Spot-Line Chromatography: A Technique for Studying Interactions between Proteins and Dyes

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It has been suggested (Klotz, Burkhard & Urquhart, 1952*a*) that the difficult problem of mapping the surface of a soluble protein molecule could be approached by studying the binding of other molecules, particularly polyfunctional ones, by the protein. Analytically, this is a somewhat indirect approach, but from a biological point of view it has the advantage that the information obtained is likely to be directly relevant to the function of the protein molecule.

A more immediate application of binding studies is in characterizing empirically proteins or protein mixtures which may be encountered in biochemical, genetic or pathological work: even where the electrophoretic or ultracentrifugal mobilities of different proteins are similar, their ability to bind dyes, or other easily detectable substances, may differ substantially. Binding studies may also provide a method of characterizing different conformational states (Eyring & Lumry, 1954) of a protein, since changes in the folding of a protein molecule may affect its ability to bind other molecules or ions (Kauzmann, 1959; Klotz, Burkhard & Urquhart, 1952*b*).

This paper describes a small-scale technique for detecting and comparing binding between proteins and dyes. The technique depends on measuring the movement of dye molecules, which may be imparted to them by moving protein. If a spot of protein is made to move through a line of dye drawn at right angles to the direction of movement of the protein, then, if there is any binding between the protein and dye, a distortion or 'loop' may be formed in the line. Generally, the greater the binding power of a given protein spot the greater will be the area of the loop formed in a given line of dye.

EXPERIMENTAL

Materials. Crystalline bovine serum albumin was obtained from The Armour Laboratories. Ovalbumin was prepared from 1-day-old eggs according to the method of Kekwick & Cannan (1936) and recrystallized four times. Protein solutions were prepared as required by dissolving a known amount (usually about 50 mg.) in the buffer being used for the run and making up the solution to 1 ml. Except in method 1, where any volatile solvent could be used, dye solutions were prepared in the relevant buffer.

British Drug Houses Ltd. dyes, most of them standard microscopical stains, were used.

A phosphate buffer (pH 6.8: 0.055 M- Na_2HPO_4 -0.044 M- KH_2PO_4) was used unless otherwise stated. Solutions in this buffer containing 50 mg./ml. of ovalbumin or bovine serum albumin had a pH of about 6.6. A borate buffer (pH 8.5: 0.022 M- H_3BO_3 -0.0088 M- NaOH) was used in the starch-gel runs. The other buffers used were borate (pH 7.8: 0.16 M- H_3BO_3 -0.01 M- $\text{Na}_2\text{B}_4\text{O}_7$), diethylbarbiturate (pH 8.6: 0.05 M-sodium diethylbarbiturate-0.01 M-diethylbarbituric acid) and acetate (pH 4.0: 0.16 M-acetic acid-0.04 M-sodium acetate).

Unless otherwise stated, the paper used was Whatman no. 1 'for chromatography'. Gels were prepared according to the method of Smithies (1955) from hydrolysed starch obtained from the Connaught Laboratories.

Method 1. A simple apparatus for paper electrophoresis (Kunkel & Tiselius, 1951) was set up, a broad paper strip being held between glass plates with the ends of the strip dipping into triple-compartment electrode troughs. First, lines of dye were drawn on the dry paper with a capillary tube and the solvent was allowed to evaporate. The paper was then wetted carefully by dipping the centre in a pool of buffer on the lower glass plate of the apparatus and lowering the ends into position. Spots containing about 200 μg . of protein were placed 1 cm. behind the dye lines. The upper glass plate was then put in position and a potential difference of 100 v applied across the 30 cm. of paper strip. With a phosphate buffer at pH 6.8 both bovine serum albumin and ovalbumin are anionic and move at comparable rates. They would overtake most of the dyes completely in a few hours. The final positions of the protein spots could be determined by staining with ethanolic bromophenol blue (about 1%) saturated with mercuric chloride, and washing with running water.

