

## Interactions of Proteins with other Polyelectrolytes in a Two-Phase System containing Phenol and Aqueous Buffers at Various pH Values

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1. Interactions of proteins with neutral polysaccharides and such polyacids as polygalacturonic acid, chondroitin sulphate, RNA and DNA in a two-phase system composed of phenol and aqueous buffers in the pH range 1.5–10 were studied. 2. Analysis of the products of the interaction was facilitated by the absolute preference of the proteins studied for the phenol-rich phase at all pH values. 3. The polyacids, on the other hand, in the absence of interactions were recovered mainly from the aqueous phases. 4. The interaction, the extent of which was mainly determined by the pH-dependent ionization state of the reacting partners, followed the patterns of antigen-antibody interactions with a well-defined equivalence point (maximum point of precipitation) and with the formation of soluble complexes. 5. The soluble complexes formed below the equivalence point were composed of proteins with small amounts of polyacids attached, and so passed into the phenol-rich phase; those formed above the maximum precipitation point were polyacidic in character and found in the aqueous phases. 6. Glycoproteins, with small amounts of covalently linked sugar residues, passed quantitatively into the phenol-rich phases. 7. The possibilities of developing a method for the analysis of glycoproteins and other applications are discussed.

The theoretical and practical importance of molecular interactions between proteins and other materials is being increasingly recognized. These interactions can be divided roughly into two major groups: first, electrostatic interactions of proteins with other electrolytes, and secondly, interactions with non-electrolytes through hydrogen-bonding, hydrophobic interactions, van der Waals forces etc., utilizing valences that ordinarily hold together the secondary structure of proteins (Tanford, 1958; Kauzmann, 1959). Some of these interactions are strong enough to survive, at least partially, the fractionation procedures used for isolating proteins, and consequently small amounts of 'non-protein' components, peptides (Synge, 1953, 1955, 1959), sugars (e.g. Rigas & Osgood, 1955; Clamp, Bernier & Putnam, 1964) etc., usually accompany purified proteins. It is laborious and occasionally very difficult to come to a firm conclusion as to the nature of the combination and the genuineness of these minor components. It is even more difficult to find ways of removing them.

The existence of strong interactions between proteins and phenols has long been recognized. Runge, the discoverer of phenol, was also the first

to notice its coagulating effect on aqueous solutions of proteins (Runge, 1834*a,b*). Anhydrous phenols and their water-saturated solutions are, however, good solvents for various vegetable and animal proteins (Kjeldahl, 1896; Mathewson, 1906; Reichel, 1909; Cooper, 1912) and can also be used for extracting them from biological sources (Tsvett, 1899; Tswett, 1900).

In contrast with its strong solvent action on proteins phenol has little tendency to dissolve polysaccharides or mucopolysaccharides (Palmer & Gerlough, 1940; Morgan & Partridge, 1940; Morgan & King, 1943; Westphal, Lüderitz & Bister, 1952), RNA (Gierer & Schramm, 1956; Kirby, 1956) or DNA (Kirby, 1957). In addition to its selectivity as a solvent, phenol is one of the strongest dissociating agents known to decrease to a minimum molecular interactions between proteins and other materials (Craig, 1962; Bagdasarian, Matheson, Synge & Youngson, 1964). Recent work, however, has drawn attention to the fact that some interactions between nucleic acids and proteins may persist even in solvent mixtures containing phenol (Brattsten, Synge & Watt, 1964, 1965). Doubtless other types of interactions may also persist in

phenol-containing solvents, and even new types of combinations, unknown in aqueous solvents, may be brought about by the action of phenol on proteins and other macromolecules.

The present paper describes the results of an investigation into the nature and the extent of some of the interactions between proteins, e.g. bovine serum albumin or cytochrome *c*, and other poly-electrolytes in a two-phase system containing phenol and aqueous buffers at various pH values. No effort was made, however, to conduct a physical study, because of the occasional difficulties in achieving equilibrium conditions. The behaviour of a few glycoproteins was also investigated under similar conditions. As a conclusion, the possibilities of developing a method for the analysis of the true sugar content of glycoproteins and other likely applications are discussed.

## MATERIALS

*Bovine serum albumin.* This was a crystalline preparation (lot no. BF1670) from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex. It contained 13.5% of N (Kjeldahl), uncorrected for moisture content, less than 0.2% of neutral sugars expressed as mannose and no phosphorus.

