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Displacement Fractionation of Deoxyribonucleoproteins by Heparin and Dextran Sulphate

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(*Received* 14 *January* 1957)

Recent investigations have shown that the total deoxyribonucleic acid, isolated from a single biological source, may be regarded as a group of closely related polymeric species having significantly different compositions.

The methods of fractionation commonly employed hitherto fall into two groups: (i) those dependent on the extraction of denatured deoxyribonucleoproteins with solutions of sodium chloride, either on a concentration gradient (Crampton, Lipshitz & Chargaff, 1954*a*, *b*; Chargaff, Crampton & Lipshitz, 1953; Lipshitz & Chargaff, 1956) or on a time gradient (Lucy & Butler, 1955), and (ii) methods involving the interaction of isolated deoxyribonucleic acid with proteins (Brown & Watson, 1953), polypeptides (Spitnik, Lipshitz & Chargaff, 1955) or ionic-exchange reagents (Bendick, Fresco, Rosenkranz & Beiser, 1955).

The present studies are concerned with an alternative means of fractionation of undenatured deoxyribonucleoproteins from calf-thymus gland and ox spleen, founded on the stepwise displacement by strong anionic polymers of the protein moiety of the nucleoproteins, and the accompanying liberation of deoxyribonucleic acid fractions. It was shown by Chargaff & Olson (1938), in another connexion, that protamine combines with heparin yielding an insoluble complex conjugate. We have now shown that isolated calf-thymus histone behaves in a similar way and is precipitated in 0·14 M-sodium chloride by titration with heparin or with dextran sulphate. Addition of sub-equivalent quantities of these polysulphates to deoxyribonucleoprotein in 0·14 M-sodium chloride leads to the liberation of deoxyribonucleic acid in solution. Unchanged nucleoproteins and the insoluble conjugate formed were removed by centrifuging. Repetition of the process thus led to the preparation of a series of deoxyribonucleic acid fractions.

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EXPERIMENTAL

Materials

Calf-thymus deoxyribonucleoprotein. The material was extracted from minced glands (obtained immediately after slaughter) by the method of Daly, Allfrey & Mirsky (1949). The product was reprecipitated four times from M-NaCl soln. and obtained as a gel by centrifuging at 81 000 *g*. Where necessary, this was stored at −18°.

Ox-spleen deoxyribonucleoprotein. Fresh tissue (150 g.) was homogenized in a Waring Blendor for 6 min. in 350 ml. of 0·14 M-NaCl soln. containing 0·01 M-trisodium citrate. The solid material was separated by centrifuging (4000 *g* for 30 min.) and washed twice with 3 vol. of NaCl–trisodium citrate soln. The washed solid was then mixed rapidly in a Waring Blendor with M-NaCl (3 vol.). After

1 min., the resulting viscous liquid was centrifuged (65 000 *g* for 30 min.) and the clear supernatant poured, with stirring, into water (6 vol.). The ropy product was again reprecipitated from M-NaCl. The resulting gel (11·67 g., moisture content 87·3%) was collected and stored at −18°. All manipulations were carried out at 0°. A specimen of deoxyribonucleoprotein was treated with chloroform–pentanol by the method of Sevag, Lackman & Smolens (1938) in order to obtain unfractionated deoxyribonucleic acid (DNA).

Calf-thymus histone. The corresponding nucleoprotein (6 g., 1·3 g. dry wt.) was treated according to the method of Brunish, Fairley & Luck (1951). The freeze-dried product (14·9% N) was free from DNA as judged by a modification of the Dische diphenylamine test (Allerton, Overend & Stacey, 1952).

Sodium heparinate was supplied by British Drug Houses Ltd. and had an anticoagulant activity of 130 units/mg.

Dextran sulphate was kindly given by Dr C. R. Ricketts. The sample contained 13·6% of S.

Methods

Pyrimidine and purine contents. Deoxyribonucleic acid fractions were hydrolysed with 72% (w/v) perchloric acid for 1 hr. at 100° and the bases in the resulting hydrolysate were separated by the method of Wyatt (1951).

