
b)
500
400
300
cpm
200
100
0
0
10
20
30
40
50
Vol(mls)

shows a ratio for serine : adenine : ribose : phosphate of 1 : 1 : 2 : 3 as predicted from the proposed structure. ADPribose was identified from a hydrolysate (0.3N HCl for 5 minutes at 30°C) of the monomer preparation, by paper chromatography against an authentic specimen with isobutyric acid/NH_4OH as solvent (Nishizuka et al, 1967).

Since nucleotide material is frequently found as a contaminant of histone preparations (Greenaway and Murray, 1973, Shepherd et al, 1970) it might be argued that the serine-phosphate-ADPribose was only casually associated with the histone. However, histone F1 is completely excluded from Sephadex G75, and if labelled with ^{32}P *in vivo*, the radioactivity elutes from the column quantitatively with the protein (Fig. 3:6a). If labelled serine-phosphate-ADPribose is added to this histone complete separation of the components is achieved under the same conditions, showing that serine-phosphate-ADPribose has no tendency to 'stick' to the protein (Fig. 3:6b). On incubation of histone F1 labelled *in vivo* with ^{32}P, with neutral hydroxylamine, (2M, 37°C, 30 minutes) it was found that all the radioactivity remained with the histone when run on a Sephadex G75 column under the same conditions as above or on precipitation with 25% TCA. Material labelled *in vitro* has also been shown to be hydroxylamine stable (Smith and Stocken, 1973). In addition to the quantitative results obtained with the phosphoseryl-ADPribose from histone F1, phosphoserine has been identified as a hydrolysis product of the polymer fraction isolated from F1, as well as from the material retained on a Dowex-1-formate column from a pronase leucineaminopeptidase

digest of the non-histone fraction containing P1. None of the radioactivity incorporated in vivo into a crude 'F1' preparation was found to be hydroxylamine labile.

Because of the acid lability of the proposed ribose-1-phosphate band, it is possible that some of the ADPribose might be lost from histone F1 during the extraction with perchloric acid. In consequence, nuclei were extracted with 0.6M NaCl, to remove histone F1 under neutral conditions, although 25% TCA was used to precipitate the histone extract to minimise the exposure time to nuclear nucleases. (The total exposure time to TCA was 20 minutes at $0^{o}C$). The non-histone proteins also extracted were removed on DEAE Cellulose, and the phosphate content of the F1 was found to be 18.7nM per mg protein, of which the free phosphoserine content was 1.55nM/mg protein. (Values obtained from a pronase-leucine-aminopeptidase digest, separated on DEAE Sephadex). The implication is that the amount of unsubstituted phosphoserine on histone F1 is very low in the normal, unstimulated rat liver. Now, the salt extracted histone F1 has 1.55nM phosphate/mg protein present as phosphoserine, leaving 17.15mM/mg as phospho-seryl ADPribose. One third of the latter would remain as phosphoserine on acid hydrolysis, giving a total of 7.3nM phosphate/mg protein which corresponds well to the value of 5.0nM/mg (0.11moles/mole F1) obtained by Balhorn et al, (1971) for their histone F1 purified on Amberlite IRC50, after 'extensive dialysis against 0.4N H_2SO_4'. If histone F1 is purified on Amberlite IRC50 in the same manner as Balhorn et al, (1971), but without dialysis against acid, a similar

Fig. 3:7

Separation of tryptic peptides of histone F1

Rat liver histone F1 incubated for 60 minutes with ^{32}P in vivo was digested with 5% trypsin for 5hrs at 25°C, and the peptides separated.

a) on a DEAE Sephadex column (A25) as Fig. 3:5, except that the urea was omitted, and the gradient run from 0.1N to N NaCl.

b) by electrophoresis on paper in 10% (v/v) pyridine acetic acid buffer, pH 6.5 for 1hr at 3½Kv.
The arrow at the bottom indicates the position of the orange G marker.

a) and b) The peptides referred to in Table 3:3 are indicated by numbered arrows.

Open circles = OD at 280nm

Closed circles = cpm/fraction.

Fig 3:7

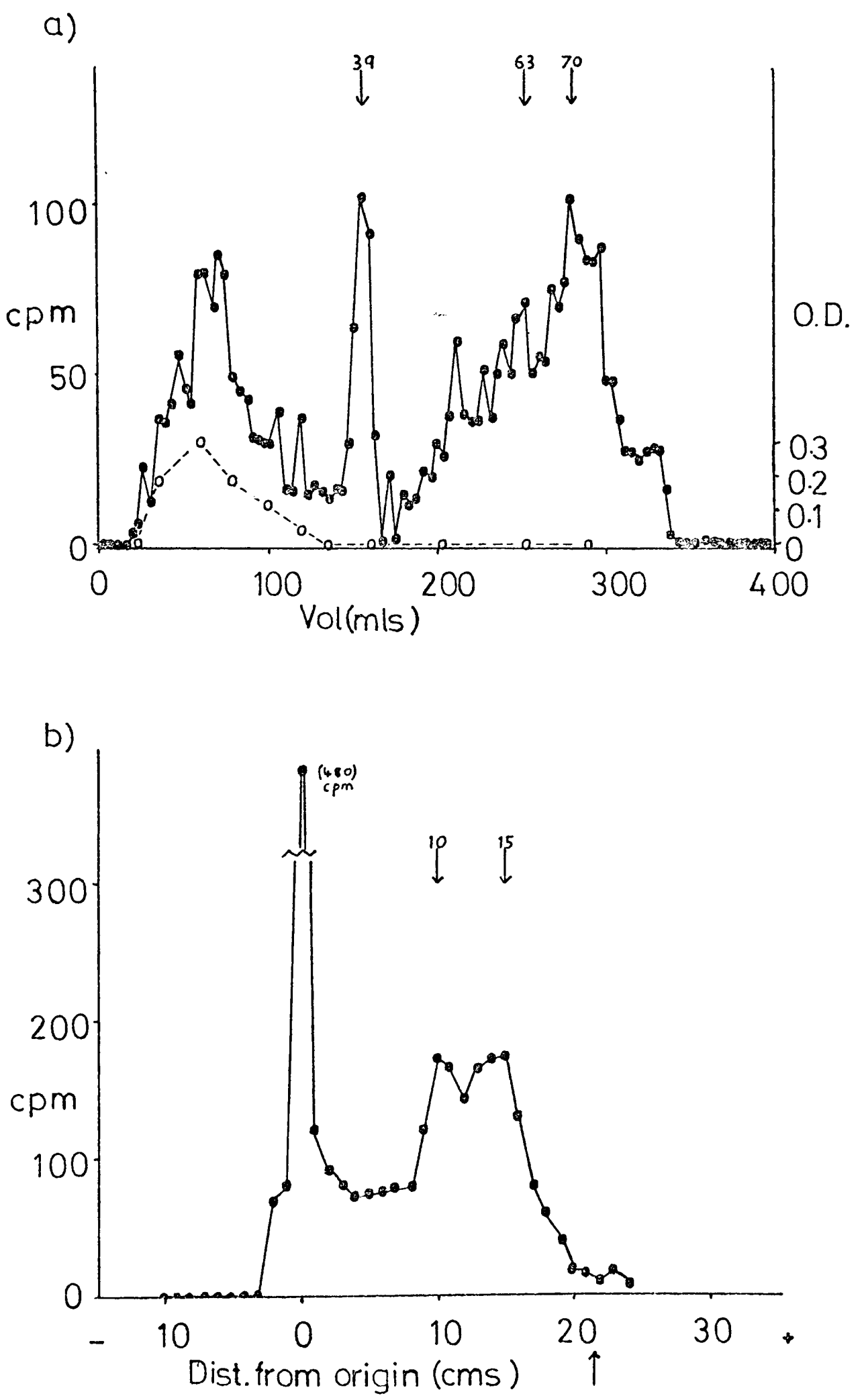

a)
39
63
70
100
cpm
50
0
O.D.
0.3
0.2
0.1
0
0
100
200
300
400
Vol(mls)
b)
(480)
cpm
10
15
300
200
cpm
100
0
-
10
0
10
20
30
+
Dist. from origin (cms)

Table 3:3

Amino Acid Analyses of Trypic Peptides of Histone F1, Bearing ADPribose

	Analysis									
Peptide	Asp	Thr	Ser	Glu	Pro	Gly*	Ala	Val	Leu	Lys
39	3.0(3)	1.4(1)	2.0(2)	4.1(4)	3.8(4)+	1.4(1)	8.4(8)	0.6(1)	0.6(1)	2.1(2)
63	2.9(3)	0.7(1)	2.0(2)	3.8(4)	-	1.3(1)	1.4(1)	-	0.5(1)	1.2(1)
70	2.4(2)	0.2(0)	2.0(2)	5.2(5)	-	1.7(1)	0.9(1)	0.4	0.5(1)	8.6(9)+
10	2.0(2)	1.2(1)	2.0(2)	2.9(3)	-	2.3(2)	3.0(3)	0.9(1)	0.9(1)	1.0(1)
15	3.1(3)	1.3(1)	2.0(2)	4.1(4)	-	2.3(2)	2.1(2)	0.8(1)	0.8(1)	0.8(1)

+Values possibly inflated by artefact.

*Adenine gives a glycine peak of 0.5moles/mole Adenine under these conditions.

Figures in brackets indicate probable numbers of residues per peptide.

separation of 'nucleotide' and histone takes place, except that a large proportion of the histone is found with the nucleotide in the fraction eluted with 3% NaCl (F1a), while the remaining histone (F1b) has some phosphoserine, but no nucleotide.

An attempt was made to locate the position of the ADPribose on the histone molecule. To this end, rat liver histone F1 labelled with ^{32}P *in vivo* was digested with trypsin, and the digest fractionated either on a DEAE Sephadex column, with a salt gradient (Fig. 3:7a) or by electrophoresis on paper (Fig. 3:7b). In either case, an easy separation of the ADPribose-bearing peptide from the bulk of the digest would be expected, owing to the high negative charge of the nucleotide. Amino acid analyses were performed on fractions with high levels of radioactivity, where these were not contaminated with the bulk of peptide material. The presence of ADPribose was indicated by a peak of optical density at 260nm. The peptides isolated appear to contain variations of a single sequence (Table 3:3). The two peptides separated on paper differ in the substitution of an alanine for an aspartate plus a glutamate. The wide spread of radioactivity observed on the DEAE Sephadex, between 160 and 340mls of eluant, may indicate that digestion is not complete. Of the peptides isolated on DEAE Sephadex, only '39' is likely to be reasonably pure. Trypsin resistant runs of lysine and alanine residues which might be indicated from these peptides are known in F1 around residue 157 of trout testis (Dixon et al, 1973). The common sequence does not occur on the known part of the F1 sequence (Bradbury and Rattle, 1972, Jones et al, 1974), but

this does not preclude ADPribosylation of the known phosphorylation sites, since only the more acidic peptides were separated by the methods used.

The analyses of these peptides are not typical of histone F1, which contains few acidic amino acid residues. However, this cannot be taken as evidence that the peptides are not derived from a subfraction of histone F1, since few have been even partially sequenced.

Discussion

Why is it that covalently-bound ADPribose is the only nucleotide found in these preparations of histone F1, when others have found quantities of assorted nucleotide material? Partly, the answer is, that loose nucleotides will become attached to DEAE Cellulose when the non-histone proteins are removed, but as observed in Chapter 2 no significant nucleotide peak is removed from this resin. The main reason probably lies in the method of preparation. Both salt and acid solubilise nucleotides from the nucleus, and it is important to precipitate the histone while leaving the nucleotides in solution. Now, it is most likely that a nucleotide small enough to be soluble in 5% PCA is also going to be soluble in 25% TCA, with which the protein is precipitated, while the high concentration of acetone or ethanol (95 and 100%) required to precipitate the histone are likely to bring down oligonucleotides as well: lyophilising the solution ensures the retention of all small nucleotides.

As mentioned in the general introduction, the literature regarding well attested nucleotide-protein bonds is somewhat sparse, but a certain amount of information is available concerning chemically synthesised bonds between nucleotide and peptide units (reviewed by Shabarova, 1970). In particular, serine-nucleotide links have been synthesised via the 5' phosphate of UMP to the amino, and hydroxyl groups of serine, and their properties investigated. The phosphodiester linkage is alkali labile, unless the serine amino group is free, in which case the phosphate, and its attached nucleoside, migrate to form the more alkali stable phosphoamide bond (Yuodka et al, 1968). The phosphoamide bond is cleaved by neutral hydroxylamine, while the phosphodiester bond is hydroxylamine stable (Berkowitz and Bendich, 1965, Yuodka et al, 1969). The phosphoester derivative is fairly acid stable, but breaks down slowly in N HCl at 37°C to give the nucleoside and O-phospho-serine. It should be noted that the phosphoamide derivative, which is more acid labile, also yields O-phospho-serine as a hydrolysis product. Acid hydrolysis is much reduced in the absence of an adjacent -OH or -COOH group, as for instance if the ribosyl 2'OH is methylated.

It will be noted that the structure tentatively proposed in Fig. 3:4 has the phosphoserine joined to ADPribose via the ribosyl 1'OH. The ribose-1-phosphate bond is extremely acid labile, being hydrolysed by 0.3N HCl at 30°C with a half life of 1 minute (Kalckar, 1945). When the 5'OH is also phosphorylated, as in ribose 1,5 diphosphate, the half life of the bond in acid is doubled (Klenow, 1953). Thus the

proposed molecule might be sufficiently stable to survive PCA extraction, while still being very acid labile. It would be alkali labile, but stable to hydroxylamine, as found. Reports of hydroxylamine lability of some of the protein bound ADPribose of the nucleus (Nishizuka et al, 1969, Ueda et al, 1972, Dietrich et al, 1973, Dietrich and Siebert, 1973) may imply that some of the material is located close enough to a free amino group (e.g. ε lys) such that migration may occur to form a hydroxylamine labile phosphoamide bond, for proteins other than F1.

It is now possible to reconcile the results in Table 3:2a, which indicate that ADPribose is present as a short oligomer with a phosphorus to ribose ratio of roughly 1 : 1, with those of Table 3:2b which show that the majority of the material, being present as the monomer has a phosphorus to ribose ratio of 3:2. On separation of the nucleotide bearing fraction of histone digest on Dowex 50, some of the seryl-nucleotide collected will be hydrolysed at the 1'OH of the ribose by 50mM HCl to give free ADPribose and O-phospho-serine. The latter will not be retained by the Dowex-1-formate when the nucleotide is reconcentrated, so the 3M formic acid eluate will contain a mixture of free ADPribose and phosphoseryl-ADPribose. If the serine and phosphate are added back to give a serine : adenine ratio of 1 : 1, the original content of the nucleotide fraction becomes serine 0.89, ribose 1.73, phosphate 2.50, adenine 0.89, and the ribose phosphate ratio is 2 : 2.89, in close agreement with the expected figure.

CHAPTER 4

INTRODUCTION

Having identified ADPribose covalently bound to histone F1, it is now appropriate to see whether other protein acceptors are to be found. This question may be resolved into three parts:

(i) With which nuclear extracts is ADPribose associated?

(ii) Is it free, or covalently bound to protein?

(iii) If the ADPribose is covalently bound, is the acceptor a protein typical of the fraction, or has it been extracted only because it is non-covalently complexed, or aggregated with those proteins.

The first part of the question is readily answered. Everyone who has labelled nuclei with [^{3}H]Adenine - NAD (Nishizuka et al, 1968, Otake et al, 1969, Nishizuka et al, 1969, Burzio and Koide, 1971, Ueda et al, 1972, Koide and Burzio, 1972, Dietrich and Siebert, 1974) has found ADPribose in every nuclear extract investigated.

