The Properties of a Nuclear Acidic Protein Fraction that Binds [6,7-³H]Oestradiol-17β

By R. J. B. KING, J. GORDON and A. W. STEGGLES
*Department of Hormone Biochemistry, Imperial Cancer Research Fund, Lincoln's Inn Fields,
London W.C.2*

1. Additional evidence was obtained that the nuclear oestradiol-17β receptor is an acidic protein. Partial purification of the receptor protein was obtained by chromatography on hydroxyapatite and it contains protein-bound phosphate. 2. The nuclear '5s' and cytoplasmic '9·5s' and '5s' receptors from uterus, dimethylbenzanthracene-induced mammary adenocarcinoma and kidney are precipitated together with bound oestradiol-17β by protamine sulphate. This common property suggests that the nuclear and cytoplasmic receptors are related to each other. 3. The properties of two acidic protein fractions from both liver and dimethylbenzanthracene-induced mammary adenocarcinoma are described. Fraction 1 contains two major components and fraction 2 contains one component, as judged from polyacrylamide-gel electrophoresis. Fraction 2 contains RNA and both fractions contain protein-bound phosphate. 4. These fractions form insoluble complexes with calf thymus histone, protamine sulphate and poly-L-lysine. The formation of these complexes is markedly affected by ionic strength and pH. Ionization of both the ε-amino group of lysine and carboxyl group are involved. RNA and DNA do not appear to be involved. The interaction is not affected by EDTA or 1mM-Na⁺, -K⁺, -Ca²⁺, -Mg²⁺ or -Mn²⁺. Per unit weight, whole histone has 4–5 times as many binding sites for the acidic proteins as the latter have for the former. 5. No convincing evidence was obtained for DNA–acidic protein interaction, but, as judged from precipitation experiments, there was competition between DNA and acidic protein for histone. 6. Relatively large amounts of acidic protein partly relieved the histone inhibition of the template activity of DNA for *Escherichia coli* RNA polymerase (EC 2.7.7.6).

Previous studies from this laboratory have shown that, in oestrogen-sensitive tissues, [6,7-³H]-oestradiol-17β is non-covalently bound to an acidic protein component of both euchromatin and heterochromatin and that this component can be extracted with sodium chloride (King, Gordon, Cowan & Inman, 1966; King & Gordon, 1968). The present paper describes the further characterization of this material.

Certain nuclear acidic proteins form insoluble complexes with histones and alter the template activity of DNA–histone for RNA polymerase (Langan, 1967; Wang & Johns, 1968; Wang, 1968). These properties of the protein fractions described below were also investigated.

MATERIALS AND METHODS

Tissue fractions. Mammary tumours were induced by intragastric administration of 50mg. of dimethylbenzanthracene in corn oil (Huggins, Briziarelli & Sutton, 1959;

Young, Cowan & Sutherland, 1963). ³H-labelled mammary tumours for the fractionation studies were obtained from intact female rats 1hr. after the subcutaneous injection of [6,7-³H]oestradiol-17β (0·1μg./100g. body wt.). Homogenates (10%, w/v) were prepared in 0·25M-sucrose containing 3mM-CaCl₂, and nuclei were isolated either by centrifugation at 700g for 10min. (method A) or by density-gradient centrifugation (Allfrey, Littau & Mirsky, 1964) (method B). In both cases the nuclear fraction was washed twice with 10mM-tris–HCl buffer, pH7·4, containing 3mM-CaCl₂ (tris–Ca²⁺). This removed negligible amounts of radioactivity.

When non-radioactive tissues were used, nuclei were prepared from 40–80g. of tissue by method A, washed twice with tris–Ca²⁺ and resuspended in 2·2M-sucrose containing 3mM-CaCl₂. This was centrifuged at 28000rev./min for 1hr. in a Spinco no. 30 rotor and the nuclear pellet was washed twice with tris–Ca²⁺ (method C).

For the experiments with protamine sulphate, kidneys and mammary tumours were obtained 1hr. after the subcutaneous injection of 0·2μg. of [6,7-³H]oestradiol-17β/100g. body wt. The rats had been ovariectomized 8 days before use and all the tumours decreased in size during that

period. Homogenates (approx. 30%, w/v) in 10mM-tris–1·5mM-EDTA–2mM-β-mercaptoethanol, pH 7·0, were prepared by using a Silverson homogenizer (Silverson Machines Ltd., London S.E.1) at maximum speed for 1 min. The homogenate was divided into two equal parts. The first was centrifuged at 10^5g for 1 hr. to give a clear supernatant fraction. The second part was diluted with 0·36M-sucrose to a final concentration of 0·25M with respect to sucrose. This was centrifuged at 500g for 10 min. and the crude nuclear pellet washed once with 10 ml. of tris–Ca^{2+}. The pellet was extracted with 0·5M-KCl–10mM-tris–1mM-EDTA, pH 7·0, for 15 min. (0·5 ml. for the kidney, 1·0 ml. for the tumour) and centrifuged at 3000g for 30 min. The supernatant was called the nuclear extract. Samples (0·2 ml.) of both the nuclear extract and supernatant fractions were applied directly to linear 5–20% (w/v) sucrose gradients. For the supernatant fractions the gradient was made up in 10mM-tris–HCl buffer, pH 7·4, but the nuclear gradients also contained 0·3M-KCl. The sedimentation coefficients quoted in this paper are those reported by Jensen et al. (1968). Another sample (0·4 ml.) of each fraction was added to 0·2 ml. of protamine sulphate (7·5 mg./ml. water) and kept at 0° for 10 min. The bulky precipitate was sedimented at 10^3g for 10 min. and 0·2 ml. portions of the supernatant were applied to the sucrose gradients. Portions were also taken for measurement of radioactivity. The pellet was dissolved in 1M-NaOH and its radioactivity measured.