Method 2. A strip of paper wetted with buffer was placed on the lower glass plate with the ends of the paper dipping into the electrode troughs. A polythene sheet was placed over the paper and air bubbles were carefully pressed out. A dye line was applied by making a slit in the sheet with a razor, and rubbing the slit lengthwise with a piece of cotton wool wet with a solution of the dye in the buffer. In this way a fine uniform line could be formed. The system was left for half an hour to allow the amount of buffer in the paper to reach a steady value. Pin-holes were then made in the polythene sheet 2 cm. behind the dye line. With an Agla micrometer syringe, small measured volumes of protein solutions (10–100 mg./ml.) were placed on the pin-holes. Most of the protein then soaked into the paper. The small amounts remaining on the surface of the polythene were washed in with two small volumes each of buffer. A potential gradient of between 5 and 10 v/cm. was then

applied until the protein spots had overtaken the dye completely.

The areas of the loops formed were estimated by drawing a line through the centre of the zone of highest concentration in the dye line, and then either tracing on paper, which was cut out and weighed, or, in favourable cases, measuring the area of a closely fitting triangle.

Method 3. Here the dye was applied by cutting 4 mm. wide strips from the paper, and soaking these in a dye solution (about 0.01%) for a known time (about 30 sec.). The strips were then placed in position on the glass plate together with the rest of the paper soaked in pure buffer. Method 2 was then followed. The advantage of this modification was that closely comparable dye lines could be applied in different runs.

Method 4. Starch gel could be used as an alternative to paper as the supporting medium. Gels were prepared in shallow (12 in. $\times$ 6 in.) Perspex trays. Here the selected procedure for testing interaction between protein and dye depended on the relative mobilities of the two. Where the protein moved more rapidly than, but in the same direction as, the substrate (as, e.g., with bovine serum albumin and Biebrich scarlet) the following procedure was used. The gel was cut at right angles to the direction of the current and one of the two pieces removed from the tray. A few millilitres of dye at a known concentration (about 0.01%) were run into the empty part of the tray, which was tilted slightly so that the dye solution did not come into contact with the gel. The tray was then carefully tilted so that the dye came into contact with the cut edge of the gel for a given time (about 30 sec.). The stained edge of the gel was then washed with two small portions of buffer. The other piece of gel was replaced and a current passed for a few minutes so that the dye line which had been formed moved into one of the pieces of gel. The protein was inserted by the technique, described by Smithies (1955), of placing on the cut edge of the gel small rectangles of filter paper soaked in the protein. In this way a very fine initial dye line could be formed under reproducible conditions and a measured amount of protein placed immediately behind it. Modifications of this procedure were used where the relative mobilities of the dye and protein were qualitatively different from that described. If, for example, the dye moved faster than the protein, the latter was run into the gel first, the paper with which the protein was applied removed, and then the dye line was formed as before.

RESULTS AND DISCUSSION

Qualitative experiments

A number of dyes were tested by method 1 with bovine serum albumin and ovalbumin. Most of these dyes are listed in Table 1, together with estimates of their binding by the two proteins. Such estimates can only be approximate since the effective concentrations of the dyes were not known and differences in adsorption of the dyes on the paper were not accurately accounted for. In some cases the dye was too insoluble in the buffer or too strongly adsorbed on the paper to allow the formation of clear loops: Sudan II and III, Congo

red and Congo rubin all interact with bovine serum albumin, but this was shown only by a slight smearing of the dye line in the path of the protein (Fig. 1f). At the other extreme, picric acid, fluorocene or bromophenol blue diffused too readily to maintain a clear line. Chromotrope also was unsatisfactory because it moved at about the same rate as the proteins. Orange G and Naphthol green B both move faster than the proteins under the conditions of the experiment; but reverse loops could be obtained with these dyes by allowing them to overtake bovine serum albumin spots (Fig. 1g).

