241. *Researches on Monolayers. Part VII.*[1] *Reactions of Casein with Dyes and other Aromatic Solutes and their Relation to Adsorption by Protein Fibres.*

By A. Cameron, C. H. Giles, and T. H. MacEwan.

The effect of aromatic sulphonates, including some dyes, upon casein monolayers spread on acid or on buffer solutions near the isoelectric point has been studied. Large dye molecules with a non-ionic polar group at each end have an effect on the film similar to that of tannic acid. Surface-active monobasic dye molecules with weak hydrogen-bonding groups penetrate the film and at high surface pressures increase its solubility. It is suggested that the latter effect may be due to the adsorption of a layer of dimerised dye molecules below the film.

Small sulphonate molecules and disulphonates with weak hydrogen-bonding centres (*e.g.*, anthraquinonedisulphonates) are probably adsorbed beneath the film. The results are used as a basis for the hypothesis that the affinity of monobasic anions for protein fibres arises from their own mutual attraction, which assists them in forming a monolayer or a layer of micelles adsorbed on the fibre, rather than from specific anion–fibre attraction.

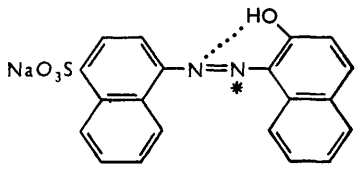
The interaction of organic ions with casein monolayers has been investigated to assess the rôle of polar and non-polar forces in the adsorption of dyes by protein fibres. Organic ions of high molecular weight are readily adsorbed by protein fibres, their affinity generally increasing with the size of their organic residue,[2,3] but the source of this affinity has not been precisely defined. It has been suggested that the major contribution to the affinity is non-polar van der Waals attraction by the hydrophobic portions of the protein molecule (see, *e.g.*, ref. **4**), and certain experimental results have been interpreted as evidence that hydrogen bonds do not operate between protein fibres and the anions of sulphonated dyes.[5,6] It has however been pointed out also that dye affinity is not as closely related to molecular weight as it would be if only non-polar forces were acting.[7] There is moreover positive evidence in favour of the hypothesis of hydrogen-bond adsorption of many non-ionic organic solutes, and a few ionic ones, by proteins, *e.g.* the action of tanning agents on protein monolayers on water,[8,9] the adsorption of un-ionised or weakly ionised organic solutes by wool,[3] the interaction of serum albumin or casein with simple azo-compounds,[10] the interference of fibrinogen clotting by thrombin caused by the presence of urea and several alcohols,[11] and complex-formation between casein, edestin, or gelatin and several solutes, including anionic dyes, detected by refractometry.[12,13]

Previous Work on Dye–Protein Monolayer Interactions.—A few authors have examined reactions between monolayers and dyes dissolved in the water beneath. Thus Wunderby[14] removed protein monolayers from the surface of solutions of several azo-dyes by lifting them on a glass slide, then redissolved the dye-protein combinations in alkali and examined the solutions spectrophotometrically. He considered that the extent of reaction depended

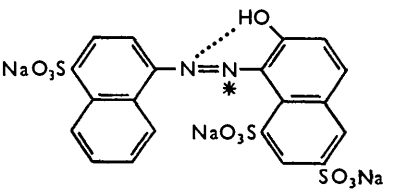
[1] Part VI, Cameron, Giles, and MacEwan, *J.*, 1957, 4304.
[2] Steinhardt, Fugitt, and Harris, *J. Res. Nat. Bur. Stand.*, 1940, **25**, 219; 1943, **30**, 123.
[3] Vickerstaff, "The Physical Chemistry of Dyeing," Oliver and Boyd, Ltd., Edinburgh, 2nd edn., 1954.
[4] Meggy, *J. Soc. Dyers Colourists*, 1950, **66**, 510.
[5] Derbyshire and Marshall, *Discuss. Faraday Soc.*, 1954, **16**, 140.
[6] Derbyshire, *Trans. Faraday Soc.*, 1955, **51**, 909.
[7] Vickerstaff, *J. Soc. Dyers Colourists*, 1953, **69**, 279.
[8] Schulman and Dogan, *Discuss. Faraday Soc.*, 1954, **16**, 158.
[9] Ellis and Pankhurst, *Discuss. Faraday Soc.*, 1954, **16**, 170.
[10] Klotz and Ayers, *Discuss. Faraday Soc.*, 1953, **13**, 189.
[11] Shulman, *Discuss. Faraday Soc.*, 1953, **13**, 109.
[12] Campbell, Cathcart, and Giles, *J. Soc. Dyers Colourists*, 1957, **73**, 546.
[13] Bruce, Giles, and Jain, *J.*, in the press.
[14] Wunderby, *Experientia*, 1951, **7**, 296.