Estimation of histone. The following modification of the Sakaguchi reaction was employed. Histone dissolved in 0·14M-NaCl (1 ml.) was mixed with 2·5M-NaOH (0·2 ml.) and ethanolic α-naphthol (1 mg./ml.; 0·2 ml.). After 5 min. at 27°, 0·06N-sodium hypochlorite (0·2 ml.) was added followed immediately by 3·3M-urea (0·4 ml.) (cf. Weber, 1930; Albanese & Frankston, 1945; Rosenberg, Ennor & Morrison, 1956). The tubes were mixed for 20 sec. at 27° and degassed for 30 sec. on the water pump. The colour was measured within 3 min. in 1 cm. cells with a Hilger Spekker absorptiometer with Ilford filter 605 (transmission max. 530–500 mμ). Controls were performed simultaneously, only the histone being omitted.

Under the controlled conditions employed, histone was determined accurately by the above modification of the Sakaguchi reagent (linear in the range 25–250 μg.). The main sources of error were the fading of the final colour and fluctuation in its intensity during photometry. We have made a study of the structure of the coloured compound formed in the reaction and this will be described later. Histone preparations prepared on different occasions by the same method gave substantially the same colour yield (100 μg. of histone ≡ 0·080 Spekker units, Ilford 605 filter).

Determination of histone content of deoxyribonucleohistone. Calf-thymus deoxyribonucleoprotein (0·336 g. = 73·9 mg. dry wt.) was dissolved in M-NaCl (40 ml.) containing streptomycin–penicillin (1:100 000, w/v, each). Samples (0·1–0·3 ml.) of the solution were removed and the histone content was determined.

Reaction of heparin with calf-thymus histone. To a series of tubes each containing the histone (114 μg. dry wt.) in 0·14M-NaCl (0·5 ml.), graded volumes of heparin in the same solvent were added. The volume in each tube was made up to 1·3 ml. by addition of 0·14M-NaCl. After 40 min. the tubes were centrifuged (2000 rev./min.) briefly and 1 ml. of the supernatant fluid was withdrawn for histone estimation.

Effect of electrolyte concentration on the solubility of the heparin–histone complex. The effect of increasing concentrations of sodium chloride was studied in the following way.

To a series of seven tubes, each containing 114 μg. dry wt. of histone in 0·14M-NaCl, M-NaCl was added in varying amounts. The final volume in each tube was adjusted to 1 ml. before addition of heparin (19·2 μg./0·3 ml. of 0·14M-NaCl). Each tube was centrifuged and the soluble histone determined.

Fractionation of calf-thymus deoxyribonucleohistone with heparin. Nucleoprotein gel (20·17 g., containing 78% of moisture) was suspended in 0·14M-NaCl (45 ml.) and heparin (82·3 mg./5 ml. of 0·14M-NaCl) was added slowly with gentle agitation for 30 min. The supernatant, obtained by centrifuging at 59 000 *g* for 30 min., was removed and its extinction at 260 mμ was measured. The liberated DNA was isolated by treatment with chloroform–pentanol (Sevag *et al.* 1938).

The unchanged nucleoprotein plus histone–heparin conjugate was resuspended in 0·14M-NaCl (0·45 ml.) and treated in the same way with 82·3 mg. of heparin. Repetition of this process yielded ten fractions of DNA. The purine and pyrimidine contents of these were determined.

Fractionation of ox-spleen deoxyribonucleohistone with heparin. Nucleoprotein gel (9·34 g. dry wt.) was suspended in 0·14M-NaCl (50 ml.) at 2° and treated successively, as in the previous experiment, with sodium heparinate (80 mg./5 ml. of 0·14M-NaCl.) The unchanged nucleoprotein and formed conjugate were separated after each stage by centrifuging at 4000 *g* for 15 min. (at 2°). Five fractions of DNA were obtained.

Determination of dextran sulphate–histone equivalent. The interaction of the protein and polysulphate was investigated in the same way as the protein–heparin interaction. Additions were made of histone (0·296 mg./ml. of 0·14M-NaCl) and dextran sulphate (0·134 mg./ml. of 0·14M-NaCl) in the first experiment and histone (0·514 mg./ml.) and dextran sulphate (0·210 mg./ml.) in the second.