Fig. 1 illustrates some of the effects produced. Often these showed characteristic features. In the loops formed by bovine serum albumin with Biebrich scarlet, when the dye was at a relatively high concentration, there were two distinct zones: the main band had a 'flame' smeared out in front of it (Fig. 1a), suggesting different binding sites on the protein, from some of which the dye is released more slowly. At lower concentrations of dye this effect became less pronounced. The effect was shown also with Orange G, where bovine serum albumin spots remained coloured with the dye for some time after the two had apparently separated (Fig. 1g).

The sample of Trypan blue which was used contained a minor purple impurity. Under the conditions of the experiment, separation of the two

Table 1. *Binding of dyes by bovine serum albumin and ovalbumin as indicated by loop formation*

S, M and W indicate strong, moderate and weak effects respectively; O indicates that no effect was detected.

No.	Name	Bovine serum albumin	Ovalbumin
1	Neutral red chloride	O	O
2	Methylene violet	O	O
3	Safranine	O	O
4	Nigrosine C	S	O
5	Acridine orange	O	O
6	5-Aminoacridine	O	O
7	Pyronine G	W	M
8	Brilliant cresyl blue	W	W
9	Celestine blue	O	O
10	Gentian violet	M	O
11	Brilliant green	M	O
12	p-Aminoazobenzene	M	W
13	Chrysoidine	M	O
14	Congo red	M	O
15	Congo rubin	M	O
16	Benzopurpurine 4B	M	O
17	Orange G	S	O
18	Biebrich scarlet	M	O
19	Trypan blue	M	O
20	Methyl orange	M	O
21	Aurantia	M	M
22	Eosin	M	O
23	Naphthol green B	S	O

components by normal electrophoresis was barely visible even after several hours. The passage of bovine serum albumin through the line, however, separated the components very clearly, two distinct loops being formed (Fig. 1e).

Where the dye was fairly strongly adsorbed by the paper, quite good loops could be obtained by

setting up the experiment as described earlier, and then allowing the buffer to siphon slowly through the paper without any electric current. This could be done simply by having a difference of a few centimetres in the level between the buffer solutions in the electrode troughs. This emphasizes that the effects described here are essentially chromato-

Fig. 1. (a) Effect produced when bovine serum albumin (left) and ovalbumin (right) move through a line of Biebrich scarlet. The initial positions of the dye (---) and the proteins (+) are shown. (b), (c) and (d) Effects produced with these proteins and *p*-aminoazobenzene, Aurantia and Pyronine G respectively. (e) and (f) Effects produced by bovine serum albumin with Trypan blue and Congo rubin respectively. (g) Illustrates the reversed loop formed when a line of Orange G overtakes a spot of bovine serum albumin.

graphic: an electric current is simply one of a number of possible devices for making the protein move: loops result from a partition of the dye between a moving and a stationary component.

With starch gel as the supporting medium, very clear loops could be obtained, provided that the runs were fairly short; but this medium had the disadvantage that diffusion of the dye tended to be greater than on paper, and that the loop areas were not so conveniently measured.

Discussion of qualitative results. Goodman (1958) has envisaged the serum-albumin molecule as a transport vehicle for fatty acids. The ability of moving serum-albumin molecules to transport other molecules is very clearly illustrated in the experiments described here and, as shown particularly by the resolution of two components in Trypan blue microscopical stain, a solution of bovine serum albumin may act as a fairly selective chromatographic solvent. The relatively high concentration of serum albumin in the blood must increase considerably the efficiency and selectivity of blood as a solvent.

According to Klotz, Gelewitz & Urquhart (1952), the exceptional binding power of the serum albumins is restricted to anions and neutral molecules. Certainly, in the experiments described here, all the anions tested which gave definite results (14–23 in Table 1) showed some binding with bovine serum albumin whereas only some of the cations did. This is particularly striking since the protein was anionic in these, as in most previous experiments (Klotz & Urquhart, 1949). The cationic triphenylmethane dyes gentian violet and Brilliant green, however, were bound moderately by bovine serum albumin.