on the colloidal nature of the dye. While the present work was in progress, Harrap [15] reported experiments on spreading a monolayer of a soluble wool keratin derivative on buffered solutions of Orange II. He observed effects indicating interaction between the protein derivative monolayer and the dye, beyond simple additive penetration. Interaction due to ionic binding occurs below pH 4. At high dye concentrations in the aqueous solution (0·1—0·5M) increased interaction takes place, apparently owing to the dye's being able to aggregate and orient at the surface, and to penetration's being facilitated by the increased screening of negatively charged protein side-chains when the ionic concentration rises.

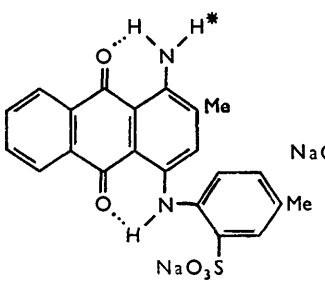
Present Work.—The present work was undertaken to define more clearly the source of affinity of organic ions for protein fibres and to resolve some of the apparently conflicting evidence regarding the rôle of polar and non-polar forces in adsorption of dyes by proteins. Surface-pressure and area measurements were made on casein monolayers spread upon water and aqueous solutions of a variety of solutes, including polar non-ionic compounds and a number of aromatic ionic compounds of various molecular sizes and containing different numbers and types of potential hydrogen-bonding group. Non-ionic solutes used were: tannic acid (I), glucose (II), and *m*-inositol (III), and anionic ones were either (i) of small molecular area or with no strongly hydrogen-



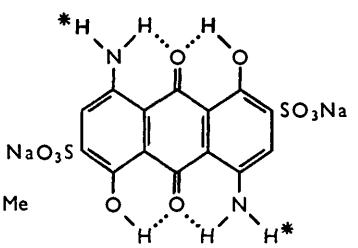
(X) C.I.15620 (ref. 16)



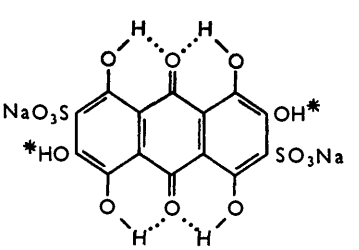
(XI) C.I.16255



(XII) C.I.62085



(XIII) C.I.63010



(XIV) C.I.58610

The asterisks indicate the probable hydrogen-bonding centres, based in the case of (XII) and (XIII) on direct determination of complex-ratios with phenol in aqueous solution.

bonding groups [sodium benzenesulphonate (IV), sodium naphthalene-2-sulphonate (V), sodium 2-hydroxynaphthalene-7-sulphonate (F-salt) (VI), and sodium anthraquinone-2-sulphonate (VII) and -1 : 5- and -2 : 7-disulphonates (VIII), (IX); also (XI), (XIII), (XIV)], or (ii) monobasic, having a large hydrophobic residue (*i.e.*, weakly surface-active) [(X), (XII)].

Results and Discussion

The results are shown in Fig. 1 and the following interpretations are given.