Fractional displacement of histone from calf-thymus deoxyribonucleohistone by dextran sulphate. Nucleoprotein gel (21 g. containing 414 mg. of histone) was suspended in 0·14M-NaCl (70 ml.) and dextran sulphate (15 mg./5 ml. of 0·14M-NaCl) was added slowly with agitation for 45 min. The resulting mixture was centrifuged (72 000 *g*) for 45 min. and the supernatant liquid containing liberated DNA was separated. After measurement of the extinction of this solution at 260 mμ, the contained DNA was isolated as in the heparin fractionation.

Seven fractions of DNA were obtained by further additions of dextran sulphate to the residual nucleoprotein resuspended in 0·14M-NaCl (50 ml.).

RESULTS

Stepwise addition of sodium heparinate to histones in 0·14M-NaCl resulted in precipitation of the protein (Fig. 1). At the end point, 1 μg. of sodium heparinate was equivalent to 4·5 μg. of histone (dry wt. basis), assuming that all the heparin and histone present have then formed a complex. Further addition of heparin beyond the end point resulted in some solubilization of the complex. The

reaction was carried out in 0·14M-NaCl soln. At higher salt concentrations, the histone–heparin was progressively more soluble (Table 1).

In view of the difficulties in obtaining pure specimens of heparin, the behaviour of dextran sulphate was investigated. Like heparin, this polysulphate did not interfere in the colorimetric estimation of histone and it gave linear precipitation of this protein, forming a dextran sulphate–histone complex insoluble in 0·14M-NaCl (Fig. 1). At the end point, 1 μg. of dextran sulphate reacted with 4·55 μg. of histone (dry wt.). The resulting complex became more soluble on addition of excess of the polysulphate. Similar findings have been reported (Krebs, 1954; Madsen & Cori, 1954) for the interaction of phosphorylases with salmine.

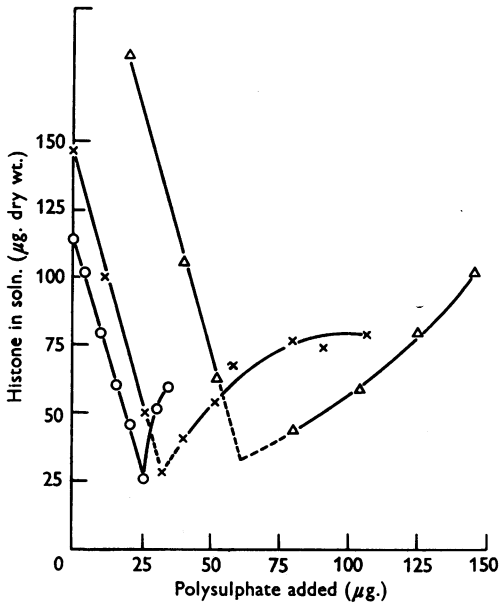

Fig. 1. Titration of calf-thymus histone by polysulphates. ×, △, Dextran sulphate (148 and 257 μg. of histone were taken respectively); ○, sodium heparinate (114 μg. of histone was taken). Ordinate is amount of histone remaining in solution after centrifuging off the polysulphate–histone complex. For details see text.

Table 1. *Effect of sodium chloride concentration on the solubility of heparin–histone conjugate*

Each tube contains 114 μg. of histone in 0·14M-NaCl (0·5 ml.). The heparin solution contained 64 μg. of heparin/ml. of 0·14M-NaCl. Final volume, 1·3 ml.

Tube	Sodium chloride (M)	Heparin (ml.)	Histone (μg./ml.)
1	0·14	0·3	29·2
2	0·27	0·3	64·2
3	0·40	0·3	57·3
4	0·46	0·3	65·4
5	1·0	0·3	71·2
6	0·14	0	80·5
7	0·67	0	81·6

Assuming molecular weights of 20 000 for heparin (reviewed by Foster & Huggard, 1955) and 15 000 for calf-thymus histone (Neurath & Bailey, 1953), the results indicate a molecular composition of 1:6 (heparin:histone) for the end-point complex. It is likely that the main cationic groups in the histone, contributing in its reaction with polysulphates, arise from arginine residues of the polypeptide chain. Analysis of calf-thymus histone by Daly *et al.* (1949) gave an arginine content of 10·8 % (w/w). Thus in the histone–heparin complex virtually complete neutralization of guanidino groups by sulphate groups would seem to have occurred.