That a proportion of this material is covalently bound is indicated by its tenacious binding to the protein (Nishizuka et al, 1968), by the effect of trypsin (Ueda et al, 1972), and by the liberating effect of hydroxylamine (Nishizuka et al, 1969, Dietrich and Siebert, 1973). The proportion of bound material is of the order of 80% (Shall, 1973).

A number of attempts have been made at purification of the acceptor protein(s). Material absorbing at 260nm has been noted in association with histone F3 (Hnilica and Bess, 1965)

Table 4:1

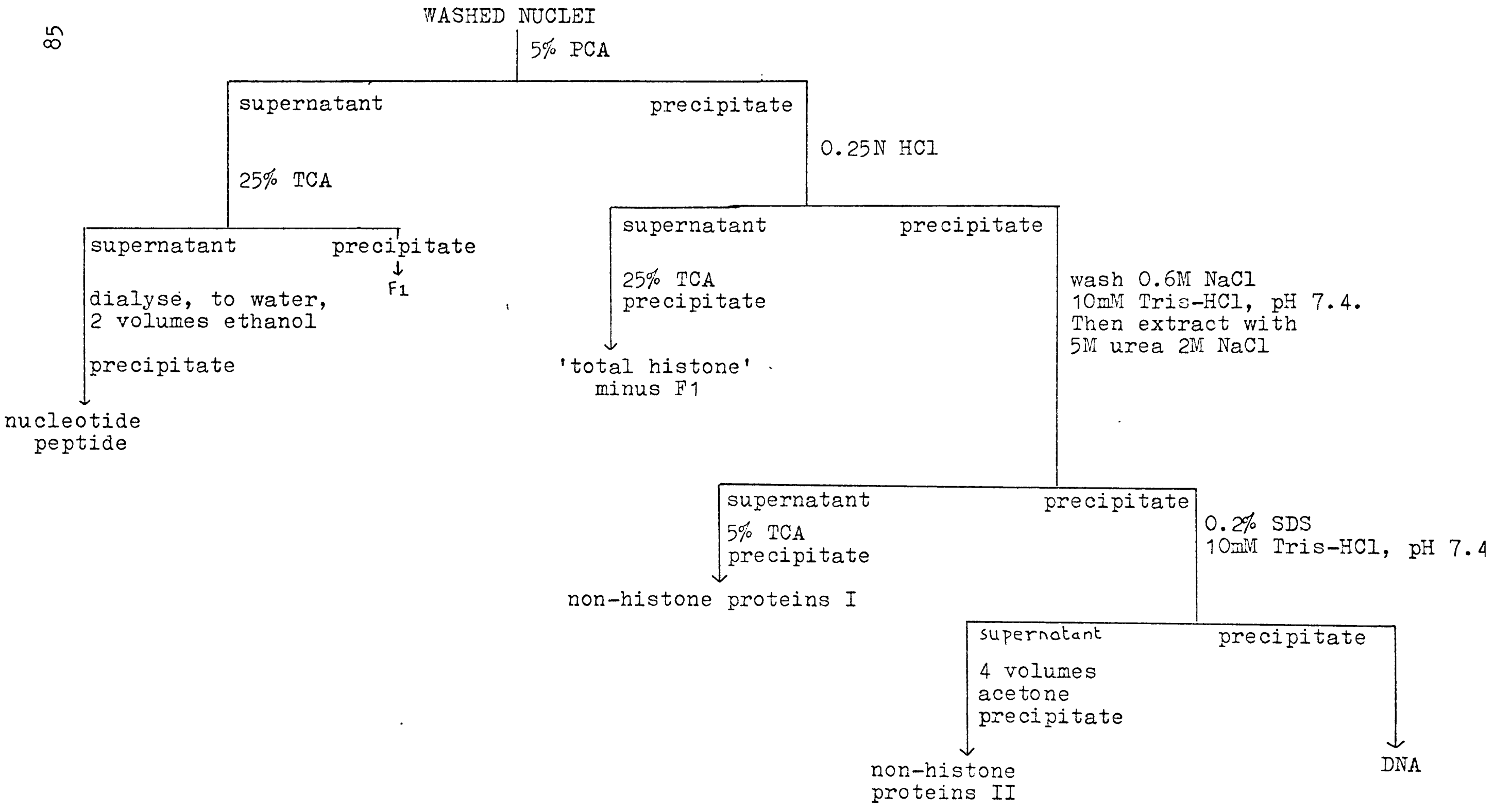

WASHED NUCLEI
5% PCA
supernatant
precipitate
0.25N HCl
25% TCA
supernatant
precipitate
F1
dialyse, to water,
2 volumes ethanol
precipitate
nucleotide
peptide
supernatant
precipitate
25% TCA
precipitate
'total histone'
minus F1
wash 0.6M NaCl
10mM Tris-HCl, pH 7.4.
Then extract with
5M urea 2M NaCl
supernatant
precipitate
0.2% SDS
10mM Tris-HCl, pH 7.4
5% TCA
precipitate
non-histone proteins I
supernatant
precipitate
4 volumes
acetone
precipitate
non-histone
proteins II
DNA

Table 4:2 (a)

Ascites Cells Labelled in vivo with ^{32}P

Fraction	Protein (mgs)	^{32}P cpm	of which ADPribose
'F1'	4.62	49,700	6,400
'Histones minus F1'	26.5	44,700	3,100
'Non-histones' (I)	33.7	46,500	7,400
'Nucleotide peptide'	0.14	105,000	104,000

(a) 8 day ascites hepatoma cells, labelled for 17 hours - rats injected with [^{32}P] orthophosphate (1mCi/Kg body weight). Cells from 3 rats were used.

(b) Rat Liver Nuclei Labelled in vitro with [^{3}H] Ade NAD

	cpm	Protein (mgs)
'F1'	170,000	2.1
'Histones minus F1'	74,360	9.0
'Non-histones' (I + II)	69,450	15.6
'Nucleotide peptide'	31,000	0.08

(b) Nuclei from livers of 6 rats were incubated with 25μCi [^{3}H] Ade NAD for 30 minutes at 37°C.

as has [^{14}C]Adenine radioactivity (Ord and Stocken, 1967). Nishizuka et al, (1968) and Otake et al, (1969) separating labelled histones on CM Cellulose, found radioactivity in all three fractions. Separation of histones on Amberlite CG50, caused a proportion of the ADPribose to elute with the run-off protein, as well as with the histone fractions (Koide and Burzio, 1972). On separation of the histones on CM Cellulose with ethanolic formate, the ADPribose containing material was separated from the bulk of the histones, and a phosphodiesterase and alkaline phosphatase digest of this material yielded a mixture of all the histones (Ueda et al, 1972). The evidence, then, is that the ADPribose-bound protein is complexed with the histones, but is insufficient to show whether the bound protein itself is histone.

Results

In starting the search for ADPribosylated proteins in vivo, it is first necessary to check that there is a significant amount of ADPribose present, and that it is not simply an artefact produced in incubating nuclei. 8-day ascites hepatoma cells were used, initially, to allow labelling with a high specific activity. 17 hour incubations with [^{32}P] orthophosphate were used. The nuclei were extracted according to the scheme in 'Table' 4:1. It appears to be important to extract with 5% (w/v) perchloric acid first, since this precipitates the nucleic acids, which do not subsequently interfere with the extractions until SDS is used. The yields of protein and ^{32}P radioactivity are shown in Table 4:2. The distribution of ^{32}P radioactivity between phosphoserine and ADPribose was determined by separation from

Fig. 4:1

Densitometer scans of polyacrylamide gels, run at pH 8.9 in the presence of SDS

a) Total histone minus F1.

b) 5M urea, 2M NaCl extract.

c) 0.2% SDS extract.

The amount of DNA from which the protein loaded is derived is a) 0.5mgs, b) 0.67mgs, c) 2mgs.

The gels are run from left to right, and the Bromophenol Blue marker ran 6cm.

Fig 4:1

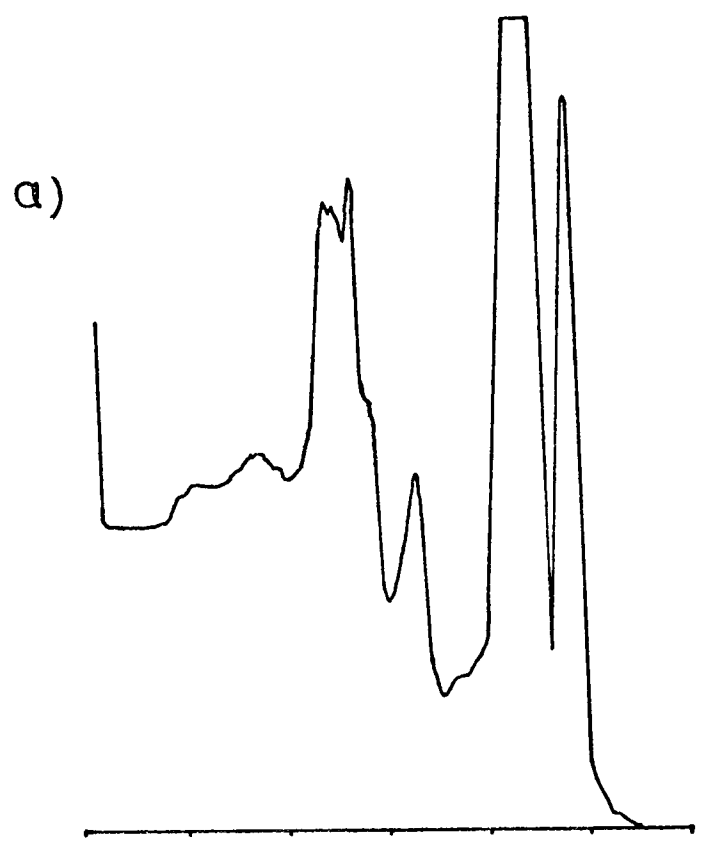


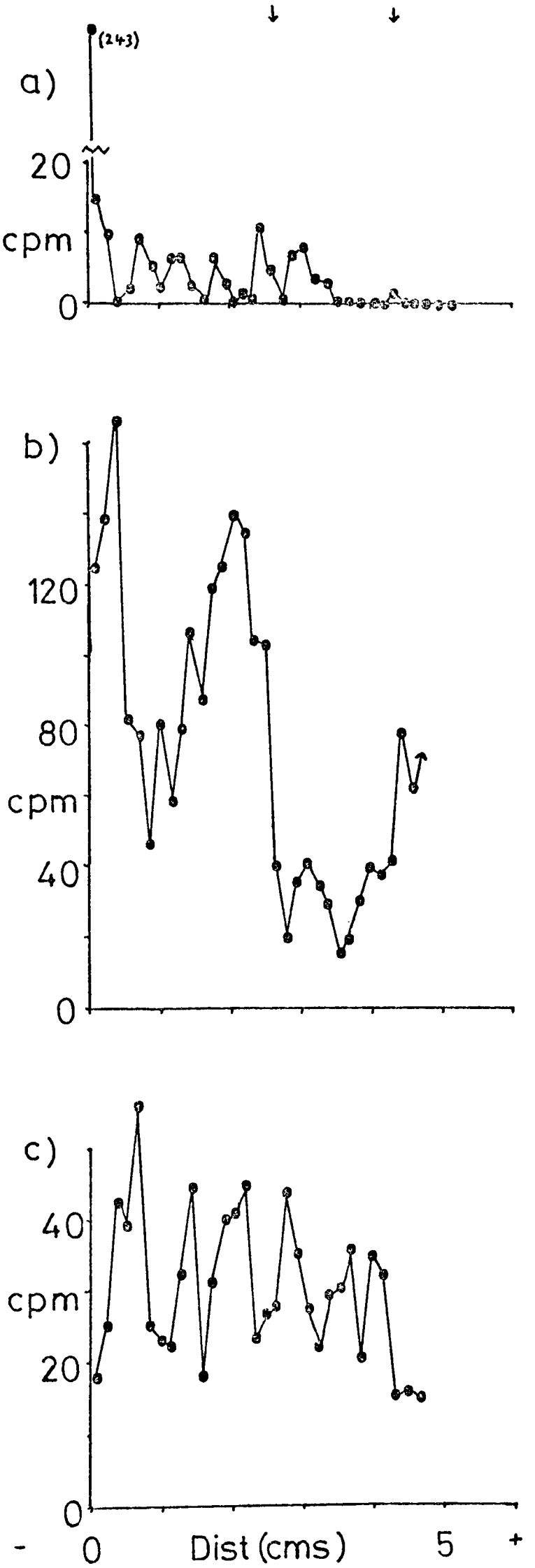
Fig. 4:2

^{32}P Radioactivity associated with the proteins of Fig. 4:1

cpm = cpm per 1½mm slice.

In vivo labelling with [^{32}P] orthophosphate was performed for 18hrs at a dose of 500μCi per rat (2mCi/kg).

Fig 4:2



an alkaline digest of the protein on a Dowex-1-formate column, eluting with a formic acid gradient as before, (Chapter 3). ADPribose was identified in all fractions investigated, including the wash with 0.6M NaCl, while RNA bases were essentially absent. So much RNA was present in the SDS extract however, that it was not examined (since UMP elutes with ADPribose).

The figures obtained by incubating rat liver nuclei with [^{3}H]Adenosine NAD are shown for comparison. Over 80% of the ^{3}H radioactivity in the HCl extract separates with F2b if the procedure of Oliver et al, (1972) is used: the non-histone proteins are also found in this fraction.

Having confirmed the presence of the ADPribose, the next problem is to separate the histones cleanly from the non-histone proteins. These experiments were performed with rat liver nuclei, labelled with ^{32}P <u>in vivo</u>. The proteins from 0.25N HCl, 5M urea/2M NaCl, and 0.2% SDS extracts were analysed by polyacrylamide gel electrophoresis in the presence of SDS, at pH 8.9, (Fig. 4:1). The supposed non-histone fractions contain low molecular weight bands, running with the histones, while the histone fraction contains a heterogeneous collection of proteins of higher molecular weight, although these are not too significant from a quantitative point of view. The ^{32}P radioactivity of the histone fraction, on the other hand is all associated with the non-histone proteins, except where it remains at the top of the gel, (Fig. 4:2a). The non-histone proteins, although present in smaller quantities evidently have some members which are highly phosphorylated (Fig. 4:2b,c).

Table 4:3

Separation of Proteins on Biorex 70

Extract	Fraction		% cpm in histone fraction
	'Histone'	'Non-histone'	
'0.25N HCl'	F1, F2a1, F2a2 F2b, F3	non-histones only	84
'5M urea 2M NaCl'	F1, F2b	none	22
0.2% SDS	F1, F2a1, F2a2 F2b, F3	non-histones only	6

If the proteins are now separated into histones and non-histones on Biorex 70, (applying the protein in 5M urea, 50mM NaCl, 10mM Tris-HCl pH 7.4, thereby retaining only the histones, which are eluted by raising the salt concentration to 2M; after Richter and Sekeris, (1972)), and the histones identified by their mobility on polyacrylamide gel electrophoresis in the presence of urea and acetic acid, it is found that a complete separation is obtained (Table 4:3). It should be noted that while the major forward band of the urea-salt extract (Fig. 4:2b) is histone F2b, as identified on the urea-acetic acid gels, only a small proportion of the SDS low molecular weight proteins are histones - they are still present on SDS gels after removal of the histones with Biorex 70, and have an acidic amino acid analysis (cf Elgin and Bonner, 1970). The highly labelled non-histone proteins from the urea-salt extract do not seem to run when electrophoresed on urea/acetic acid gels. In all cases, the ADPribose is associated with the non-histone fraction.

The gels shown in Fig. 4:1 were run with protein that had not been precipitated, but had simply been dialysed against gel sample solution, with a view to minimising the likelihood of aggregation. In consequence, there are many phosphate counts in the low molecular weight region of the gel of the urea-salt extract:- these are due to the phospholipid which is present in this extract, and which is normally lost on precipitation of the protein with TCA.

