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Part I of this thesis is concerned with the adsorption of non-ionic solutes on protein substrates. The refractive index method has been used with dilute aqueous solutions of a variety of simple monosaccharides and disaccharides many in D- and L- configurations on the one hand, and several proteins, and various model compounds, on the other. The results show that: all pentoses, and also meso-inositol and mannitol appear to react with each protein tested; no disaccharide reacts; hexoses may or may not react, according to the particular stereo-isomer used and the nature of the protein. It is suggested tha a molecular sieve process is in operation, such that only carbohydrate molecules below a limiting size can penetrate the dissolved protein aggregates and thus form a hydrogen-bond complex. Hexose molecules ar near the critical size for entry and whether they combine or not is determined by steric factors. It is also suggested that L-sorbose exists in water, and combines with other solutes, as an aggregate of eight molecules.

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STUDIES IN SOLUTION ADSORPTION AT BIOLOGICAL SURFACES.

BY

ROBERT BRUCE McKAY

A Thesis submitted to the University of Glasgow in accordance with the regulations governing the award of the Degree of Doctor of Philosophy in the Faculty of Science.

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December, 1962.

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ACKNOWLEDGMENTS.

The author wishes to thank the following: Dr. C.H. Giles, for encouragement and guidance throughout this investigation; Professor P.D. Ritchie, F.R.S.E., for his interest; Dr. J.R. Baker, F.R.S., for instruction in microscopy and biological microtechnique and for guidance throughout the work described in Part II, Section 2, which he suggested, and also Professor J.W.S. Pringle, F.R.S., for facilities to carry out the work described in this section, in the Cytology Laboratory, Department of Zoology, Oxford; Professor P.J. Le Fevre (University of Missouri, U.S.A.) for suggesting the project described in Part I, Section 2, and for communications; Professor E.L. Hirst, F.R.S. (University of Edinburgh) and Dr. B. Lustig (Clairol Inc., Stamford, Conn., U.S.A.) for discussions and suggestions on this topic; Professor E.O. Morris for discussions and criticism on Part II, Introduction and Section 1; Dr. A.C. Syme for micro-analyses and for determining the molecular weight of L-sorbose in dilute aqueous solution; Miss S.P. McGoldrick and Mrs. B.M. Luke (Oxford) for experimental assistance; and the Department of Scientific and Industrial Research for a scholarship and the Bursaries Committee of the Royal College of Science and Technology for a Bellahouston Travelling Scholarship.

The author also wishes to thank Professor A. Yabe (Ochanomizu University, Tokyo, Japan) and Dr. K.V. Datye for discussions.

PUBLICATIONS.

The researches described in this thesis have been presented in the following papers.

General Introduction.

"Studies in Adsorption. Part XII. Mechanism of Adsorption of Aromatic Hydrocarbons by Chromatographic Alumina", by C.H. Giles and R.B. McKay, J. Chem. Soc., 1961, 58; this work is also included in Chap. 4, "Adsorption", by C.H. Giles in "Chromatography", ed. Heftmann, Reinhold Publishing Corporation, New York, 1961.

Part I, Section 1.

"Studies in Hydrogen-Bond Formation. Part X. Complex-formation between a variety of Organic Solutes in Carbon Tetrachloride", by C.H. Giles, R.B. McKay and (in part) W. Good, J. Chem. Soc., 1961, 5434.

Section 2.

"Studies in Hydrogen-Bond Formation. Part XI. Reactions between a Variety of Carbohydrates and Proteins in Aqueous Solutions," by C.H. Giles and R.B. McKay, J. Biol. Chem., 1962, 237, 3388.

Part II, Section 1.

"The Dye-Staining of Micro-Organisms. A Review", by R.B. McKay (submitted).

"Studies in Adsorption. Part XVIII. The Adsorption of Cationic Dyes by Yeast Cells," by C.H. Giles and R.B. McKay, (submitted)

Section 2.

"An Investigation of the Anomalous Staining of the Chromatin in the Spermatogenetic /...

Spermatogenetic Cells of the Mouse by the Acid Dyes Methyl Blue and Aniline Blue", by R.B. McKay, Quart. J. micr. Sci., in the press.

Other Publications (not included in this thesis).

The author has contributed some experimental work to "Identification of the Application Class of a Colorant on a Fibre", by C.H. Giles, M. Bashir Ahmad., S.D. Dandekar and R.B. McKay, J. Soc. Dyers Colourists, 1962, 78, 125, and a "Note on Photochemistry" to "The Light-Fading of Dyes. A Review", by C.H. Giles, N. Macauley, R.B. McKay and S.M.K. Rahman, accepted for publication by the Textile Research Journal.

SUMMARY.

