# CLXXII. COMBINATION OF PROTEINS WITH PHTHALEIN DYES.

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Many properties of proteins have been ascribed to adsorption phenomena and surface effects—grouped under the name of colloidal properties. Loeb [1922] and others have shown that many properties of proteins may be explained by the orthodox laws of chemical combination. In this paper an attempt is made to explain the effect of proteins on phthalein dyes as a chemical phenomenon.

The interesting observation was made by Rosenthal [1926] that when a small amount of protein has been added to a dilute rose bengal solution the deep pink colour is not discharged as usual when the solution is acidified. This he ascribes to the fact that the dye behaves as though it were not in solution. It was thought that this observation deserved further attention and it was found that if the colour of a dilute rose bengal solution were discharged by acidification with hydrochloric acid and then a small amount of protein were added and the solution warmed, a rose-red colour soon developed. The combination of protein and dye resulted in colour change, so that the simple explanation of the dye behaving as though it were not in solution is hardly accurate since the same colour is obtained when the dye is undoubtedly in solution and already decolorised. The reaction between the protein and acidified dye proceeds very slowly in the cold, probably because an intramolecular change takes place as will be shown later.

The question arose as to whether rose bengal (tetraiodotetrachlorofluorescein) was unique in its behaviour or whether all fluorescein and phenolphthalein derivatives behaved similarly. The first dyes examined fell into two classes:

(I) *Colour discharge prevented by protein.*

Rose bengal, eosin (tetrabromofluorescein), erythrosin (iodofluorescein) and phloxin red (dichlorotetrabromofluorescein).

(II) *Colour discharged even when protein is present.*

Phenolphthalein, tetrachlorophenolphthalein, tetrabromophenolphthalein, phenolsulphonephthalein and bromothymol blue.

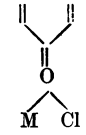
It would thus seem that fluorescein derivatives behaved in one way and phenolphthaleins and sulphonephthaleins in the other. But the deep yellow colour of alkaline fluorescein solutions changed to the pale yellow colour when

acidified even when protein was present. Thus some other explanation was necessary since fluorescein behaves differently from its derivatives. It was noticed that the usual change of colour of all the dyes of class I takes place at reactions on the acid side of the isoelectric point of the protein ($p_H$ 4·8), while the usual change of colour of dyes of class II occurs on the alkaline side of the isoelectric point. The behaviour of thymol blue (thymolsulphonephthalein) supports this observation. This dye changes from blue to yellow in alkaline solution ($p_H$ 9·6) and from yellow to red in acid solution ($p_H$ 2·8). When protein was added the change from blue to yellow on adding acid was not interfered with, but the change from yellow to red did not take place when small amounts of serum-proteins were present before acid was added. The $p_H$ at which the dye changes colour, therefore, determines its behaviour.

Table I. *Reaction of colour change of dyes.*

| Reaction at which colour begins to change when acid is added. | | | |
|---|---|---|---|
| Colour retained in acid protein solution | | Colour discharged by acidification | |
| Dye | $p_H$ | Dye | $p_H$ |
| Eosin ... ... ... | 3·0 | Thymol blue—alkaline range | 9·6 |
| Erythrosin ... ... | 3·6 | Fluorescein ... ... ... | 5·6 |
| Phloxin red ... ... | 3·6 | Bromothymol blue ... ... | 7·6 |
| Rose bengal ... ... | 4·6 | Phenolsulphonephthalein ... | 8·4 |
| Bromophenol blue ... | 4·6 | Tetrabromophenolphthalein | 9·0 |
| Thymol blue—acid range | 2·8 | Tetrachlorophenolphthalein | 9·4 |
| | | Phenolphthalein ... ... | 10·0 |

From this it is evident that acid protein ($PrNH_3^{\cdot} + Cl'$) and coloured (quinonoid) dye must co-exist if colour discharge on acidification is to be prevented. Now Meyer and Hantzsch [1907] describe complexes of phthalein dyes and heavy metal salts (*e.g.* $SnCl_4$) which are red in colour, and therefore have the quinonoid structure, and are probably oxonium salts of the type I.

