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Nucleoproteins

2. FRACTIONATION OF DEOXYRIBONUCLEOPROTEINS INTO ACIDIC PROTEINS AND LYSINE-RICH HISTONES ASSOCIATED WITH DEOXYRIBONUCLEIC ACID*

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A study of the action of salts and phenol on mammalian tissues has shown that the ability to separate the DNA from the protein depends on the nature of the salt used (Kirby, 1957). Hydrophilic salts (0.3M) released very little DNA, whereas lipophilic salts released DNA to which some protein remained attached. Salts that had both lipophilic and chelating properties liberated DNA with the minimal amount of residual protein.

Sodium benzoate in combination with phenol released a DNA-protein complex from rat liver, the proportion of protein being dependent on the molarity of the benzoate used (Kirby, 1958). This reaction has been extended to other tissues and compared with the reaction of sodium phenolphthalein diphosphate.

Amino acids were previously estimated (Kirby, 1958) as their Cu^{2+} ion complexes after separation by paper chromatography and paper electrophoresis. The colours tended to fade and the results were not always reproducible. Solvent systems have been found that will separate all the amino acids by two-dimensional paper chromatography, and the ninhydrin products formed complexes with Cd^{2+} ions that were stable for 24 hr. This method of estimating amino acids in nucleoprotein complexes is much more reliable than the previous one.

* Part 1: Kirby (1958).

MATERIALS AND METHODS

Purification of alkoxyethanols and the origin of the transplantable tumours were as described by Kirby (1959).

Estimation of amino acids. Deoxyribonucleoprotein (9–10 mg.) was hydrolysed with 5.8N-HCl (0.5 ml.) in a sealed tube at 110° for 24 hr. The tube was opened, centrifuged to remove insoluble material, the supernatant quantitatively transferred to a small flask (B10 joint) and the HCl removed by rotary evaporation at 40° for 15 min. Acetic acid (20%, v/v) was added several times and evaporated to remove residual HCl. The residue was dissolved in 20% acetic acid (0.1 ml.) and 0.01 or 0.02 ml. was used for each chromatogram (on Whatman no. 1 paper). The solvent for first-dimensional chromatography was butan-2-one-acetic acid-water (14:6:5, by vol.). After upward development for 16 hr. the papers were dried and developed in the second dimension with butan-2-one-2-methylpropan-2-ol-water-aq. NH_3 (sp.gr. 0.88) (60:16:24:3, by vol.). This solvent was made up at least 24 hr. before use, and 2 hr. before chromatography a small beaker containing aq. NH_3 (sp.gr. 0.88) was placed in the tank and then removed when the papers were immersed.

The papers were dried in a fume cupboard for 48 hr. at 20° (drying for a shorter time led to high blank values because of residual NH_3) and dipped in a solution of ninhydrin (0.2%) in acetone. The colours developed during 48 hr. at 20°. The purple spots were cut out and the colours eluted with a mixture of 5 ml. of a solution of CdCl_2 (analytical reagent) (1 g.) in water (5 ml.), redistilled 2-methoxyethanol (95 ml.) and acetic acid (1 ml.). After 1 hr.

the colours (which were stable for 24 hr.) were read against an appropriate blank at 510 m μ .

Tryptophan was estimated by the method of Spies & Chambers (1949), and total protein by the Folin method as described by Lowry, Rosebrough, Farr & Randall (1951).

Other analytical methods. DNA was determined by Burton's (1956) modification of the Dische (1930) reaction and the determination of phosphorus was as described by Kirby (1957).

Preparation of the deoxyribonucleic acid-protein complexes. The methods were the same as described by Kirby (1958), either sodium benzoate or sodium phenolphthalein diphosphate (15 mM; pH 8) being used to homogenize the tissue. After extraction with 90% (w/v) phenol considerable care was taken in centrifuging, to avoid losses of the DNA-protein complexes. Centrifuging at 20000 rev./min. was carried in a Spinco model L centrifuge.

Rat-liver nuclei. These were prepared by the method of Dounce (1955), Mirsky & Pollister (1946) or Kay, Simmons & Dounce (1952).