The adsorption of non-ionic and ionic solutes from aqueous solution at selected biological surfaces has been investigated by direct and indirect methods. In preliminary experiments, π-electron complex formation which might operate in the adsorption, has been investigated; also the "method of continuous variations" with refractive index as the independent variable has been shown to be satisfactory for determining intermolecular-complex formation in binary mixtures of solutes dissolved in hydrogen-bonding and non-bonding solvents.

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GENERAL INTRODUCTION.

The broad aim of the author's work was to initiate a research programme designed to throw light on some of the chemical mechanisms of adsorption at biological surfaces, of which two aspects are dealt with here. The chemical mechanism of cell staining, in particular, has not previously been exhaustively investigated and it was hoped that techniques used in this laboratory to investigate adsorption at non-biological surfaces would prove useful. In preliminary work, π-electron complex formation, which might operate in the adsorption, was investigated. The results are discussed in this Introduction. In addition, the reliability of the method of continuous variations with refractive index as the independent variable to investigate intermolecular complex-formation in polar and non-polar solvents was confirmed. This aspect is presented in Part I, Section 1.

Biological substrates may be regarded as heterogeneous systems, which consist of several solid phases, the physical and chemical properties of which may vary widely. Information about the actual mechanism of adsorption, when the active constituent of the substrate is known, can be obtained by examining the interactions of carefully selected model compounds. This technique is used in Part I to investigate the adsorption of non-ionic solutes. Much information about the location of the sites of adsorption can be obtained by observing the effect produced by the removal or deactivation of a constituent phase. This technique is employed in Part II, Section 2, to investigate the adsorption of anionic solutes.

The adsorption of cationic solutes has been investigated quantitatively by determining adsorption isotherms, and is described in Part II, Section 1.

All /...

All biological processes, and the staining processes with which this thesis is concerned, take place in aqueous media. The present series of investigations is thereby concerned with adsorption from dilute aqueous solution.

ADSORPTION.

Adsorption at the Solid-Liquid Interface.[1,2,3.]

Adsorption is essentially an interfacial phenomenon. This thesis is concerned with adsorption at the solid-liquid interface and, in particular, with systems in which the liquid phase is a dilute solution of one or more dissolved solutes.

Matter is composed of constituent particles (atoms, molecules, ions or free radicals), each of which exerts in all directions attractive forces. In the gaseous state these particles have high energy and the attractive forces have only a minor influence on their behaviour. In the liquid phase, where the particles have lower energy, the influence is greater and the particles tend to exist closer together in a more ordered manner. Nevertheless, in both the gaseous and liquid states the particles have translational, vibrational and rotational freedom. In the solid phase, however, the constituent particles have much lower energy and the influence of the attractive forces surrounding each is so great that they are drawn together into a rigid ordered structure. They may have rotational and vibrational freedom, but they have no freedom of translation. In consequence, all interfaces between a solid and an adjoining phase are immobile.

In /...

In the bulk of the solid phase, the attractive forces which each constituent particle (atom, ion or molecule) exerts in all directions are balanced by the attractive forces of its neighbours. Several types of forces (e.g.,electrostatic, homopolar, physical) are known to operate, often simultaneously, although usually one type predominates.

At the surface of the solid phase the attractive forces are balanced only on the side nearer the bulk of the phase and unbalanced forces thereby remain to attract the constituent particles of the adjoining phase. If this phase is liquid, the constituent particles (molecules, ions or free radicals), which have translational freedom and which also have surrounding fields of forces, will tend to satisfy the unbalanced forces by moving toward the interface, with the result that their concentration there will be greater than in the bulk of the liquid. This phenomenon is called adsorption. The solid phase is called the adsorbent. If the liquid phase is a solution it will have two or more components, all of which compete for a place at the interface (see later). The component which is preferentially adsorbed is called the adsorbate.

Adsorption is usually a reversible process and the adsorbate particles, after a period of time, leave the interface and return to the liquid. Equilibrium is established when the number of adsorbate particles entering the interface in a given time is equal to the number of particles leaving in the same time.

<u>The Interface : The Electrical Double Layer and Zeta Potential.</u>[2]

An interface between two phases is remarkable, for although it is only a few molecular diameters in thickness, yet it is macroscopic in length and breadth. /...

breadth. The difference in electrical potential between two phases in contact is therefore of the greatest importance in adsorption. It is intimately associated with the formation of an electrical double layer, i.e. an unsymmetrical distribution of electrically charged particles (ions or electrons) near the surface with excess of positive charges directed towards the phase which assumes the positive potential and excess of negative charges directed towards the phase which assumes the negative potential.