Uteri from eight to 12 immature Sprague–Dawley rats were incubated with 5 ml. of 1nM-[6,7-^{3}H]oestradiol-17β in Krebs–Ringer phosphate medium II (Dawson & Elliott, 1959) at 4° for a total time of 2 hr. The medium was replaced twice during this period. The uteri were washed twice with 5 ml. of the homogenizing medium and a homogenate (approx. 30%, w/v) was prepared as detailed above that is essentially the same as that described by other workers (Toft & Gorski, 1966; Jensen et al. 1968; Rochefort & Baulieu, 1968).

Columns (3 cm. × 4·5 cm.) of hydroxyapatite (Bio-Gel HTP; BioRad Laboratories, Richmond, Calif., U.S.A.) were prepared in 1mM-potassium phosphate buffer, pH 7, as described by Velle & Engel (1964). For routine experiments the column was eluted with 140 ml. of 1 mM-potassium phosphate buffer, pH 7, followed by 0·1M-potassium phosphate buffer, pH 7. Fractions (20 ml.) were collected until no further material absorbing at 280 nm. was eluted (usually about 100 ml.). The concentration of potassium phosphate buffer, pH 7, was then changed to 0·2M and the process repeated, and the column was eluted with 0·4M-potassium phosphate buffer, pH 7, in the same way. The column was washed with 1M-potassium phosphate buffer, pH 7, exhaustively washed with 1mM-potassium phosphate buffer, pH 7, and stored in this solution at 4° until re-used.

The material eluted with 0·1M-phosphate (fraction 1) was 50% saturated with $(NH_4)_2SO_4$ at pH 7·0, the precipitate dissolved in 10mM-tris–HCl buffer, pH 8·5, and the solution dialysed overnight against the same buffer. The material eluted with 0·2M-phosphate (fraction 2) was treated in the same way. In a typical preparation from 80 g. of liver, fractions 1 and 2 contained 4 mg. and 5 mg. of protein respectively. From 70 g. of tumour, fractions 1 and 2 contained 3 mg. and 4 mg. of protein respectively.

[^{3}H]Tryoptphan-labelled fractions 1 and 2 were obtained from livers taken 30 min. after the intraperitoneal injection of 100 μC of L-[^{3}H]tryptophan (specific radioactivity 2c/m-mole; The Radiochemical Centre, Amersham, Bucks). Only 10 g. of liver was used for each of these experiments. After elution from the hydroxyapatite fractions 1 and 2 were exhaustively dialysed against water at 4°, freeze-dried and redissolved in 10mM-tris–HCl buffer, pH 8·5.

Histone–acidic protein interaction. Unless stated otherwise, this was carried out in 10mM-tris–HCl buffer pH 8·5, in a final volume of 0·25 ml. at 0° for 10 min. The acidic protein was always added last. The complex was sedimented at 1000g for 10 min. and washed twice with 0·5 ml. of water, and protein in the pellet was determined.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystallized bovine serum albumin (Armour Pharmaceuticals Ltd., Eastbourne, Sussex) as standard. This standard was used throughout these experiments, so that quantitative comparisons cannot be made between the amounts of protein precipitated with protamine and poly-L-lysine. DNA was measured by the diphenylamine reaction (Burton, 1956) and phosphate by a micro modification of the method described by King & Wooton (1956). Protein-bound phosphate was released by treatment with 1M-NaOH for 10 min. in a boiling-water bath (Langan, 1967). Control determinations were carried out both on protein samples that had not been hydrolysed and on solutions that contained no protein but had been hydrolysed. The orcinol reaction for RNA was carried out by the method of Greenbaum & Slater (1957).

Gel electrophoresis was carried out (1) towards the anode at pH 8·5 in gels containing 7·5% (w/v) polyacrylamide (Cyanogum 41; British Drug Houses Ltd., Poole, Dorset) and (2) towards the cathode at pH 4 in 15% (w/v) polyacrylamide gels as described by McAllister, Wan & Irvin (1963) for histones. They were stained with Coomassie Blue for protein (Crambach, Reisfeld, Wyckoff & Zaccari, 1967) or Acridine Orange for RNA (Richards, Coll & Gratzer, 1965).

RNA polymerase (EC 2.7.7.6) (ribonuclease-free) was prepared from *Escherichia coli* strain M.R.E. 620 up to stage 3 of the method described by Chamberlin & Berg (1962). The template activity of the DNA (salmon sperm) was measured by the same method, with a 20 min. incubation period and 10 μg. of DNA/tube.

'Melting' profiles of DNA were measured in a Gilford model 2002 spectrophotometer fitted with an ethylene glycol-heated cell housing. The measurements were made in 10mM-tris–HCl buffer, pH 8·5.

The effect of DNA on the sedimentation of [^{3}H]tryptophan-labelled acidic proteins was carried out by equilibrating 50 μg. of calf thymus DNA with either fraction 1 or fraction 2 in 0·2 ml. of 10mM-tris–HCl buffer, pH 7·4, for 10 min. at 0° and then layering it on top of a linear 5–20% (w/v) sucrose gradient prepared in 10mM-tris–HCl buffer, pH 7·4. This was spun at 39000 rev./min. for 5 hr. in a Spinco SW 39 rotor.

Materials. Protamine sulphate (salmine sulphate) was purchased from British Drug Houses Ltd. Calf thymus histones, either unfractionated or the F1 and F3 fractions, were kindly given by Dr A. J. MacGillivray and had been prepared by method 2 of Johns (1964). The F1 fraction had a lysine/arginine molar ratio of 21·6:3·6% and the F3 fraction had a lysine/arginine molar ratio of 11·9:9·9%.