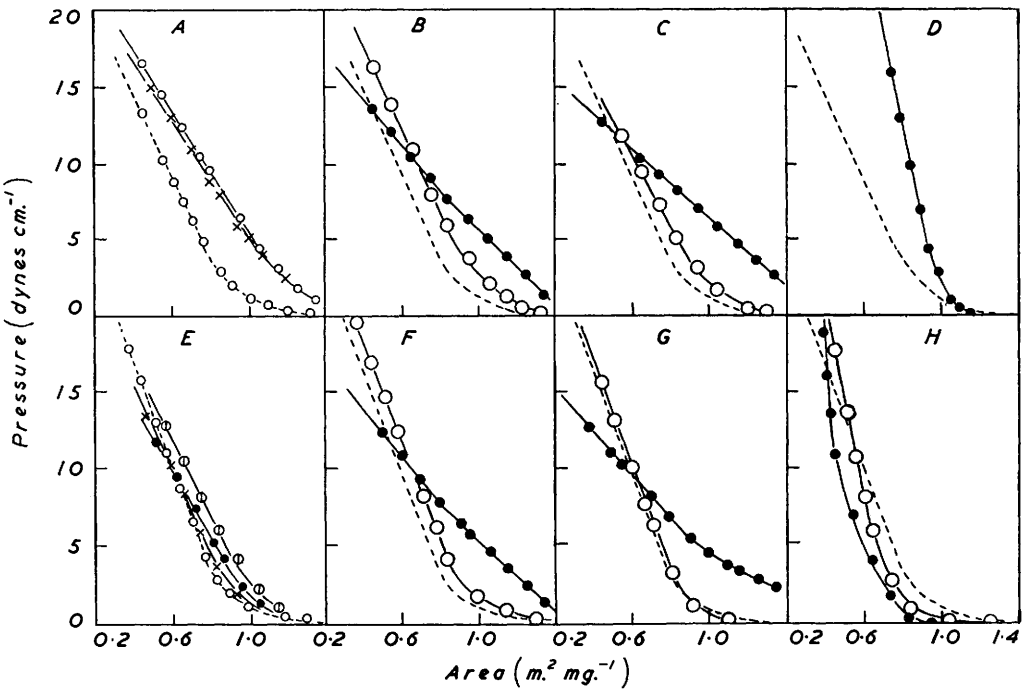
Effect of Non-ionic Compounds.—Tannic acid has the same effect upon casein (Fig. 1*H*) as it has on collagen monolayers; [9] it condenses the film, making it more rigid, *i.e.*, less

[15] Harrap, *Proc. Second Internat. Congr. of Surface Activity*, 1957, Preprint, **2**, 370.

[16] "Colour Index," Society of Dyers and Colourists, Bradford, and American Association of Textile Chemists and Colourists, Lowell, Mass., 2nd edn., 1957.

compressible. The hydroxy-groups in the tannic acid molecule form hydrogen bonds with specific groups in a number of protein chains, the whole complex forming a raft-like structure in which the tannic acid molecules lie horizontally in the water below the film. This type of complex can form only with large multifunctional solute molecules [9] and not with the small molecules of glucose and inositol. These compounds (0·01M, pH 4·34) have no apparent effect on the casein film (Fig. 1*E*). Refractometric tests also suggest that they do not form complexes with casein (in alkaline solution).[13]

FIG. 1. *Force–area curves for casein monolayers on water and various substrates.*



A; ○ with broken line, Acid alone, ○ with full line, (IV), × (VII)—(IX). All 10^{-3}M and in 1·0N-HCl.
B; ○ (XIII) (10^{-3}M), ● (XII) (10^{-5}M). Both in 1·0N-HCl.
C; ○ (XI), ● (X). Both in 1·0N-HCl.
D; ● (XIV) (10^{-3}M) in 1·0N-HCl.
E; ○ Acid alone, coinciding with (II) and (III) (10^{-2}M); ⊙ (V), ● (IX), × (VI). All 10^{-3}M in buffer pH 4·34.
F; ○ (XIII) (10^{-3}M); ● (XII) (10^{-4}M). Both in buffer pH 5·1.
G; ○ (XI), ● (X). Both 10^{-3}M in buffer pH 5·1.
H; ○ (XIV) (5×10^{-4}M), ● (I) (43 mg. $l.^{-1}$). Both in buffer pH 4·34.
For comparison the control curve is repeated (broken) in curves *B*—*H*.

Effect of Anionic Solutes in Acid Solution.—Hydrochloric acid alone, at pH 1·0, has little effect on the film (Fig. 1*E*), but when an aromatic anionic solute is present considerable expansion occurs, with in most cases some change in compressibility (Fig. 1*A–D*). Ellis and Pankhurst [9] found that basic chromium sulphate expands a gelatin monolayer without causing any noticeable change in compressibility and attributed the effect to the action of coulombic forces between the solute ions and oppositely charged side-chains of the protein. The expansion of the present casein films is attributable also to coulombic attraction, but the compressibility changes are probably due to forces acting between the protein and the organic residues of the solute ions in the water below the film (Fig. 2*A*). The expansion may then be the result of the interposition of the solvated water atmospheres around the individual ionic centres.

