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A New Method for the Isolation of Deoxyribonucleic Acids: Evidence on the Nature of Bonds between Deoxyribonucleic Acid and Protein

By K. S. KIRBY*
Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, S.W. 3

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When homogenized mammalian tissue was extracted with a two-phase phenol–water mixture at room temperature ribonucleic acids (RNA) were released into the aqueous layer, whereas deoxyribonucleic acids (DNA) remained completely insoluble with the denatured protein. This method has been developed as a preparative procedure for RNA (Kirby, 1956*b*).

Replacement of the water by solutions of certain anions brought DNA as well as RNA into the aqueous layer and the effectiveness of some of the salts used has been reported in a preliminary note (Kirby, 1956*a*).

Most of the tests have been carried out with rat liver, but similar results have been obtained with rat kidney, spleen, testis, a hepatoma and calf thymus glands. The tissue was homogenized with isotonic solutions of the salts, adjusted to pH 6·5 in order to avoid complications by release of nuclear materials through osmotic or pH effects. DNA was liberated when potassium cyanide or ethylenediamine was used at pH 10, but remained insoluble if the pH was 6·5.

EXPERIMENTAL

Test for the release of deoxyribonucleic acids

The salt solutions were prepared either directly from the sodium salt or by weighing the acid, titrating it with N-NaOH to pH 6·5 and adjusting to the correct volume with water. When the sodium salt was insoluble at 0·15M-concentration, as with riboflavin phosphate, nitroso-2-naphthol-3:6-disulphonate (nitroso R salt) and spermine phosphate, a saturated solution was used. Complexing agents which were insoluble in water were dissolved in 1 or 2 ml. of 2-methoxyethanol, then water was added and the mixture used with any precipitate present.

Pieces of tissue (1·5 g.) were treated with the solution of the compound to be tested (20 ml.) in an all-glass tissue grinder and the mixture was poured off from any fibrous matter which remained at the bottom of the tube. Phenol [(90%, w/w); 20 ml.] was added to the tissue mixture and the two-phase system was stirred or shaken vigorously for 1 hr. at room temperature. The extract of phenol and water was then centrifuged at 1000 *g* for from 10 min. to 1 hr., depending upon the viscosity of the aqueous layer. A volume (1 ml.) of this aqueous supernatant layer was withdrawn. Dische's (1930) reagent (2 ml.) was added and the mixture was heated on a boiling-water bath. The development of a blue colour was a positive indication of DNA. Another 1 ml. portion of the aqueous layer was withdrawn, 1 ml. of ethanol added and the mixture shaken vigorously. A fibrous precipitate showed that DNA was present. The precipitation test was essential where the Dische colour was inhibited by the salt in solution, as for example with KSCN, $Na_2S_2O_3$ and *p*-aminosalicylate. The Dische test was then applied to the precipitate after washing and dissolution.

It was essential to homogenize the tissue in the salt solution first and then to treat with the phenol, since no DNA was released by treating tissues with water and phenol and subsequently adding the effective salts.

Extraction of liver homogenates with n-*butanol and protein denaturants*

Rat liver (7·5 g.) was homogenized in water (100 ml.); 20 ml. portions were taken and the various compounds shown in Table 1 added, and each was then shaken with *n*-butanol (20 ml.). When the liver was homogenized in 0·15M-sodium salicylate and treated with the same additives as above, exactly the same results were obtained.

Rat liver (1·5 g.) was homogenized in 20 ml. of 0·15M-sodium salicylate; resorcinol (10 g.) was added and the mixture shaken, but no DNA could be detected after removal of insoluble material by centrifuging. Addition of *n*-butanol (8 ml.) and *n*-hexylresorcinol (6 g.) with the same homogenate and subsequent separation resulted in a positive test for DNA being given by the aqueous layer.

* British Empire Cancer Campaign Research Fellow.

Table 1. *Tests for release of deoxyribonucleic acids with different protein denaturants*

Rat liver (7·5 g.) was homogenized in 100 ml. of water. Portions (20 ml.) were treated with the additives shown and then shaken with 20 ml. of *n*-butanol.

Additive	Dische reaction	Addition of ethanol
None	+ −	−
Sodium dodecyl sulphate (1 g.)	+ + +	Fibrous
Cetyltrimethylammonium chloride (1 g.)	−	−
Urea (4·8 g.)	−	−
Guanidine hydrochloride (7·65 g.)	+ −	−

Absorption of deoxyribonucleic acid by collagen

Collagen (standard hide powder) was extracted with cold water in a Soxhlet apparatus overnight and then well washed with water, most of which was removed by suction at the pump.

For treating the collagen 0·1 M-solutions of potassium citrate ($K_3C_6H_5O_7,H_2O$), sodium acetate ($NaOAc,H_2O$), cobalt acetate [$CO(OAc)_2$], manganese acetate [$Mn(OAc)_2$] and zinc acetate [$Zn(OAc)_2$] were used. Copper acetate [$Cu(OAc)_2,H_2O$] and ferrous sulphate ($FeSO_4,7H_2O$) were adjusted as follows: copper acetate (10 g.) and potassium citrate (8·8 g.) were dissolved in water, N-NaOH (26 ml.) was added and the solution was made up to 500 ml. Ferrous sulphate (13·9 g.) was dissolved in 250 ml. of 0·1 M-potassium citrate; N-NaOH (10 ml.) was added and the mixture was made up to 500 ml.

The wet hide powder (4 g.) was mixed with 100 ml. of each of the above salt solutions and the mixtures were shaken occasionally during 1 hr., after which the solutions were filtered off. The metal–collagen complexes were suspended in water (100 ml.) and filtered off four times during the day, before being allowed to remain in water (100 ml.) overnight. Each collagen was then filtered off and used in this state.