Phosvitin was obtained from Mann Research Laboratories, New York, N.Y., U.S.A. Yeast 'soluble' RNA was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Poly-L-lysine (mol.wt. 2500), bovine pancreatic ribonuclease (type 1A), pancreatic non-crystalline deoxyribonuclease 1 and calf thymus DNA (type 1) were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Salmon-sperm DNA was obtained from Calbiochem Ltd., London W.1.

The [6,7-^{3}H]oestradiol-17β (specific radioactivity 235μc/μg.) was prepared and purified as described by King & Gordon (1966).

Measurement of radioactivity. This was carried out in a Packard Tri-Carb model 3003 liquid-scintillation spectrometer. The phosphor had the following composition: 100g. of naphthalene, 3g. of 2,5-diphenyloxazole, 230ml. of ethanol, 385ml. of xylene and 385ml. of dioxan. With the exception of the sucrose gradients (expressed as c.p.m.) the radioactivity of all samples was corrected for quenching by a channels-ratio method (Baille, 1960) and values are expressed as d.p.m.

RESULTS

Fractionation of the bound [6,7-^{3}H]*oestradiol-17β from mammary-tumour nuclei.* Optimum extraction of bound [6,7-^{3}H]oestradiol-17β from nuclei (isolated by method A) occurred with 2ml. of 1M-sodium chloride/g. original wt. of tissue. When nuclei were prepared by method B, 1ml. of 1M-sodium chloride/g. original wt. of tissue was used. This extracted 80% of the nuclear [6,7-^{3}H]oestradiol-17β. Dilution of this extract to 0.15M-sodium chloride by addition of 1mM-potassium phosphate buffer, pH7, precipitated over 90% of the DNA together with 11% of the protein without precipitation of radioactivity. In later experiments it was found that dilution to

0.4M-sodium chloride gave similar results, and this was then used as a routine.

Fractional precipitation with ammonium sulphate did not give further purification and also partly dissociated the oestradiol–protein complex. It was not used further with [6,7-^{3}H]oestradiol-17β-labelled fractions. The extract in 0.15M-sodium chloride solution was applied directly to the hydroxyapatite column and eluted successively with 1mM-, 50mM-, 0.1M-, 0.2M- and 0.4M-potassium phosphate buffer, pH7 (Fig. 1). The radioactivity eluted in the 1mM-phosphate fraction was due to free [6,7-^{3}H]oestradiol-17β. Radioactivity, together with material absorbing at 280nm., was eluted with each of the 0.1M-, 0.2M- and 0.4M-phosphate fractions. For the large-scale isolation of fractions 1 and 2 from both tumour and liver, the diluted sodium chloride extract was 80% saturated with ammonium sulphate, the precipitate dissolved in 1mM-potassium phosphate buffer, pH7, and applied to the column.

Composition of fractions 1 and 2. Only the 0.4M-phosphate fraction contained small amounts of DNA, and this fraction was not investigated further. Similar results were obtained when the nuclei were isolated by method B except that all the fractions contained less protein and RNA.

Analysis showed that fractions 1 and 2 from liver and tumour both contained about 10μg. of protein-bound P/mg. of protein. In six separate estimations on liver this varied from 7 to 44μg. of P/mg. of protein. Fraction 2 gave a positive orcinol test for RNA but fraction 1 did not.

In view of the possible presence of contaminating basic proteins in the 0.4M-phosphate extract, the
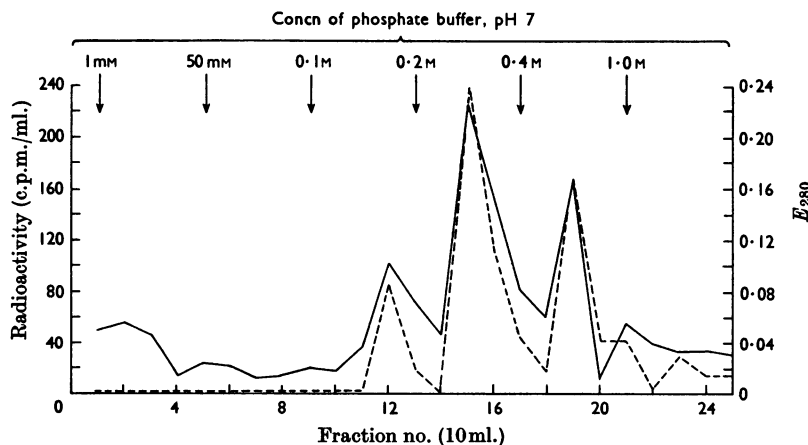

Fig. 1. Fractionation of NaCl extract of nuclei from mammary tumour labelled *in vivo* with [6,7-^{3}H]oestradiol-17β on hydroxyapatite. The column (3cm. × 4.5cm.) was eluted with a discontinuous gradient of potassium phosphate buffer, pH7, the concentration of which is indicated by the arrows. -----, E_{280}; ———, Radioactivity.

affect of adding 1g. of the cation-exchange resin Amberlite CG-50 (Na$^+$ form) to the 0·4M-phosphate extract was tested. This removed about 30% of the protein present at that stage but influenced neither the final yield of fractions 1 and 2 nor their phosphate content or ability to complex with histone. This stage was therefore omitted from routine preparations. Polyacrylamide-gel electrophoresis patterns of the liver preparations are shown in Fig. 2. At pH 8·5 fraction 1 contained two main components and several fast-moving minor ones, whereas fraction 2 contained one main component with a mobility similar to that of component 2 of fraction 1. Addition of 8M-urea to the gels did not alter the properties of the bands. Neither fraction contained detectable amounts of protein that migrated at pH 4. As judged from Acridine Orange staining, no RNA-containing fraction migrated at pH 8·5. Similar results were obtained with mammary tumour.