It may be stated parenthetically, that phospholipids are found in small quantities in all extracts except 5% PCA extracts even after precipitation with TCA. They are extractable by

Fig. 4:3

Sephadex G75 chromatography of in vivo ^{32}P labelled total histone minus F1

15mg protein, labelled for 18hrs with ^{32}P *in vivo*, chromatographed in 50mM Tris-HCl, pH 7.4.

Column dimensions: 35cm x $10cm^2$.

Flow rate: 20mls/hr.

Breakthrough peak contains 26% OD_{280} absorbing material.

Fig 4:3

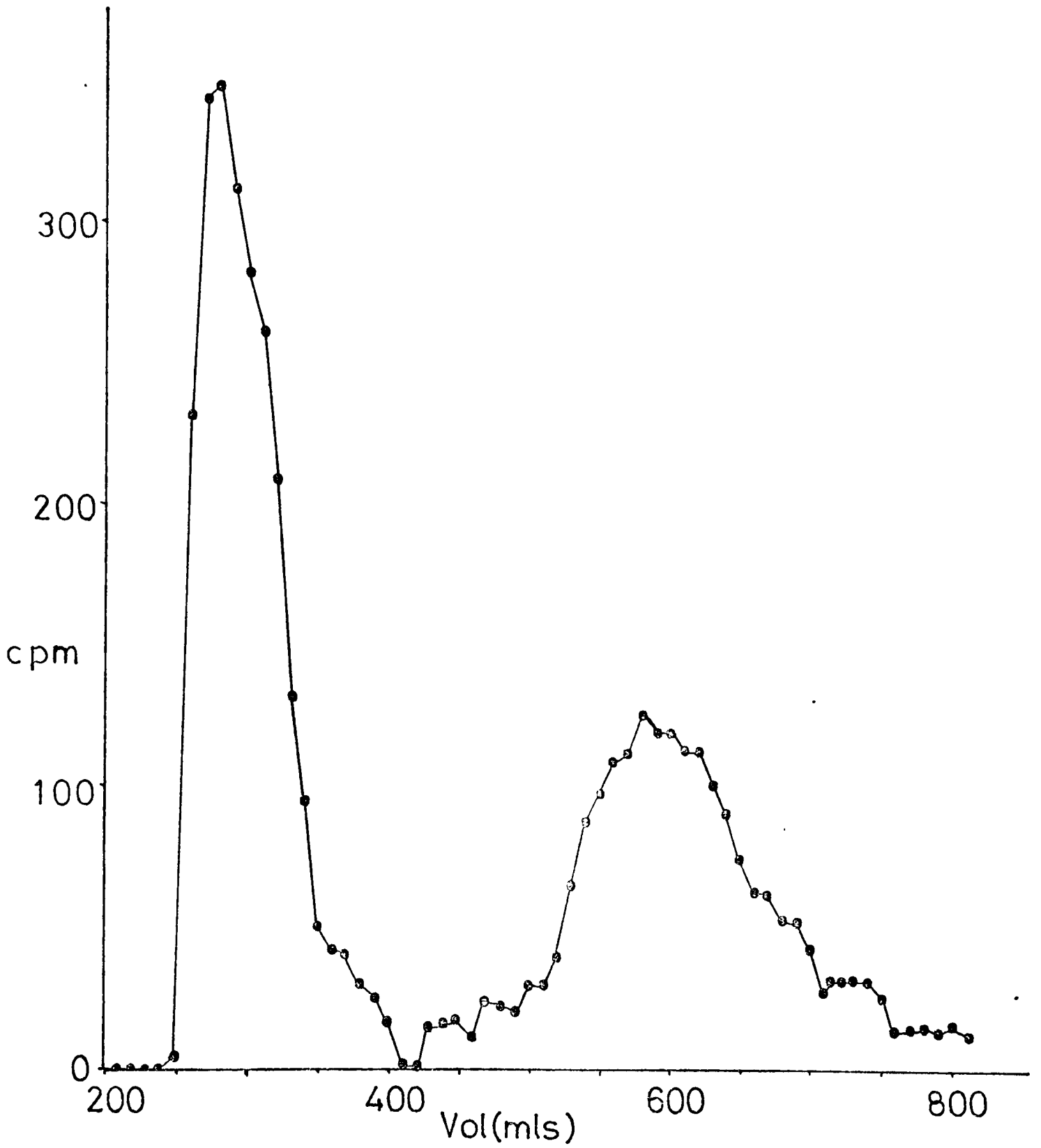


Table 4:4 (a)

Amino Acid Analyses of Possible ADPribose Acceptors

	A.	B.	C.	D.	E.	F.
Asx	9.11	6.75	6.21	5.74	7.97	8.38
Thr	5.04	4.81	4.52	5.34	3.89	1.65
Ser	7.77	5.67	18.40	20.94	22.80	15.46
Glx	10.31	10.17	14.29	15.22	13.84	17.12
Pro	5.94	4.88	3.23	2.81	2.36	2.89
Gly	11.54	9.89	18.12	16.52	19.71	10.91
Ala	10.21	11.57	9.30	9.18	10.41	9.50
Cys	0.41	0.22	-	-	-	trace
Val	5.42	5.19	3.78	3.79	0.52	2.85
Met	0.30	0.83	0.61	0.75	0.78	1.04
Ile	4.03	3.96	2.46	2.25	1.52	2.02
Leu	7.75	8.43	3.93	3.42	2.35	2.63
Tyr	2.26	1.77	1.59	1.55	0.52	1.00
Phe	2.76	1.91	1.72	1.64	1.10	2.79
His	2.35	2.89	3.62	3.93	4.04	7.15*
Lys	7.52	12.58	4.60+	4.50++	1.80+++	11.97
Arg	7.24	8.89	3.62	2.42	1.86	2.67

+ Also an artefact equivalent to 6.13 which may be derived from nucleotide.

++ Also an artefact equivalent to 3.15 " " " " " "

+++ Also an artefact equivalent to 4.57 " " " " " "

* Skewed peak, probably containing both histidine and artefact.

A. Total histone excluded from Sephadex G200 in 50mM Tris-HCl buffer, pH 7.4.

B. Total histone excluded from polyacrylamide gel.

C. Total histone excluded from Sephadex G200 in 8M urea, 50mM Tris-HCl buffer, pH 7.4. - 'Histone' on Biorex 70.

D. Total histone excluded from Sephadex G200 in 8M urea, 50mM Tris-HCl buffer, pH 7.4. - 'Non-histone' on Biorex 70.

E. Aggregating protein.

F. P1-associated material.

Table 4:4 (b)

ADPribose Acceptors

	F1a	Nucleotide-peptide from Sephadex G75	Nucleotide-peptide from gel band
Asx	2.31	12.06	13.05
Thr	5.79	4.40	4.37
Ser	7.41	9.77	16.30
Glx	3.46	15.33	14.74
Pro	6.80	0	1.58
Gly	6.66	18.11	24.17
Ala	24.65	9.76	8.45
Cys	0	0	0
Val	4.22	6.64	3.87
Met	0	0	0
Ile	1.29	2.49	2.38
Leu	4.01	4.93	4.44
Tyr	0.58	0.93	0
Phe	0.47	1.43	0
His	0.34	6.38	2.24
Lys	29.72	5.34	3.87
Arg	2.20	2.44	0.67

chloroform: methanol (2:1) and if separated by thin layer chromatography by the method of Skipski et al, (1964) are found to contain phosphatidyl - inositol, - serine, - ethanolamine, and - choline.

Attempts were also made to separate the ADPribose containing proteins on Sephadex, bearing in mind that ADPribose appears to lead to aggregation of protein. A separation of aggregated protein material from the bulk of a total histone - minus - F1 fraction from in vivo ^{32}P labelled rat liver is shown in Fig. 4:3. The high molecular weight peak contains 55% of the radioactivity, but only 26% of the protein as measured by OD at 280nm. This is a maximum estimate of the protein content as the peak of uv absorbance for this fraction is at 260nm. Nucleotide is found only in the first peak. However, SDS polyacrylamide gel electrophoresis gives the same pattern as the original extract, showing that the aggregation is random. Similar results are obtained using non-histone extracts, or using 7M urea and 0.2% SDS as eluent. Where urea or SDS are used in the solvent, the distinction between the high molecular weight and the low molecular weight peaks is blurred, as material, presumably non-histone protein, is released from the aggregate, leaving material with high contents of serine, glycine and glutamate still excluded (Table 4:4a). The unwillingness of the aggregated material to disaggregate (and indeed, even to go into solution) may well be due to denaturing of proteins, particularly the non-histones on acid extraction (Graziano and Huang, 1971).

One method to overcome this problem is suggested by the fact that proteins from the aggregate will run on polyacrylamide gel electrophoresis, since they are clearly being pulled off the ADPribosylated proteins by this treatment. The material removed from the top of the gel after electrophoresis has been analysed (Table 4:4). The analysis is compared with that of material from an acid extract which was totally excluded from Sephadex G200 in the absence of urea or SDS, and which separated as non-histone on Biorex 70. The shortcoming of both preparations is that they will contain high molecular weight non-histone proteins quite unrelated to the ADPribose acceptors.

The other possibility for avoiding aggregates caused by denaturation, is to extract the non-histone proteins prior to acid extraction. This is conveniently performed by extracting first with 8M urea, 50mM Tris-HCl, pH 7.4 (cf Gronow and Griffiths, 1971). A number of problems are encountered: the chromatin partly solubilises so that the DNA/histone is difficult to precipitate rapidly, and considerable amounts of RNA are extracted with the proteins. The first problem is minimised by accepting a less than quantitative yield of protein. The DNA is fairly well precipitated by centrifugation for 1 hour at 25,000g and the top 80% of solution may be removed without disturbing the gel. On dialysing against 50mM Tris-HCl, pH 7.4, much of the protein and nucleotide material comes out of solution, but this protein readily redissolved in 0.2% SDS, 8M urea, 50mM Tris-HCl, pH 7.4, leaving any DNA as precipitate. The remaining chromatin, after washing with 8M urea, 50mM Tris-HCl, pH 7.4, is extracted with 0.25N HCl as usual. The HCl extract

was precipitated with 25% TCA, redisolved, and dialysed against 50mM Tris-HCl, pH 7.4. After fractionation on Sephadex G200, the aggregated (excluded) material was made 0.2% with SDS, 8M urea, and 1mM with βmercaptoethanol, and rechromatographed on Sephadex G200. All fractions were dialysed against water, lyophilised, and separated into histone and non-histone fractions on Biorex 70. All fractions contained protein, to a greater or lesser extent, with the amino acid analysis shown in Table 4:4a. Most of the protein is of low molecular weight but with a certain amount still excluded. RNA is found in the higher molecular weight non-histone fractions. The urea extracted non-histones gave similar analyses. If these analyses are compared with those from acid extracted material excluded from Sephadex G200 in the presence of urea or SDS (Table 4:4a) it will be seen that the latter contain mainly the same (aggregating) protein, together with small amounts of histone or non-histone protein. The high serine and glycine content is not due to the presence of nucleotide, as the analysis is the same for the non-histone fractions which are heavily contaminated with RNA. However, an artefactual peak which elutes close to lysine is observed, and this is found in RNA digests. Since a uniform analysis is found on proteins derived from aggregated material, the strong possibility must be entertained that this protein is the ADPribose acceptor. This possibility is enhanced by the observation that the ADPribose bearing material associated with P1 (Chapter 3) has a high serine and glycine content, while bands comparable to those of P1 are observed on polyacrylamide gels.

Fig. 4:4

Sephadex G75 chromatography of in vivo labelled nucleotide-peptide

Total nucleotide-peptide from 8 rats labelled with 250µCi ^{32}P each (1mCi/kg) for 90 minutes in vivo. Chromatographed in 50mM Tris-HCl, pH 7.4.

Column dimensions: 20cm x $2\frac{1}{2}cm^2$.

Flow rate: 20mls/hr.

Closed circles = cpm/3ml fraction.

Open circles = OD at 260nm.

Fig 4:4

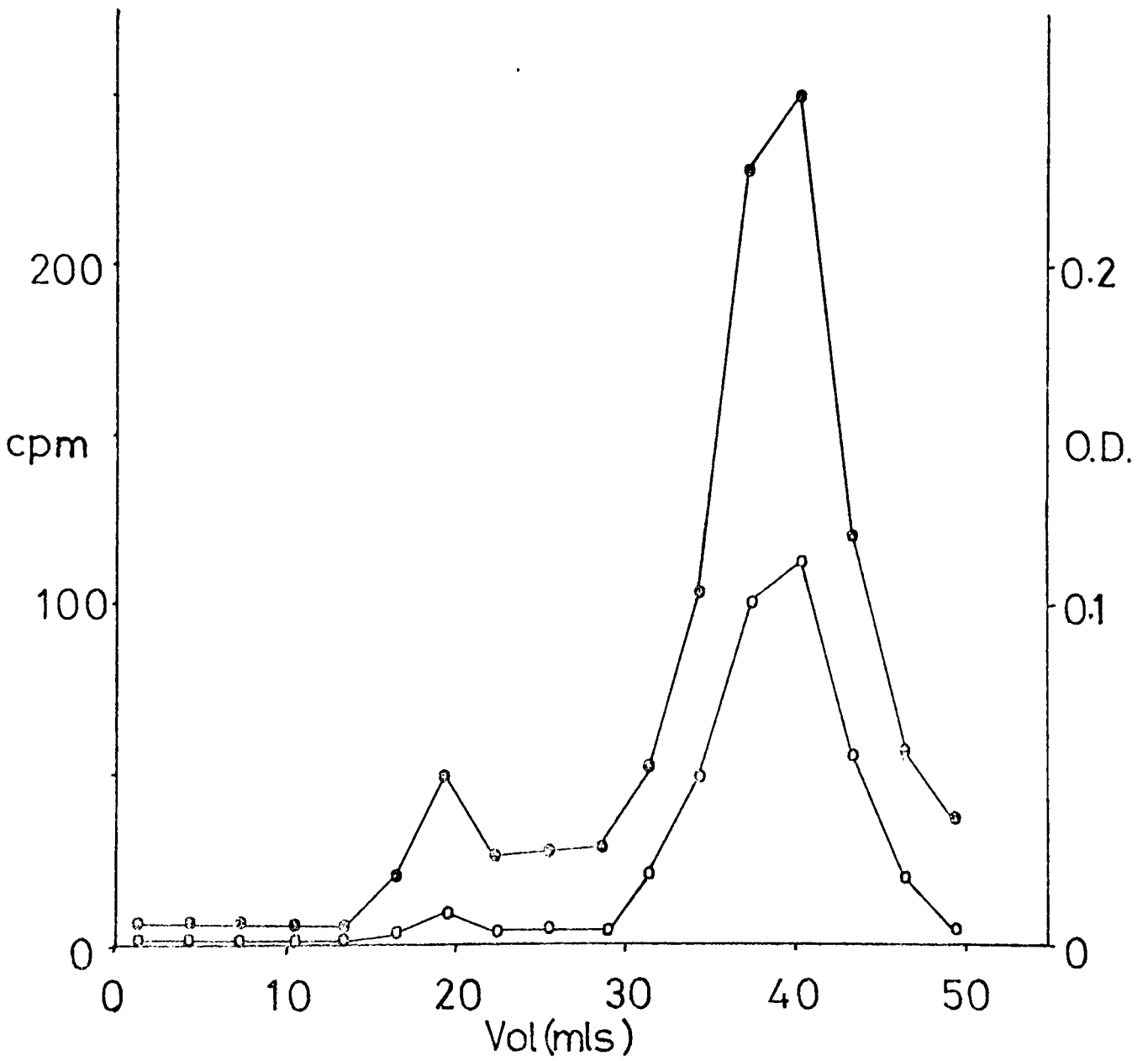


Fig. 4:5

Ultraviolet absorption spectra of ADPribose derivatives

a) Total histone minus F1, material excluded from Sephadex G200 in 7M urea 50mM Tris-HCl, pH 7.4.
Solid line = 'non-histone' on Biorex 70.
Dotted line = 'histone' on Biorex 70.