The compressibility of the casein films varies in an interesting manner with the nature of the solute anion, and the variations suggest differences in the location of the attached anions. Three effects can be distinguished.

(i) *Slight increase in compressibility.* This occurs with the small monobasic anion, *i.e.*, sodium benzenesulphonate (Fig. 1*A*), and with the di- or tri-basic anions which have no strongly hydrogen-bonding centres and no very large unsulphonated residue, *i.e.*, the anthraquinonesulphonates (VII—(IX) (Fig. 1*A*) and the azo-dye (XI) (Fig. 1*C*). These have neither surface activity nor cross-linking power, so they must be located in the water just below the film (Fig. 2*A*).

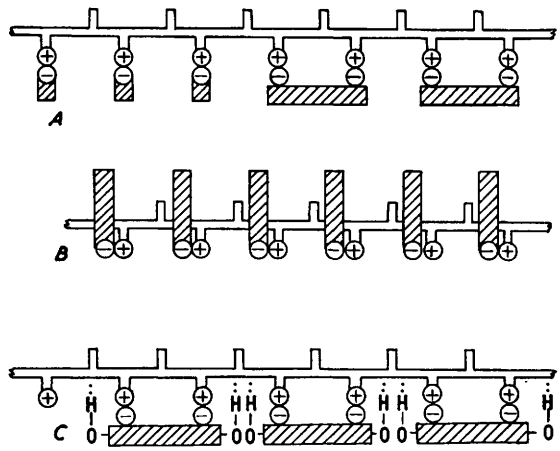
(ii) *Great increase in compressibility.* The two monobasic dyes (X) and (XII) cause considerable increase in film area and compressibility (Fig. 1*C*, *B*). The liquid-expanded type of film obtained resembles those observed when one monomeric surface-active compound penetrates a film of another (cf. ref. 17).* There is clearly ion–ion association between the charged cationic groups of the protein and the dye sulphonate groups. In the greatly expanded state the dye molecules must be lying flat on the surface, their large area causing the protein chains to be widely separated.

FIG. 2. *Schematic diagram of suggested mechanism of casein–dye attachment under acid conditions.*

Ion–ion bond with small uni- or bi-valent anions.

Ion–ion bond and film penetration with large univalent anions at moderate pressure.

Ion–ion and hydrogen bonds with bivalent anions containing at least two bonding groups.



It was also found that injection of a solution (final concn. in the trough 0·00004M) of the dye X below a casein film on pH 4·34 buffer, previously compressed at 8 dynes/cm., caused the film to expand to the area obtained at that pressure when it is initially spread on dye solution (cf. Fig. 1*G* and p. 1228). This evidence of penetration of a film of one compound *A* under pressure by another compound *B* injected below it implies that a stable complex is formed between *A* and *B*.[19] The complex appears to be stable over the whole pressure range examined, for the curve for the mixed film intersects that for casein alone at high pressures (Fig. 1*C*). This implies that the film does not return to its normal state under high pressure but remains actually more soluble that it is in the absence of dye, and that therefore sulphonate groups in the complex must be in contact with the water.

The mechanism of association at the higher pressures may well resemble that postulated by Pankhurst [20] for gelatin–sodium alkyl sulphate detergent complexes formed in solution. Under acid conditions the ion–ion attraction causes a monolayer of detergent to be built

* For a general discussion of some effects of penetration of films, see Schulman.[18]

[17] Pankhurst, *Proc. Roy. Soc.*, 1941, **179**, *A*, 393.
[18] Schulman, *ibid.*, 1936, **155**, *A*, 701.
[19] Schulman and Hughes, *Biochem. J.*, 1935, **29**, 1243.
[20] Pankhurst, "Surface Chemistry," Butterworths, London, 1949, p. 109.

up on the protein, with the polar groups oriented towards the protein and the hydrocarbon groups directed outwards, giving the complexes oil-solubility. Further detergent anions are adsorbed in the reverse manner, being attached to the first layer by van der Waals forces between the hydrocarbon chains. A reverse-adsorbed layer of this nature, beneath the casein film, would give the latter a closely-packed surface of sulphonate groups, which would account for its high solubility. The two dyes (X) and (XII) which cause the increased film solubility each have a structure which would favour dimerisation by van der Waals attraction, giving a complex with a sulphonate group at each end. A similar dye (Orange II, sulphanilic acid→2-naphthol) has been shown by Derbyshire [6] to exist in dilute aqueous solution largely in such a form.