*Cytochrome c.* Horse-heart cytochrome *c* (type II; lot nos. 74B-7110 and 33B-802) was purchased from Sigma Chemical Co., London. It had 15.0% of N (Kjeldahl) and no measurable amount of sugar or phosphorus.

*Ribonucleic acid.* This was an alkali-degraded yeast RNA preparation from British Drug Houses Ltd., Poole, Dorset. It gave on analysis 13.4% of N (Kjeldahl), 7.5% of P and 20.0% of ribose.

*Deoxyribonucleic acid.* This was a preparation of highly polymerized calf-thymus DNA (sodium salt) (type I; lot no. 104B-0960) from Sigma Chemical Co. It gave on analysis 12.0% of N (Kjeldahl) and 8.06% of P.

*Chondroitin sulphate.* This was a preparation from bovine nasal-septa cartilage (grade I; lot no. C79-53) from Sigma Chemical Co. It contained 3.0% of N (Kjeldahl).

*Polygalacturonic acid.* This was a preparation of unspecified source from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

*Starch.* A somewhat degraded but not wholly soluble preparation from British Drug Houses Ltd. was used.

*Glycoproteins.* The following glycoproteins were also used: ovalbumin, a crystalline salt-free preparation (lot no. 17102-250) from Sigma Chemical Co.; bovine serum  $\gamma$ -globulin (lot no. HA0271) from Armour Pharmaceutical Co. Ltd.; kidney-bean glycoprotein (I), prepared by the method of Pusztai (1965*b*); kidney-bean glycoprotein (II), prepared by A. Pusztai (unpublished work).

*Chemicals.* Phenol, the buffer salts and other chemicals used were of A.R. quality. Phenol solutions were always made up fresh before the start of each experiment and were completely colourless.

## METHODS

*Kjeldahl nitrogen, amino sugars and phosphorus.* These were all estimated as described by Pusztai (1965*a*).

*Neutral sugars.* These were estimated by the phenol- $\text{H}_2\text{SO}_4$  method of Dubois, Gilles, Hamilton, Rebers & Smith (1956).

*Uronic acids.* These were determined by the carbazole- $\text{H}_2\text{SO}_4$  technique (Dische, 1950).

*Deoxypentoses.* These (for DNA analysis) were estimated by the diphenylamine reagent of Seibert (1940).

*Cytochrome c.* This was estimated by measuring its extinction at  $538\text{m}\mu$  by a method similar to that of Margoliash & Frohwirt (1959).

*Standard partitioning procedure.* Proteins and other materials were accurately weighed and placed in conical (approx. 10 ml.) glass centrifuge tubes. The contents of the tubes were dissolved or dispersed by gently shaking with the appropriate buffer solutions (2 ml. of each). The following buffer solutions in a final concentration of 0.05 M were used: HCl (0.03 N)-NaCl (0.02 M), pH 1.5; citric acid-sodium citrate, pH 2.6-6.0, or occasionally, when no phosphorus estimations were required, citric acid- $\text{Na}_2\text{HPO}_4$ , pH 2.6-7.0; pH values above 8.0 were attained by using sodium borate buffers. For the preparation of the above buffer solutions see Gomori (1955). When the buffer solutions contained additives such as NaBr the pH of the resulting solution was carefully readjusted to the original pH value. When the dispersion of the reacting components was judged to be complete and the appropriate reactions had also taken place, solid phenol (2 g.) was added and the mixtures were quickly warmed to about 70° on a water bath. The contents of each tube were thoroughly mixed with a glass rod and the resulting single-phase liquids were rapidly cooled to room temperature by immersing the tubes in a cold-water bath. The separation of the two phases was aided by centrifuging, after which the clear upper phases were carefully removed by siphoning. The removal of the aqueous phases in this way is not complete and for this reason the bottom layers, after the addition of fresh buffer solutions (2 ml. to each tube) of the original pH value, were again subjected to the same procedure. In most cases three successive extractions sufficed and the removal of the materials soluble in the aqueous phases could be regarded as complete. The combined aqueous phases from the successive extractions were passed through a small funnel drawn out from a piece of narrow glass tubing and containing a firmly pressed glass-wool plug to filter out any floating precipitate. Each filter was thoroughly washed with small amounts of buffer and the washings were combined with the appropriate aqueous phases. The same filters were then used for filtering the phenol-rich phases. In this way all materials insoluble or precipitated as a result of the interaction were collected into one fraction. The funnels were washed with aq. 90% (w/v) phenol and the filtrates were added to the appropriate phenol-rich phases. Both the aqueous and the phenol-rich phases were evaporated *in vacuo* with repeated additions of water. The final residues were taken up in 0.1 N-NaOH (usually 2.5 ml.) and these solutions were used for analysis. When the precipitates were required for analysis the filter pads containing the precipitate together with the narrow drawn-out part of the funnels were transferred to separate tubes, a solution of 0.1 N-NaOH (2.5 ml. to each tube) was added and the contents of the tubes were shaken for 30 min., centrifuged and the clear supernatants were subjected to various analyses. Occasionally the total amount of a precipitate was transferred straight into a Kjeldahl diges-