The electrical double layer was at first considered to be a parallel-plate condenser,[4] the separation being one molecular dimension. Later views supported the idea of a diffuse double layer. In 1924, however, Stern[5] combined the characteristics of both these views to give the concept which has become generally accepted today. He suggested that at the solid surface there is a single layer of firmly bound charged particles which gives rise to a sharp fall of potential and that next to this there is a diffuse layer which extends for some distance into the liquid phase. The component particles of this layer are believed to have considerable translational freedom, but unlike the bulk of the liquid phase the distribution of positively and negatively charged particles is not uniform, since the electrostatic field at the solid surface will result in a preferential attraction of those of the opposite sign. The result is a gradual fall of potential into the bulk of the liquid where the charge distribution is uniform. The observed zeta potential is the fall of potential across this diffuse layer.

The electrical double layer at the solid-liquid interface may arise from /...

from the preferential adsorption of ions of one sign, or from the orientation of neutral molecules which contain electrical dipoles. The concept of a diffuse layer on the solution side of the interface applies well in systems where adsorption is by strong electrostatic attraction. This type of force is long-range and influences particles of the liquid phase at some distance from the interface. The concept, however, is less satisfactory when adsorption is due to short-range physical attraction which only influences particles within a few Ångström units from the solid surface. It is believed that adsorbed particles are located in the diffuse layer.

The magnitude of the zeta potential varies from system to system. Its sign, however, is negative for most solids in water, for example chromatographic alumina (in its original alkaline state, containing sodium carbonate)[6] silica (see ref. 7 and other references quoted there), and cellulose[8], but there are many exceptions, for example, the film of alumina produced on an aluminium anode immersed in chromic acid[9].

Types of Adsorption Forces.

In recent years much evidence has been presented in the literature to suggest that several types of retention forces operate in adsorption at the solid-liquid interface. The principal types are: van der Waals (physical) attraction, hydrogen bonding, π-electron complex formation, covalent bonding and ion exchange. It is clear that more than one type of force may operate simultaneously and that the nature of the forces which predominate depends on the adsorption system. The various types of forces are best illustrated by considering specific examples.

Van der Waals /...

Van der Waals Attraction. - Van der Waals attraction is a term collectively applied to a group of short-range physical forces. This group includes orientation forces (attraction between permanent dipoles), induction forces (attraction between permanent dipoles and induced dipoles), attraction between ions and dipoles and dispersion forces (quantum-mechanical interaction between momentary dipoles).

The anions of acid dyes are adsorbed to a much greater extent than inorganic anions by wool and nylon from mildly acid solutions, and this must be attributable largely to van der Waals attraction between the aromatic nuclei of the dye and the hydrophobic parts of the protein chain molecules of the wool[10]. Two examples may be quoted in support of this belief: (a) it has been shown that the affinity for wool of a series of acid dyes and of certain organic acids rises almost linearly with increase in molecular weight[11]; (b) dry wool and nylon under certain conditions can adsorb from non-aqueous solvents appreciable amounts of certain aromatic compounds, e.g., benzene or stilbene, which most probably are attracted to the fibre by van der Waals forces only.[12]

The mechanism of retention of direct cotton dyes to cellulose is not yet fully understood. Nevertheless, certain observations indicate that van der Waals attraction and not only hydrogen bonding must play some part: e.g., anthracene-α-sulphonic acid, which has no hydrogen bonding groups, and haematoxylin, which has four strong (phenolic) and two weak (alcoholic) hydrogen bonding groups, are both adsorbed by cellulose and the former has the higher affinity.[13]

The /...

The retention of organic anions by chitin under acid conditions appears to be due mainly to van der Waals attraction between the anions and the water-solvated molecular chains of chitin. The affinity appears to rise markedly with increase in size of the aromatic portions of the anions.[14]

The retention of aromatic hydrocarbons on graphite has been attributed to van der Waals attraction. Recent evidence, however, shows this can only be partly true for benzene is adsorbed more strongly than cyclohexane, which it closely resembles in size and shape.[15] This suggests that the π-electron system of aromatic compounds is also involved.

Hydrogen Bonding : Hydrogen Donation by Solute. - All polar and unsaturated non-polar organic solutes are retained by an alumina column. Strong adsorption is favoured generally by high polarity, by an increase in the number of polar substituents or of aromatic nuclei, and by the absence of internal chelation.[6,16,17,18] Recent detailed work has confirmed that all compounds with a hydrogen atom capable of bonding are adsorbed strongly by alumina.[6,9,19,20] Such compounds include phenols, amines and aldehydes, for which the respective hydrogen-bonding mechanisms may be shown as $-OH\cdots O$, $>NH\cdots O$, $\geq CH\cdots O$. The solute molecules are adsorbed rapidly and appear to cover the whole external surface of the alumina, most probably as a monolayer.[6,20]

It is believed that silica adsorbs phenols, amines, etc., from water and non-aqueous solvents by hydrogen bonding, hydrogen atoms being donated from the solute to the surface oxygen atoms.[7]