Three separate batches (0·5 g. in each) of each of the treated collagens were weighed out, and to the first was added water (10 ml.), to the second rat-liver DNA solution (10 ml.) and to the third calf-thymus DNA solution (10 ml.) were added, and the mixtures were allowed to remain in a cold room. The light absorption at 258 mμ was measured after bringing the solutions to room temperature and centrifuging. The water from the collagen treated with the appropriate metal was used as a blank experiment in each case.

Isolation of deoxyribonucleic acids from rat liver

It was advantageous to allow the rats to fast for 24 hr. before removing the livers. The animals were killed by breaking their necks, the livers were removed and immediately dropped on to solid CO_2. The pooled rat livers (75 g.) were broken down in a high-speed mixer (45 sec.) with a solution of sodium *p*-aminosalicylate (600 ml. of 6%, w/v; not necessarily all added at once). The mixture was poured through a Büchner funnel to remove fibres and debris, and the filtrate was stirred while 600 ml. of 90% (w/w) phenol was added quickly. Stirring was continued for 1 hr., after which the mixture was centrifuged in an International centrifuge at 0° (200 rev./min. for 1 hr.). The supernatant was pale yellow and reasonably clear, and was removed by suction. The phenolic layer and the insoluble material were washed once with a small quantity of 6% (w/v) sodium *p*-aminosalicylate solution, and the aqueous layer was separated by centrifuging. The combined aqueous layers (400 ml.) were stirred and 2-ethoxyethanol (400 ml.) was added. The fibrous precipitate was removed with a glass rod and placed in water (100 ml.) immediately. The DNA dissolved after shaking for 30 min. and sodium *p*-aminosalicylate (6 g.) was added and the DNA was precipitated again with 2-ethoxyethanol (100 ml.). In each case a flocculent precipitate of RNA remained in the water–ethoxyethanol mixture. The DNA precipitate dissolved quickly in water (100 ml.); sodium acetate ($NaOAc,H_2O$; 4g.) was added and the DNA precipitated with 2-ethoxyethanol (100 ml.). This precipitate was dissolved in 50 ml. of water, sodium acetate (2 g.) and ribonuclease (1·5 mg. in 1 ml. of water) were added and the mixture was allowed to remain at 2° for 16 hr. The DNA was precipitated by 2-ethoxyethanol (50 ml.) and as much as possible of the solvent removed from the precipitate before it was dissolved in water (33 ml.). When dissolution was complete (15–30 min.) potassium phosphate [33 ml. of 2·5 M-K_2HPO_4 and 1·65 ml. of 33% (v/v) H_3PO_4] and 2-methoxyethanol (33 ml.) were added, and the mixture was well shaken and allowed to stand until the layers separated, when the bottom layer was run off. The top layer was separated from any insoluble material and centrifuged in polyethylene centrifuge tubes in a Servall centrifuge at 10000 *g* for 1 hr. The clear organic layer was carefully poured from any insoluble sediment, a few drops of toluene were added and the mixture was dialysed twice against water (2 l. each time), and twice against 1% sodium acetate (2 l. each time). The contents of the bag were then removed, centrifuged, made up to 4% (w/v) with respect to sodium acetate (the volume is usually about 100 ml.), and the DNA was precipitated with an equal volume of 2-ethoxyethanol. The fibrous precipitate was removed, washed twice with ethanol–water (3:1), once with ethanol and then dried over $CaCl_2$ in a vacuum desiccator. The product was a white fibrous felt ($\lambda_{max.}$ 258 mμ, moisture content 15%) which dissolved readily in water (less than 1 hr.). The yield was about 200 mg./100 g. of rat liver, which indicated that there were few losses, as Thomson, Heagy, Hutchison & Davidson (1953) reported values of 21·0–29·7 mg. of DNA P/100 g. of rat liver.

DNA has also been prepared in exactly the same way except that the livers were broken down in sodium trichloroacetate (0·3 M) or sodium benzoate (0·3 M). The yields were about the same.

DNA which had been precipitated each time with ethanol instead of 2-ethoxyethanol could be separated from ribonucleotides present by dissolution in water and precipitating twice with 2-ethoxyethanol in the presence of sodium acetate.

Determination of base ratios

The base ratios of the DNA were determined by hydrolysis with formic acid in the following manner (Vischer & Chargaff, 1948; Wyatt, 1951): DNA (2·5 mg.) was weighed into a test tube made of heavy-walled Pyrex glass (9 mm. × 70 mm.); the neck was constricted and formic acid (AnalaR; 0·5 ml. of 90%) was added. The tube was sealed at the constriction and allowed to remain until the DNA had dissolved. The tube was then placed in a large test tube containing 1:2-dichlorobenzene and fitted with a reflux condenser. The

dichlorobenzene was raised to boiling point (176°) during about 20 min. and heating was continued for another 40 min. The contents of the tube were by then a pale yellow, but no insoluble matter had separated. The tube was opened carefully, after freezing in solid CO_2, and the contents were quantitatively transferred to a small flask (B 10 standard ground-glass joint) which was attached to a rotary evaporator, and the formic acid removed at 40°. The residue was dissolved in 0·1 ml. of N-HCl, and 0·01 ml. of this solution was used for each spot on the paper for chromatography (Whatman paper no. 4). The solvents used were methanol–11·6N-HCl–water (7:2:1) (Kirby, 1955). After development (16 hr.) the paper was dried at room temperature, the spots were marked with the aid of a Hanovia Detectolite lamp (Hanovia Ltd., Slough, Bucks) and the bases eluted with 5 ml. of solvent. To elute thymine, cytosine and adenine 0·1N-HCl was used, and 0·5N-HCl for guanine. The estimations were carried out in the usual way. When P was determined, 0·02 ml. of the same solution as was used for determination of the base ratios was used, and the phosphate colour developed by addition of $HClO_4$ (60%) (in place of conc. H_2SO_4), ammonium molybdate and the reducing agent of Fiske & Subbarow (1925).