Of the neutral and cationic phenazines and acridines investigated (1–6 in Table 1) only Nigrosine C was bound by bovine serum albumin, but this was bound very strongly. It may be significant that of this group of substances Nigrosine C alone contains a *p*-quinonoid electronic system similar to those in the triphenylmethane dyes.

Derivation of loop area

In deriving an expression for the area of the loop formed when a spot of protein moves through a line of dye, it is convenient to consider first the case where the protein moves into a uniform broad band of dye of molecular concentration $[X]$ (see Fig. 2). It will be assumed that the relative movement is slow enough for the protein and dye always to be in equilibrium, and that binding of dye does not significantly alter the rate of the protein molecules.

As the protein spot enters the band, the total concentration of dye will rise in the front element of the spot if there is any protein-dye binding

(Fig. 2b). It will continue to rise until the rate at which the dye molecules are entering the first element equals the rate at which they are leaving it and entering the second, i.e. until the concentration of unbound dye within the first element rises to the value $[X]$. Similar steady states will be reached successively in each element until finally dye will be leaving the spot at the concentration $[X]$, which will be the concentration of unbound dye throughout the spot. Then, provided that the protein molecules bind independently of each other, the average number of dye molecules bound by each protein molecule will be the same, and independent of the protein concentration. By this stage a 'wedge' will have been formed in the band (Fig. 2c). Since the free dye concentration will be the same at each point in the spot as in the band as a whole, the bound dye will be equal to that missing from the wedge. The area of the wedge will thus be proportional to the number of protein molecules, and independent of the size, shape or concentration distribution of the spot: i.e. for a given protein, and for a given band of dye:

$$A = \text{constant} \times W, \quad (1)$$

where W is the weight of protein in the spot and A the area of the wedge.

If $[X_B]$ is the concentration of bound dye in a uniform spot with a protein concentration of $[P]$, and B is the area of the spot, then

$$[X_B]B = [X]A. \quad (2)$$

Fig. 2. Protein spot entering a broad band of dye. For further explanation see text.

Hence:

$$A = \frac{[X_B]}{[P]} \cdot \frac{W}{t.m.[X]}, \quad (3)$$

where t is the effective thickness of the buffer in the supporting medium and m is the molecular weight of the protein.

Goodman (1958) and Scatchard, Coleman & Shen (1957) showed that if there are several classes of independent binding sites on the protein molecules, and n_i sites with an apparent binding constant K_i , then the relationship

$$\frac{[X_B]}{[P]} = [X] \sum_i \frac{n_i K_i}{1 + K_i [X]} \quad (4)$$

is approximately true; hence from (3):

$$A = \frac{W}{t.m} \sum_i \frac{n_i K_i}{1 + K_i [X]}. \quad (5)$$

If a proportion of the unbound dye molecules are adsorbed by the supporting medium, and $[X']$ is the concentration available for binding by the protein, then $[X']$ must be substituted for $[X]$ in (4), so that (5) becomes

$$A = \frac{[X']}{[X]} \frac{W}{t.m} \sum_i \frac{n_i K_i}{1 + K_i [X']} \quad (5')$$

but, provided that there is no competition between the protein and the dye for adsorption sites on the supporting medium, the general conclusion (1) should still hold.

Under the reversible conditions postulated, a protein spot emerging from a band of dye would deposit a wedge exactly similar to that removed on entry. The looped band so formed could be regarded as being made of a number of parallel elements each acting independently: the area of the loop formed when a spot of protein moves through a band of dye should thus be independent of the width of the band.