Association with polycations. Both fractions formed insoluble complexes with calf thymus histone, protamine sulphate and poly-L-lysine (Table 1), and this was influenced by ionic strength (Fig. 3) and pH (Fig. 4). The decrease at pH 11 indicated that the lysine ε-amino group might be involved, and the effect at acid pH could be due to carboxyl-group ionization. Changes at about pH 6 and 7·5 also affected the interaction. The interaction was unaffected by 1mM-EDTA, 1mM-potassium chloride or 1mM-sodium chloride, all at

pH 8·5. With the same molarity of calcium chloride, magnesium chloride or manganous chloride no consistent results were obtained: they either had no effect or gave a small inhibition. As fractions 1 and 2 contained both RNA and protein-bound phosphate it was possible that phosphate ionization might explain this effect at neutral pH, and this was investigated by using the model compounds phosvitin and 'soluble' RNA. Phosvitin did form complexes with poly-L-lysine at pH 5·5, but not at 7·5, and these were not affected by EDTA. At pH 8 1mM-magnesium chloride enhanced RNA–poly-L-lysine interaction, which indicated that neither of these two types of interaction could

Table 1. *Precipitation of acidic protein by polycations*

Results are expressed as μg. of protein precipitated and represent the means ± S.E.M. of at least four determinations. A 50μg. portion of each component was present in a final volume of 0·25ml. of 10mM-tris–HCl buffer, pH 8·5.

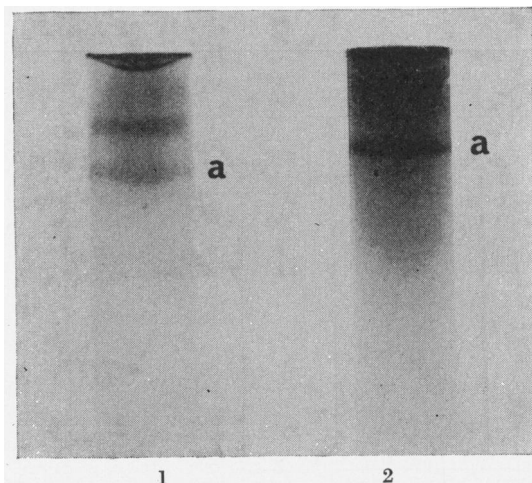| Acidic protein	Histone	Protamine	Poly-L-lysine
Liver			
Fraction 1	41±4	31±4	18±2
Fraction 2	46±6	33±5	19±5
Mammary tumour			
Fraction 1	41±1	—	—
Fraction 2	48±6	—	—

Fig. 2. Polyacrylamide-gel electrophoresis of acidic protein fractions 1 and 2 from liver nuclei. A 100μg. portion of each fraction was applied to the gels (7·5% polyacrylamide, pH 8·5), which were run for 45min. at 7mA/gel. 'a' indicates the protein band common to both fractions 1 and 2.
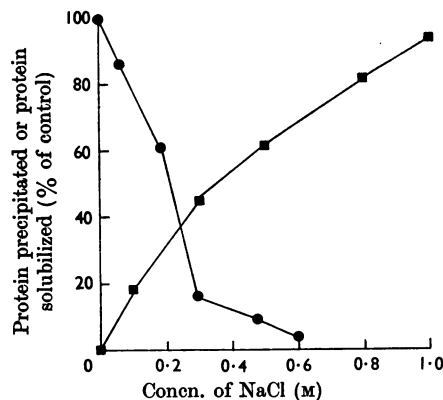

Fig. 3. Effect of NaCl on histone–acidic protein interaction. ●, Effect on complex-formation. Equal amounts (50μg.) of fraction 2 and calf thymus histone were mixed in a final volume of 0·5ml. of NaCl. The amount of protein precipitated relative to that in 10mM-tris–HCl buffer, pH 8·5, was measured. ■, Solubility of the complex. The histone–fraction 2 complex was formed in 10mM-tris–HCl buffer, pH 8·5, and sedimented at 800g for 10min. Then 0·5ml. of NaCl was added and the amount of protein solubilized relative to that in the control (0·5ml. of water) was measured.

explain the EDTA effect on poly-L-lysine–acidic protein interaction.

Incubation of fraction 2 with either ribonuclease or deoxyribonuclease for 15 min. at room temperature before addition of the histone did not diminish the amount of complex formed or its E_{280}/E_{260} ratio of 1·3.

The effect of titrating 50 μg. of the acidic proteins with histone is shown in Figs. 5(a) and 5(b). The arginine-rich F3 histone was more efficient at formation of complexes with the acidic protein than was the lysine-rich F1 fraction. At optimum histone concentrations more protein was precipitated with
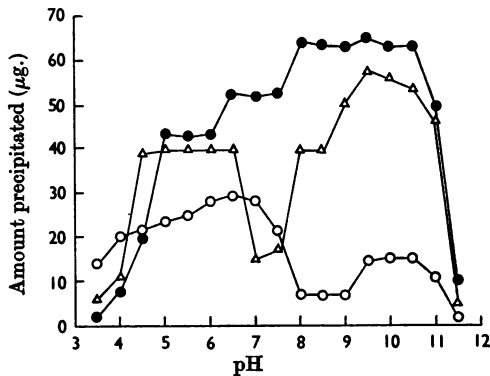
fraction 2 than with fraction 1. Maximum precipitation occurred with a whole histone/acidic protein weight ratio of about 1:1.

When 50 μg. of histone was titrated with increasing amounts of acidic protein (Fig. 6), there was a linear increase in the amount of protein precipitated up to a ratio of 1 μg. of histone to 4–5 μg. of acidic protein, indicating that, per unit weight, the histone contained 4–5 as many binding sites for the acidic protein as the latter had for the former.

The complex was soluble in 10 mM- and 0·2 M-hydrochloric acid and 1 M-sodium hydroxide and in high concentrations of salt (Fig. 3). When poly-L-lysine was used, the pH–solubility profile was characterized by a marked decrease at pH 7 that was eliminated if 1 mM-EDTA was present.