Estimation of amino acids

DNA (15 mg.) was weighed into a tube and mixed with 5·8N-HCl (0·5 ml.). The tube was sealed and the mixture allowed to stand until the DNA had dissolved, after which it was heated at 100° for 24 hr. The tube was opened, centrifuged to remove insoluble matter and the supernatant transferred to a small flask (B 10 standard ground-glass neck) which was attached to a rotary evaporator and the HCl removed at 40°. Water (0·5 ml.) was added several times until no HCl was left. A volume (0·1 ml.) of water–acetic acid (4:1, v/v) was added, and when as much as possible of the residue had been dissolved it was transferred to a small tube and centrifuged. A portion (0·01 ml.) of this solution was used for the determination of P.

Paper chromatography was carried out with Whatman no. 1 paper. A sheet 47 cm. × 16·5 cm. was used for single-way chromatography and one 47 cm. × 35 cm. for chromatography and electrophoresis. A volume (0·02 ml.) of the hydrolysis solution was applied to each paper and both were chromatographed by upward development along the longer length for 20 hr. A mixture of ethyl methyl ketone–acetic acid–water (3:1:1, by vol.) was added as a developing solvent, which rose about 40 cm. in the development time. The papers were dried at room temperature. The smaller paper was sprayed with ninhydrin solution, and the colour was allowed to develop during 48 hr., after which the paper was sprayed with a solution of copper acetate (100 mg.) in water (6 ml.) and ethanol (94 ml.), and the spots corresponding to lysine (R_F 0·11) and arginine (R_F 0·16) were removed for quantitative estimation.

The larger paper was prepared for electrophoresis as follows: molten paraffin wax was applied to a depth of about 8 mm. along the shorter lengths of the paper; holes were punched at the centres of the waxed edges and, after spraying the paper evenly with 0·033M-acetic acid, it was hung by two hooks in a Perspex electrophoresis cabinet with 0·033M-acetic acid as the conducting solution. Electrophoresis (0·8 mA, at about 260 V, constant current) was allowed to proceed for 15 hr., when the paper was removed, dried at room temperature and sprayed with ninhydrin solution and the colours were developed in the dark for 48 hr.

The ninhydrin colours were converted into their copper complexes. The pink spots were cut out, the colour was eluted with 80% (w/w) methanol–water (5 ml.) and the absorptions were read at 500 mμ on a Unicam spectrophotometer.

Standard solutions of the amino acids (0·2 or 0·1%) were chromatographed in triplicate, and the colours developed and eluted exactly as described above to relate the intensity of the colour to the amount of amino acid. The following E values were found for 0·01 mg. of amino acid in 5 ml. of 80% methanol: lysine, 0·045; arginine, 0·062; serine, 0·08; aspartic acid, 0·042; glutamic acid, 0·107; tyrosine, 0·037; phenylalanine, 0·035; leucine, 0·035.

Test for the presence of ribonucleotides in deoxyribonucleic acid preparations

DNA preparation (7·5 mg.) was heated in a sealed tube with N-HCl (0·05 ml.) for 1 hr. at 100°. A portion (0·01 ml.) of this solution was chromatographed on Whatman no. 4 paper with the solvent system ethyl methyl ketone–acetic acid–water (3:1:1, by vol.).

Ribose, if present, was detected by spraying the paper with aniline hydrogen phthalate (Partridge, 1949) and heating at 100° for 5 min.

Examination of the developed, dried paper with a Hanovia Detectolite lamp showed a brilliantly blue fluorescent spot (R_F 0·51) in addition to a number of other absorbing spots. A blue fluorescent spot with similar paper-chromatographic and ultraviolet-light-absorbing characteristics was produced by heating guanine (1 mole) with deoxyribose (3 moles) with N-HCl at 100° for 1 hr. This blue fluorescent spot had the same R_F in the above solvent and in a non-acidic one (ethyl methyl ketone–*tert.*-butanol–water 60:25:15) (R_F 0·4) as that produced by heating DNA from rat liver, calf thymus or herring sperm with N-HCl. The nature of the compound is unknown, but adenine, cytosine and thymine when heated with deoxyribose in N-HCl at 100° produced no fluorescent material. The blue fluorescence was quenched by ammonia, but the substance was eluted with N-HCl, and had $\lambda_{max.}$ 259 mμ, $\lambda_{min.}$ 240 mμ. In alkali $\lambda_{max.}$ was 235 mμ and an inflexion appeared at 260 mμ.

This fluorescent component is possibly the same as that described by Dunn (1955) and by Levy & Snellbaker (1955). These authors reported a white fluorescent spot which appeared on paper chromatography of a hydrolysate of calf-thumus DNA by N-HCl. Dunn was of the opinion that the substance was a guanine derivative but reported that guanine deoxyriboside did not produce a fluorescent component with N-HCl unless phosphoric acid was present.

RESULTS

The compounds that have been tested with rat liver are shown in Table 2.

Absorption of DNA by collagen. The ability of some bivalent metal ions to promote the absorption of DNA by an insoluble protein was studied by measuring the uptake of DNA by collagen which had been treated with various metal ions. The results are shown in Table 3.