In the dextran sulphate–histone titration, assuming a molecular weight of 30 000 for the former (Ricketts, 1951), the end-point complex has a molecular composition of 1:9 (dextran sulphate:histone). A second modification of the dextran sulphate–histone complex also appears to be formed at higher polysulphate concentrations, having the composition 75 μg. of polysulphate to 148 μg. of histone, corresponding to a molecular composition of 1:4 with a S:guanidino group ratio of 3·2:1. It is assumed that the S atoms are in the form of available sulphuric acid esters.

In a trial experiment the histone content of calf-thymus deoxyribonucleohistone was found experimentally to be between 68·5 and 70·0 %. Stepwise addition, in one-tenth portions, of the calculated amount of heparin necessary to precipitate totally the histone in a fixed amount of the nucleohistone yielded DNA fractions with compositions of the order 1·37–3·78 % P and 4·42–8·75 % N (Table 2*a*). These low values were traced to impurities in the heparin, some specimens of which were contaminated with uracil-containing substances, as shown by hydrolysis and chromatography. There was some evidence of fractionation in variations in the base ratio of the fractions and in their disproportionate yields. With pure heparin, successful fractionation of ox-spleen DNA was achieved. Five fractions were isolated, with the properties shown in Table 3.

Under similar conditions, dextran sulphate gave seven fractions of DNA from calf-thymus deoxyribonucleohistone (Table 2*b*). The products were free from the sulphur-containing impurities and, after hydrolysis, gave no evidence of the presence of uracil. Control experiments showed that, in the conditions described, no nucleic acid was liberated in the absence of either polysulphate.

Table 2. *Displacement of fractionation of calf-thymus deoxyribonucleohistone with polysulphates*

E_C represents the absorption at 260 mμ of each fraction in 75 ml. of 0·14 M-NaCl.

	Fraction no.										Unfractionated DNA
	1	2	3	4	5	6	7	8	9	10	
	(*a*) With heparin										
P (%)	3·19	3·78	1·89	2·15	1·93	1·87	1·70	1·37	3·08	3·27	14·90
N (%)	8·60	8·75	8·21	8·07	6·02	6·31	5·44	4·42	7·60	7·90	8·55
Adenine + thymine / Guanine + cytosine	0·95	1·02	0·89	0·78	1·02	1·05	0·97	1·15	1·15	1·12	1·27
DNA recovered (mg.)	26·3	33·8	14·6	1·68	26·2	36·0	27·4	34·4	38·4	44·2	—
E_C	2·73	3·08	2·93	2·66	2·20	6·25	2·37	3·15	7·75	6·64	—
	(*b*) With dextran sulphate										
Adenine + thymine / Guanine + cytosine	1·21	1·10	1·02	1·04	1·13	1·14	1·16	—	—	—	1·03
DNA recovered (mg.)	9·9	14·1	17·0	33·9	24·3	14·3	20·4	—	—	—	—
E_C	4·58	9·75	21·5	13·8	10·6	6·15	8·9	—	—	—	—

Table 3. *Displacement fractionation of ox-spleen deoxyribonucleohistone with heparin*

Values for a repeat experiment are shown in parentheses.

	Fraction no.					Unfractionated DNA
	1	2	3	4	5	
N (%)	14·90	14·71	14·82	14·86	14·79	14·86
P (%)	8·47	8·52	8·56	8·63	8·68	8·51
N/P atomic ratio	3·89	3·82	3·84	3·81	3·77	3·87 (3·87)
Purine/pyrimidine	1·13	1·07	1·06	1·05	0·95	1·03 (1·05)
Adenine/guanine	0·94	1·01	1·08	1·07	1·22	1·10
Thymine/cytosine	0·91	1·17	1·06	1·37	1·50	1·30
Adenine + thymine / Guanine + cytosine	0·93 (0·98)	1·1 (1·2)	1·13 (1·3)	1·20 (1·30)	1·36 (1·40)	1·2 (1·24)
Adenine/thymine	1·15	1·00	1·08	0·95	0·88	0·96
Guanine/cytosine	1·12	1·16	1·05	1·20	1·07	1·1