Association with DNA. Table 2 shows the interaction with DNA either alone or in the presence of histone. This experiment was set up such that there was an excess of DNA relative to histone. Some competition existed between the DNA and acidic protein for histone, as the amount of protein precipitated with the DNA was less than in its absence. Similarly the amount of DNA precipitated in the presence of histone and acidic protein was less than with histone alone. The amount of protein precipitated/μg. of DNA increased in the presence of the acidic protein, indicating the formation of a DNA–histone–acidic protein complex. This experiment was repeated a further three times with similar results, except that the actual amount of protein precipitated varied from batch to batch of the acidic protein.

Owing to the crudeness of the estimation methods, this did not provide conclusive evidence as to whether the acidic fraction did or did not form complexes with DNA. This was checked by using

Fig. 4. Effect of pH on protein–polycation interaction. Equal amounts (50 μg.) of the two reactants were mixed in a final volume of 0·5 ml. of buffer and the precipitate was sedimented at 800g for 10 min. The following buffers were used: pH 3·5–6·5, 10 mM-sodium citrate–HCl; pH 7·0–9·0, 10 mM-tris–HCl; pH 9·5–11·5, 10 mM-glycine–NaOH. ●, Fraction 2 + calf thymus histone; △, fraction 2 + poly-L-lysine; ○, phosvitin + poly-L-lysine.
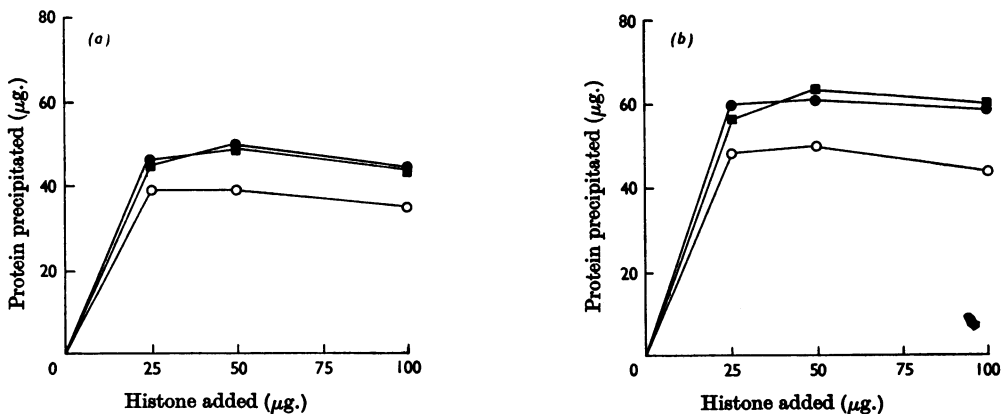

Fig. 5. Effect of increasing amounts of calf thymus histone on its interaction with acidic protein fraction 1(a) or fraction 2(b). Calf thymus histone was mixed with 50 μg. of acidic protein in a final volume of 0·5 ml. of 10 mM-tris–HCl buffer, pH 8·5. ■, Whole histone; ●, histone F3; ○, histone F1.

Table 2. *Interaction of DNA, histone and acidic protein*

DNA (50 μg.) was added to each tube except those shown in the bottom line. The reaction was carried out in 0·25 ml. of 10 mM-tris–HCl buffer, pH 8·5.

Precipitate	Histone (50 μg.)	No acidic protein	Fraction 1 (50 μg.)	Fraction 1 (250 μg.)	Fraction 2 (50 μg.)	Fraction 2 (250 μg.)
Total protein (μg.)	+	52	76	122	84	202
	−	0	10	8	8	10
Total DNA (μg.)	+	17	13	10	15	13
	−	0	2	2	2	2
Protein (μg.)/DNA (μg.)	+	3·1	5·9	12·2	5·7	15·5
Protein precipitated in the absence of DNA (μg.)	+		176		226	

fractions that had been labelled *in vivo* with [³H]-tryptophan. The presence of DNA did not influence the sedimentation pattern of either fraction 1 or fraction 2 in a 5–20% sucrose gradient, which supports the conclusion that there was no DNA–acidic protein interaction under these conditions. Neither fraction showed any definite sedimentation peaks, most of the radioactivity being in the upper third of the gradient. Under these conditions the DNA migrated half way down the gradient.

Further evidence for the lack of DNA–acidic protein interaction was the observation that fraction 2 did not alter the T_m of DNA (75 μg. of protein + 75 μg. of DNA).

Influence of fraction 2 on the template activity of DNA for RNA polymerase. These experiments were set up to give maximal histone inhibition (20 μg. of histone + 10 μg. of DNA). All the reactants except the DNA, [³H]UTP (obtained from The Radiochemical Centre) and RNA polymerase were mixed and kept at 0° for 10 min. to allow histone–acidic protein interaction to occur. The other reactants were then added and the tubes incubated for 20 min. at 37°. In separate experiments it was shown that this incubation medium (minus DNA) allowed complete interaction between the two types of protein, whereas the medium of higher ionic strength described by Fuchs, Millette, Zillig & Walter (1967) did not. Histone/acidic protein ratios of 1:1 and 1:5 were tested. Low concentrations of fraction 2 had very little effect on the template activity of DNA in either the presence or the absence of histone (Table 3). The higher concentration of fraction 2 occasionally had a small inhibitory effect on its own but gave some release of the histone inhibition. This experiment was repeated a further three times with each tissue with essentially similar results, except that the effect of the 100 μg. of acidic protein on the DNA–histone varied from 28% to 76% for liver and from 28% to 44% for the tumour. Different batches of acidic fraction were prepared for each experiment.
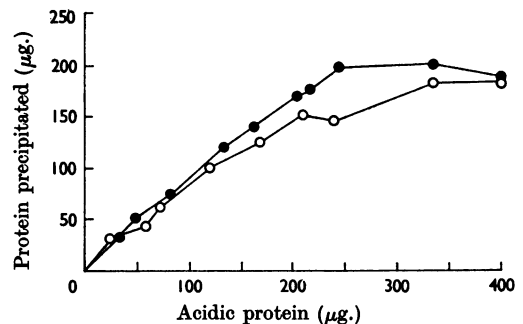

Fig. 6. Effect of increasing amounts of acidic protein on their interaction with calf thymus histone. Details are as given in Fig. 5. ○, Fraction 1; ●, fraction 2.