Table 1. Partitioning of various polyelectrolytes between phenol and aqueous buffers at different pH values

Experimental conditions are given in the text. The results are expressed as percentages (w/w) of the original material recovered from (a) the aqueous and (b) the phenol-rich phases calculated on the basis of N (Kjeldahl), neutral sugar, deoxyxypentose, P etc. values. Occasional low recoveries are due to the formation of small amounts of insoluble material under certain conditions.

Material subjected to standard extraction procedure	Recovery of materials (% w/w)													
	At pH1.5		At pH2.5		At pH4.0		At pH6.0		At pH8.0		At pH9.2		At pH10.0	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Bovine serum albumin	0	100	0	100	0	100	0	100	0	100	0	100	0	100
Cytochrome c	0	100	0	100	0	100	0	100	0	100	0	100	0	100
RNA	95	7	98	2	100	0	100	0	100	0	100	0	100	0
DNA	74	21	89	13	99	1	100	0	100	0	100	0	100	0
Polygalacturonic acid	12	8	14	6	16	0	33	0	82	0	92	0	94	0
Chondroitin sulphate	69	22	69	7	71	2	74	0	80	0	84	0	90	0
Starch	94	2	98	0	99	0	99	0	99	0	102	0	103	0

tion flask and washed over with the acid used for the digestion.

## RESULTS

In the first instance all materials to be studied were subjected to the standard partitioning procedure on their own and the results of these experiments are summarized in Table 1. As both proteins were recovered quantitatively from the phenol-rich phases, the range of the aqueous solutions used was extended on the acid side to include 0.1N-, 1.0N-, 2.0N- and 5.7N-hydrochloric acid and on the alkaline side to include 0.1N- and 0.3N-sodium hydroxide. The cytochrome c used in this experiment, however, was again quantitatively recovered from the phenol-rich phases.

Next, the comparatively simple case of the possible reaction between bovine serum albumin and an uncharged polysaccharide, namely starch, was studied. No interaction was observed between these two materials in the pH range studied; the protein was quantitatively recovered from the phenol-containing phases, and starch nearly quantitatively from the aqueous phases.

The results of the experiments on mixtures of the various polyelectrolytes and proteins are given in Figs. 1-6, where the recoveries of the amounts of the various reacting partners in percentages of their original amounts in the separated phases or precipitates are plotted against the pH of the aqueous phases.

The reaction between cytochrome c and the nucleic acids was studied in greater detail. Fig. 7 shows the amount of DNA recovered from the phenol-rich and aqueous phases and from the insoluble precipitate formed as a function of the amount of cytochrome c in the reaction mixture at constant DNA concentration, pH (2.8) and ionic

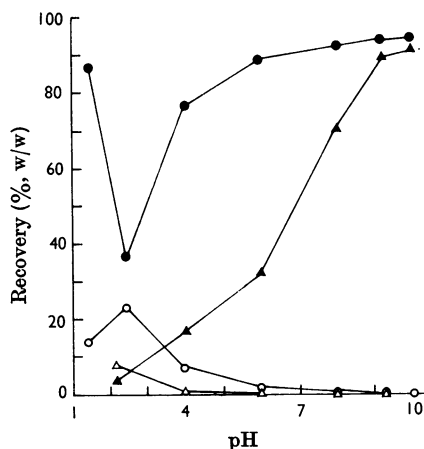


Fig. 1. Recovery of RNA and polygalacturonic acid from artificial mixtures with bovine serum albumin. RNA (5 mg.) or polygalacturonic acid (2.5 mg.) was mixed with bovine serum albumin (5 mg.) and subjected to the standard extraction procedure. RNA recoveries (●, aqueous phase; ○, phenol-rich phase) are calculated on the basis of P and ribose estimations, and polygalacturonic acid recoveries (▲, aqueous phase; △, phenol-rich phase) from the results of the carbazole-H<sub>2</sub>SO<sub>4</sub> test. The results are expressed as percentages (w/w) of the original amounts.