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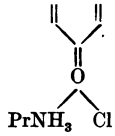


II

It is suggested therefore that we are dealing with dye-protein chloride compounds of the type II. Very strong support for this view is found in the fact that alkaloids similarly interfere with the colour discharge of certain phthalein dyes [Messner, 1903], as does benzidine, as pointed out to me by Dr Marrack. In the case of simple aromatic amines there can be little doubt that the mechanism is chemical combination and it seems highly probable that the same applies to proteins and dyes.

Further evidence for the view of oxonium salt formation is found in the fact that the colour change of rhodamine B is not prevented by adding protein before acidifying. Rhodamine B changes colour on the acid side of the isoelectric point of proteins ($p_H$ 1·2) but the dye does not possess a quinonoid structure and the chromophoric group is a diethylimino-group.

Fluorescent dyestuffs no longer fluoresce when combined with protein in acid solution. When serum-albumin and acid are added to eosin and erythrosin the colour deepens, so that the acid solution is actually more deeply coloured than the original alkaline solution. In addition to this the tint is altered on adding protein and acid. The absorption spectra of the compounds were therefore studied. The author is indebted to Prof. Roaf for allowing him to use his spectrometer and calibrations.

Table II. *Absorption bands.*

Wave lengths in Ångström units.

| Dye | | Alkaline dye | | Acid-protein-dye | | Shift in band |
|---|---|---|---|---|---|---|
| | | Limits | Mean | Limits | Mean | |
| Rose bengal (0·01 %) | 1st band | 4978–5186 | 5080 | 5220–5360 | 5290 | 210 |
| | 2nd band | 5378–5522 | 5450 | 5580–5700 | 5640 | 190 |
| Eosin (0·008 %) | | 5000–5270 | 5135 | 5290–5490 | 5390 | 255 |
| Erythrosin | | 4980–5300 | 5140 | 5290–5480 | 5395 | 255 |
| Phloxin | 1st band | 5047–5175 | 5111 | — | — | — |
| | 2nd band | 5313–5453 | 5383 | 5443–5615 | 5529 | 146 |

These dyes when acidified possessed no prominent bands in the visible region of the spectrum. There is thus a shift of 150–250 Å. in the absorption bands when protein and hydrochloric acid are added to an alkaline dye. So marked an alteration in the absorption spectrum suggests the formation of a new chemical compound with properties different from its components rather than loose physical attachment or adsorption of the dye by protein micellae. One might note at this point that Rosenthal assumed that the colour of rose bengal did not change when combined with acid protein and calculated the amount of dye combined by comparison of the colour of the acid-protein dye with that of alkaline rose bengal. As there is certainly a change in tint and probably in intensity of the colour this seems hardly justifiable. His method of protein determination in the cerebrospinal fluid by this method may also be criticised on the ground that a very large difference in protein concentration has a very small effect on the colour produced.

As we have seen, closely defined conditions as to the critical reactions of the dye and protein must be observed in order that the phenomenon of colour retention in acid solution should occur. This specificity supports the view of chemical combination, rather than physical adsorption. Further support for this view is found in the shift of the absorption band which one would not expect with a purely surface effect.

If the combination of dye and protein were purely an adsorption phenomenon and surface effect it would be expected that surface-active substances, *i.e.* those lowering interfacial surface tension, would have a great effect on the combination. Ten liquids not miscible with water were chosen, viz. amyl and capryl alcohols, chloroform, ether, liquid paraffin, oleic acid, olive oil, light petroleum, toluene and turpentine. The interfacial tension of these liquids against water varies from about 4 to 40 dynes per cm. [Du Noüy,

1926], and one would expect differences in their effect on an adsorption system. It was found, however, that none of the liquids had any appreciable effect on the combination of eosin with proteins whether the liquid was shaken with the acid-protein-dye solution, or with the eosin before the protein and acid were added, except that some of the liquids extracted a small amount of acid-eosin.

Rosenthal [1925] states that 10 mg. of sodium taurocholate prevented the combination of rose bengal with one drop of serum (*i.e.* about 3 mg. of protein). Apart from the fact that this seems an excessive amount of bile salt to have to use if a pure surface effect were involved, it was found that if 10 mg. of sodium taurocholate or saponin were added to one drop of horse serum, and then 10 cc. of rose bengal and 4 cc. of $N/10$ hydrochloric acid were added, the solution remained deep pink in colour. The colour of the solution had been slightly reduced by the surface-active substance but the combination was only very partially inhibited. Possibly the result described by Rosenthal was due to the effect of the bile salt on the membranes he used. The absence of any very great effect of surface-active substances on the dye-protein combination is further evidence that the effect is not purely surface adsorption.