Table 3. *Effect of acidic and basic proteins on the template activity of DNA for RNA polymerase*

All tubes contained 0·1 μmole each of ATP, GTP, CTP and [³H]UTP, 1·25 μmoles of MnSO₄, 5 μmoles of MgCl₂, 10·5 μmoles of tris buffer, 5 μmoles of β-mercaptoethanol and 10 μg. of DNA in a final volume of 0·25 ml. In the absence of the additions shown in the table this incorporated 340 pmoles of UMP/20 min.

Additions	Template activity (% of that with DNA alone) Liver	Template activity (% of that with DNA alone) Tumour
Histone (20 μg.)	14	18
Fraction 2 (20 μg.)	96	84
Fraction 2 (100 μg.)	79	108
Histone (20 μg.) + fraction 2 (20 μg.)	23	8
Histone (20 μg.) + fraction 2 (100 μg.)	76	44
Histone (20 μg.) + fraction 2 (100 μg.); no polymerase	10	4
Histone (20 μg.); no polymerase	10	4

Precipitation of bound [6,7-³H]*oestradiol-17β with protamine sulphate.* As the nuclear oestradiol-binding protein fraction from mammary tumour formed an insoluble complex with protamine sulphate it was decided to test whether the oestradiol-binding proteins from other tissues reacted in the same way.

Table 4 shows that protamine sulphate precipitated an appreciable amount of the radioactivity from nuclear extracts of uterus, kidney and mammary tumour. The precipitation step was carried

Table 4. *Precipitation of radioactivity from tissue extracts by protamine sulphate*

A 0·4ml. portion of each fraction was mixed with 0·2ml. of protamine sulphate (7·5mg./ml.) at 0° for 10min. Results represent the means ± s.E.M. of at least four determinations.

Tissue	Fraction	% of radioactivity in fraction precipitated with protamine sulphate
Uterus	Nucleus	60·6 ± 3·0
	Supernatant	86·8 ± 1·8
Mammary tumour	Nucleus	37·5 ± 3·1
	Supernatant	31·3 ± 4·4
Kidney	Nucleus	43·8 ± 3·2
	Supernatant	34·0 ± 1·5
Serum	—	5·7 ± 0·3
Bovine serum albumin	—	0·7 ± 0·1

out in a final concentration of 0·32M-potassium chloride, which appreciably inhibits histone–acidic protein interaction (Fig. 3). It is likely therefore that the amounts of protein and radioactivity precipitated in these experiments represent submaximal values.

Radioactivity was also precipitated from the supernatant fractions, but as these were carried out at lower ionic strength than the nuclear extracts these values should represent maximal precipitation. The absence of precipitation of radioactivity from solutions of bovine serum albumin and rat plasma containing comparable amounts of protein and [6,7-³H]oestradiol-17β indicates that this is not a non-specific interaction. To check this in a more definitive manner the effect of protamine sulphate precipitation on the sedimentation behaviour of the radioactive material on sucrose gradients was investigated (Fig. 7). As far as the supernatant fractions were concerned, protamine sulphate precipitated all of the 9·5s component from both tumour and uterus and a large part of the radioactivity in the 3–6s region. This was virtually complete with uterus but less so with tumour and kidney, which agrees with results given in Table 4. This difference may be due to albumin binding. Serum albumin does bind [6,7-³H]-oestradiol-17β, it sedimented in this region of the gradient and was not affected by protamine
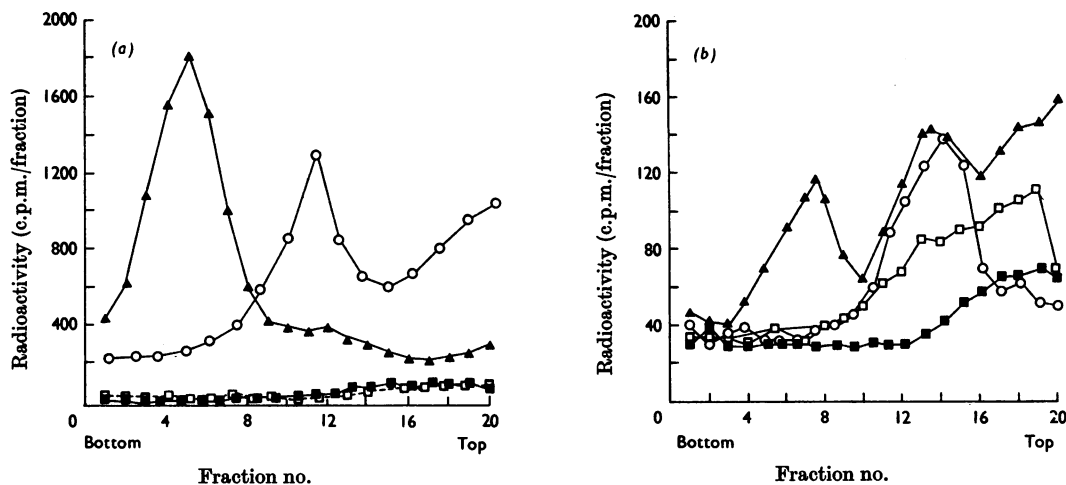