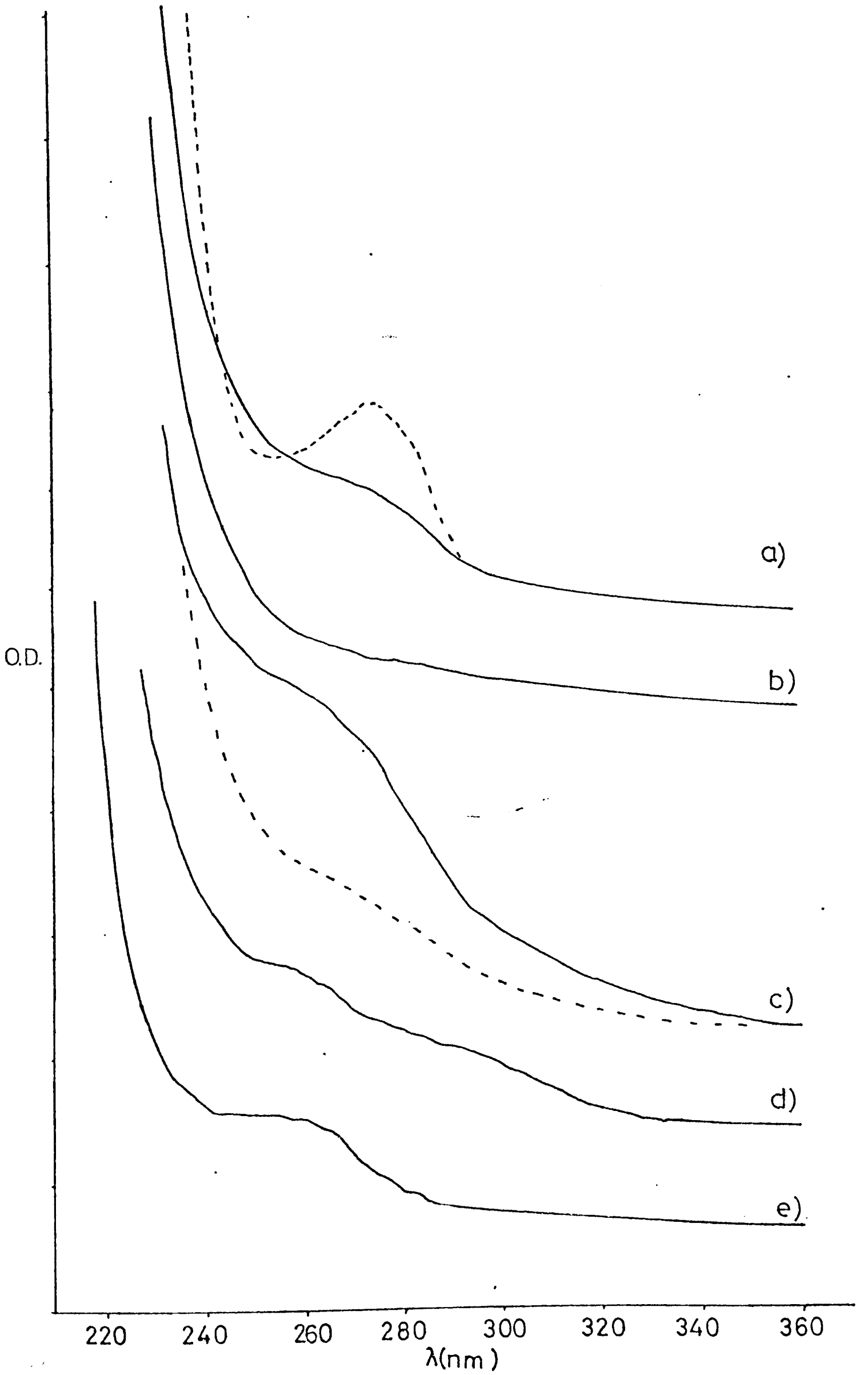
b) Total histone minus F1, extracted after removal of non-histones, excluded from Sephadex G200 in 50mM Tris-HCl, pH 7.4, included in Sephadex G200 in the presence of SDS urea.
'Histone' on Biorex 70.

c) Serine-phosphate-ADPribose, derived from F1.
(Dotted line = lower concentration of same).

d) ADPribose-peptide (15) derived from F1.

e) Nucleotide-peptide.

Fig 4:5



Ultraviolet absorption spectra of the supposed acceptor for ADPribose, together with other known cases of ADPribose bearing materials are shown in Fig. 4:5. Genuine absorption peaks at 260nm are only observed after release of the nucleotide either as ADPribose, or as adenine. The close similarity of the spectra supports the notion that the aggregating protein contains bound ADPribose. The fact that this protein can be found in the histone fraction from Biorex 70 indicates extensive amidation of the acidic amino acid residues. Such a fraction of proteins corresponds with the H3Q1 fraction of MacGillivray and Rickwood, (1973).

An ADPribose acceptor which is quite distinct from those proteins already considered, is that designated nucleotide peptide in Table 4:1. It is extracted in 5% PCA, but not precipitated with 25% TCA. It is therefore prepared by dialysing the 25% TCA supernatant from the preparation of F1 against water, and precipitating with 2 volumes ethanol (Islam and Kay, 1972). The nucleotide peptide is purified by redissolving the material in water and reprecipitating with ethanol. It is a small molecule, and is not at all excluded from Sephadex G75 (Fig. 4:4). It runs close to the Bromophenol Blue marker dye on polyacrylamide gel electrophoresis at pH 8.9, in the presence or absence of SDS. (The nucleotide peptide stains rather poorly with Naphthalene Black after gel electrophoresis). The gel band contains all the radioactivity of this fraction, whether the material was labelled with ^{32}P *in vivo* or with [^{3}H] Adenine labelled NAD *in vitro*. The amino acid analysis of nucleotide peptide as purified on Sephadex, or by gel

electrophoresis at pH 8.9 in the absence of SDS, is given in Table 4:4. Adenine as determined by OD at 260nm is present to an extent of 1 mole to approximately 25 moles amino acid.

ADPribose has been identified on Dowex-1-formate, as for F1, but a second peak is observed just behind the elution position of ADP, which accounts for 27% of the counts. Adenine has been isolated by paper chromatography of acid digests of nucleotide peptide, and is accompanied by a second uv absorbing spot which runs behind it, with an r.f. value corresponding to that of cytosine for the system of Lane, (1963) or of guanine for the isopropanol-HCl system. This is tentatively regarded as a substituted adenine base, and may account for the second peak on Dowex-1-formate. A ratio of ribose : phosphate of 1 : 1 was obtained for the ADPribose product of a pronase-leucineaminopeptidase digest, collected on Dowex-1-formate. A small amount of serine phosphate was also identified, but the quantitative studies required to establish if the nucleotide-peptide bond is the same as for F1 have not been performed.

It might be considered that quantitative studies of the phosphoserine and bound nucleotide could be performed simply by the method of Berenblum and Chain, (1937). An alkaline digest of the protein fraction would yield inorganic phosphate (from phosphoserine), and the free nucleotide. The inorganic phosphate would then be extracted with isobutanol, and if performed rapidly, would be separated before the acid present in the molybdate containing aqueous layer liberated the phosphate from the ribose-1-OH. This phosphate could then also be extracted with isobutanol, leaving the nucleotide phosphate in

the aqueous phase. Unfortunately, although possible in principle, this method is subject to two difficulties. Firstly, phosphomolybdate formed in the aqueous phase, prior to extraction with isobutanol, binds strongly to peptide material present in the digest, and forms an insoluble complex at the phase boundary. This problem is potentially soluble, in that these complexes can be filtered out from the aqueous phase. The second difficulty is that more phosphate (monitored as ^{32}P) is released from a total histone minus F1 preparation into the isobutanol phase in the absence of digestion than is the case after alkaline digestion, or indeed after protease digestion (L.A. Stocken unpublished observation). This presumably indicates that some nucleotide-phosphate is present in a chemically unstable form on the intact protein, due to the conformation of the protein, but a detailed interpretation is not yet possible.

Although neutral hydroxylamine (2M) is without effect on F1-bound ADPribose, it does liberate approximately 30% of <u>in vivo</u> incorporated ^{32}P radioactivity from high molecular weight material of a 'total histone minus F1' preparation into low molecular weight material. This is in line with the results of Dietrich and Siebert, (1973) who find 60% lability of [^{3}H] NAD labelled nuclear ADPribose to hydroxylamine, or of Nishizuka et al, (1969) who find release by hydroxylamine of total histone associated ADPribose counts which survive acid extraction and dialysis.

Discussion

It is probable that the histones other than F1 are free of covalently bound ADPribose, but bind in hydrophobic complexes to the substituted acceptor, and the difficulty of removing the histones present is a secondary effect due to denaturation of the non-histone proteins on acid extraction. The problem of aggregation is such that it becomes necessary to separate the acceptor protein from other complexed proteins under conditions in which aggregation is prevented. This is not easy, since charge differences are minimised in conditions of high ionic strength, or in the presence of ionic detergents. However, if proteins are used which have not been acid-precipitated, the aggregated material in both urea and HCl extracts does appear to be similar, and not subject to further division. While the ADPribose-protein bond has not been fully investigated, the lability to hydroxylamine argues that it is different from that to F1; this is not surprising since the bond proposed for F1-ADPribose would not survive extraction by strong acid.

The nucleotide-peptide is rather better characterised, although its purity is difficult to ascertain in view of the small quantities of material present in the original extract. Assuming one ADPribose to a molecule, a molecular weight of about 3,000 daltons may be estimated, if the amino acid content does not include serious contamination by peptide material. The molecule is very highly labelled in ascites cells, although this is not the case for resting liver, raising the possibility that it is associated with condition of growth.

There would appear, then to be at least four classes of bound ADPribose; that bound to F1, that bound to the low molecular weight peptide, the material bound to aggregating protein in a hydroxylamine labile fashion, and the material bound to aggregating protein by a hydroxylamine stable bond. For the time being, the ADPribose which is associated with P1 during most of the purification procedure will be placed in the last category.

CHAPTER 5

INTRODUCTION

One of the few ways in which a reasonably synchronous cell population may be obtained with mammalian tissue in situ is by partial hepatectomy. 70% hepatectomy, performed by the method of Higgins and Anderson, (1931) triggers, by an unknown mechanism, a period of hepatic growth, until the original weight of the liver is regained. The first cell cycle, involving parenchymal hepatocytes, is reasonably synchronous, allowing observation of the various stages of cell division.

A number of studies on the phosphorylation of histones during liver regeneration after partial hepatectomy have been performed, with variable results. Thus, for instance Guttierez-Cernosek and Hnilica, (1971) find an increase in $[^{32}P]$ phosphate incorporation in all fractions, starting within the first four hours, and continuing steadily for thirty hours, while Letnansky, (1973), relating his results to sham-operated animals, finds an early 1-2 hour peak for all fractions except F3 together with peaks for various fractions at times from 10 to 30 hours after the operation. Piantanelli et al, (1974) find an early (6 hour) peak, and a 20 hour peak for all fractions, with the first peak being less significant in older rats. In each of these cases, $[^{32}P]$ phosphate radioactivity is followed, without correction for the specific activity of the incorporated phosphorus. In studies where total phosphate was measured, no early increase was found, except for F3, which contains the acid soluble non-histones in this method of separation (Ord and Stocken, 1969). Similarly, where changes in electrophoretic

Fig. 5:1

Change in ^{32}P specific radioactivity of histone F1 after partial hepatectomy

Specific activity is cpm/mg protein, relative to ^{32}P uptake into cell.

Points are mean ± SD of 4 rats (3 at 21½ and 24½hr points).

Fig 5:1

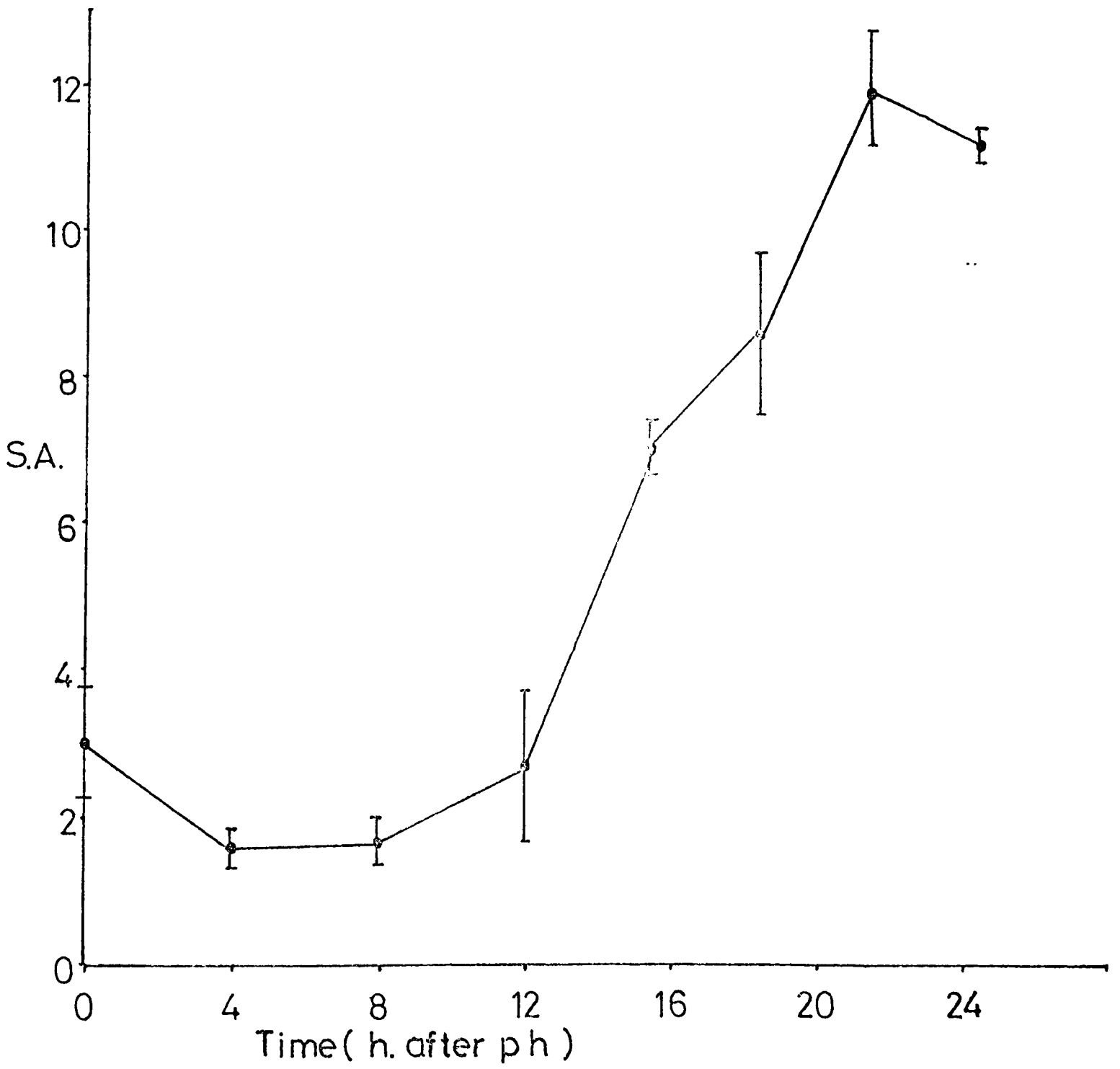


Fig. 5:2

Change in incorporation of ^{32}P radioactivity of

a) F1b.

b) F1a.

after partial hepatectomy.