strength. Fig. 8, on the other hand, shows the effect of increasing DNA concentration on the recovery of cytochrome c at constant protein concentration, pH (5.0) and ionic strength. In Fig. 9 the composition and weight of the insoluble complexes formed from cytochrome c and DNA or RNA respectively are plotted against the pH of the aqueous phases. In Table 2 the effects of increasing

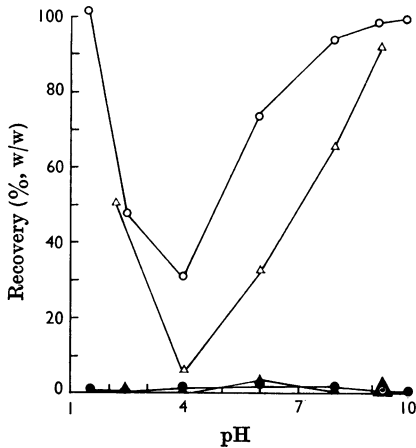


Fig. 2. Recovery of bovine serum albumin from its artificial mixtures with RNA or polygalacturonic acid. For the conditions of the experiments see Fig. 1 and the text. For the RNA-serum albumin mixture the protein recoveries were calculated from N (Kjeldahl) values after an allowance was made for the N content of RNA calculated on the basis of P and ribose estimations (●, aqueous phase; ○, phenol-rich phase). No allowance had to be made when the protein recoveries were calculated in mixtures containing polygalacturonic acid and serum albumin (▲, aqueous phase; △, phenol-rich phase). The recoveries are expressed as percentages (w/w) of the original values.

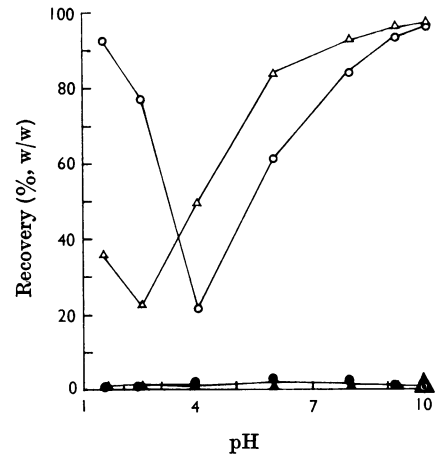


Fig. 4. Recovery of cytochrome c from its artificial mixtures with DNA or chondroitin sulphate. For the conditions of the experiments see Fig. 3 and the text. The recoveries are calculated from N (Kjeldahl) values after an allowance was made for the N content of DNA (●, aqueous phase; ○, phenol-rich phase) or that of chondroitin sulphate (▲, aqueous phase; △, phenol-rich phase). The results are expressed as percentages (w/w) of the original protein values and plotted against the pH of the aqueous phases.

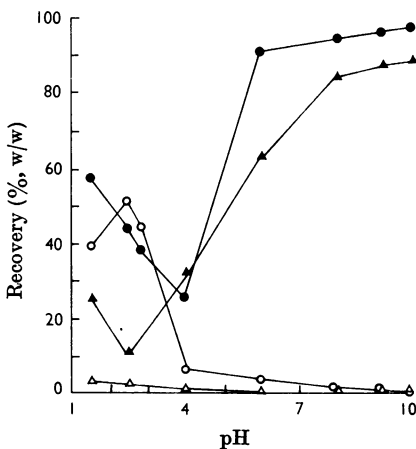


Fig. 3. Recovery of DNA and chondroitin sulphate from artificial mixtures with cytochrome c. DNA (4.2 mg.) or chondroitin sulphate (5 mg.) was mixed with cytochrome c (5 mg.) and subjected to the standard extraction procedure. DNA recoveries are calculated on the basis of P and deoxy-pentose estimations (●, aqueous phase; ○, phenol-rich phase), and chondroitin sulphate recoveries from the results of the carbazole- $\text{H}_2\text{SO}_4$  test (▲, aqueous phase; △, phenol-rich phase). The results are expressed as percentages (w/w) of the original values and plotted against the pH of the aqueous phases.