The argument applied to the formation of a cut-out wedge in a broad band of dye would still apply where there was rapid but irreversible binding of dye at n_0 sites on the protein molecules, except that the term $n_0/[X]$ would have to be added to the sum in (5). The wedge deposited as the protein left the band would be smaller than the cut-out wedge and would be due only to the reversible sites: (5) would still apply to the area of this wedge. If, however, the width of the band were to be reduced so that there was insufficient dye to saturate the irreversible sites (i.e. if the cut-out wedge due to the irreversible sites alone were greater than the width of the band) then no loop, or an incomplete loop, would be formed. In the quantitative experiments described below with the bovine serum albumin-Biebrich scarlet system, such rapid irreversible binding did not occur since at the end of each run complete loops had been

formed, showing that any irreversible sites must have been saturated, and no dye was visible on the protein spots. The concentration of the protein, and the limit of visibility of the dye on the paper (about 0.0005 %), were such that a dye concentration of below one-tenth of that required to saturate only one site on the protein would have been visible.

Quantitative experiments

A series of experiments (method 2) were carried out in which different known amounts of bovine serum albumin were run through a uniform line of Biebrich scarlet. As expected, low dye concentrations and large amounts of protein favoured large loops. The relationship (1) was approximately true for this system, except that plots of A against W generally cut the x axis well above the origin: there appeared to be a minimum threshold amount of protein required for loop formation. That this threshold depended on C , the area of paper covered by the protein before reaching the dye line, is suggested by Fig. 3; these results can be explained if it is assumed that the loop area was proportional to the effective weight of protein overtaking the dye line, but that this effective weight was decreased by about 20 $\mu\text{g./cm.}^2$ of

Fig. 3. A is the area (cm.^2) of the loop formed in a line of Biebrich scarlet by a spot ($W \mu\text{g.}$) of bovine serum albumin which has traversed $C \text{ cm.}^2$ of paper. Relative concentrations of dye in three runs, as judged visually: $\Delta > \circ \approx \bullet$.

paper covered by the protein before reaching the dye. In these experiments variations in C resulted mainly from variations in the widths of the spots, each of which was placed a known distance of about 2 cm. behind the dye line.

In another series of experiments (method 3), spots containing the same weight of protein were made to move through oblique lines of dye. Fig. 4 illustrates the effect produced in such runs. These experiments showed clearly that, initially, decay in binding power depends on the distance moved by the protein before reaching the dye line. In a number of runs with different dye concentrations, and with spots containing between 100 and 300 $\mu\text{g.}$ of protein, the initial rates of decay were equivalent to the loss of between 10 and 30 $\mu\text{g.}$ of active protein/ cm.^2 of paper traversed. The decay then became less pronounced, until finally loop areas were approximately constant (usually at somewhat below 10% of their initial values). Fig. 5 (open symbols) shows a typical plot of loop area against area of paper swept out by the protein before this reaches the dye. When a number of spots (300 $\mu\text{g.}$) of the protein were placed 2 cm. behind a dye line, considerable variations were observed in the areas of the loops subsequently formed; but when small variations in the width of the spots were taken into account, and A was plotted against C , a straight line was obtained

Fig. 4. Typical decay pattern formed when spots containing the same amounts of bovine serum albumin move through oblique lines of Biebrich scarlet. Protein starting points: +.

Fig. 5. Binding of Biebrich scarlet shown: (a) by spots (200 $\mu\text{g.}$) of bovine serum albumin which have moved different distances before reaching the dye (○); (b) same, with paper previously washed with a solution of the protein (●).

Fig. 6. Binding of Biebrich scarlet shown by spots (300 $\mu\text{g.}$) of bovine serum albumin with variations in the widths of the spots. In the first run (a, ●) spots moved through one dye line at 2 cm. from the protein starting line. In the second run five spots moved through two dye lines at 2 cm. (b, ▲) and 14 cm. (c, ○) from the protein starting line.