Action of deoxyribonuclease. The DNA-protein complex (160 mg.; protein content approx. 1%; prepared by the action of 0.35 M-sodium benzoate and phenol on rat liver) was dissolved in 1 mM-sodium acetate, made up to 160 ml. (with 10 mM-sodium acetate) and MgSO₄ (32 mg.) added. Deoxyribonuclease (1.9 mg.) was added and there was an immediate fall in viscosity and separation of an insoluble material. The mixture was shaken for 15 min., the solid separated by centrifuging, washed with ethanol-water and ethanol and dried over CaCl₂ *in vacuo*. The reaction was similarly carried out with DNA-protein complexes in which 0.8, 26.0 and 30.0% of protein was present (the latter two were prepared from rat liver by the action of 70 mM-sodium benzoate and phenol).

Action of trypsin. The DNA-protein complex (86 mg.) was dissolved in water (50 ml.), and 0.134 M-Sørensen phosphate buffer, pH 7.7 (50 ml.), and trypsin (1.3 mg.) were added. The mixture was incubated at 37° for 19 hr., after which the fragmented peptides were either removed by dialysis or extraction with 90% phenol. The DNA was finally precipitated by 2-ethoxyethanol.

Preparation of 2,4-dinitrophenyl derivatives. The DNA-protein complex (100 mg.) was dissolved in water (50 ml.), and a solution of NaHCO₃ (200 mg.) in water (5 ml.) and 1-fluoro-2,4-dinitrobenzene (200 mg.) in 2-methoxyethanol (5 ml.) were added. The solution was shaken for 4–5 hr. and then left for 16 hr. at 2°. The pH was brought to 6 by the careful addition of 20% acetic acid, then dinitrophenol was

removed by extraction with butanol, and the dinitrophenylated DNA-protein complex was precipitated as yellow fibrous material by adding an equal volume of 2-ethoxyethanol. The product was isolated and dried as usual. The yield was about 90 mg.

The dinitrophenylated complex (70 mg.) was hydrolysed with 5.8 N-HCl (1 ml.) for 20 hr. at 105°. The HCl was removed, and after solution in water (0.5 ml.) the extract was separated into ether-soluble and water-soluble fractions. The DNP-amino acids were extracted from the aqueous phase with butan-1-ol or butan-2-ol. The DNP-amino acids were chromatographed on paper by using butan-2-ol saturated with 50 mM-phthalate buffer, pH 6 (Phillips, 1958), 3-methylbutan-1-ol saturated with 1% (v/v) acetic acid or butan-1-ol-acetic acid-water (5:1:4, by vol.). Standard preparations of DNP-amino acids were run on the same paper. ϵ -DNP-lysine was identified by its R_f value and its reaction with ninhydrin.

RESULTS

Amino acids. The following values were found for the extinction of 0.01 mg. of each amino acid dissolved in 5 ml. of the cadmium chloride solution: histidine, 0.20; lysine, 0.25; arginine, 0.26; aspartic acid, 0.19; glutamic acid, 0.315; alanine, 0.50; serine, 0.45; threonine, 0.39; tyrosine, 0.22; leucine, 0.38; isoleucine, 0.40; phenylalanine, 0.19; methionine, 0.36; valine, 0.42. After hydrolysis of bovine plasma albumin and use of the above values for estimating the proportions of the amino acids, good agreement was found with the proportions given by Moore & Stein (1949). The recovery of amino acids was about 75–80%.

Isolation of deoxyribonucleic acid-protein complexes. The yields and analyses of the DNA-protein complexes isolated from rat liver by the action of 70 mM-benzoate and phenol are shown in Table 1. No DNA-protein was isolated if the concentration of the benzoate was decreased to 35 mM.

When phenolphthalein diphosphate (15 mM) was used to extract the complex the yields were as follows: rat liver, 200 mg.; rat spleen, 1000 mg.; MA and QS tumours, 500 mg. The complexes from rat liver and spleen each had about 0.2% of protein whereas those from the tumours had 4–8% of protein. The amino acid compositions of the DNA-protein complexes isolated by using 70 mM-benzoate and phenol are shown in Table 2. Estimation of tryptophan in the DNA-protein complex of rat liver showed that about 1% of tryptophan was present in the protein.