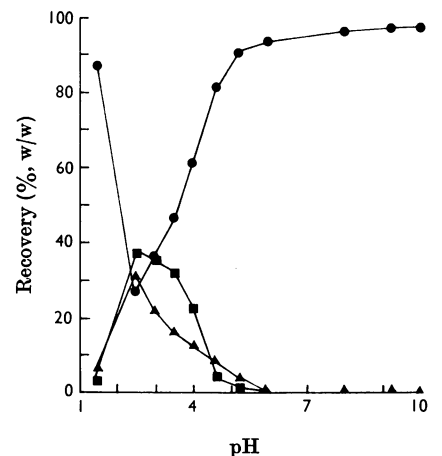


Fig. 5. Recovery of RNA from its mixtures with cytochrome c on partitioning between phenol and aqueous buffers at various pH values. RNA (5 mg.) and cytochrome c (5 mg.) were mixed and subjected to the standard extraction procedure. RNA recoveries are calculated on the basis of N (Kjeldahl), P and ribose estimations from the aqueous phase (●), the phenol-rich phase (▲) and the precipitate (■). The results are expressed as percentages (w/w) of the original values and plotted against the pH of the aqueous phases.

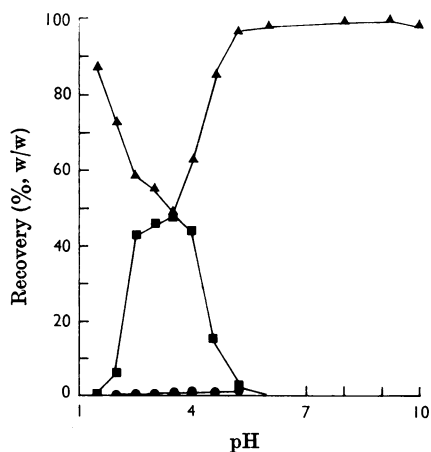


Fig. 6. Recovery of cytochrome *c* from its artificial mixtures with RNA. For experimental conditions see Fig. 5 and the text. Protein recoveries are calculated on the basis of extinction values at  $538\text{m}\mu$  and N (Kjeldahl) estimations on materials recovered from the aqueous phase (●), the phenol-rich phase (▲) and the precipitate (■). The results are expressed as percentages (w/w) of the cytochrome *c* recovered and plotted against the pH of the aqueous phases.

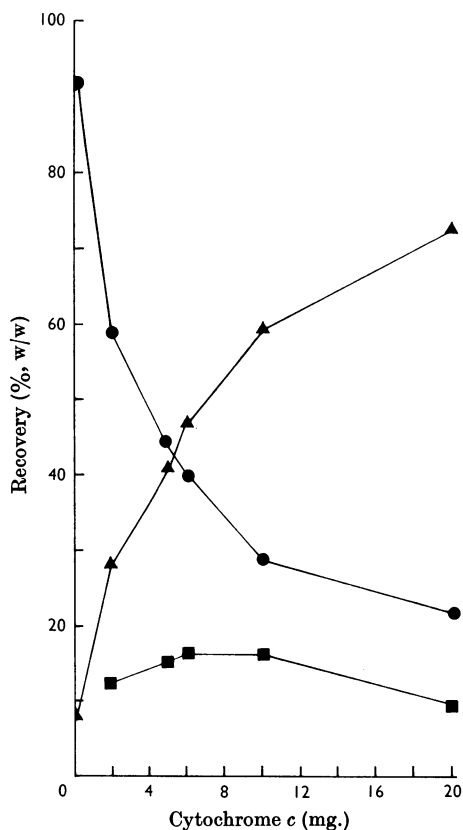


Fig. 7. Effect of increasing cytochrome *c* concentrations on the recovery of DNA at constant DNA concentration, pH (2.8) and ionic strength. DNA (5 mg.) and cytochrome *c* (0–20 mg.) were mixed, 0.05 M-citric acid-sodium citrate buffer (2 ml.) and phenol (2 g.) were added and the mixtures were subjected to the standard partitioning procedure. DNA recoveries (●, aqueous phase; ▲, phenol-rich phase; ■, precipitate) were calculated from P and deoxyribose values and expressed as percentages (w/w) of the original values.

ionic strength on the recovery of cytochrome *c* and DNA from the insoluble precipitates are recorded.