At an intermediate stage between the liquid-expanded film and the final state, the aromatic portion of the dye molecules may be forced partially out of the film to stand vertically upwards (Fig. 2*B*). If this does occur, then with increasing compression the orientation of the adsorbed dye molecules may be envisaged as passing through three stages with possible " buckling " of the film in each.

(iii) *No change, or decrease in compressibility.* The anthraquinone dye (XIV) produces a marked decrease in compressibility (Fig. 1*D*), doubtless a " tanning " effect caused by hydrogen-bond cross-linking of protein chains with active phenolic groups at opposite ends of the dye molecule (cf. Fig. 2*C*).* This dye expands films of monomeric non-ionic surface-active substances, by cross-linking,[21,22] but in these monomeric films the compressibility is greatly increased by cross-linking.

The other anthraquinone dye with two hydrogen-bonding centres (XIII) produces no change in film compressibility (Fig. 1*B*). Its effect is thus intermediate between those of, say, (IX) and (XIV), and it is probably causing a small degree of hydrogen-bond cross-linking, the affinity of its bonding centres under acid conditions being low, and only slightly greater than that of the quinone groups.

Effect of Anionic Compounds near the Isoelectric Point.—The isoelectric point of casein is at pH 4·6, and on buffer solutions in this region the ion–ion attraction is low and any changes in the films must be due to non-ionic and some ion–dipole forces. The compressibility changes are similar to those observed with acid solutions, but the expansions are less (see Fig. 1*E*).

The form of the curves obtained with the two monobasic dyes (X) and (XII) (Fig. 1*G*, *F*) is very similar to that obtained with these dyes on acid solutions, and a sequence of orientation changes can be suggested to occur here similar to those on acid solutions. Pankhurst [20] observed that the sequence of changes in the properties of detergent–gelatin complexes is much the same above as below pH 2, but in the latter conditions the detergent : protein ratio required to give maximum hydrophobic properties is higher, and some inorganic salt must also be in the solution; the anions are probably first adsorbed at the keto-imide groups in the backbone of the protein, rather than on side-chains. In the present case the buffer gives the required salt effect.

Nature of Non-ionic Dye–Protein Reactions.—The present results throw some light on the non-ionic reactions of dyes and other organic ionised solutes with protein fibres and help to reconcile some of the apparently conflicting evidence obtained by earlier investigators. It appears first that non-ionic polar groups in sulphonate anions can form hydrogen bonds to casein in water over quite a wide pH range. In anions with weak hydrogen-bonding power, however, bonding effects with the casein monolayer are masked by other effects produced either by surface activity in " unsymmetrically " sulphonated dyes [*e.g.*, (X) and (XII)] or by high water-solubility in " symmetrically " sulphonated compounds [*e.g.*, (VII)—(IX) and (XI)]. If it is assumed that suitable polar groups are

* The rigidity of the " tanned " film can be seen by sprinkling powdered talc on the surface; the " penetrated " films are markedly less rigid.

[21] Giles and Neustädter, *J.*, 1952, 3806.

[22] Allingham, Giles, and Neustädter, *Discuss. Faraday Soc.*, 1954, **16**, 92.

as accessible in wool as in casein it follows that there can be hydrogen-bond adsorption by that fibre of anions with active polar groups.

From a consideration of the present results and those, for example, of Pankhurst's work on gelatin complexes [20] the known phenomena of anion affinity for wool can be accounted for by the following tentative hypothesis.