Analyses of the DNA-protein complexes isolated by the methods of Dounce (1955) and Kay *et al.* (1952) from rat-liver nuclei are shown in Table 3.

When the DNA-protein complexes were centrifuged at 20000 rev./min. for 5 hr. sedimentable and supernatant fractions were obtained which contained different proportions of DNA. The amounts obtained after fractionation are shown in Table 4.

Table 1. *Composition of deoxyribonucleic acid-protein complexes*

The complexes were isolated by the action of 70 mM-benzoate and phenol. The proportion not accounted for was water.

Tissue	Yield of DNA-protein complex (mg./100 g. of tissue)	Composition of DNA- protein complex (%)	
		Protein	DNA
Rat liver	237	26.7	53
Rat spleen	920	3.4	81
Mouse liver	180	19.7	61
Mouse spleen	1350	12.5	73
MA tumour	350	17.0	65

Table 2. *Amino acid compositions of deoxyribonucleic acid-protein complexes in Table 1*

Amino acids are expressed as moles/100 moles of all amino acids found. Cystine and proline were present but were not determined.

Tissue	...	Amino acid composition of DNA-protein complex				
		Rat liver	Rat spleen	Mouse liver	Mouse spleen	MA tumour
Histidine		3.7	5.3	3.6	3.2	4.1
Lysine		10.2	13.2	14.8	14.3	14.2
Arginine		5.6	4.4	5.2	4.0	7.6
Aspartic acid		16.2	19.4	12.8	8.3	13.6
Glutamic acid		13.6	14.9	14.0	10.4	15.9
Alanine		12.7	10.0	11.9	13.9	13.6
Serine		8.4	9.6	8.4	8.0	8.2
Threonine		5.7	6.2	5.7	9.0	5.9
Tyrosine		4.7	3.4	2.4	4.1	0.0
Valine		4.5	4.4	6.4	6.5	5.9
Leucine + isoleucine		10.6	7.0	11.5	12.2	8.9
Phenylalanine		4.1	2.2	3.3	6.1	2.7

Table 3. *Amino acid composition of deoxyribonucleic acid-protein complexes from rat-liver nuclei*

Amino acids are expressed as moles/100 moles of all amino acids found. Amino acids not in the Table were present in similar proportions to those given in Table 2.

Method of preparation	...	Amino acid composition of DNA-protein complex	
		Dounce (1955)	Kay <i>et al.</i> (1952)
Yield of DNA-protein complex (mg./100 g. of tissue) ...	...	89	138
Protein (%) ...	...	13.8	13.4
Histidine		2.7	2.3
Lysine		14.7	12.0
Arginine		5.4	5.8
Aspartic acid		14.4	10.3
Glutamic acid		14.7	14.0
Alanine		16.0	13.8

Table 4. *Fractionation of deoxyribonucleic acid-protein complexes by centrifuging*

The DNA-protein complexes were all prepared by the action of 70 mM-benzoate and phenol. Fractionation was carried out by centrifuging solutions of the complexes (1–2 mg./ml.) in 10 mM-sodium acetate for 5 hr. at 20000 rev./min. The values in parentheses indicate the percentage of DNA in the fraction.

Tissue	Composition of DNA-protein complex (%)	
	Sedimentable fraction	Supernatant fraction
Rat liver	45.4 (28.0)	54.6 (74.9)
Mouse liver	31.0 (27.2)	69.0 (71.5)
Mouse spleen	22.5 (37.7)	77.5 (68.5)
MA tumour	32.0 (25.0)	68.0 (68.0)

The amount of protein associated with DNA in the supernatant fraction was about 2%. The proportions of amino acids in the sedimented complex were similar to those of the starting material, and

the proportions of amino acids in the supernatant fractions were as shown in Table 5.

The proportions of amino acids present in the supernatant fractions from the DNA-protein complexes of rat-liver nuclei isolated by the two methods are shown in Table 6.

The amount of protein associated with the DNA was about 2.0%. A different type of protein remained with the DNA when 280 mM-benzoate was used compared with 70 mM-benzoate.