When considerable amounts of concentrated acids, *e.g.* 30 % hydrochloric acid, 20 % salicylsulphonic acid, are added to the acid-protein-dye solutions the colour is discharged, probably because the dye cannot exist in the coloured quinonoid form, even when combined with protein, in strong acid solution. In the case of salicylsulphonic acid the protein is, of course, precipitated, but, even when the sparse precipitate is dissolved by warming, the colour is not regained.

In all these experiments serum, serum-albumin and globulin, egg-albumin and gelatin behave similarly except that gelatin manifests less dye-combining power, possibly due to the molecular aggregation of this protein as suggested by Rosenthal [1926].

The main points supporting the view of chemical combination between the dyes and protein rather than the idea of surface adsorption are as follows.

(1) The specificity and rigidly defined conditions of $p_H$, etc., for combination to occur.

(2) The analogy between proteins and simple organic bases in their behaviour towards dyes.

(3) The shift in absorption band which suggests a chemical rearrangement rather than loose physical forces being called into play.

(4) The lack of effect of surface-active substances on the combination.

Small strips of silk and wool were immersed in fairly concentrated (*ca.* 1 %) alkaline solutions of the dyes, the temperature was rapidly raised to the boiling-point, the solutions were cooled and the material immediately removed from the bath, rinsed with water and immersed in $N/10$ hydrochloric acid. The results were as follows.

| Dye | Silk | Wool |
|---|---|---|
| Eosin | Deep pink | Scarlet |
| Erythrosin | Magenta | Deep red |
| Rose bengal | Magenta | Deep magenta |
| Thymol blue | Yellow | Deep yellow |
| Phenolphthalein | Colourless | Colourless |
| Fluorescein | Colourless | Almost colourless |

These results are thus in complete accord with those obtained with serum-proteins, egg-albumin and gelatin. Those dyes which give coloured compounds with the proteins in acid solution dye the fabrics and the dye is acid-fast, whilst dyes which do not give coloured solutions with acid-proteins do not dye the fabrics to any extent.

Similarly it was found when paraffin sections of various tissues were stained that the same tissues were stained by eosin, erythrosin and rose bengal, and the differences in tint with the different dyes were similar to those with wool and silk. When the stained sections were immersed in $N/20$ hydrochloric acid the stain was not removed. Evidently this histological protein staining is another manifestation of the chemical phenomenon described. Differential staining by treating with acids after the phthalein dyes is therefore due to the stability of the protein-dye compound in acid solution.

Rous [1925] has injected phthalein dyes into living animals and from the colour assumed by the various organs, draws important conclusions regarding the $p_H$ of different tissues. The greatest caution should be exercised in drawing such conclusions since many of the phthalein dyes combine with proteins, and the tint of some of these compounds is slightly different from that of the dye itself, and the colour of the compound varies little with change of $p_H$.

It will be obvious also that many of these dyes cannot be used as indicators as the "protein error" is very great. The case of bromophenol blue may be cited as an example. In series I, one drop of the dye solution was added to 5 cc. of acetate buffer of each $p_H$. In series II, a small amount of serum was added to the dye solution, and one drop of the alkaline dye-protein solution was added to each buffer.

| | $p_H$ | 3·2 | 3·4 | 3·6 | 3·8 | 4·0 | 4·2 | 4·4 | 4·6 |
|---|---|---|---|---|---|---|---|---|---|
| I. Dye alone | ... | yellow | | green-yellow | green-blue | | | blue-violet | |
| II. Protein present | | green-blue | | | blue | | | | |