The affinity for a protein fibre P of surface-active anions $R{\cdot}SO_3'$, *i.e.*, those having their ionic group or groups at one end of the molecule, and having only weak hydrogen-bonding power, *arises from the mutual forces between the anions themselves, i.e.*, the forces (largely van der Waals forces) between R and R, rather than between R and P. These mutual forces assist the adsorbed anions to form either a condensed monolayer or micelles. Evidence in favour of this suggestion is: (i) The rise in affinity for wool of monobasic aromatic anions with increase in their molecular size [3, 7] (the affinity of some dyes rises linearly with increase in length of an attached alkyl chain). If the affinity were due to R–P attraction it would be unlikely to rise regularly with size of R, because the protein molecule would become more and more inaccessible to the anions as they increased in size (cf. ref. 23). (ii) The similarity in heat of reaction in water of the dye Orange II (as the free acid) with wool (−9·27 kcal./mole) and (as the sodium salt) with itself (−10·48 kcal./mole).[6] If the reaction were between the dye anion and hydrophobic parts of the protein molecule the " heat of dyeing " would be expected to differ from the heat of dimerisation, *e.g.*, the heat of reaction of the dye anion with several amino-acids, even including tyrosine, whose side-chain, being aromatic, is nearest in type to the dye itself, is only about −1 kcal./mole.[5] (iii) The behaviour of a monolayer of long-chain alkyl sulphate ($C_{22}H_{45}SO_4Na$) when a protein (hæmoglobin) is injected beneath it. No penetration occurs at the isoelectric point of the protein, but it does occur under acid conditions, where ion–ion attraction between sulphate and protein is powerful enough to expand the film.[24] This suggests that the attraction of one alkyl chain for another in the condensed monolayer is greater than its attraction for the protein. (iv) The character of the surface of wool dyed with surface-active sulphonated dyes containing paraffin chains. A brief report by Preston [25] is that when dyed under acid conditions the fibre surface is hydrophobic and when dyed neutral it is hydrophilic. In acid solution sulphonate groups of the dye are attached to the charged groups in the fibre and the alkyl chains directed outwards; in neutral solution the sulphonate groups are not attracted by the uncharged fibre, but are directed towards the water. These observations suggest that the dye anions on the the fibre surface, in both sets of conditions, are present either in a condensed monolayer oriented perpendicular to the surface, or in micelles. The mutual attraction between the hydrophobic portions of the anions themselves must therefore be greater than their attraction for the hydrophobic parts of the wool molecule.

The affinity of non-surface-active anions for protein must arise largely from dye–protein attraction, due partly to hydrogen-bond forces and partly to van der Waals attraction, but the quantitative data available are inadequate to confirm this point.

Hydrogen-bonding and Interanion Attraction with Dissolved and Fibrous Proteins.—A surface-active ion with weak hydrogen-bonding centres may form hydrogen-bonded complexes with proteins when both are in solution, but at a solid protein surface it may be adsorbed so that the aromatic residues of the dye ion are associated with themselves alone and not hydrogen-bonded to the protein. This explains why casein–surface-active dye hydrogen-bonded complexes appear to exist in solution (at least in alkaline solution *) [12] but not always when the dye is adsorbed at the casein monolayer.

* Calorimetric measurements give no evidence of hydrogen-bond interaction between amino-acids or dipeptides and the free acid of Orange II in very low concentration in acid solution.[5] Under these conditions the ion–ion reaction must preponderate over the competing dipole–dipole (hydrogen-bond) reaction.

[23] Pankhurst, *Discuss. Faraday Soc.*, 1949, **6**, 52.
[24] Matalon and Schulman, *Discuss. Faraday Soc.*, 1949, **6**, 27.
[25] Preston, *Hexagon Digest*, 1952, No. 11, 31.

Experimental.—The apparatus has been described.[1] The dyes and other reagents were purified by normal methods and were dissolved in distilled water. Casein was B.D.H. " light white soluble " quality, spread from a 0·15% solution in pH 9 buffer by dropping on the water surface from an " Agla " micrometer syringe. Preliminary tests showed that the film reached equilibrium in about 15 min., but in each case after spreading 30 min. were allowed before the film was compressed. Subsequent stages of compression were measured after intervals of 3 min., which was sufficient to allow the film to regain equilibrium. During this time the pressure decreases slightly, possibly owing to reorientation of side-chains.

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