The DNA-protein complexes isolated from MA and QS tumours with 15 mM-phenolphthalein di-phosphate and phenol did not sediment as easily as the complexes isolated with 70 mM-benzoate, and it was necessary to centrifuge for 12 hr. at 30000 rev./min. (Spinco model L). Amino acid analyses of the fractions (see Table 7 for results with material from MA tumour) indicated there was little difference between them. About 50% of the material from the MA tumour sedimented and contained about 17% of protein; the supernatant fraction had 5% of protein. Amino acid analyses of the fractions from QS tumour were almost the same and the sedimented material (34%) had 11% of protein and the supernatant fraction (66%) had 4%.

Action of deoxyribonuclease. The addition of deoxyribonuclease brought about an immediate precipitation of material that proved to be protein to which about 1% of DNA remained attached. Table 8 shows that the precipitation of protein was almost quantitative. The amino acid analyses of the protein which separated were almost the same as those in Table 2.

Action of trypsin. The addition of trypsin to a solution of the DNA-protein complexes from rat liver (isolated with 70 mM-benzoate) clarified the solution. When ethoxyethanol was added the DNA which separated contained about 8% of protein, but when the material was extracted with phenol first and ethoxyethanol added to the aqueous phase DNA was isolated with about 2% of protein.

Table 5. *Amino acid compositions of deoxyribonucleic acid-protein complexes of the supernatant fractions*

The fractions were isolated from the supernatant solution after centrifuging solutions of the complexes at 20000 rev./min. Amino acids are expressed as moles/100 moles of all amino acids found. Amino acids not in the Table were present in similar proportions to those given in Table 2.

	Tissue	...	Amino acid composition of DNA-protein complex			
			Rat liver	Mouse liver	Mouse spleen	MA tumour
Histidine			2.2	1.5	1.8	0.3
Lysine			13.7	15.9	28.0	19.6
Arginine			4.1	4.5	2.4	5.2
Aspartic acid + glutamic acid			47.6	34.9	16.1	23.1
Alanine			11.8	12.3	26.2	20.8

Table 6. *Amino acid composition of the deoxyribonucleic acid-protein complex from the supernatant fraction from rat-liver nuclei*

The fractions were isolated from the supernatant solution after centrifuging solutions of the complexes at 20000 rev./min. Amino acids are expressed as moles/100 moles of all amino acids found. Amino acids not in the Table were present in similar proportions to those reported for rat liver in Table 2.

Method of isolation of nuclei	...	Amino acid composition of DNA-protein complex		
		Mirsky & Pollister (1946)	Kay <i>et al.</i> (1952)	
Extraction of DNA-protein complex	...	70 mM-Benzoyate	70 mM-Benzoyate	280 mM-Benzoyate
Histidine		1.6	1.2	3.7
Lysine		26.4	25.9	14.0
Arginine		4.4	2.9	7.4
Aspartic acid + glutamic acid		22.3	14.8	32.2
Alanine		26.1	29.6	12.5

Table 7. *Amino acid composition of deoxyribonucleic acid-protein complex from MA tumour*

The complex was isolated by the action of 15 mM-phenolphthalein phosphate and phenol. The amino acids are expressed as moles/100 moles of all amino acids found.

	Amino acid composition of DNA-protein complex	
	Supernatant fraction	Sedimentable fraction
Histidine	1.1	3.5
Lysine	21.1	21.0
Arginine	9.3	12.4
Aspartic acid	15.1	11.0
Glutamic acid	17.7	18.3
Alanine	15.5	10.0
Threonine	5.8	5.0
Valine	4.7	6.0
Isoleucine	3.0	3.7
Leucine	5.8	6.6
Phenylalanine	1.3	2.5

incubated at 37° for 24 hr. and the insoluble precipitate was isolated by centrifuging, washed with water and then ethanol, and dried. The yield was 61 mg. (3.48 % of P; 58 % of protein; 11 % of moisture; thymine : cytosine ratio 1.42; traces of adenine and guanine were also present).

Dinitrophenyl complexes. 1-Fluoro-2,4-dinitrobenzene reacted readily with the DNA-protein complexes, but did not react at all with DNA that was free from protein. A solution of the dinitrophenylated complex in 0.2M-sodium chloride had $\lambda_{\max}$ at 258 m μ and 360 m μ .