(Fig. 6a). In another similar run the widths of the spots were varied deliberately over a much larger range by using different amounts of buffer to wash the protein on to the paper. The spots were then made to move through two parallel lines at 2 cm. and 14 cm. from the protein starting line. These results, which are similar to those obtained by varying the length of the protein run, are plotted in Fig. 6(b) and (c). A somewhat greater initial loss of binding power is indicated here (about $40 \mu\text{g./cm.}^2$ of paper traversed).

Quantitative runs with ovalbumin were unsatisfactory since both Aurantia and Pyronine G moved and diffused too quickly to maintain clear loops over a long period. The results of runs with ovalbumin and oblique lines of Aurantia were very indefinite, although they suggested that binding decay, if it occurs at all in ovalbumin, is not so pronounced as in bovine serum albumin.

*Further investigation of binding decay
in bovine serum albumin*

Results so far considered suggest that there is a relationship between the loss of binding power of bovine serum albumin for Biebrich scarlet and the area of paper traversed by the protein: that the phenomenon is due to some kind of interaction between the protein and the paper. Results of 'control' experiments designed to detect alternative causes are now considered briefly.

Dilution of the protein spots, as they move across the paper, might account for the apparent decay in binding power which is observed (if, for example, there is competition between the protein and the dye for adsorption sites on the paper). But a spot containing $25 \mu\text{g./cm.}^2$ of paper could still give a larger loop in the first of two dye lines through which it passed, and 'decayed' spots with concentrations of over $50 \mu\text{g./cm.}^2$ could be obtained. A dilution effect, if operating at all, cannot account for the whole of the decay.

Dissociation of different components within the protein spot might affect its loop-forming ability. Such dissociation could be increased both as a result of increasing the width of the spot (a dilution effect) and, through electrophoretic separation of the components, by increasing the distance moved on the paper. However, binding power was not restored when 'decayed' spots were reversed in their tracks, as would have been expected if electrophoretic separation was an important factor. Also, decay patterns (like Fig. 4) were produced even when the protein was made to move simply by siphoning the buffer through the paper.

Prolonged pre-dialysis of the protein had little effect on decay patterns: the decay cannot be due to the loss of a loosely bound small molecule or ion from the protein.

Time and temperature. A run with oblique lines was carried out in which the protein was allowed to stay on the paper for 2 days before the current was switched on. In another similar run an alternating current was first passed for the same period. In both of these experiments normal decay patterns resulted. Evidently the protein must move before extensive decay in its binding power occurs: contact with a limited amount of paper is insufficient to produce the effect, as is rise in temperature due to the passage of current.

In another experiment protein spots were run on paper as usual for long enough to induce maximum decay. Then new spots were placed in line with the original ones, and both sets allowed to move through a line of Biebrich scarlet. The new spots gave much larger loops than the original ones, demonstrating that the decay is not due to changes in the dye, buffer or paper during the runs.

Buffers and pH. Decay patterns were produced with phosphate (pH 6.8), borate (pH 7.8) and diethylbarbiturate (pH 8.6) buffers. With an acetate buffer at pH 4.0, with the protein moving towards the cathode, loop areas again decreased with the area of paper traversed; but this may have been due entirely to the comparatively heavy loss of protein on to the paper which occurs under these conditions.

Supporting medium. The areas of loops formed in starch gel were difficult to estimate owing to relatively fast diffusion of Biebrich scarlet in this medium; but, except where the protein was placed immediately behind the dye line, loops of about the same area were produced in a dye line when spots containing the same amount of protein were made to overtake the dye simultaneously after moving different distances. Spots with very short distances to move before reaching the dye (about 1 mm.) gave larger loops than the others by a factor of 1.5-2.0.

The decay phenomenon was observed with each of six different grades of paper (Whatman nos. 1, 2, 3, 4, 7 and 84).