DISCUSSION

It is envisaged that two factors (Lipshitz & Chargaff, 1956) participate in stabilizing the complex formed between DNA and proteins: (*a*) ionic or electrostatic bonds of different strengths between the acid and the polybase; e.g. nucleic acid rich in guanine and cytosine may be expected to undergo weaker ionic interactions than DNA rich in adenine and thymine by reason of internal bonding which can exist between the 2-amino group of guanine and the oxo-group of cytosine (Brown & Watson, 1953); (*b*) secondary valence forces arising from the molecular configuration of nucleotides.

Results of existing fractionations of DNA by sodium chloride extraction suggest that guanine–cytosine-rich DNA is released preferentially. For the most part, these procedures are concerned with the solubility properties of denatured nucleoproteins. The polysulphate-displacement technique can be regarded as essentially an ion-exchange procedure and presents an approach to fractionation of undenatured nucleoprotein. With ox-spleen DNA the results are similar to previous findings, early fractions having (adenine + thymine)/(guanine + cytosine) values of 0·93, and later fractions rising to 1·36. With calf thymus, an early fraction, (adenine + thymine)/(guanine + cytosine) = 1·21, represents only a small quantity of the whole DNA, the predominating fraction having a base ratio of 1·02.

In no case, however, has the recovery of DNA been quantitative. In heparin fractionations, 62% of the DNA was recovered and in dextran sulphate fractionations, 75% was obtained. As with other investigators (Crampton *et al.* 1954*b*), difficulty was experienced in obtaining complete release of DNA. Crampton *et al.* (1954*b*) produced evidence that the yield of DNA from denatured nucleoprotein is related to the degree of polymerization of the nucleic acid.

Since the polysulphate fractionation consists in protein precipitation, it seems likely that the separation involves further considerations, particularly regarding the nature of the protein moiety. It has been shown (Stedman & Stedman, 1951; Davison & Butler, 1954) that at least two protein species differing markedly in their arginine and lysine contents are present in isolated calf-thymus deoxyribonucleoprotein.

Lucy & Butler (1955) concluded that guanine–cytosine-rich DNA was associated with the lysine-rich histone and the adenine–thymine-rich DNA with the arginine-rich histone. Later it was shown (Lucy & Butler, 1956) that the initial fractions obtained by extraction with sodium chloride contained a large excess of protein over DNA. It was concluded that this excess of histone was uncombined with DNA, and that it had become detached from DNA which appeared in subsequent fractions. If this explanation is correct and detachment occurs during isolation of deoxyribonucleohistone, it is likely that unattached free histone would be detected in the polysulphate displacement. In each case, however, the initial addition of polysulphate is accompanied by precipitation of the histone complex and by the liberation of DNA, which would be unlikely if free histone were present. It is tentatively suggested that detachment of histone may occur in denaturing the nucleohistone before salt extraction.

Though the 'histone' used in the present work almost certainly contains the two forms mentioned above, it is surprising that some distinction is not apparent in the titration with heparin or dextran sulphate, in view of the different pK of the guanidine group of arginine and the amino group of lysine. The present results would agree with the notion of displacement of the weak cationic protein (accompanied by liberation of guanine–cytosine-rich DNA) followed by displacement of a more strongly ionic protein and the liberation of the adenine–thymine-rich DNA. Further clarification of this hypothesis could be obtained by examination of the content of basic amino acids in successive polysulphate–histone precipitates.

SUMMARY

1. Histones form conjugates with sodium heparinate and with dextran sulphate which are insoluble in 0·14 M-sodium chloride.

2. Stepwise addition of subequivalent quantities of these polysulphates to undenatured deoxyribonucleohistone preparations results in the liberation of deoxyribonucleic acid fractions.

3. The fractions from calf thymus have base ratios (adenine plus thymine:guanine plus cytosine) in the range 0·78–1·21, and those from ox spleen have values between 0·93 and 1·40.

4. Results suggest that all the histone is combined with deoxyribonucleic acid in isolated deoxyribonucleoprotein.

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