After hydrolysis with 5.8N-hydrochloric acid for 20 hr. at 105° and separation of DNP-amino acids into ether-soluble and water-soluble fractions, the ether-soluble fractions contained two components that could be separated by paper chromatography. The major one had the same R_f as DNP-glutamic acid in three different solvent systems; the minor one has not yet been identified.

It appears from the amino acid analysis of the dinitrophenylated DNA-protein complex (Table 9) as if histidine, lysine and tyrosine had reacted. ϵ -DNP-lysine could be isolated from the water-soluble fraction after hydrolysis, and it was clear from the amount that most of the yellow colour in the complex was due to this component.

Action of hydrochloric acid. The DNA-protein complex (108 mg.; isolated from rat liver with 70 mM-benzoyate and phenol; 4.75 % of P; 31.2 % of protein; thymine : cytosine ratio 1.49) was dissolved in distilled water (100 ml.) and 0.1N-hydrochloric acid (100 ml.) was added. The mixture was

Table 8. *Action of deoxyribonuclease on deoxyribonucleic acid-protein complexes*

The protein determination on the whole complex (column 2) is compared with the amount of insoluble material (column 3) separated by centrifuging after the action of deoxyribonuclease. The proportion of DNA in the insoluble material was determined by the method of Burton (1956).

Wt. of DNA-protein complex (mg.)	Wt. of protein (Lowry estimation) (mg.)	Wt. of insoluble material (mg.)	DNA in insoluble material (%)
126	1.0	0.84	0.7
161	1.6	2.1	2.1
134	34.8	23.6	0.9
144	44.6	48.7	0.9

Table 9. *Amino acid composition of dinitrophenylated deoxyribonucleic acid-protein complex from rat liver*

Amino acids are expressed as moles/100 moles of all amino acids found. Amino acids not listed were present in similar proportions to those reported for rat liver in Table 2.

Amino acid composition of dinitrophenylated DNA-protein complex	
Histidine	0.6
Lysine	1.8
Arginine	6.1
Aspartic acid	10.7
Glutamic acid	14.0
Tyrosine	1.5

DISCUSSION

DNA-protein complexes have been isolated from all tissues by the action of dilute salts and phenol, but it was clear that the amount and nature of the protein associated with the DNA that had been extracted depended very much on the tissue and the salt used for the extraction. Though the proportion of the protein in the complex from rat liver decreased as the molarity of the benzoate increased, the amount of protein associated with DNA from rat spleen was about the same whether 70 mM-benzoate or 0.3 M-*p*-aminosalicylate was used for the extraction. Moreover, extraction of MA tumour with 15 mM-phenolphthalein diphosphate yielded a complex with a higher lysine content than the product isolated by extraction with 70 mM-benzoate and yet this diphosphate removed nearly all the protein from rat-liver DNA.

The amino acid compositions of the complexes described in Table 2 showed a number of similarities but, with the exception of the complex from mouse spleen, there was a preponderance of acidic over basic amino acids. There was always less arginine than lysine, but the aspartic acid: glutamic

acid ratio varied with the tissue. When a DNA-protein complex was isolated from rat-liver nuclei with 70 mM-benzoate and phenol the amount of protein associated with the DNA was relatively richer in lysine and alanine (Table 3) but the proportion associated with the DNA was less.

Fractionation of the complexes by centrifuging showed that one fraction, containing 12–25 % of the DNA and most of the protein, could be sedimented, and that the rest of the DNA, containing about 2 % of protein, remained in the supernatant fraction.

The composition of the protein that sedimented was similar to that of the whole complex but there were considerable differences in the amino acid compositions of the supernatant fractions. There was a slight increase in the lysine content of the proteins associated with the supernatant fractions of DNA from rat and mouse livers, but the proteins associated with the supernatant fraction from mouse spleen and MA tumour were largely or partly lysine-rich histones.

The behaviour of the DNA-protein complex from rat-liver nuclei was similar to that of mouse spleen since, on fractionation by centrifuging, DNA associated with a lysine-rich histone was found in the supernatant fraction. This result was only obtained when the nuclei were extracted with 70 mM-benzoate, for when 280 mM-benzoate was used for the extraction a DNA-protein was obtained with about 3–4 % of protein and no lysine-rich histone was associated with the DNA that remained in the supernatant fraction after centrifuging this complex. This result should be compared with that of DNA-protein from rat spleen where only 3–4 % of protein remained with the DNA and no fractionation was achieved by centrifuging.