Incorporation is in cpm incorporated into the fraction/mg DNA, relative to ^{32}P uptake into the cell.

c) % of incorporated cpm of histone F1 as F1b.

Fig 5:2

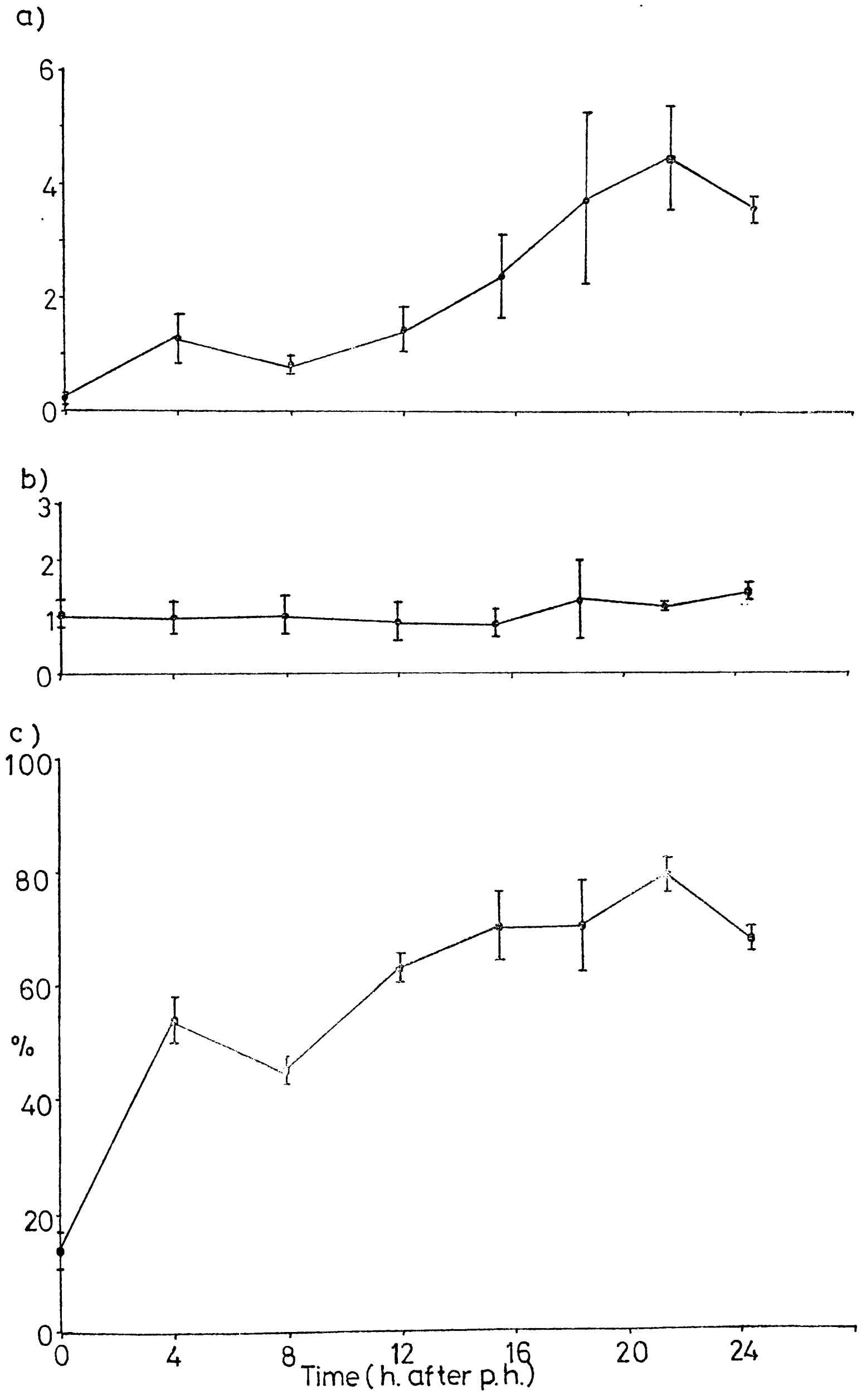


Fig. 5:3

Change in ^{32}P specific radioactivity after partial hepatectomy of

a) Total histone minus F1.

b) Non-histone proteins (extracted with 2% SDS, 7M urea).

Specific activity is on the same scale as Fig. 5:1.

Fig 5:3

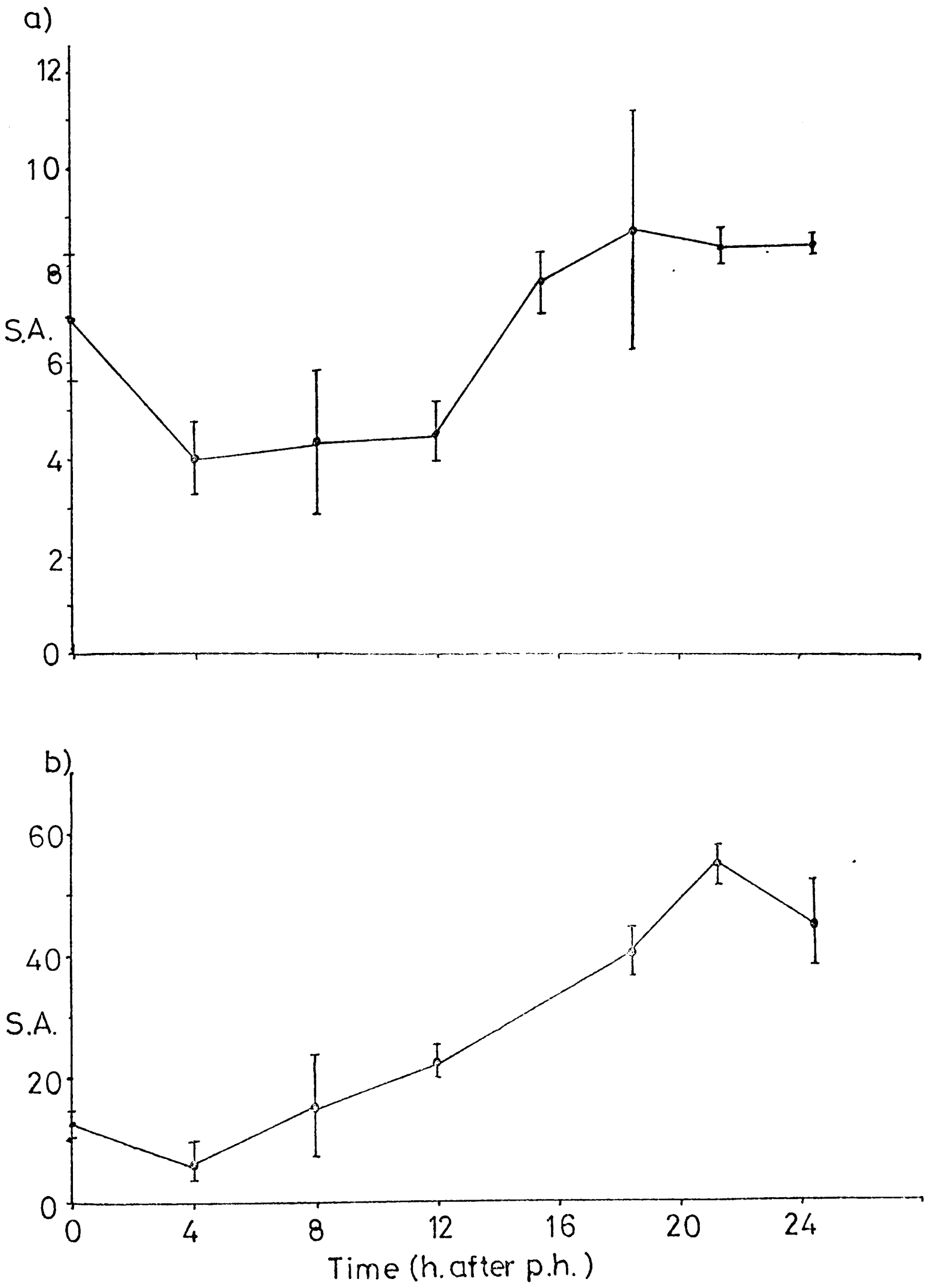


Fig. 5:4

Change in ^{32}P specific radioactivity after partial hepatectomy of

a) Nucleotide-peptide.

b) DNA.

Specific activity for a) is on the same scale as Fig. 5:1. Specific activity for b) is cpm/mg DNA relative to ^{32}P uptake into cell.

Fig 5:4

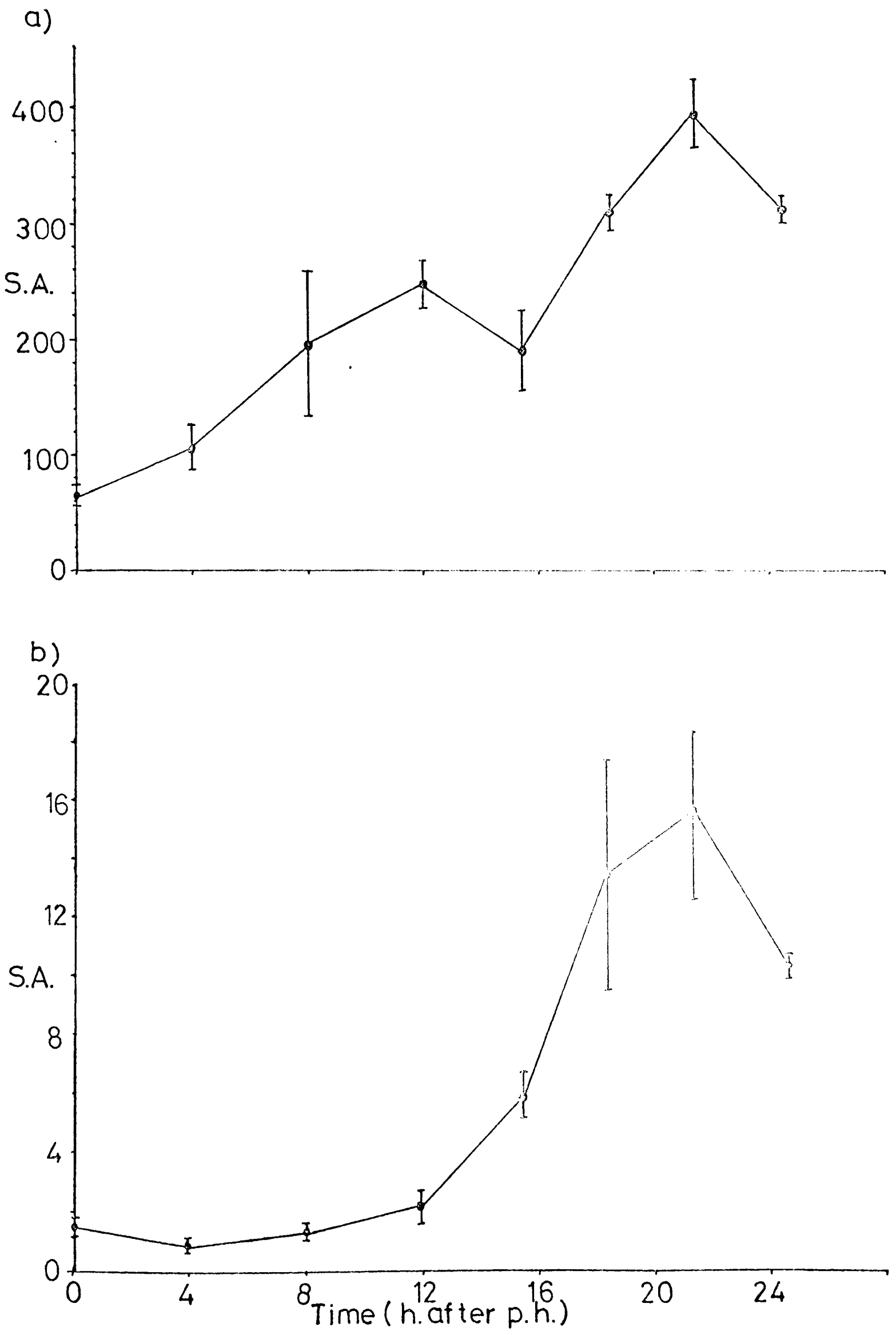

a)
400
300
S.A.
200
100
0
b)
20
16
12
S.A.
8
4
0
0
4
8
12
16
20
24
Time (h. after p.h.)

Fig. 5:5

Urea-acetic acid polyacrylamide gels of a) F1, b) total histone minus F1 from control and 21hr regenerating rat liver.

Open circles: 21hr regenerating liver.

Closed circles: resting liver.

^{32}P activity is expressed as cpm/(1½mm) slice.