Next, the behaviour of a few glycoproteins on partitioning between phenol and neutral or slightly alkaline buffer solutions (pH 8.0) was studied. Glycoproteins (5 mg. of each) were dissolved in the buffer (4 ml.) and then solid phenol (4 g. to each) was added. The procedure from then on was similar to that described above. There was no insoluble material formed in these mixtures and only the phenol-rich phases were recovered. The phenol was removed *in vacuo* and the contents were dissolved in 0.1 N-sodium hydroxide, with the exception of ovalbumin, for which 0.3 N-sodium hydroxide and vigorous shaking were needed to bring the denatured protein into solution. Nitrogen, neutral sugar and hexosamine estimations were performed on these solutions and the results are compared with the results of analyses on the original glycoproteins (Table 3).

## DISCUSSION

The results of the experiments described in Table 1 in most respects support accepted views on the distribution of various classes of materials between phenol and water. Polysaccharides (Westphal *et al.* 1952), chondroitin sulphate (Partridge & Davis, 1958), RNA (Kirby, 1956; Schuster, Schramm & Zillig, 1956) and DNA (Kirby, 1957) at most pH values remain insoluble in the phenol-

rich phase and are usually recovered from the aqueous phases when the pure components are subjected to partitioning between phenol and aqueous buffers in the pH range 3–10. At pH values below 3, however, small amounts of the polyacids studied were recovered from the phenol-rich phases (Table 1), and this tendency seemed to increase as the pH of the aqueous phases was lowered. This may be connected with the progressive suppression of the ionization of the negatively charged anionic groups in these materials with a subsequent change in their molecular conformation. In addition, the possibility of the splitting of a few primary linkages may also contribute to this partial change in solubility of the polyacids studied.

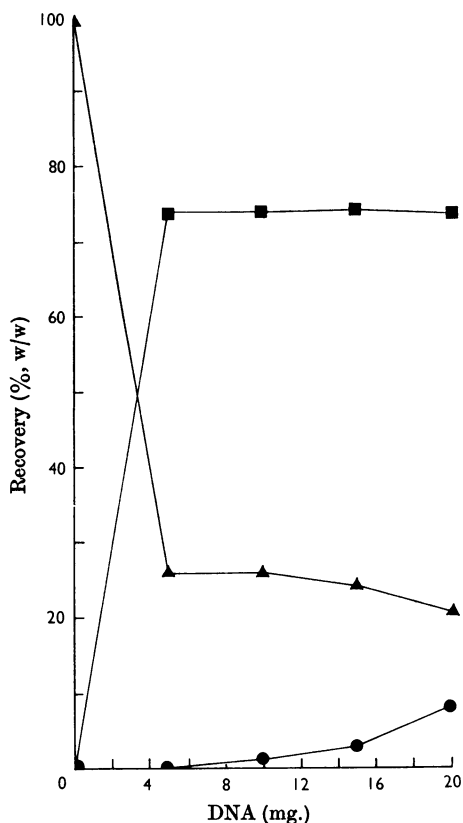


Fig. 8. Effect of increasing DNA concentration on the recovery of cytochrome *c* at constant pH (5.0), ionic strength and protein concentration. Cytochrome *c* (4mg.) and DNA (0–20mg.) were mixed, 0.05M-citric acid–sodium citrate buffer, pH5.0 (2ml.) and phenol (2g.) were added and the standard partitioning procedure was carried out. The protein recoveries (●, aqueous phase; ▲, phenol-rich phase; ■, precipitate) were calculated on the basis of extinction values at 538m $\mu$  and N (Kjeldahl) estimations and expressed as percentages (w/w) of the original protein.

The two proteins, serum albumin and cytochrome *c*, studied in the present work showed an absolute preference for the phenol-rich phase at all pH values. This observation is in line with a previous finding (Woodman & Gallagher, 1929) that gelatin, when partitioned between a mixture of isomeric cresols and water, preferentially passed into the cresol-rich phase. In addition to proteins, certain peptides also partitioned in favour of the phenol-rich phase in two-phase systems (Grassmann & Deffner, 1953). Similarly, phenol is a good solvent for synthetic polyamides, nylon, Perlon etc., and fibres can be cast from their phenolic solutions (Watkins, 1947). This preferential solubility is poorly understood, although it has been attributed to the strong