Since DNA was so easily liberated from tissues by the method described a number of preparations have been made from rat liver. The cell nuclei were not separated before the extraction. Sodium trichloroacetate, sodium *p*-aminosalicylate and sodium benzoate were chosen as salts for the preparation of homogenates since the first compound was a protein precipitant, the second had metal-complexing properties, whereas the third was not known to possess either of these properties but produced a very viscous aqueous layer. Sodium *p*-aminosalicylate was used rather than salicylate since, after centrifuging, the aqueous layer was much clearer and easier to separate from the interfacial insoluble material. This separation proved much more difficult with the mixture produced from the treatment with sodium benzoate because of the high viscosity of the aqueous layer.

Table 2. *Compounds tested for release of deoxyribonucleic acids*

Rat liver was homogenized with 0·15 M-solutions of the above compounds and the homogenate shaken with an equal volume of 90% phenol. After centrifuging, the aqueous layer was tested for DNA. A strong Dische colour was produced by treatment with the effective compounds; a weaker colour by moderately effective compounds and in these the precipitate was less fibrous and the amount variable.

Anions which were effective	Anions which were moderatively effective	Compounds which released no DNA
m-Aminobenzoate	0·66 M-Chloride	Acetate
2-Amino-1-naphthylsulphate	0·3 M-Chloride + *o*-phenanthroline	Acetylacetone
p-Aminosalicylate	Decanoate	Adrenaline
Aurine tricarboxylate	Diphenylacetate	Ascorbate
Azide	2-Ethylbutyrate	0·3 M-Chloride
Benzoate	Gentisate	Cholate
p-tert.-Butylbenzoate	Hexahydrobenzoate	Citrate
Cupferron	*m*-Hydroxybenzoate	Cyanide
1:2-*cyclo*Hexyldiamine-*NNN'N'*-tetracarboxylate	Naphthalene-2-sulphonate	Cysteine
Diethyldithiocarbamate	1-Naphthol-4-sulphonate	3:4-Dihydroxybenzoate
Diphenate	1-Naphthylamino-4-sulphonate	1:8-Dihydroxynaphthalene-3:6-disulphonate
2:6-Dipicolinate	*iso*Nicotinate	Dihydroxyphenylalanine
Flavianate	Perfluoro-octanoate	1:2-Dimercaptopropanol
Fluoride	Sulphosalicylate	3:4-Dimercaptotoluene
3-Hydroxyanthranilate		*NN*-Dimethylglycine
Kynurenate		$\alpha\alpha'$-Dipyridyl
Methylene disalicylate		Dithio-oxamide
Methyl orange		Ethyl acetoacetate
Nicotinate		Ethylenediamine
2-Nitroso-1-naphthol-4-sulphonate		Ethylenediamine-*NNN'N'*-tetra-acetate
Orange II		Glycerophosphate
N-Phenylanthranilate		Glycine
2-Phenylphenanthrene-3:2'-dicarboxylate		Hexametaphosphate
Phenylphosphate		Histidine
Phthalate		*p*-Hydroxybenzoate
Picolinate		8-Hydroxyquinoline
Quinaldinate		8-Hydroxyquinoline-5-sulphonate
Quinolinate		Kynurenine
Salicylate		Methylmalonate
Sorbate		2-Naphthylamine-4:8-disulphonate
Thiocyanate		1-Nitroso-2-naphthol-3:6-disulphonate
Trichloroacetate		4-Nitrosoresorcinol
Xanthurenate		Orotate
		Oxalate
		Perchlorate
		o-Phenanthroline
		Phosphate
		Proline
		Pyridoxine
		Pyrophosphate
		Riboflavin phosphate
		Salicylaldoxime
		Spermine phosphate
		Tartrate
		Thioacetamide
		Thioglycollate
		Tryptophan

Table 3. *Absorption of deoxyribonucleic acids by collagen*

	Rat-liver DNA					Calf-thymus DNA				
Cation	Original optical density (258 mμ)	Optical density after 2 days	4 days	7 days	14 days	DNA left in soln. (% after 14 days)	Original optical density (258 mμ)	Optical density after 2 days	4 days	7 days	14 days	DNA left in soln. (% after 14 days)
Cu^{2+}	0·725	0·54	0·495	0·435	0·37	49·9	0·655	0·545	0·455	0·38	0·31	47·5
Co^{2+}	0·725	0·62	0·565	0·535	0·50	67·8	0·655	0·525	0·485	0·45	0·41	62·5
Fe^{2+}	0·725	0·67	0·610	0·590	0·53	72·3	0·655	0·585	0·55	0·47	0·42	64·9
Mn^{2+}	0·725	0·61	0·595	0·505	0·43	58·2	0·655	0·535	0·48	0·44	0·39	59·5
Zn^{2+}	0·725	0·60	0·555	0·520	0·47	64·5	0·655	0·51	0·46	0·43	0·38	58·5
Na^{+}	0·405	—	—	0·417	0·41	100	0·53	—	—	0·517	0·50	93·5
K^{+}	0·405	—	—	0·405	0·405	100	0·53	—	—	0·525	0·53	100·0

Table 4. *Analyses and base ratios of deoxyribonucleic acids extracted in different ways*

Preparations were made with: (column *a*), 0·3 M-trichloroacetate; (column *b*), 0·3 M-*p*-aminosalicylate; (column *c*), 0·3 M-benzoate. Base ratios are expressed as moles/100 moles of total bases.

	a	*b*	*c*
Guanine (base ratio)	21·1	21·3	22·1
Adenine (base ratio)	29·3	29·1	29·5
Cytosine (base ratio)	20·2	20·1	19·8
Thymine (base ratio)	29·6	29·5	28·8
N (%)	13·20	11·9	12·5
P (%)	8·02	7·24	7·22
N/P	1·64	1·64	1·73
$\epsilon_{(P)}$ (258 mμ) (in 0·1 N-NaCl)	5900	6340	6580
$\epsilon_{(P)}$ (258 mμ) (in water)	7700	8150	8000
P (atoms/100 moles of total bases)	—	97	—