Fig 5:5

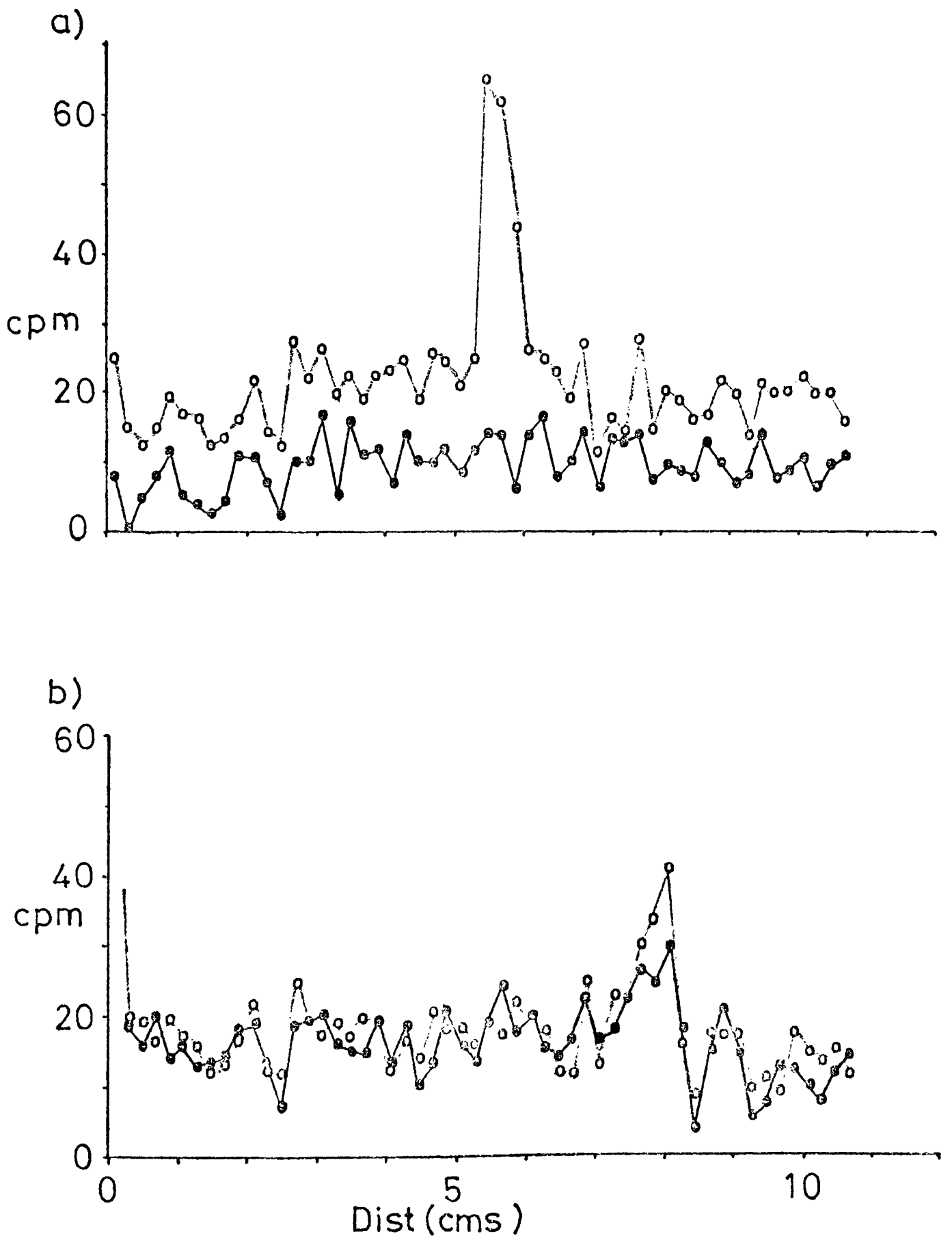


Fig. 5:6

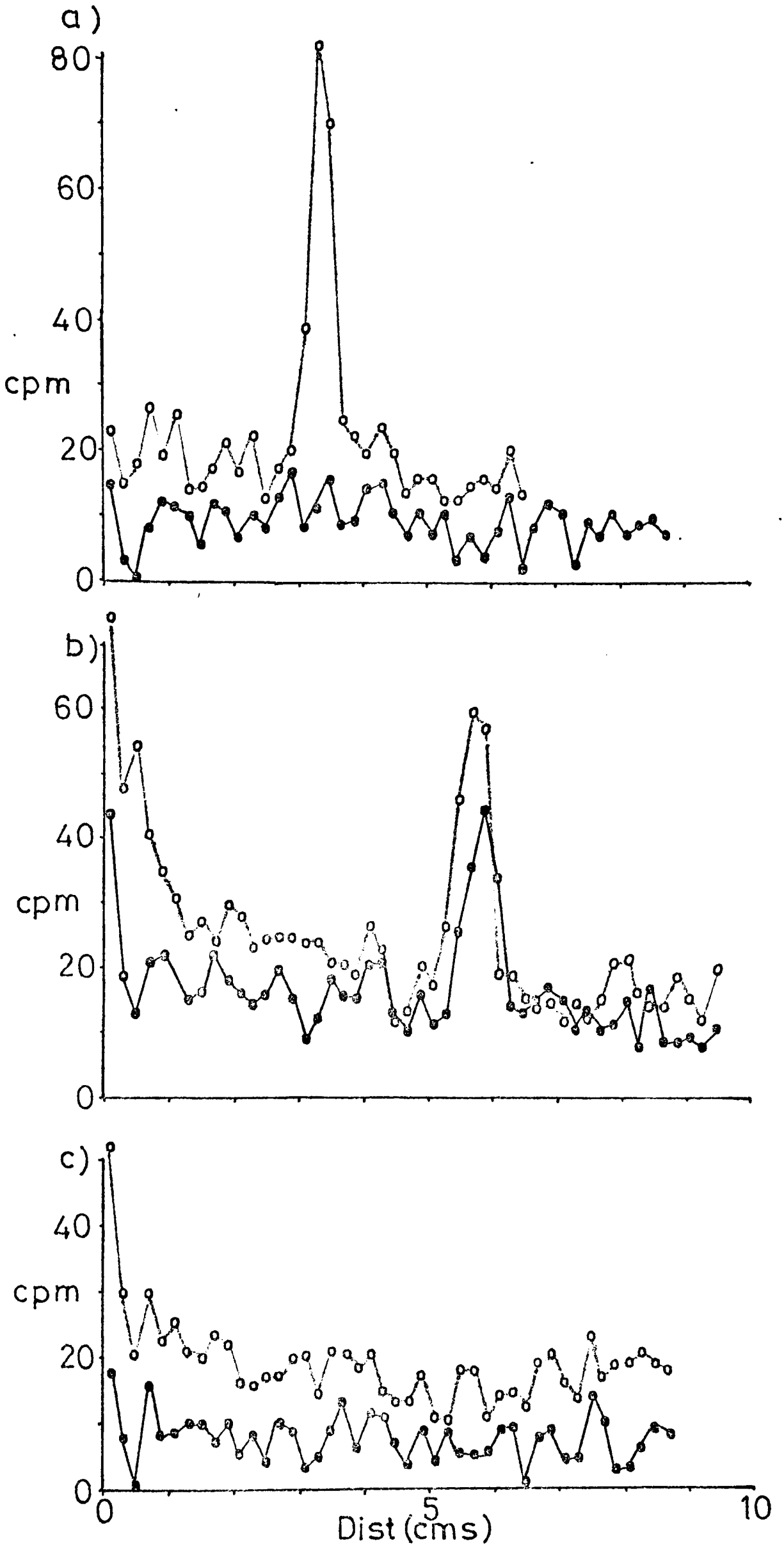
<u>SDS polyacrylamide gels (pH, 8.9) of a) F1, b) Total histone minus F1, c) non-histone protein of resting and 21hr regenerating rat liver</u>.

Open circles: 21hr regenerating liver.

Closed circles: resting liver.

^{32}P radioactivity is expressed as cpm/(1½mm) slice.

Fig 5:6



mobility of F1 due to phosphorylation is followed, no early increase is noted (Balhorn et al, 1971).

Results

The results shown in the first four figures are for 180-200g rats (4 to each point) partially hepatectomised between the hours of 10.00 a.m. and 2.00 p.m, injected with 250μCi [^{32}P] orthophosphate 90 minutes before being killed. In the case of the control (zero time) rats, the same lobes of the liver were used as remained after partial hepatectomy.

The specific activity of the various crude fractions: 'histone F1', 'total histone minus F1' and 'non-histones' shows a similar general sort of curve; an initial decline, which reflects the increased uptake of phosphate into the liver, a period without change, followed by a rise which peaks at mid S. (Fig. 5:1, 5:3a, 5:3b). The differences lie in the degree of stimulation (approximately 7-fold for F1, 2-fold for TH minus F1, 7-fold for non-histones) and the time at which the increase commences (12 hours for F1, TH minus F1, 4-8 hours for non-histones). A general picture would seem to say that the histones except F1 were simply maintaining their level of phosphorylation, while F1 and the non-histones show a definite increase in level of phosphorylation. In fact, the synthesis of histone F1 is parallel to its phosphate incorporation, so that even here there might not be an increase in phosphate content if turnover is low.

The problem of heterogeneity can be overcome to some extent by separating the proteins by polyacrylamide gel electrophoresis (Figs. 5:5, 5:6). The dose of radioactivity was here

increased to 1mCi per rat to give a sufficiently high specific activity. Certain features now became clear: firstly, the phosphate migrating with histone F1 is considerable at 21 hours, while being negligeable in the control. The increase associated with F2a2 however, is of the same order as the increase in uptake of labelled phosphate into the cell (80% higher). Differences which may be significant are observed, however, in the higher molecular weight acid soluble non-histone proteins. Possible differences may also be observed in the non-histone fraction, although the specific activity of these fractions is insufficient for certainty in these cases.

In order to obtain satisfactory results, it is clearly going to be necessary to separate out each population of phosphate molecules. This is most coveniently performed for the histone F1 fraction, by separation of the non-histone material first, and then dividing the histone itself into F1a and F1b on the Amberlite IRC50 column (Chapter 3). All the ADPribose is contained in the F1a fraction, while most of the plain phosphoserine is in F1b. These populations may now be considered separately (Fig. 5:2a,b). It will be noted that the incorporation into F1a is constant, per unit DNA, as found for ADPribose polymerase by Hilz and Kittler, (1971). The curve for F1b, on the other hand, shows an early rise, with a final stimulation of incorporation in the order of 30-fold. What is notable is the enormous change in the pattern of incorporation into F1a and F1b in regenerating as compared to normal rat liver. A change from 15% of the radioactivity finding its way into F1b in resting liver, to 50% by 4 hours,

Table 5:1

^{32}P and ^{3}H Uptakes into Rat Liver Histones

0-2 and 18 hours Post Hepatectomy

^{3}H as cpm/μmole lysine, relative to plasma [^{3}H] lysine x 10^{3}

^{32}P as cpm/mg protein, relative to plasma ^{32}P

	Hours Post Hepatectomy		
	0-2	18	
Histone	^{32}P	^{32}P	^{3}H
F1a	6.5	3.8	4.7
F1b	0.6	26.1	7.4
F2b	0	1.4	0
F2a2	8.4	24.5	7.3
F3	0	0	20.6
F2a1	(+)	2.4	13.6

is observed (Fig. 5:2c), and much of the F1a protein is now found in the F1b fraction. Thus the phosphorylation of histone F1, besides loosening its binding to DNA would also appear to reduce the tendency to aggregate around ADPribose-bearing F1. The pattern for F1 associated non-histones is similar to that for F1a, save for an increase at 18 hours. The figures are too low for an accurate assessment to be made. The time course of incorporation for the other separable ADPribose acceptor - the nucleotide-peptide, is quite different from that of F1a, showing an early increase which continues until mid S (Fig. 5:4a).

The remaining histones are also separable; if the arginine-rich and lysine-rich histones are extracted according to Johns, (1964), F2a1, F3 and F2a2 can be separated on Biogel P10 (Sung et al, 1971) if reducing conditions are used. The differences found between 2 hour and 18 hour regenerating liver are shown in Table 5:1. F3 is notable in incorporating no phosphate at all, while F2a2 has a high turnover, even in resting liver. Both F2a1 and F2b show phosphorylation in the regenerating liver which is absent in resting liver, or at early times. Changes in chromatin structure associated with S might be expected to involve these fractions, together with F1b. The differences in lysine activity reflect different time courses of synthesis in the fractions, (Ord and Stocken, unpublished). Thus, a complicated pattern emerges, from the apparently simple overall picture. This situation might be expected to apply to an even greater degree for the non-histone proteins, and a meaningful result awaits their separation.

Table 5:2

a) Specific Radioactivities (cpm/μg protein) of Phosphate in PCA Soluble Proteins from Normal and 21hr Regenerating Rat Liver

Fraction	Phosphoserine	ADPribose	
F1	0.20	0.31	normal
	2.40	0.23	regenerating
'P1'	0.00	5.50	normal
	9.75	3.25	regenerating

Rats were injected ip with 250μCi ^{32}P 17hrs before being killed. The ADPribose was separated (after alkaline hydrolysis) on Dowex-1-formate.

b) Phosphate Contents of PCA Soluble Proteins

Fraction	Phosphoserine	ADPribose	
F1	4.5	10.6	normal
	12.3	12.3	regenerating
'P1'	0.0	51.0	normal
	86.4	59.7	regenerating

Phosphate derived from phosphoserine by alkaline hydrolysis was separated into isobutanol by the method of Berenblum and Chain, (1937), allowing only 15 seconds in the acid phase prior to separation.

Having noted an increase in incorporation of radiophosphate in certain fractions, it is next necessary to discover whether this represents a genuine increase in the amount of phosphate or merely increased turnover. One way in which this question can be approached is by labelling *in vivo* for a sufficiently long period for the label to reach an equilibrium with the unlabelled material. This has been attempted by injecting ^{32}P *in vivo* 17 hours before killing. The results obtained for the PCA soluble proteins are shown in Table 5:2a. Large increases are observed in the phosphoserine levels for both F1 and the associated non-histones, while a slight decline in ADPribose levels is found in each. The decline in nucleotide specific activity probably reflects the presence of new, as yet unsubstituted protein, rather than an actual loss of material. For the other histones, the specific activity of the F2a histones as separated by the method of Johns, (1964) remains constant (normal:- 0.42, regenerating:- 0.39), while the F2b fraction, which also contains the acid soluble non-histone proteins contains 80% of the radioactivity in the regenerating liver, compared to 27% at zero time, which is an 8-fold increase in specific activity.

The alternative approach is to measure phosphate directly. Table 5:2b shows the results for the PCA soluble proteins. Phosphate which is present as phosphoserine is converted into inorganic phosphate by alkali, and on acidification forms phosphomolybdic acid with molybdate. The phosphomolybdic acid can then be extracted into isobutanol, and the phosphate

estimated (Berenblum and Chain, 1937). However, the ribose - 1-phosphate bond, postulated (in Chapter 3) to join the ADPribose to histone F1 is extremely acid labile, and to avoid this phosphate also being extracted the separation must be performed rapidly. Phosphate associated with protein material precipitated at the interface has been considered as phosphoserine derived. Given the reservations regarding this method, the results of Table 5:2b are in borad agreement with those of Table 5:2a. If F1b is separated, and its phosphate content measured, it is found to increase from 2.4nM/mg in normal to 28.8nM/mg in 18 hour regenerating liver, while the F1a content remains steady at a level of 15nM/mg ($\pm$ 5nM/mg depending on the period of extraction, and subsequent treatment). Ribose estimations are unreliable in the presence of protein, the characteristic green product of the orcinol being sometimes obliterated by a brown colour. Where this has not occurred, the figures show a slight decline in the F1a, and an increase (from 2.7 to 4.8nM/mg) in the total histone minus F1 extract.

The pattern of phosphorylation of nuclear proteins during liver regeneration can be summarised as follows: by 4 hours, two changes have been observed, an increase in the activity of nucleotide-peptide, and a change of much histone F1a into F1b. During S, increased levels of phosphate are found in F1b, F2a1 and F2b, and the activity of the nucleotide-peptide is also increased. F2a2 increases its rate of phosphorylation to maintain constant levels of phosphate, which would otherwise be depleted due to synthesis of unsubstituted

histone. Incorporation into F1a remains constant, but the ADPribose levels associated with acid extracted proteins is increased. A large increase in phosphate is found in the acid extracted non-histone proteins. A general increase of phosphate into the non-histone proteins occurs but its distribution is as yet obscure.

Discussion

The part of the cell cycle which has been followed in this Chapter contains the change from Go to G1, and on as far as late S. Two sorts of change might be expected to take place within the nucleus during this period; that concerned with the transition from Go to G1, and that concerned with S.

The early changes, as far as they are reflected in nuclear phosphate metabolism, are few. One is a rapid increase in the rate of [^{32}P] phosphate uptake into the cell, which increases 60% within the first 4 hours, and is maintained at a level about 80% higher than normal for the remainder of the period studied. Another is the increase in incorporation into nucleotide-peptide, which is also noticeable by 4 hours. Finally, there is an 8-fold increase in the incorporation of phosphate into F1b, into which fraction protein is released from F1a. It is possible that the increase in F1 phosphorylation is caused by the wave of cAMP production which occurs from 2-4 hours after partial hepatectomy (MacManus et al, 1972). It so, the phosphorylation is likely to be qualitatively different from that found during S, and may serve the purpose of preventing aggregation of F1 about the ADPribosylated histone, as is found *in vitro*. Given a model of chromatin

in which F1 is a cross-linking protein which is extra to the basic chromatin fibre, an aggregation of F1 about the ADPribose derivative would cause a drawing together of fibres into a rather insoluble aggregate in the Go nucleus, which would be loosened by the early phosphorylation of F1.

Of the later phosphorylations, those of F2a1 and F2b are likely to be directly concerned with the synthesis of new DNA, probably with the laying down of new histone on the newly synthesised DNA as suggested by Dixon et al, (1973). The phosphorylation of F1b might have a similar function; this would not rule out a different phosphorylation effect at mitosis as postulated in Physarum by Bradbury et al, (1974). It is as well to observe at this point, that while, for instance, Balhorn et al, (1971) find a peak of F1 phosphorylation at mitosis (29 hours), in Fig. 5:2a a drop is noted at 24½ hours, compared with 21½ hours. This drop is also seen from the individual points on the graph of Balhorn et al, although it is not large, and the suggestion would be that the location of the phosphate at mitosis is different from that at S.