Fig. 7. Sedimentation profiles of tissue extracts labelled with [6,7-³H]oestradiol-17β. The gradients [5–20% (w/v) sucrose in 10mM-tris–HCl buffer, pH7·4] were centrifuged for 16–18hr. at 39000rev./min. in the Spinco SW39 rotor. The gradients for the nuclear extracts in addition contained 0·3M-KCl. (a) Uterus labelled *in vitro* with 1nM-[6,7-³H]oestradiol-17β. ▲, 10⁵g supernatant; ■, 10⁵g supernatant after protamine sulphate treatment; ○, 0·5M-KCl extract of nuclei; □, 0·5M-KCl extract of nuclei after protamine sulphate treatment. (b) Mammary tumour labelled *in vivo* with [6,7-³H]oestradiol-17β. ▲, 10⁵g supernatant; ■, 10⁵g supernatant after protamine sulphate treatment; ○, 0·5M-KCl extract of nuclei; □, 0·5M-KCl extract of nuclei after protamine sulphate treatment.

sulphate. As judged from the haemoglobin colour, the tumour and kidney supernatant fractions contained some blood whereas the uterus contained very little.

Under the conditions used for extraction of the nuclei, only a 5s component was obtained and the amount of it was markedly diminished by protamine sulphate treatment.

DISCUSSION

The evidence presented here provides further proof that the nuclear oestradiol-receptor protein in uterus, kidney and dimethylbenzanthracene-induced rat mammary adenocarcinoma is acidic (King, 1967). The ability of both it and the cytoplasmic receptor to form complexes with protamine sulphate indicates some similarity in the properties of the nuclear and cytoplasmic receptor(s). It is noteworthy that the androgen-receptor protein in the cytoplasm of rat prostate gland is also precipitated by protamine sulphate (W. I. P. Mainwaring, personal communication).

The presence of protein-bound phosphate in the nuclear oestradiol receptor fraction is of interest in view of the possible role of these proteins in metabolic control processes (Kleinsmith, Allfrey & Mirsky, 1966; Langan, 1967), but it remains to be seen whether the phosphate is actually present in the binding protein or merely a contaminant of this, as, yet, impure fraction. The enzyme phosphoprotein phosphatase does not alter the binding characteristics of either the nuclear or the cytoplasmic receptors, which indicates that phosphate is not involved in oestradiol-17β binding (R. J. B. King, unpublished work).

The acidic protein fractions described here represent a very small proportion of the nuclear acidic proteins present in the nucleus. The total nuclear acidic protein in liver is about 2mg./g. wet wt. (Steele & Busch, 1963) and in mammary tumours about 8mg./g. wet wt. (Smith, King, Meggitt & Allen, 1966). Allowing for a 50% yield of nuclei in our experiments, the amount of protein recovered in fractions 1 and 2 combined would represent about 10% of the total nuclear acidic protein. Even so, the two fractions are still heterogeneous. Fraction 1 contained a number of minor protein bands on polyacrylamide-gel electrophoresis in addition to the two main components. Although fraction 2 ran as a single component on polyacrylamide gel, it too is heterogeneous, as judged from its sedimentation behaviour in a sucrose gradient. The ready solubility of both fractions in dilute tris and the absence of detectable protein migrating at pH 4 indicated that very little, if any, basic protein was present.

The interrelationship of fractions 1 and 2 is not clear. In mammary tumour they both contained bound oestradiol-17β and, as judged from their electrophoretic mobility, fraction 1 contains one component that is the same as fraction 2. However, this cannot be explained by poor fractionation on the hydroxyapatite because, if fraction 1 is rechromatographed on a fresh column, it is still eluted with 0·1 M-phosphate. Both fractions react with polycations, although fraction 1 does this less efficiently than fraction 2. This may be due to the greater heterogeneity of this fraction rather than to an intrinsic property of the acidic protein present in it.

The ability to form insoluble complexes with polycations is similar to that reported by other workers (Langan, 1967; Wang & Johns, 1968), but differs in the greater efficiency of precipitation in our experiments. This may be due to the study of different proteins or to more homogeneous preparation. The formation of this complex is markedly affected by ionic strength and pH. The interaction is completely inhibited by 0·6 M-sodium chloride, and 85% inhibition occurs with 0·3 M-sodium chloride. Once the complex has been formed a higher salt concentration is required to dissociate it. The solubility of the complex in salt solutions would explain the previous observation that a high salt concentration was required to solubilize the nuclear oestradiol-17β-receptor protein even after removal of DNA with deoxyribonuclease (King & Gordon, 1967). The effect of ionic strength on the association suggests that ionic bonds are primarily involved, and this is supported by the pH effect. Carboxyl-group ionization could account for the dissociation in acid. Changes occurring at about pH 6 and 7·5 are also involved. The differences in the pH curves for the interaction of acidic protein with whole histone and with poly-L-lysine suggest that some of the interaction at intermediate pH values is due to non-lysine residues of the histone. As both fractions contain RNA and protein-bound phosphate it is possible that these may also play a part in the interaction. Bonner (1967) has described an acidic protein present in chromatin that contains RNA, and the protein described here has some of the properties of this 'histone-binding protein'. However, the lack of effect of ribonuclease and the enhancement of RNA–histone interaction by Mg^{2+} suggests that RNA is not involved in the present experiments. As no RNA was detected in the protein band on gel electrophoresis it is possible that the RNA in these fractions is an impurity rather than being associated with the protein. The involvement of protein-bound phosphate is less clear. The phosvitin–poly-L-lysine interaction has some of the characteristics of the poly-L-lysine–acidic protein interaction, but differences do exist. The decrease in binding of phosvitin at neutral pH cannot be

overcome by EDTA whereas that of the acidic protein can. The ability of the acidic proteins to form complexes with at least three polycations indicates that no great specificity is involved as far as the cation is concerned, but some specificity must exist for the acidic protein because acidic proteins such as serum albumin do not form insoluble complexes with the polycations used in these experiments.