The tints of the tubes of series II are in no case the same as those of series I and are deeper, but apart from this not even an approximate idea of the $p_H$ can be gained from the colour of the tubes of series II. If to each of the dye-buffer solutions of series I one drop of ten times diluted serum is added the colours alter slightly, but even after 24 hours' standing at room temperature the colours are not the same as those of series II; but if the tubes are plunged in a boiling water-bath for a few seconds and then cooled, the colours of series I and II are indistinguishable. In the tubes of series II, the dye is in the quinonoid form when brought into contact with the acid protein, hence coloured

oxonium salt formation occurs immediately. In the tubes of series I, however, the acid protein is brought into contact with the dye, mainly in the phenolic form, hence combination is slow, but on warming some of the phenolic form changes into the quinonoid form and this combines with the acid protein. Change of phenolic into quinonoid dye in the cold is slow under these conditions but when warmed more quinonoid dye is formed—if a greenish solution of bromophenol blue is warmed the solution becomes blue while hot, but greenish again on cooling. The formation of some quinonoid dye from phenolic dye on heating is a property of many phthalein dyes.

The dyes bromophenol blue, rose bengal, phloxin red, erythrosin, eosin and thymol blue (the last-named in its acid range) cannot therefore be used as indicators in the presence of proteins. Since combination of the protein occurs only with the quinonoid dye, different end-points are obtained when solutions containing protein are titrated, using these dyes as indicators, according to whether acid is added to the alkaline solution or alkali to the acid solution.

In connection with these phenomena an observation of Deutsch [1927] is of interest. He observes that when an aqueous solution of thymol blue, just on the alkaline side of its range of colour-change from the yellow quinonoid to the red phenolic form, is shaken with benzene, a reddish violet colour appears, which disappears when the liquid settles down. This he ascribes to alteration of the dissociation constant of the dye when adsorbed at the benzene-water interface. There is no evidence for this assumption and another explanation is possible. Only the phenolic red form of thymol blue is soluble in benzene in which it gives a reddish violet solution. It is probable therefore that only the phenolic form is adsorbed at the benzene-water interface. In the solution used by Deutsch there is a small amount of the red form in equilibrium with a large amount of the yellow form. When the fluid is shaken the benzene-water interface is greatly increased and all the red form present is adsorbed; some of the yellow form in the aqueous solution then changes over to the red form, to maintain the equilibrium in the water, and this is promptly adsorbed and so on, the reddish colour of the dye adsorbed at the interface predominating over the yellow colour of the aqueous solution. When allowed to settle down the adsorption of the red form becomes negligible since the interface has decreased enormously, and when it passes into the water it is mostly changed to the yellow quinonoid form to give the original equilibrium. There is therefore no need to postulate a change in dissociation constant to explain the behaviour of the dye.

Up to now we have been dealing with very dilute solutions of dyes (*ca.* 0·01 %). When more concentrated solutions are employed another phenomenon has been observed. If serum-proteins are added to a fairly concentrated aqueous dye solution, *e.g.* 0·25 % eosin, and then dilute hydrochloric acid is added gradually, a reddish precipitate first forms and then, on adding more acid, the precipitate dissolves, giving a clear, deeply-coloured solution. If the dye alone is acidified a yellow precipitate is formed and this does not dissolve

in excess acid. This behaviour is not confined to the dyes which remain coloured in acidified protein solutions, but occurs with all the dyes investigated, *i.e.* phenolphthalein and its derivatives, phenolsulphonephthalein and its derivatives, and fluorescein and its derivatives. Those dyes which do not remain deeply coloured in acidified protein solutions do not give deeply coloured precipitates, *e.g.* phenolphthalein gives a colourless precipitate soluble in excess acid. Serum, serum-albumin, serum-globulin, egg-albumin and gelatin all behave in the same way when sufficient protein is added. When an insufficient amount of protein is employed a clear solution in excess acid is not obtained. The precipitation of yellow phenolsulphonephthalein-serum-albumin occurred, although both the dye and the protein alone are soluble at all reactions.