The early rise in incorporation into nucleotide peptide continues to a maximum at S: this molecule seems to be associated with growth in some way, as its activity in ascites cells is many times higher than in regenerating liver. Its function is not known, but Islam and Kay, (1972) report that added nucleotide-peptide collects in the nucleus (of ascites cells) and inhibits DNA synthesis in a reversible and non-toxic manner.

The contrast between the incorporation patterns of nucleotide-peptide, and F1a, make it clear that their ADPribosylation is controlled in different ways and presumably that different enzymes are involved. The ADPribosylation of the non-histone protein, if it is a single species, would appear to be intermediate, to judge by the counts left at the top of polyacrylamide gels, showing a 2 or 3-fold rise during S, similar to that observed by Lehmann et al, (1974) for poly ADPribose polymerase in lymphocytes. The increase in incorporation into the non-histone fraction is earlier than S; Kostraba and Wang, (1973) find a change in a 'non-histone protein' extract, which contains also RNA, by 6 hours after partial hepatectomy, which causes an increase in chromatin template activity for RNA polymerase. For the only non-histone protein for which total phosphate figures have been obtained i.e. those associated with histone F1, and the ADPribose level remains roughly constant, while the level of phosphoserine increases from virtually nothing to 100nM/mg.

CHAPTER 6

In recent years, considerable progress has been made in the characterisation of histones, and F2a1, F2a2, F2b and F3 have been isolated and sequenced. Histone F1 has been prepared free from contaminants and separated into 4 or 5 subfractions, but these have not yet been completely sequenced. Of the non-histone proteins, only P1 has been isolated pure in reasonable yield, although Goodwin et al, (1973) have obtained a reasonable separation of the main 0.35M saline soluble proteins on Bio Gel P60.

Many of the proteins of the cell nucleus have the ability to accept phosphate as phosphoserine. It has been suggested that the phosphorylation of histones could be a mechanism either for opening up the chromatin structure during S, or for bringing about chromosome condensation, during mitosis. Since histone F1 is also phosphorylated in response to hormonal stimulation, it is likely that any gross alteration in the structure of chromatin can be brought about by suitable phosphorylation. While there is a considerable amount of phosphate associated with non-histone nuclear protein fractions, it is not at all clear which proteins carry the phosphate, or what role the phosphate plays. The control of nuclear function by phosphorylation is in turn dependent on the activities of the nuclear protein kinases. The number, and varying specificity of these kinases are becoming apparent (Kish and Kleinsmith, 1974).

In addition to the simple phosphorylation of nuclear proteins, the addition of a residue of ADPribose is recognised. Histone F1 and a small peptide are acceptors, together with non-histone nuclear protein(s). The nucleotide is bound via phosphoserine in the case of the histone, but the finding that a proportion of the non-histone bound material is hydroxylamine labile argues that in the latter case there are two distinct modes of binding. It would seem likely that the hydroxylamine stable material is bound by the same bond as for histone F1, since no hydroxylamine stable radioactivity is found by workers who first dialyse their proteins against acid (Nishizuka et al, 1969). These authors find that the rate of release of (poly) ADPribose with hydroxylamine is the same as that for amino acyl tRNA, and propose that the ADPribose is similarly bound via a carboxylic ester bond.

As with the results presented for histone F1 in Chapter 3, Nishizuka et al, (1969) find that the ADPribose monomer is a major species of their hydroxylamine labile material, although they find a higher proportion of radioactivity in the high molecular weight fraction. While it is not surprising to find different ratios of products when comparing two different populations of molecule, particularly when one was labelled *in vivo*, and one *in vitro*, the vast excess of monomer over dimer and trimer products in both cases might be significant. It suggests that two different enzymes are involved in each case; the first being concerned with attaching the first residue, and the second being an elongation enzyme, which could be common to both types of attachment. In any event, the elongating

enzyme appears to act preferentially on the smaller oligomers, since the pool of dimers, trimers etc. is very small.

The peptide-ADPribose bond present in nucleotide-peptide has not been investigated; however, the method of isolation of this molecule involves prolonged exposure to acid, so the linkage must be fairly acid stable.

The complexity of chromatin makes it difficult to study any one function in isolation; however, a number of correlations have been observed which it is claimed give an indication of the types of processes involved. Thus, phosphorylation of histone correlates with an extended (Louie and Dixon, 1973) or condensed (Bradbury et al, 1974) state of chromatin, and more histone phosphate is found in rapidly than in slowly dividing tissues. The thiol/disulphide ratio in histone F3 is similarly altered during the process of cell division. Perhaps it should not be considered surprising that increased phosphorylation of histone F1 can be found both in connection with DNA synthesis and mitosis, in spite of the difference in state of the chromatin. If, indeed, histone F1 is concerned with higher order structure of chromatin, it might be expected that any change of chromatin state would be brought about by a modification of F1; for instance, by phosphorylation of a different site, or of a different subfraction.

It is of interest, therefore, to find the protein P1 which is subject to phosphorylation complexes to histone F1 in a manner which is dependent on its redox state. Through complexed proteins of this sort, changes in the redox state of the nucleus will affect the redox state of P1, which in turn

will change the state of F1, and hence the conformation of chromatin. Since P1 is also phosphorylated, possibly by a different kinase, the possibility is introduced that non-histone kinases can also have an indirect effect on F1.

A model for the action of F1 in maintaining chromatin structure during the cell cycle may be formulated as follows. In the Go cell, both the ADPribosylated F1 and the P1 protein act as centres of aggregation for the normal chromatin bound unmodified F1, keeping the chromatin in a fairly condensed state. When the cell moves in to G1, the low level of phosphorylation of F1 becomes sufficient to break up these aggregates. The shift into G1 is marked by an increase in template activity for RNA synthesis, in the absence of DNA synthesis (McArdle and Creaser, 1963). A second type of phosphorylation of F1 is then associated with S phase, and is probably concerned with ensuring the correct binding of new F1, or with maintaining an open structure at the replicating point. Finally, a third phosphorylation takes place, which triggers mitosis, causing the ordered condensation of the chromosomes.

It may be noted in passing, that the species specificity of binding of F1 to DNA is dependent not on F1 itself, but on a species which is separated on Amberlite (Gottesfield et al, 1972) and might reflect ADPribosylation of F1, or the specificity of the associated non-histone proteins.

As far as the other histones are concerned, the phosphorylation of F3 during mitosis (Gurley et al, 1974) might reasonably be concerned with chromosome condensation, but the phosphorylations observed on other fractions during S

are more difficult to evaluate. The phosphate contents of histones F2a1 and F2a2 have been found to be elevated at 4 and 13 hours after partial hepatectomy, times which correspond to the peaks of cAMP contents in liver (McManus et al, 1972). The pattern for F2b appears to be similar, but the values here are much lower (Ord and Stocken, in preparation). Histone F3 is not phosphorylated prior to mitosis. The figures for the incorporation of ^{32}P into the individual histone fractions do not necessarily reflect the phosphate contents at the time of examination. The changes observed are much smaller than those found for F1b, or for phosphoserine in P1. When histone fractions are isolated on Bio Gel, a partial separation of the phosphorylated species is obtained. The use of material labelled *in vivo* with [^{3}H]lysine shows that the newly synthesised histone is preferentially phosphorylated.

The ADPribose associated with histone F1 maintains a steady level of incorporation throughout the cell cycle, and is hence presumably structural, or passive, as found for their ADPribose by Hilz and Kittler, (1971) and Leiber et al, (1973). That associated with the non-histone proteins shows an increase in incorporation which is probably in excess of that required to maintain phosphate levels on those proteins, which is as would be expected from the increase in poly ADPribose polymerase in PHA stimulated lymphocytes (Lehmann et al, 1974).

While the function of the ADPribosyl non-histone protein is not known, its association with a single protein species, and the increase in level during S phase, at a time when non-specific nuclear activity is taking place, suggests that

its function involves bringing about structural changes in the state of chromatin.

The ADPribose found on the small perchloric acid soluble nucleotide-peptide is different again, metabolically. It is particularly highly labelled in ascites cells. When isolated from ascites cells by a similar procedure, nucleotide-peptide added exogenously was concentrated into the cell nucleus, causing a reversible inhibition of DNA synthesis (Islam and Kay, 1972). The early, continuous increase in synthesis, peaking during S, allow the possibility that the molecule is produced to terminate a round of DNA synthesis, as its concentration becomes sufficient to precipitate the DNA of the nucleus. This would be compatible with its high rate of synthesis in rapidly dividing cells. It is not ruled out that this species is the one responsible for inhibiting DNA synthesis in nuclei incubated with NAD, (Burzio and Koide, 1970, 1971, 1972), although the authors, who examined only histone-associated material suggest the acid soluble ADPribosylated non-histones as the agent.

While we are far from a complete understanding of the mechanism of nuclear function, a general picture is available of chromatin as a nucleohistone complex, whose basic structure is maintained by histones F2 and F3, with the higher order structure being dependent on the state of phosphorylation of histone F1, which form bridges from strand to strand of the chromatin. Tissue and species specificity, although shown by histone F1 subfractions, is probably mainly due to the

non-histone proteins of the nucleus. ADPribosyl proteins would appear to play important roles in the structure of chromatin, and of its function on the level of DNA synthesis and cell division.

APPENDIX - METHODS

PREPARATION OF NUCLEI

a) From sheep thymus.

Sheep thymus nuclei were prepared by the method of Allfrey, Littau and Mirsky, (1964). The thymus was removed from the animal as soon as possible after death, and placed immediately on ice. The connective tissue was removed, and 100g of the gland was minced with scissors, and blended at top speed in a Waring blender for five minutes with 900mls of 0.25M sucrose, 10mM Tris-HCl buffer, pH 7.2, 5mM $MgCl_2$. The homogenate was filtered through two layers of muslin, and centrifuged at 500g for fifteen minutes in an M.S.E. Mistral 6L centrifuge. The pellet of nuclei was resuspended in 150mls 0.25M sucrose, 10mM Tris-HCl buffer, pH 7.2, 5mM $MgCl_2$, and centrifuged again for fifteen minutes at 500g. All operations were carried out between 0^{o} and $4^{o}C$.

b) From ascites hepatoma.

Rat hepatoma 223 originated as dimethylaminobenzene-induced tumour (Mansbridge and Korner, 1966). It was maintained intraperitoneally in male Wistar rats (body weight 180-200g) and transferred every 7 days by intraperitoneal injection of 2mls ascitic fluid which had been taken from two donors and mixed in a sterile syringe. Rats were killed, 7 days after tumour transfer, by cervical fracture. The cell suspension (about 10^{8} cells/ml) was drained from the peritoneal cavity, and filtered through two layers of cheese cloth into ice-cooled

beakers. Cells were pelleted by centrifugation at 1,500g x 4 minutes in an M.S.E. bench centrifuge and washed three times in 5mM $CaCl_2$. The cells were resuspended in Cemulsol medium, using about 30mls per rat and nuclei prepared as for rat liver.

c) From rat liver.

(i) Rat liver nuclei were prepared by the method of Dabeva and Tsanev, (1966). 10g rat liver were homogenised with 75mls 0.25M sucrose, 10mM Tris-HCl buffer, pH 7.2, 5mM $MgCl_2$ 0.2% Cemulsol NPT6 and NPT12. The homogenate was underlayed with 5mls 1.4M sucrose, 10mM Tris-HCl buffer, pH 7.2, 5mM $MgCl_2$, and 20mls 1.6M sucrose 10mM Tris-HCl buffer, pH 7.2, 5mM $MgCl_2$, and centrifuged at 1,500g (3,000rpm) for 20 minutes in an M.S.E. Superspeed centrifuge. The pellet of nuclei was resuspended in 50mls 0.25M sucrose, 10mM Tris-HCl buffer, pH 7.2, 5mM $MgCl_2$ and centrifuged for 5 minutes at 6,000rpm in a Baro Gyro centrifuge.

(ii) For large scale preparations of rat liver nuclei, 1Kg rat liver was homogenised with 6L 0.25M sucrose, 10mM Tris-HCl buffer, pH 7.2, 5mM $MgCl_2$, 0.2% Cemulsol NPT6 and NPT12 (Nonidet NP40 may be used instead of Cemulsol). The homogenate was centrifuged at 1,000g for 20 minutes in an M.S.E. Mistral 6L centrifuge. The nuclear pellet was resuspended in 500mls 1.8M sucrose 10mM Tris-HCl buffer, pH 7.2, 5mM $MgCl_2$, and centrifuged for 30 minutes at 1,500g (3,000rpm)

in an M.S.E. Superspeed centrifuge. The pellet was resuspended in 0.25M sucrose, 10mM Tris-HCl buffer, pH 7.2, 5mM $MgCl_2$ and centrifuged for 5 minutes at 6,000rpm in a Baro Gyro centrifuge.

(iii) Rat liver nuclei were also prepared by the method of Chauveau et al, (1956). The liver was homogenised initially in 7 volumes ice cold 2.2M sucrose, 5mM $MgCl_2$, 10mM Tris-HCl buffer, pH 7.4. The homogenate was placed in Spinco model L centrifuge tubes, underlayed with 5ml 2.2M sucrose medium, and centrifuged at 40,000g (20,000rpm) for 60 minutes using a 30 rotor. The white pellet was resuspended in 20 volumes 0.25M sucrose, 5mM $MgCl_2$, 10mM Tris-HCl buffer, pH 7.4, and centrifuged at 3,600g for 2 minutes in a Baro Gyro centrifuge.

Extraction of nuclei

Nuclei were washed twice with 5mM $MgCl_2$, 10mM Tris-HCl buffer, pH 7.2, and once with 1mM HCl, the nuclei being recovered each time by centrifugation at 3,000g for 5 minutes.

Histone F1 was prepared by method I of Johns (1964) by extraction with 5% (w/v) Perchloric acid for 20 minutes, and centrifuging at 3,000g for 5 minutes. The supernatant was made 20% with Trichloracetic acid, and the precipitate collected by centrifugation for 20 minutes at 3,000rpm in an M.S.E. Superspeed centrifuge. The precipitate is redissolved in a minimal volume of distilled water, and the solution dialysed overnight against

100 volumes water.

The remaining histones were prepared by extraction of the nuclei, after a wash with 5% Perchloric acid, with 0.25M HCl. The suspension was centrifuged at 3,000g for 5 minutes and the supernatant made 15% with Trichloracetic acid. The TCA precipitate was collected by centrifugation at 3,000g for 10 minutes.

After extraction of histones, the remaining nucleoprotein was suspended in 7M urea 2M NaCl, 10mM Tris-HCl buffer, pH 7.2. The suspension was centrifuged for 20 minutes at 10,000g, and the supernatant made 5% with Trichloracetic acid. The Trichloracetic acid precipitate was collected by centrifugation at 3,000g for 10 minutes. The urea-salt precipitate was re-extracted in 2% sodium dodecyl sulphate, 50mM Tris-HCl buffer, pH 7.2. Non-histone proteins were precipitated with 5 volumes acetone, and collected by centrifugation for 10 minutes at 2,000g.

Partial hepatectomy

Male Wistar rats (body weight 180-200g) were partially hepatectomised (Higgins and Anderson, 1931) under ether anaesthesia. 70% of the liver is removed leaving the right lateral, caudate and accessory lobes. Solid food and 10% sucrose to drink were available to the rats after the operation.

^{32}P Labelling in vivo

Rats were injected intraperitoneally with carrier free [^{32}P]orthophosphate (specific radioactivity 67Ci/mg of P) in 0.3ml of 0.9% NaCl. The dose and period of incubation are given for each experiment.