The stoicheiometry of the histone–acidic protein interaction is also of interest as, on a weight-for-weight basis, the whole histone has about 4 times as many binding sites for the acidic protein as the latter has for the former.

The evidence presented here suggests that these particular acidic proteins do not associate with DNA, as do some of the bacteriophage (Ptashne & Hopkins, 1968) and liver nuclear acidic proteins (Wang, 1967), but that triplex DNA–histone–acidic protein molecules can be formed with some competition between the DNA and acidic protein for the histone binding sites. The precipitation experiments indicate that DNA–histone interaction takes preference over histone–acidic protein interaction. This is supported by the experiments on the template activity of the DNA. Even though histone–acidic protein interaction was allowed to take place before addition of the DNA template, a fivefold weight excess of acidic protein to histone was required to produce any marked relief of the histone blocking of the DNA template.

As far as can be judged, the acidic protein fractions from dimethylbenzanthracene-induced mammary tumour possessed similar properties to those from liver. As judged by the precipitation of oestradiol-17β-receptor protein from uterus and kidney by protamine sulphate the same is true of these tissues, but the relevance of the properties reported in this paper to the mechanism of oestradiol action remains to be investigated. It is compatible with an effect of the hormone on the chromatin (King, 1967; Warren & Barker, 1967; Hamilton, 1968), and it is tempting to speculate that oestradiol-17β may alter the structure of the chromatin by affecting the interaction of DNA, histone and the acidic proteins.

REFERENCES

Allfrey, V. G., Littau, V. C. & Mirsky, G. E. (1964). *J. Cell Biol.* **21**, 213.

Baille, L. A. (1960). *Int. J. appl. Radiat.* **8**, 1.

Bonner, J. (1967). In *Biochim. biophys. Acta Library vol. 20: Symposium on Regulatory Mechanisms in Nucleic Acid and Protein Synthesis*, p. 211. Ed. by Koningsberger, V. V. & Bosch, L. Amsterdam: Elsevier Publishing Co.

Burton, K. (1956). *Biochem. J.* **62**, 315.

Chamberlin, M. & Berg, P. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 81.

Crambach, A., Reisfeld, R. A., Wyckoff, M. & Zaccari, J. (1967). *Analyt. Biochem.* **20**, 150.

Dawson, R. M. C. & Elliott, W. H. (1959). In *Data for Biochemical Research*, p. 208. Ed. by Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. Oxford: Clarendon Press.

Fuchs, E., Millette, R. L., Zillig, W. & Walter, G. (1967). *Europ. J. Biochem.* **3**, 183.

Greenbaum, A. L. & Slater, T. F. (1957). *Biochem. J.* **66**, 155.

Hamilton, T. H. (1968). *Science*, **161**, 649.

Huggins, C., Briziarelli, G. & Sutton, H. (1959). *J. exp. Med.* **109**, 25.

Jensen, E. V., Suzuki, T., Kawashima, T., Stumpf, W. E., Jungblut, P. W. & DeSombre, E. R. (1968). *Proc. nat. Acad. Sci., Wash.*, **59**, 632.

Johns, E. W. (1964). *Biochem. J.* **92**, 55.

King. E. J. & Wooton, I. D. P. (1956). *Micro-analysis in Medical Chemistry*, pp. 75–81. 3rd ed., London: J. and A. Churchill Ltd.

King, R. J. B. (1967). *Arch. Anat. microsc. Morph. exp.* **56**, 570.

King, R. J. B. & Gordon, J. (1966). *J. Endocrin.* **34**, 431.

King, R. J. B. & Gordon, J. (1967). *J. Endocrin.* **39**, 533.

King, R. J. B. & Gordon, J. (1968). *J. Endocrin.* **40**, 195.

King, R. J. B., Gordon, J., Cowan, D. M. & Inman, D. R. (1966). *J. Endocrin.* **36**, 139.

Kleinsmith, L. G., Allfrey, V. E. & Mirsky, A. E. (1966). *Proc. nat. Acad. Sci., Wash.*, **55**, 1182.

Langan, T. A. (1967). In *Biochim. biophys. Acta Library vol. 20: Symposium on Regulatory Mechanisms in Nucleic Acid and Protein Synthesis*, p. 232. Ed. by Koningsberger, V. V. & Bosch, L. Amsterdam: Elsevier Publishing Co.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

McAllister, H. C., Wan, Y. C. & Irvin, J. J. (1963). *Analyt. Biochem.* **5**, 321.

Ptashne, M. & Hopkins, N. (1968). *Proc. nat. Acad. Sci., Wash.*, **60**, 1282.

Richards, E. G., Coll, J. A. & Gratzer, W. B. (1965). *Analyt. Biochem.* **12**, 452.

Rochefort, H. & Baulieu, E. E. (1968). *C. R. Acad. Sci., Paris*, **267**, 662.

Smith, J. A., King, R. J. B., Meggitt, B. F. & Allen, L. N. (1966). *Brit. J. Cancer*, **20**, 335.

Steele, W. J. & Busch, H. (1963). *Cancer Res.* **23**, 1153.

Toft, D. & Gorski, J. (1966). *Proc. nat. Acad. Sci., Wash.*, **55**, 1574.

Velle, W. & Engel, L. L. (1964). *Endocrinology*, **74**, 429.

Wang, T. Y. (1967). *Arch. Biochem. Biophys.* **122**, 629.

Wang, T. Y. (1968). *Exp. Cell Res.* **53**, 288.

Wang, T. Y. & Johns, E. W. (1968). *Arch. Biochem. Biophys.* **124**, 176.

Warren, J. C. & Barker, K. L. (1967). *Biochim. biophys. Acta*, **138**, 421.

Young, S., Cowan, D. M. & Sutherland, L. E. (1963). *J. Path. Bact.* **85**, 331.