After the separation, DNA, RNA and small amounts of polysaccharides were present in the aqueous layer. Addition of ribonuclease brought about the breakdown of RNA, and DNA was separated from polysaccharides by extraction with 2-methoxyethanol from potassium phosphate solutions, in exactly the same manner as described earlier for RNA (Kirby, 1956*b*). To judge from the Dische reaction DNA was entirely extracted into the organic layer, which was dialysed to remove particles of small molecular weight, and the DNA was finally precipitated by ethanol. The product was shown by hydrolysis to contain amino acids and ribonucleotides, and DNA prepared by the sodium benzoate method had the highest content of amino acids. Treatment with sodium dodecyl sulphate made little difference to the amino acid content, although there was some loss of material by this procedure. The amino acid content could be considerably or almost completely removed by increasing the concentration of the salts used for homogenizing from 0·15 M to 0·3 M. After this treatment DNA prepared with the aid of sodium benzoate contained approximately 1% of amino acids. The use of sodium trichloroacetate reduced this content somewhat, and DNA prepared with sodium *p*-aminosalicylate contained only the faintest traces of amino acids. One difficulty arose in that, although it was not possible to detect ribonucleotides by the usual colour reaction, hydrolysis of the DNA with N-HCl at 100° for 1 hr. followed by paper chromatography of the solution showed the presence of ribose. Obviously all the ribonucleotides had not been separated by dialysis despite the high ionic content. Complete separation of the fragments containing ribose was achieved by precipitating DNA with 2-ethoxyethanol instead of with ethanol.

Analyses of DNA. The base ratios and other analytical figures are shown in Table 4.

The $\epsilon_{(P)}$ (Chargaff, 1955) value is an indication that DNA was not degraded by treatment with phenol. The sodium benzoate preparation had a slightly higher guanine content than the DNA prepared with the aid of the other salts.

The DNA prepared by the three methods yielded varying amounts of amino acids upon hydrolysis, and it is probable that these originated from a protein or polypeptide firmly bound to the DNA. The amino acids have been estimated quantitatively to discover if their ratios bore any relationship to those found in histones as normally extracted from cell nuclei. Because of the large amount of glycine produced by decomposition of adenine and guanine under the hydrolytic conditions, it was essential to have a system that gave a good separation of this amino acid from those to be estimated. Paper chromatography with ethyl methyl ketone–acetic acid–water, followed by paper electrophoresis in 0·033 M-acetic acid at right angles to the direction of

chromatography, was found to be very suitable, and a diagram of the amino acids separated by this system is shown in Fig. 1. Lysine and arginine were estimated after single-way chromatography. Estimations were made with the coloured ninhydrin–copper complexes of the amino acids (cf. Fischer & Dörfel, 1953).

The analytical figures for the amino acids present in rat-liver DNA prepared by treatment with sodium benzoate (0·3 M) are shown in Table 5. The molar ratios of the amino acids present in rat-liver histone (Brunish, Farley & Luck, 1951) are included for comparison. Other amino acids which were also detected were valine, methionine, alanine, serine and threonine. The glycine spot was large and tended to trail into the serine and threonine spots. The essential difference between the results and those described previously for rat-liver nucleohistone was that there was relatively less lysine and arginine compared with aspartic and glutamic acids.

Calf-thumus DNA, prepared by the sodium dodecyl sulphate method, has also been hydrolysed and the amino acids were separated and estimated. The results are shown in Table 6. Although the results are probably not of a very high degree of accuracy, the amino acids attached to the DNA bore little relation to those in calf-thumus histone (Tristram, 1953), and the relatively high content of acidic and basic amino acids was noteworthy.

The results with herring-sperm DNA were surprising in that lysine, aspartic acid, glutamic acid and phenylalanine were present. These amino acids are absent from herring-sperm protamine. The results for herring-sperm DNA are shown in Table 7.

Rat-liver DNA prepared by treatment with 0·3 M-sodium *p*-aminosalicylate and phenol had only traces of amino acids. No ninhydrin colours developed before about 40 hr. after spraying, and only lysine, arginine, glutamic and aspartic acids could be recognized. The amounts were too small to measure accurately but indicated about a mole of amino acid in a particle weight of $1–2 \times 10^6$ DNA.

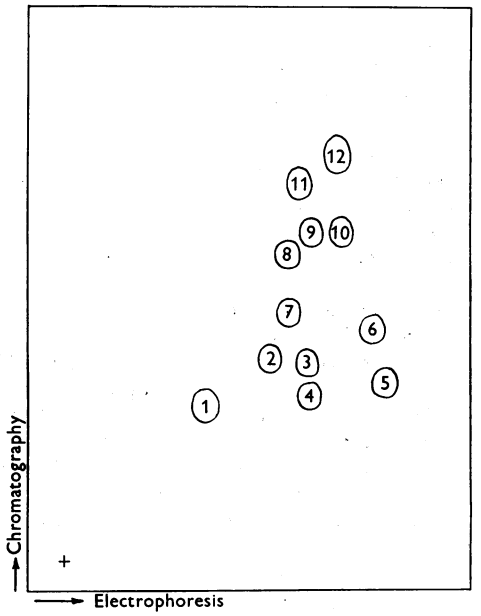

Fig. 1. Appearance of amino acids after chromatography and electrophoresis. 1, Aspartic acid; 2, glutamic acid; 3, threonine; 4, serine; 5, glycine; 6, alanine; 7, proline; 8, tyrosine; 9, methionine; 10, valine; 11, phenylalanine; 12, leucine/isoleucine.