Electrometric determinations were carried out with Dr J. R. Marrack's apparatus and help to determine the reactions at which precipitation and solution occurred. Precipitation commenced near the isoelectric point of the protein, *i.e.* when the protein began to function as a base, and the precipitate dissolved, giving a clear solution at about $p_H$ 2·5. These points appeared to be independent of the nature of the dye.

| | Precipitation | | |
|---|---|---|---|
| Dye change-point | Began | Cleared | Clear |
| $p_H$ | $p_H$ | $p_H$ | $p_H$ |
| 3 eosin—serum-albumin | 4·9 | 2·9 | 2·3 |
| 3 eosin—gelatin | 4·7 | — | — |
| 8 phenol red—serum-albumin | 4·5 | — | 2·6 |

In three cases analysis of the protein-dye precipitates was conducted as follows: The protein was added to the alkaline dye solution and acid was added until a copious precipitate formed. This was allowed to stand overnight and was then filtered off on a hard filter-paper on a Büchner funnel. The dye precipitate was then dissolved in $N/10$ hydrochloric acid, the protein determined by Kjeldahl's method and the dye determined by adding 1 cc. of the solution to hot alcohol, making up the volume to 25 cc. and then comparing the colour of the alcoholic solution, when made alkaline, with that of standard alcoholic solutions of the dyes.

| Constituents | Dye % | Protein % | Mols. dye / Mols. protein |
|---|---|---|---|
| Eosin—serum-albumin | 0·27 | 0·85 | 20 : 1 |
| Rose bengal—serum-albumin | 1·765 | 2·26 | 34 : 1 |
| Phenol red—egg-albumin* | 1·1 | 4·66 | 28 : 1 |

* Assuming the molecular weight of egg-albumin to be 44,000 [Marrack and Hewitt].

It seems probable that these precipitates are due to salt formation between the dyes which have acidic properties and the proteins which on the acid side of the isoelectric point behave as bases. Chapman, Greenberg and Schmidt [1927] describe the formation of similar insoluble protein-azine-dye precipitates. The varying ratio of dye molecules to protein molecules is due to the fact that at these reactions the maximum acid-binding power of the protein is not reached. The solution of the protein-dye compound in excess acid is probably

due to the combination of the basic groups of proteins, which are not already combined with dye, with hydrochloric acid forming soluble hydrochlorides. In support of this is the observation that when excess dye is present and nearly all the basic groups are combined with dye the compound does not dissolve in excess acid.

Since the acidic groups of the dyes at these reactions are mainly phenolic it would be anticipated that phenol itself would behave similarly to the dyes, and this was found to be the case. When a small amount of protein is added to a dilute solution of phenol and acid is gradually added, a precipitate first forms and this dissolves up when more acid is added. A typical experiment with acetate buffers was as follows:

| $p_H$ | 5·2 | 5·0 | 4·8 | 4·6 | 4·4 | 4·2 |
|---|---|---|---|---|---|---|
| Precipitation | — | ± | + + + | + + | + + | – |

Hoffman and Gortner [1925] suggest that on the acid side of $p_H$ 2·5 proteins do not combine chemically with acids but form adsorption compounds. Chapman, Greenberg and Schmidt [1927] find that in solutions more acid than $p_H$ 2·5 certain dyes combine with proteins in stoicheiometric proportions giving protein-dye precipitates. The valency of the proteins in these compounds approximates closely to the maximum acid-combining power of the proteins calculated from their diamino-acid content. Gortner's [1927] reply is that these results "offer very striking substantiation of the conclusions reached by Hoffmann and Gortner." Gortner assumes that in very acid solution there are present protein cations with surface electrical charges and the number of these charges is equal to the chemical valency of the protein, and the same with the dye. These charges are neutralised and a protein-dye colloid is precipitated. It seems probable, however, that such neutralisation of ionic charges may well be chemical combination, and is in no wise different from the precipitation of barium sulphate by the neutralisation of the ionic charges of barium and sulphate ions and formation of an insoluble chemical compound. The mere fact of the protein cation being larger than the barium cation does not change a phenomenon from chemical combination to physical adsorption.

## Summary.

Phthalein and fluorescein dyes which change colour on the acid side of the isoelectric point of proteins combine with proteins in acid solution giving coloured oxonium salts. The colour of such dyes is not discharged or altered as usual on acidification and hence they cannot be used as indicators in solutions containing proteins. The "protein error" of indicators, acid-fast staining of tissue sections and acid-fast dyeing of fabrics are explained.

When any phthalein or fluorescein dye is mixed with a protein solution and acid is added, a precipitate forms at the isoelectric point of the protein and dissolves at about $p_H$ 2·5. Salt formation is indicated, and it is considered unnecessary to postulate physical adsorption to explain any of the phenomena described.

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