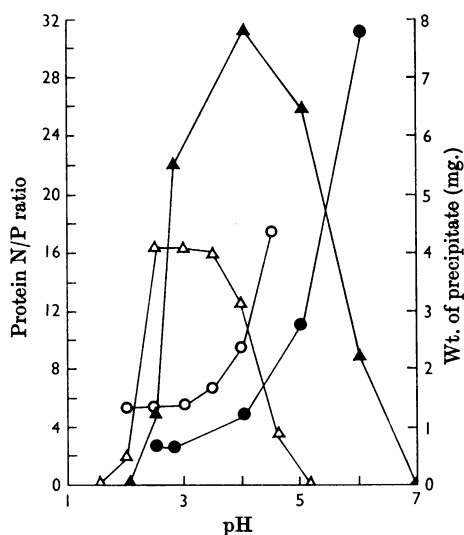


Fig. 9. Composition and weight of the insoluble complexes formed from cytochrome *c* and DNA or RNA as a function of pH. Experimental details were as in the standard partitioning procedure. Protein N/P ratios ( $\mu\text{g. atoms}/\mu\text{g. atom}$ ) were calculated for cytochrome *c* and DNA (●) and for cytochrome *c* and RNA (○) in the insoluble complexes formed at various pH values. The weights of the precipitates (mg./reaction mixture), calculated from N and P values, are also included (▲, cytochrome *c*-DNA; Δ, cytochrome *c*-RNA).

Table 2. Effect of increasing ionic strength on the formation of insoluble complexes between DNA and cytochrome *c* at constant pH

DNA and cytochrome *c* (5mg. of each) were mixed with the following solutions (2ml. of each): 0.05M-, 0.1M-, 0.2M- and 0.5M-citric acid–sodium citrate buffers and a 0.5M-citric acid–sodium citrate buffer containing NaBr (1.0M), pH4.0, and subjected to the standard partitioning procedure with phenol. The precipitates were isolated and analysed for N, P and deoxyribose. The results are given as percentages (w/w) of protein and DNA recovered from the original amount taken.

Buffer solution	Recovery of protein (% w/w)	Recovery of DNA (% w/w)
0.05M-Citrate	73.0	66.0
0.1M-Citrate	72.5	64.0
0.2M-Citrate	71.8	61.8
0.5M-Citrate	73.7	57.2
0.5M-Citrate containing NaBr (1.0M)	5.9	58.2

affinity of phenols for the amide linkage and resulting disruption of the intramolecular hydrogen-bonded structure in these macromolecules (Dawy-

Table 3. *Results of chemical analyses on glycoproteins before and after treatment with phenol*

Experimental details are given in the text. The results of analyses are given in g. of constituent/100 g. of (a) original and (b) glycoprotein recovered from the phenol-rich phase.

	Ovalbumin		Serum $\gamma$ -globulin		Kidney-bean glycoprotein (I)		Kidney-bean glycoprotein (II)	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
N (Kjeldahl)	14.4	14.3	14.0	14.0	13.8	13.8	15.7	15.6
Total neutral sugar expressed as mannose	1.99	2.05	1.07	1.10	7.79	7.91	5.72	5.46
Hexosamines expressed as glucosamine*	1.71	1.72	1.29	1.29	2.79	2.71	1.39	1.44

\* Estimated after hydrolysis with 4N-HCl at 100° for 4hr. by chromatography on an ion-exchange resin column (Pusztai, 1964).

doff, 1953; Mankash & Pakshver, 1953; Pakshver & Mankash, 1953, 1954). The results of later spectroscopic studies (Cannon, 1955), however, cast doubts on the existence of these hydrogen-bonded structures in polyamides. In complete contrast with the views above, several synthetic polypeptides in their solutions in *m*-cresol gave viscosity (Yang, 1958) and optical-rotatory-dispersion data (Urnes & Doty, 1961) consistent with their being in a completely helical form and so excluding the possibility of any interaction between the peptide linkage and the cresol. The experiments described in the present paper have shown that as a result of the action of phenol the molecular conformation taken up by the two proteins studied seems to have become incompatible with their solubility in the aqueous phase without pointing to the forces responsible for the phenomenon.