Table 5. *Amino acid analyses of rat-liver deoxyribonucleic acids prepared by the use of sodium benzoate and phenol*

Amino acids are expressed as a percentage of the DNA. Molar ratios were calculated assuming that leucine = 20.

	Amount	Molar ratios	Molar ratios of rat-liver histone
Lysine	0·22	9	14
Arginine	0·14	4	16
Aspartic acid	0·20	9	9
Glutamic acid	0·20	8	12
Tyrosine	0·09	3	5
Phenylalanine	0·11	4	3
Leucine/isoleucine	0·43	20	22

Table 6. *Amino acid analyses of calf-thymus deoxyribonucleic acids*

Amino acids are expressed as a percentage of the DNA. Molar ratios were calculated assuming that leucine = 30.

	Amount	Molar ratios	Molar ratios of calf-thymus histone
Lysine	0·20	29	11
Arginine	0·10	9	15
Aspartic acid	0·23	34	7
Glutamic acid	0·14	20	5
Phenylalanine	0·01	1	4
Leucine/isoleucine	0·20	30	30

Table 7. *Amino acid analysis of herring-sperm deoxyribonucleic acids*

Results are expressed as a percentage of the DNA.

Lysine	0·09
Arginine	0·04
Aspartic acid	0·03
Glutamic acid	0·02
Phenylalanine	0·05
Leucine/isoleucine	0·20

DISCUSSION

The separation of deoxyribonucleic acids from nucleoproteins has been the subject of several procedures, and these have been adequately discussed previously (Chargaff, 1955; Frick, 1954*a*, *b*; Jones & Marsh, 1954). A characteristic feature of the problem has been the varying degree of difficulty with which the DNA is separated from the protein in different species. Herring-sperm DNA was split quite easily from the protamine by treatment with M-NaCl. This method is not generally applicable since, for example, the nucleoprotein of *Mycobacterium phlei* was unaffected by saturated NaCl (Jones & Marsh, 1954). The application of sodium dodecyl sulphate to the separation of DNA (Marko & Butler, 1951) was a considerable advance, but here again it was much more difficult to obtain protein-free DNA from bull testis than from calf thymus gland with this method.

The present study has shown that a considerable number of salts may be used in conjunction with phenol to liberate DNA from mammalian tissues, but the salts were not equally efficacious in removing protein.

Phenol was as essential to the reaction as the salt in solution and no DNA was released when phenol was replaced by resorcinol or urea (4M), and very little when *n*-butanol or guanidine hydrochloride (4M) was used in conjunction with sodium salicylate solution. Sodium dodecyl sulphate produced a very viscous solution of DNA but cetyltrimethylammonium chloride produced none at all.

The function of the phenol is probably that of a protein solvent, in that it extracts protein which has been separated by the salt in solution. Denaturation of the proteins may be incidental to the reaction, since the more usual protein denaturants urea and guanidine had no effect. The considerable power that phenol possesses to extract proteins from aqueous solution has been demonstrated by Grassmann & Deffner (1953).

To some extent the activity of the salts is related to their ability to interact with proteins. Trichloroacetate, toluenesulphonate, fluoride and thiocyanate ions are much more strongly bound by albumin than are chloride and perchlorate (Scatchard & Black, 1949), and this difference in binding corresponds to the ability of the anions to release DNA. Naphthalene-2-sulphonate showed very little activity in the DNA test, although this anion is normally very strongly bound by proteins (Steinhardt, 1941). 1-Naphthol-4-sulphonate was not much better but 2-nitroso-1-naphthol-4-sulphonate was very much more effective, and the increased activity may be related to the presence of a metal-complexing group. Probably most of the compounds in Table 2 have some capacity for binding to proteins, although azide ions are generally known for their ability to form complexes with heavy metals. Diethyldithiocarbamate, which forms very stable metal complexes, has a similar structure to 2-ethylbutyric acid, and absorption on to the protein may be by a similar mechanism, although *NN*-dimethylglycine, with a similar type of structure to diethyldithiocarbamate, had no activity under the test conditions. The greater effectiveness of diethyldithiocarbamate, compared with 2-ethylbutyrate, may be attributed to the ability of the former to bind metal ions. Generally, non-ionic complexing agents showed no activity, but *o*-phenanthroline in combination with 0·3M-NaCl released a small quantity of DNA.

The decreased activity of *m*-hydroxybenzoate and inactivity of *p*-hydroxybenzoate compared with salicylate may also be related to the ability of the last of these compounds to form metal complexes. Evidence of the differential interaction of isomeric compounds with proteins is somewhat contradictory in that *o*-hydroxyphenylacetate had a greater affinity for albumin than the *p*-compound (Luck & Schmidt, 1948), whereas more *o*-nitrophenol than *p*-nitrophenol was absorbed by albumin (Teresi & Luck, 1948).

The inactivity of the naphthalene disulphonic acids is probably related to the highly charged hydrophilic groups at opposite ends of the molecule, which could have prevented absorption on the nucleoprotein. Orange II was the only disulphonic acid with any activity, and this compound possessed lipophilic benzeneazo and a potentially complexing *o*-hydroxyazo grouping. The dispositions of the highly charged groups could also explain the decreased activity of sulphosalicylic acid compared with salicylic acid, and the inactivity of ethylenediaminetetra-acetic acid compared with 1:2-diamino*cyclo*hexane-*NNN'N'*-tetracarboxylate.

Direct proof of the presence of metal ions in nucleoproteins is difficult to obtain, since nucleoproteins are not easy to characterize and, being insoluble, have the capacity for ion exchange.