[^{3}H]Adenosine NAD labelling in vitro

Rat liver nuclei made by the method of Chauveau were incubated in 28mM $MgCl_2$ 1mg/ml glucose, 10mM Tris-HCl buffer, pH 7.4, 25µCi [^{3}H]Ade NAD for 10 minutes at 37°C.

Formation of protein complexes - Langan and Smith, (1965)

The formation of complexes between histone and non-histone proteins was followed turbidometrically by measuring the extinction at 450nm, 15 minutes after the addition of various amounts of histone or poly L lysine to a solution of P1 (100µg in 2ml of 25mM Tris-HCl buffer, pH 7.4).

Conditions for the incubations of enzymes

(i) Histone kinase

The assay for the ability of protein to act as a substrate (phosphate acceptor) for histone kinase was similar to that of Pawse et al, (1971). A 100µg portion of substrate protein, 2.5µmol $MgCl_2$, 10µmol Tris-HCl buffer, pH 7.4, 0.21µmol of [γ-^{32}P] ATP (specific radioactivity 28,000 dpm/nmol) and 0.1

unit of the enzyme, were contained in a final volume of 0.2mls, and incubated for 10 minutes at 37°C. The tubes were then placed on ice, and 0.5mls 50% TCA added, together with 0.3mls F1 (10mg/ml) as carrier. The tubes were centrifuged for 15 minutes at 2,000g. The protein was washed twice with 25% TCA, then resuspended in 10mls H_2O for counting by the Cerenkov method. (One unit is the activity of enzyme required to transfer 1nmol of phosphate from ATP to histone F1 in 10 minutes at 37°C).

(ii) RNA polymerase

The method of Leake et al, (1968) was used. The assay was carried out in a volume of 0.5mls, containing 30µmol Tris-HCl buffer, pH 8.0, 8µmol KCl, 2µmol NaF, 7µmol βmercapto-ethanol, 0.1µmol each of ATP, CTP and UTP, 0.04µmol ^{14}C labelled GTP (2µCi/µM), 0.1µmol $MgCl_2$, 0.6µmol $MnCl_2$, 40µg DNA (from calf thymus, type V - Sigma) and 0.01ml RNA polymerase (prepared by the method of Chamberlin and Berg, (1962)). The mixture was incubated for 10 minutes at 37°C and the reaction stopped by adding 0.2ml ice cold 4M NaCl. After 10 minutes at 0°C, 0.5ml 10% TCA was added, and left for 5hrs at 0°C. Acid insoluble material was collected on millipore filters, and counted in dioxan scintillant.

(iii) Ribonuclease

The substrate in 1ml final volume was made 50mM with acetate buffer, pH 5.5, 0.1mg nuclease added (from Micrococcus - Worthington) and incubated for 30 minutes at 37°C. The reaction was terminated by addition of an equal volume 50% TCA.

(iv) Phosphodiesterase

The conditions of incubation were those of Nishizuka et al, (1967). The sample was made 10mM in $MgCl_2$, 20mM in Tris-HCl buffer, pH 7.4 in a final volume of 1ml, and incubated with 0.1mg venom phosphodiesterase (from Crotalus atrox, containing pyrophosphatase and phosphodiesterase activities, together with a low activity of phosphomonesterase) for 3 hours at 37°C.

(v) Pronase-leucine aminopeptidase

Complete enzymic digestion of protein was performed according to Balhorn et al, (1971). 10mgs protein in 5mls 30mM Tris-HCl buffer, pH 7.4, were incubated with ½mg pronase (Koch-Light) for 24 hours at 37°C. The pronase was then inactivated by heating to 100°C for 15 minutes.

Leucine aminopeptidase (Sigma) was activated by incubation of 0.2mgs enzyme in a volume of 2mls containing 60µmol Tris-HCl buffer, pH 8.6 and 2.5µmol $MnCl_2$ for 3 hours at 40°C. The activated enzyme was incubated with the pronase digest for 4 days at 37°C.

(vi) Trypsin

Histone F1 in 0.1M acetic acid made to pH 5.8 with pyridine (Sung et al, 1971) was incubated with 5% of its weight to trypsin (Worthington-treated with L (tosylamido 2 phenyl) ethyl chloromethyl ketone to inhibit chymotryptic activity) for 5 hours at 25°C. The digest was applied to paper (Whatman 3mm) and electrophoresed for 1 hour at 3½ KV in pyridine-acetic acid pH 6.5 (10 volumes pyridine, 89 volumes H_2O, 0.4 volumes acetic acid). Orange G was used as marker.

Polyacrylamide gel electrophoresis

(i) pH 7.0 in the presence of sodium dodecyl sulphate (Weber and Osborn, 1969).

Stock solutions.

A. $NaH_2PO_4 . 2H_2O$ 4.3g.
$Na_2HPO_4 . 12H_2O$ 25.7g.
SDS 2.0g.
TEMED 1.5mls.
H_2O to 1 litre.

B. 22.2g acrylamide.
0.6g bisacrylamide.
H_2O to 100mls.

C. 1.5% ammonium persulphate (freshly prepared).

Running buffer.

$NaH_2PO_4 . 2H_2O$ 2.15g.
$Na_2HPO_4 . 12H_2O$ 12.85g.
SDS 1.0g.
H_2O to 1 litre.

For 12 gels (length 7.5cm, diam. 0.5cm) 15mls solution A are mixed with 13.5mls B and deaerated. 1.5mls C are added and the gel solution transferred to 10cm long glass tubes and overlaid with 0.1ml distilled water. At least 2 hours are allowed for polymerisation. Lyophilised protein samples are dissolved in 6M urea, 1% SDS, 1% βmercaptoethanol and underlayed onto the gels. Electrophoresis at 2m.amps per gel is continued until the Bromophenol Blue tracker dye has migrated three quarters

of the gel length. The gels are removed by forcing 50% Teepol solution between gel and gel tube, using a syringe fitted with a 22swg hypodermic needle. They are fixed overnight in 25% isopropanol, 10% acetic acid, stained for 2 hours in 0.1% Coomassie brilliant blue, 7% acetic acid, and destained by diffusion into 7% acetic acid.

(ii) pH 8.9, in the presence of sodium dodecyl sulphate, (MacGillivray et al, (1972)).

Stock solutions.

A. 1M HCl 48mls.
Tris. 36.6g.
TEMED. 0.23mls.
H_2O to 100mls. (pH 8.9).

B. Acrylamide 40g.
Bisacrylamide 0.6g.
H_2O to 100mls.

C. Ammonium persulphate 0.14g.
in 100mls 8M urea, 0.2% SDS.

D. Tris. 5.98g.
TEMED. 0.46mls.
Make pH 6.7 with 1M HCl.
H_2O to 100mls.

E. Acrylamide 10g.
Bisacrylamide. 2.5g.
H_2O to 100mls.

F. Riboflavin 4mg.
H_2O 100mls.

G. 8M urea.

0.2% SDS.

Running buffer.

Tris.	3g.
Glycine.	14.4g.
SDS.	1g.
H_2O to 1 litre.	

The running gel is made by mixing 1 volume A, 3 volumes B and 4 volumes C, and deaerating. The mixture is placed in tubes of 5mm i.d. to a height of 12cm, overlaid with 0.1ml H_2O and left at least 2 hours to polymerise. When polymerisation is complete, stacking gel mixture consisting of 1 volume D, 2 volumes E and 1 volume F and 4 volumes G is added to the tubes to a height of 1.5cm. The tubes are again overlaid with distilled water and left for 1 hour under an ultra violet lamp to polymerise. The lyophilised sample is dissolved in 200µl 8M urea 0.2% SDS, and the gels run at 2mA per tube until the tracking dye (Bromophenol Blue) is within 1cm of the end of the tube. The gels are removed from the tubes, fixed overnight in 50% Methanol, 10% acetic acid and stained with 0.1% Naphthalene Black in 7% acetic acid.

(iii) pH 2.9 in the presence of urea, (Panyim and Chalkley, (1969a,b)).

Stock solutions.

A.	Acrylamide	60g.
	Bisacrylamide	0.4g.
	H_2O to 100mls.	
B.	Acetic acid	43.2mls.
	TEMED	4mls.
	H_2O to 100mls.	
C.	Urea	25g.
	Ammonium persulphate	0.2g.
	H_2O to 100mls.	

Running buffer.

5.4% acetic acid.

2 volumes A, 1 volume B and 5 volumes C are mixed and deaerated. Gel tubes of 5mm i.d. are filled to within 1cm of the top with the mixture and overlaid with 0.1ml distilled water. They are left for 1 hour to polymerise. The gels are pre-run at 2mA/tube until the tracker dye (Methyl Green) leaves the tube. The lyophilised sample dissolved in less than 0.2mls 5.4% acetic acid 2.5M urea is loaded and fresh running buffer placed in the electrode compartment. The gels are run at 1mA/tube for two lengths of the tracker dye.

(iv) pH 8.9 (derived from Davies, (1964)).

Stock solutions.

A. N HCl 48mls.
Tris 36.6g.
TEMED 0.23mls.
H_2O to 100mls.

B. Acrylamide 28g.
Bisacrylamide 0.73g.
H_2O to 100mls.

C. Ammonium persulphate 0.14g.
H_2O to 100mls.

Running buffer.

Tris 6g.
Glycine 28.8g.
H_2O to 1 litre.
(pH 8.3, dilute 1 in 10 for use).

1 volume A, 2 volumes B, 1 volume H_2O and 4 volumes C are mixed and the mixture placed in tubes of 5mm i.d. to within 1cm of the top, and overlaid with 0.1ml distilled water. 1 hour is allowed for polymerisation. The lyophilised sample is applied in running buffer made 1M in sucrose. 1ml 0.001% Bromophenol Blue is added to the upper reservoir as a tracker dye, and the gels run at 2mA/tube until the dye is within 1cm of the bottom of the tube.

(v) pH 4.3 (from Reisfield et al, 1962).

Stock solutions.

A.	N KOH	48mls.
	Glacial acetic acid	17.2mls.
	TEMED	4mls.
	H_2O to 100mls.	
B.	Acrylamide	60g.
	Bisacrylamide	0.4g.
	H_2O to 100mls.	

C. 0.28% ammonium persulphate (freshly prepared).

Running buffer.

βAlanine	31.2g.
Glacial acetic acid	8mls.
H_2O to 1 litre.	

The gels consist of 1 part A, 2 parts B, 1 part water and 4 parts C, polymerised as (iii). They are run for 3-4 hours at 3m.amps per gel.

Estimation of protein - (Hartree, 1972), derived from Lowry et al, (1951).

Stock solutions.

A. 2g Sodium potassium tartrate.
100g Sodium carbonate.
500mls N sodium hydroxide.
H_2O to 1 litre.

B. 2g Sodium potassium tartrate.
1g $CuSO_4$ $5H_2O$.
10mls N NaOH.
H_2O to 100mls.

C. Folin and Ciocalteau's reagent diluted 1 in 15 with H_2O.

To 1ml sample was added 0.9mls A, and incubated at 50°C for 10 minutes. The mixture was cooled to room temperature and 0.1ml B was added, and left at room temperature for 10 minutes. 3mls C was added rapidly, and left at 50°C for 10 minutes. The optical density was read at 650nm.

Estimation of ribose and RNA. (Hurlbert et al, 1954).

1ml sample in 0.5N Perchloric acid was added to 2mls freshly prepared 0.4% orcinol, 12N HCl, 0.04% $FeCl_3$, and incubated at 100°C for 15 minutes. Optical density was read at 660nm.

Estimation of DNA. (Burton, 1956).

Diphenylamine Reagent:

100mls glacial acetic acid.

1.5g diphenylamine.

1.5g conc. H_2SO_4.

0.1ml of 16mg/ml acetaldehyde solution is added to 20mls reagent just before use.

The sample, containing 0.30µg DNA in 0.4mls 0.5N Perchloric acid was digested at 70°C for 20 minutes. 0.8mls diphenylamine reagent is added and left 19 hours at 28°C. The optical density at 600nm was read against a 0.5N Perchloric acid blank.

Estimation of Phosphorus from Bartlett, (1959).

Fiske - Subbarow reagent.

0.5g 1 amino 2 naphthol 4 sulphonic acid.

200mls 15% Sodium bisulphite.

1g Sodium sulphite is added, the solution filtered, and stored in a dark bottle.

A sample containing 3-18nm phosphorus in 0.5ml was ashed in the presence of 0.3ml 60% Perchloric acid for not less than 5 hours at 180°C. The resultant solution was made up to 0.3mls with 60% Perchloric acid, 0.55mls distilled water was added, and the solution vortexed with 0.2mls 5% ammonium molybdate. 0.05mls Fiske-Subbarow reagent was added, and the vortexed mixture heated in a boiling water bath for 7 minutes. The sample was immediately placed on ice. Optical density was read at 820nm. Before use, the pyrex centrifuge tubes used for the estimation were ashed at 180°C for 16 hours with 2mls 60% Perchloric acid, and washed 7 times with distilled water, repeating this procedure until reagent blanks showed an optical density of 0.03-0.05.

Organic and inorganic Phosphorus were separated by the method of Berenblum and Chain, (1937). 2mls sample were shaken with 0.5mls 5N H_2SO_4, 1ml 5% ammonium molybdate, 3mls isobutanol. The isobutanol layer, which contains the inorganic phosphate is separated and washed with N H_2SO_4, and then dried down for estimation as above. The aqueous layer was used directly for estimation of organic phosphorus as above.

Staining of Phospholipids.

Reagent of Vaskovsky and Kostetsky, (1968).

16g ammonium molybdate is dissolved in 120mls H_2O. To 80mls of the solution, 40mls HCl and 10mls Mercury are added, and the solution added to 200mls conc. H_2SO_4. The remainder of the original solution is added, and made up to 1 litre.

Phospholipids, separated by thin layer chromatography were stained directly by spraying with the above reagent.

Radioactive Counting.

A Beckman Liquid Scintillation Counter was used for all estimations of radioactivity.

In experiments using ^3H a dioxan based scintillant was used:

8g Butyl PBD (5-(4-biphenylyl)-2-(4-tert-butylphenyl)-1-oxa-3,4 diazole)(CIBA Ltd.).

80g Naphthalene.

1 litre dioxan.

^{32}P was counted in water using Cerenkov emission.

Gel Densitometry.

Polyacrylamide gels were scanned using a 'Chromoscan' scanning densitometer.

Estimation of Adenine.

Adenine was estimated by its optical density at 260nm.

Amino acid analysis. (Spackman et al, (1958)).

A sample containing not more than 500µg protein was lyophilised in an acid-washed pyrex tube. 0.1ml 6N HCl (Aristar) was added, the tube sealed, and hydrolysis carried out at 110°C for 21 hours in an oil bath. The sealed tube was centrifuged to collect the solution at one end, opened, and the solution evaporated to dryness. The protein hydrolysate was analysed on a single column in a Locarte amino acid analyser. Correction is made for 5% loss of threonine and 10% loss of serine during hydrolysis.

Analysis of stained bands from polyacrylamide gel was performed by hydrolysis with 1ml 6N HCl, and the gel washed after hydrolysis with 1ml 6N HCl, the washing being added to the original hydrolysate and evaporated to dryness. A stained blank gel was run for comparison.

End Group Analysis. (Gray, 1967).

0.01µmol protein was dissolved in 0.5ml demineralised 8M urea, 0.5M in Sodium bicarbonate. 0.5ml Dansyl chloride (20mg/ml in acetone) was added and left for 1-2hours at 37°C until the yellow colour had gone. The solution was dialysed overnight against distilled water, then dried and hydrolysed in a sealed tube at 110°C with 6N HCl for 5 hours. The dansyl amino acid was identified by chromatography on polyamide tlc sheets (Woods and Wang, 1967) obtained from the Cheng Chi Trading Co., Ltd., Taipei.

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