The separations can be explained if it is assumed that some of the acidic proteins form cross-links with the DNA chains and the complex is in reality a highly swollen gel. Presumably, with rat spleen extracted with 70 mM-benzoate, rat-liver nuclei extracted with 280 mM-benzoate or the tumours extracted with 15 mM-phenolphthalein diphosphate, the proteins that form the cross-links are preferentially removed and these complexes cannot be fractionated by centrifuging.

Lysine-rich histones have previously been isolated by extracting and then fractionating histones (Phillips, 1961), and their function in the chromosomal unit is by no means clear, although Busch & Davis (1958) have reported the presence of a histone in tumours which has a high rate of incorporation of lysine.

The lysine-rich histones were associated more with spleen and tumour tissues, which have a higher mitotic rate than liver, and it is possible that the presence of these histones in rat-liver nuclei may be a response of the nucleus to damage.

It is always difficult to be certain that DNA-protein complexes of this nature are entities present in the cell nucleus. That the protein was intimately associated with the DNA was indicated by the immediate and quantitative precipitation of the protein, to which a little DNA remained attached, by the action of deoxyribonuclease. This was even the case when only 0.8 % of protein remained with the DNA. Similarly dilute hydrochloric acid precipitated protein to which some DNA remained attached, whereas DNA alone was completely solubilized by the same treatment. Trypsin clarified solutions of the gel and rendered the DNA non-sedimentable, presumably by degrading the proteins that formed cross-links. Some protein still remained attached to the DNA.

Reaction of the DNA-protein complex from rat liver (isolated with 70 mM-benzoate) with 1-fluoro-2,4-dinitrobenzene yielded a dinitrophenyl derivative in which most of the ϵ -amino groups of the lysine, histidine and tyrosine were substituted and the *N*-terminal group was mainly glutamic acid. The dinitrophenyl derivative was as stable as the original complex to extraction with phenol or with methoxyethanol and phosphate buffer and could be fractionated by centrifuging in exactly the same way as the original complex.

It is likely that the acidic protein associated with the DNA is the same as 'the residual protein' isolated from nuclei by other methods (Allfrey, Mirsky & Stern, 1955; Monty & Dounce, 1958), and this and possible relationships of these nucleoproteins to deoxyribonucleic acid polymerase and ribonucleic acid polymerase associated with the chromosomal structure have been discussed by Kirby (1961). It is clear that the chromosomes must be considered as dynamic units, as in the model proposed by Jacob & Monod (1961), and what can be isolated will depend on the activities of the units at the time of extraction. It seems possible then that the different proteins that have been found associated with the DNA may be related to the different functions of the tissues investigated; Allfrey, Littau & Mirsky (1963) have reported the differential effect of arginine-rich and lysine-rich histones on the synthesis of RNA in nuclei of calf thymocytes.

SUMMARY

1. An improved method of estimating amino acids, through their Cd^{2+} ion complexes, in nucleoproteins is described.

2. DNA-protein complexes have been isolated from liver, spleen and tumour tissues by the action of 70 mM-benzoate or 15 mM-phenolphthalein diphosphate and phenol.

3. The complexes from spleen and tumour tissues (isolated with 70 mM-benzoate) could be

fractionated by centrifuging. Most of the acidic protein sedimented with the DNA, whereas DNA associated with a lysine-rich histone remained in the supernatant solution.

4. The proportion of lysine in the supernatant fraction after centrifuging complexes from liver was increased, but neither this increase nor the change in the alanine was sufficient to suggest the presence of a lysine-rich histone. A lysine-rich histone could be separated from the DNA-protein complex of rat-liver nuclei by centrifuging.

5. Phenolphthalein diphosphate (15 mM) removed most of the residual protein from liver and spleen DNA but left a lysine-rich histone associated with the DNA from tumour tissues. These complexes could not be separated by centrifuging in the same manner as those prepared with 70 mM-benzoate.

6. It is suggested that phenolphthalein diphosphate preferentially removes acidic proteins that form cross-links with DNA and render it more readily sedimentable.

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