That transition-group metals could promote the absorption of DNA by an insoluble protein was shown by measuring the uptake of DNA by collagen which had been treated with various metals. Practically no DNA was absorbed by collagen treated with Na^+ or K^+ ions, but approximately 30–50% was absorbed when the collagen had been treated with Cu^{2+}, Co^{2+}, Mn^{2+}, Fe^{2+} or Zn^{2+} ions. The metal ions are almost certainly taken up by the carboxylate groups in the protein, and absorption of DNA could be due to some extent to a shift in the isoelectric point of the collagen, to bonding of a phosphate to a carboxylate grouping through a metal ion or to a purine or pyrimidine chelating on to

a metal–carboxylate grouping as shown in Fig. 2 (cf. Wallenfels, 1955).

Histological evidence was that tissues treated with isotonic solutions of some of the salts in Table 2, or with phenol, showed no change, but that combined treatment always resulted in extraction of DNA from rat-liver nuclei. Sections of the testis showed that only the vesicular nuclei (spermatogonia, spermatocytes and spermatids) were Feulgen-negative, whereas sperm heads remained Feulgen-positive. All the DNA was removed from the sperm heads when the testis was homogenized; the sperm heads were separated by centrifuging and given a longer treatment with the salt solution before the addition of the phenol. The sperm heads remained intact and cytologically recognizable after treatment with azide, cupferron or salicylate in conjunction with phenol.

Linkage of a protein to DNA by electrovalent bonds only would appear to provide little opportunity for specificity in the sequence of amino acids in the proteins. If, however, a number of electrovalent linkages were to be followed by a co-ordinate link of the type shown in Fig. 2 (for example), then a much greater specificity of amino acid sequence would be required in order to complete the bonding along any given polynucleotide chain, and the positions of the aspartic and glutamic acid residues would be of considerable importance.

The activities of benzoate and sorbate were noteworthy in that these compounds have little, if any, complexing abilities. The aqueous layer from the extract of liver with benzoate and phenol was much more viscous than that from trichloroacetate and *p*-aminosalicylate. This greater viscosity was not due to a higher yield of DNA, but was probably related to the presence of protein in the DNA prepared with the aid of benzoate. The protein content was higher in this preparation than in those with trichloroacetate and *p*-aminosalicylate, and this variation in protein content with the mode of preparation could well explain the considerable variations in the molecular weights reported for different samples of DNA.

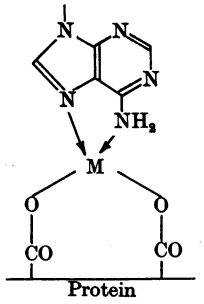

Fig. 2. Possible means by which DNA could form a complex through a metal with a protein containing aspartic or glutamic acid residues.

Analyses of the protein still attached to the DNA showed many similarities to the rat-liver histone separated by Brunish *et al.* (1951), the main difference being a decrease in the basic amino acids. Hydrolytic experiments on calf-thymus and herring-sperm DNA, both prepared by the sodium dodecyl sulphate method, have shown protein to be present, but here there were few resemblances between these amino acids and the composition of the histone and protamine respectively. Calf-thymus DNA had relatively more basic and acidic amino acids, whereas herring-sperm DNA produced, surprisingly, amino acids which are not present in herring-sperm protamine: lysine, glutamic and aspartic acids and phenylalanine. These amino acids must be examined in further preparations but these results are of considerable significance for the bonding of proteins to nucleic acids.

Proteins of an acidic nature have been extracted from nuclei previously. Stedman & Stedman (1943, 1947) isolated a protein, chromosomin, from nuclei of various tissues and found that it was characterized by high proportions of tryptophan and glutamic acid. Wang, Mayer & Thomas (1953) extracted from rat-liver nuclei a lipoprotein which was soluble in alkali and precipitated by addition of acetic acid. They believed the material may have been a portion of the nuclear membrane. The 'residue protein fraction' of Mirsky & Ris (1951) also differed from histones, which these authors claimed could be removed from the nucleus without altering the appearance of the chromosomes. The relationships of these proteins to those found firmly bonded to DNA in the present work is not clear at the moment, but all the evidence points to considerable complexity in the components of cell nuclei.

It is clear that when tissues are treated with 0·15 M-salt solution and phenol, a DNA–protein complex is liberated, and the same is probably true to some extent of the method with sodium dodecyl sulphate. The firm binding of this protein moiety, the high content of glutamic and aspartic acids and the complete removal of these by *p*-aminosalicylate are further indications that the protein is not bound to the DNA solely by electrovalent linkages, and that bonding of the DNA through a metal attached to a carboxylate group could provide an explanation of the experimental evidence.

The use of *p*-aminosalicylate and phenol has a number of advantages for the preparation of DNA from rat liver. It is not necessary to isolate cell nuclei, a procedure that must involve some losses, deoxyribonucleases are probably inactivated in a manner similar to that for ribonuclease and the yield is good. Ribonucleotides and amino acids are completely absent from the product, and the $\epsilon_{(P)}$

value is an indication that the DNA is no further degraded than by other methods of isolation.

Whatever the explanation of the differential release of RNA and DNA from tissues, the fact that no DNA is released until certain salts are present before the phenol is added suggests a fundamentally different type of bonding between each nucleic acid and its respective protein. It is suggested that RNA is bound mainly by hydrogen bonds between the bases and the proteins, whereas DNA is bound by electrovalent and possibly co-ordinated metal linkages.

The actions of ribonuclease and deoxyribonuclease on RNA and DNA is an indication of a difference in the interaction of the nucleic acids with proteins. Ribonuclease acts directly upon its substrate whereas deoxyribonuclease has no activity upon DNA until activated by bivalent cations, the most active of these being Mg^{2+}, Mn^{2+}, Fe^{2+} and Co^{2+} ions (Schmidt, 1955).