As expected, serum albumin and the neutral polysaccharide, starch, were completely separated by phenol. The results of studies on the interactions between the amphoteric proteins and various polyacids, on the other hand, presented an entirely different picture. The reaction as carried out in the standard partitioning procedure consisted of two separate steps. In the first step a chiefly electrostatic interaction between the reaction partners is brought about in dilute aqueous buffers. This interaction is very similar to those described by Anderson (1961, 1962, 1963, 1965), with a definite zone of precipitation in the pH range where the proteins are mainly positively charged and the polyacids are approaching their characteristic pK values. There is a pH value in this zone where maximum precipitation occurs and the amount of precipitated material usually decreases steeply at either side of this pH. No interaction is indicated outside this precipitation zone. The second step consists in the addition of phenol, then the rapid warming of the mixture to about 70°, thereby forming a single-phase system, and finally, after

cooling, the physical separation of the re-formed two phases. When precipitates were formed in the first step they seemed to remain largely insoluble on the addition of phenol. This supports the view that phenol, as an intermediate between the strongly and the weakly protic solvents (Singer, 1962), may have little influence over the net charge of the reacting polyelectrolytes. In the final phase of the second step the two-phase system is reconstituted, but by this time the polyelectrolytes, either free or bound, will have passed into the phase appropriate for their ionic structure, state of aggregation, conformation etc. On analysis of the contents of the two phases and the precipitated materials (Figs. 1-6) it is obvious that the interactions extend over to either side of the rather narrow precipitation zone. This is strongly implied by the recoveries of significant amounts of polyacids from the phenol-rich phases at pH values at or below the point of maximum precipitation. Although at these pH values the polyacids themselves begin to show a tendency to pass partly into the phenol-rich phase (Table 1), the presence of protein in the solution increases this to significant levels (Fig. 7). There is a similar tendency on the other side of the maximum precipitation point; increasing concentration of polyacids can draw small but significant amounts of protein into the aqueous phase at pH 5.0 (Fig. 8). The picture thus presented closely resembled that of antigen-antibody reactions with a well-defined equivalence point and 'excess' zones for the multivalent reaction partners. The composition of the complexes changes with changing conditions. Under favourable conditions (pH and concentration of the components) practically all material may become insoluble. At pH values far from the pH of maximum precipitation or large excess of one of the reaction partners or both, no precipitate may be formed at all, but a large part of the reaction partners is nevertheless combined in soluble complexes.

The salient features of the reaction are summarized in Fig. 9, where the amount and the composition of the insoluble complexes formed are plotted against the pH. The protein/nucleic acid (polyacid) ratio changes relatively little near the pH of maximum precipitation, then increases rapidly as the pH rises and the precipitate disappears. When the same ratio is examined in the soluble complexes, it is found to be high at low pH values and becomes progressively lower with increasing pH. At low pH values the soluble complexes contain large amounts of positively charged protein linked to small amounts of nucleic acid and so they pass into the phenol-rich phase in the physical partitioning stage. The insoluble complexes, if they are formed at these pH values, consequently become relatively richer in nucleic acids. The whole effect is reversed above the pH of maximum precipitation; the ionization of the positively charged groups in the proteins is progressively suppressed, and the polyacids are strongly negatively charged. Consequently, at these pH values the soluble complexes contain a large excess of the polyacids and pass into the aqueous phase. This latter phenomenon is obvious only at high polyacid concentrations. At sufficiently high pH values, where the positive charges on the proteins have largely been eliminated and the polyacids and the protein carboxyl groups are highly charged, all interactions are abolished; the proteins are recovered quantitatively from the phenol-rich phase and the polyacids from the aqueous phase.

The abolition of electrostatic and other interactions between proteins and other polyelectrolytes can have great practical importance and, although mixtures of these materials when obtained from biological sources very seldom behave in exactly the same way as the mixtures of pure model compounds, the basic principles of the interaction established in the latter will have a relevance in the former. As shown in the present work, the extent of the interaction is mainly determined by the pH-dependent ionization state of the reacting polyelectrolytes; increasing the ionic strength of the media (up to 0.5M) has but little influence over it. For the isolation of the polyacids free from proteins therefore high pH values are advantageous, preferably near or above the isoelectric point of the proteins. If the isolation of the polyacid has to be carried out at acidic pH values the use of dissociating agents such as sodium bromide (Table 2) can help to keep any potential interaction between the polyacids and any protein present to a minimum.

When, on the other hand, glycoproteins are subjected to partitioning between phenol and buffer solutions of near-neutral pH values, the protein together with the covalently linked sugar residues passes into the phenol-rich phase. When sugar-

containing materials such as starch or nucleic acids are added to the glycoprotein, the extra sugar is found in the aqueous phase. For this reason the method described in the present paper may have value in the analysis of the true sugar content of glycoproteins and, in general, in the analysis of protein preparations.

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