In view of the dependence of the decay phenomenon on the area of paper swept out by the protein, and the lack of support for alternative explanations, it appears that the effect is due to some kind of interaction with the supporting medium. If this is so there must be only a limited number of active sites on the paper, enough, that is, to induce decay in about $20 \mu\text{g.}$ of protein/ cm.^2 of paper. Five general types of interaction can be considered: (1) permanent adsorption of the protein by the paper; (2) permanent and preferential adsorption of a minor active component by the paper; (3) loss of a mordant ion or molecule to the paper; (4) competitive binding of an impurity from the paper; (5) direct interaction between protein

and paper which does not immobilize the protein but decreases its binding power.

(1) The decay patterns (Fig. 4), showing as they do rapid initial decline in binding power followed by loops of approximately constant area, are not consistent with a simple loss of total protein due to 'tailing'. A small amount of protein is lost on to the paper by permanent adsorption (Kunkel & Tiselius, 1951): by staining with bromophenol blue, a fairly uniform tail can be seen between the starting point and the final position of the spot. However, the amount of protein lost in this way is normally only about $2 \mu\text{g./cm.}^2$ of paper traversed.

(2) The protein tails were not noticeably more dense in the region of rapid decay than elsewhere. If decay is due to preferential adsorption of an active component this must be present in a very small amount.

(3) and (4) These would fit the data so far discussed. Treatment of the paper with a large number of different solvents had little or no effect on the decay. Also, washing the paper with a large excess of the protein did not prevent it (see Fig. 5b). If the decay results from binding by the protein of an interfering ion or molecule then even if the numerous solvents were not effective in removing it the protein itself should have been. In another experiment, four sets of protein spots were made to move along the same set of tracks. There was no increase in the size of the loops formed by the later spots. Interaction with the paper by any of the mechanisms (1)–(4) would have been expected to have left some sort of trail in which the paper would have been less effective in inducing binding decay in further protein.

Similar binding decay was observed with eosin or gentian violet in place of Biebrich scarlet. There is a marked change in the colour of eosin when it is bound on bovine serum albumin. The decay can be seen here by soaking the whole paper in eosin solution and watching the deeper pink of the bound eosin fade as a protein spot moves across the paper.

A series of long spots containing up to 4 mg. of bovine serum albumin were run across a piece of paper which was then sprayed with a dilute solution of bovine serum albumin (to give a concentration of about $20 \mu\text{g./cm.}^2$) followed by an eosin solution. There was no sign of an inactive trail behind the spots. This would have shown up as a deeper pink.

The decay in the binding power of bovine serum albumin for Biebrich scarlet or eosin, as the protein moves across filter paper, thus appears to be due to (5), direct protein-cellulose interaction resulting in some permanent change in the protein but not in the cellulose. The 'decayed' protein cannot be completely denatured, since denaturation gives rise to heavy tailing of bovine serum albumin spots. The much smaller normal tailing observed

with native bovine serum albumin may be due to the presence of a limited number of sites on the paper which adsorb and denature the molecules. It is suggested that there are about ten times as many adsorption sites on the paper which produce less drastic but persistent changes in the protein molecules, and that these changes result in the observed decrease in their dye-binding ability.

Decay in the binding power of bovine serum albumin has been noted on prolonged contact with 0.01N-sodium hydroxide or heat, and attributed to a denaturation effect (Klotz, Triwush & Walker, 1948).

SUMMARY

1. Binding between proteins and other molecules can be studied by allowing a spot of protein to overtake a line of a second component in a suitable supporting medium. Binding may be shown by a distortion or loop in the line. This effect has been used to screen a number of dyes for interaction with bovine serum albumin and ovalbumin.

2. It has been shown that under ideal conditions the area of the loop formed by a given protein in a given line of dye should be proportional to the total weight of active protein in the spot. This has been verified as being approximately true for the bovine serum albumin-Biebrich scarlet system, but with one important reservation.

3. As bovine serum albumin molecules move across filter paper, their binding power for Biebrich scarlet falls to about 10 % of the initial value. This seems to be due to adsorptive interaction between the protein and the paper.

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