The liberation of DNA by reaction with a salt (particularly sorbate and decanoate) and phenol has an interesting correlation with work on the bacteriophage. Jesaitis & Goebel (1955) have recently reported that the DNA of T_4 bacteriophage of phase II *Shigella sonnei* was liberated by a specific lipocarbohydrate present in the host. The lipocarbohydrate was fractionated into a lipid cofactor, which is probably palmitic acid, and an extracted lipocarbohydrate. Neither the lipid cofactor nor the extracted lipocarbohydrate alone released DNA from the bacteriophage by combined treatment, nor did addition of palmitic acid to the extracted lipocarbohydrate bring about release of DNA.

Linkage of DNA to a protein through metal ions (Ca^{2+} or Mg^{2+} ions) has been suggested previously by Mazia (1954) and Levine (1955) for *Drosophila melanogaster*, and by Steffenson (1955) for *Tradescantia*, mainly from genetic evidence. Hyde (1955) has commented on the action of ethylenediaminetetra-acetate in the presence of $CaCl_2$ and $MgCl_2$ on onion-root tip and *Vicia faba* chromosomes, and Eversol & Tatum (1956) have noted that ethylenediaminetetra-acetate or manganous chloride increased the frequency of crossing over of mutant strains of *Chlamydomonas reinhardi*. The motility of starfish sperm was increased by ethylenediaminetetra-acetate and also by Ni^{2+} and Co^{2+} ions (Metz & Birky, 1955), and Parizek (1956) showed that a nutritional deficiency of Zn^{2+} ion caused injury to the testis. Mann (1945) analysed ram spermatozoa and showed that iron, copper and zinc were present in greater amounts in the sperm heads than in the mid-pieces and tails. White (1955) had published the spermicidal activities of a number of chelating agents, and among the more active compounds of those tested were cupferron, sodium diethyldithiocarbamate, 1-nitroso-2-naphthol and *o*-phenanthroline. 1-Nitroso-2-naphthol-3:6-disulphonic acid, thioglycollic acid, 1:8-dihydroxynaphthalene-3:6-disulphonic acid and $\alpha\alpha'$-dipyridyl were among the ineffective compounds. Although the motility of spermatozoa may not be directly related to the bonding of DNA to proteins, there is an interesting parallel, if not a complete correlation, in the activities of the compounds tested.

SUMMARY

1. Deoxyribonucleic acids (DNA) can be liberated from mammalian tissues by the action of certain salt solutions and phenol. Neither alone effected the release of DNA. The effective anions have strong interactions with proteins or the ability to form metal complexes.

2. DNA can be freed completely from ribonucleic acids (RNA) by treatment with ribonuclease and precipitation with 2-ethoxyethanol.

3. DNA can be freed completely from protein by the use of *p*-aminosalicylate and phenol and this has been developed into a preparative method yielding DNA with $\epsilon_{(P)}$ value 6340.

4. The amino acids which remain bound to rat-liver DNA prepared with sodium benzoate bore some similarities to those in rat-liver histone, but fewer basic amino acids were present.

5. Calf-thymus DNA and herring-sperm DNA prepared with the use of sodium dodecyl sulphate had considerably different ratios of amino acids from those present in their respective histone and protamine.

6. DNA was absorbed by collagen treated with Cu^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+} and Zn^{2+} ions but not by collagen treated with Na^+ or K^+ ions.

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Studies with ^{15}N-Labelled Nitrogen Mustards. The Combination of Di-2-(Chloroethyl)methylamine with Proteins

By V. C. E. BURNOP, G. E. FRANCIS, DENISE E. RICHARDS and A. WORMALL
Department of Biochemistry and Chemistry, Medical College of St Bartholomew's Hospital, London, E.C. 1

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Much evidence is available that the nitrogen mustards readily react with various enzymes and other proteins. For example, hexokinase (Dixon & Needham, 1946), cholinesterase (Thompson, 1947; Adams & Thompson, 1948) and choline oxidase and choline acetylase (Barron, Bartlett & Miller, 1948) are strongly inhibited by di-(2-chloroethyl)methylamine (NM), and although this enzyme-inhibitory capacity is fairly specific, NM reacts with many widely differing proteins. It effects a slight but significant change in the immunological properties of the serum proteins and ovalbumin (Watkins & Wormall, 1952*a*), and it rapidly inactivates haemolytic complement (Watkins & Wormall, 1948, 1952*b*), a complex serum system which consists of four components which are largely or entirely of protein nature. Furthermore, there is evidence of a cross-linking effect when NM is allowed to act on proteins, and it has been shown that in the nitrogen-mustard series the presence of two halogenoalkyl groups is needed for marked biological activity (Haddow, Kon & Ross, 1948; cf. also Goldacre, Loveless & Ross, 1949; Haddow, 1949).

No precise information has yet been obtained about the nature of the particular protein groups whose reaction with NM causes inactivation of enzymes or changes in the immunological properties of proteins, but it has been shown that NM reacts with amino, carboxyl, sulphydryl and sulphide groups of proteins (see, for example, Fruton, Stein & Bergmann, 1946, and the reviews by Gilman & Philips, 1946; Boyland, 1948; Philips, 1950; Ross, 1953; Alexander, 1954). NM has a marked alkylating capacity towards the amino and carboxyl groups of proteins (see Loveless, 1951; Ross, 1953).

Evidence of a change in certain characteristic or functional groups of a protein after its exposure to NM does not, however, necessarily establish that firm combination of the vesicant with the protein has occurred, nor does the measurement of the resulting decrease in the free amino and sulphydryl groups of the protein afford precise quantitative evidence about the condensation of NM and protein molecules. The reaction between sulphur mustards and proteins has been studied quantitatively with