Hydroxy-compounds are adsorbed by wool and nylon from aqueous solution by /...

by forming hydrogen bonds probably with the enolic forms of amide or peptide groups of the fibres.[12] There is also good evidence that phenol is adsorbed from water by cellulose acetate by a hydrogen-bonding mechanism at the acetyl groups.[21] Water-soluble dyes used for cellulose acetate, e.g., the "Solacet" (I.C.I.) sulphato-ester dyes are probably adsorbed by this mechanism also.[10]

Hydrogen Bonding : Hydrogen Acceptance by Solute. - The hydrogen acceptor solutes, e.g., nitrobenzene, azobenzene, are adsorbed less by alumina than hydrogen donor solutes. The hydrogen acceptors are probably bonded through the limited number of free hydroxyl groups known to be present in the surface of the alumina.[6] An alternative view, however, is that this adsorption is due to donation of n electrons to electron-deficient sites in the alumina surface.[22]

There is also evidence to suggest that ethanol is bonded to free hydroxyl groups in the alumina surface.[15] It may be that ethanol, in part at least, acts as a hydrogen acceptor.

π-Bond Donation by Solute. - Condensed-ring polynuclear aromatic hydrocarbons can be separated on an alumina column. It has been shown that the adsorption is favoured by an increase in the number of double bonds in the solute molecule, by an increase in its degree of coplanarity and by symmetry factors[23] (see also ref. 24, not yet available). Also in isomeric hydrocarbons differing only in degree of conjugation, the most extensively conjugated isomer in the most strongly adsorbed.

The author has investigated quantitatively the behaviour of Grade I and /...

and Grade II alumina powder towards some aromatic hydrocarbons.[25,26,27] By analysis of solutions before and after shaking with the powder it was established that there is positive adsorption. It was found that 2:3-benzanthracene, phenanthrene, and pyrene are all adsorbed from xylene, naphthalene is adsorbed from 2:2:4-trimethylpentane, but not from xylene. The xylene itself must compete too strongly for the active sites, so that naphthalene is not taken up in its presence. Benzene is not adsorbed from 2:2:4-trimethylpentane, so presumably xylene is adsorbed more readily than benzene. This agrees with the observation that methyl groups assist adsorption.[23] Only a very small proportion of the available surface is covered. The amount of phenanthrene adsorbed is substantially higher on Grade I than on Grade II alumina, but the apparent affinity is actually lower.

The roasting treatment used to prepare Grade I material removes firmly-bound water. Therefore, although Grade I oxide hasaa higher affinity for water than Grade II it has a lower affinity for a hydrocarbon; yet it has more available sites. This suggests that water and hydrocarbons are adsorbed at different sites and that the chemisorbed water may screen some of the sites suitable for hydrocarbons. Tests with hydrocarbons on silica and cellulose showed that no adsorption occurs, suggesting that neither oxygen nor hydrogen atoms are the points of attachment of the hydrocarbon molecules. Therefore the reaction sites are believed to be aluminium atoms. The hydrocarbons are considered to form a π-electron complex with the small proportion of aluminium atoms that happen to be exposed at the surface, due to /...

to deformation of the crystalline structure by grinding and abrasion. These aluminium atoms will bear a partial positive charge.[28] This conclusion agrees with that of Klemm et al.[23] who suggested that the adsorption is unimolecular and takes place on "active spots" of the alumina surface in a π-type complex.

Further evidence in favour of this adsorption mechanism has been obtained from nuclear magnetic resonance studies.[29]

Few cases of this π-electron complex-formation have so far been reported. A similar mechanism may be involved in the formation of complexes between aromatic hydrocarbons and picric acid, between benzene and silver cations[30,31] and between π electrons of graphite and alkali-metal cations.[32] It has also been suggested that π-type hydrogen bonds are formed in the adsorption of direct cotton dyes on cellulose. π electrons from the long conjugated dye molecules are considered to interact with hydroxyl groups in the cellulose.[33]

Electron complex-formation may well be involved in some biological processes and in biological staining techniques, e.g., the staining of phospholipids with acid xanthene dyes, such as Eosin.

Covalent Bonding. - There are relatively few reported cases of covalent bonding in adsorption. Broadly speaking, it may be said that adsorbed molecules which are extremely fast to rinsing in both aqueous and organic solvents are retained by forces which have a high degree of covalent character. This phenomenon is well known in many forms of textile dyeing, where the last traces of an adsorbed dye are often extremely difficult to remove. /...

Perhaps the most striking case of covalent bond formation is the adsorption of reactive dyes on cellulose and nylon.[34] It is believed that a covalent bond is formed between the triazinyl nucleus (a characteristic feature of some reactive dye molecules) and hydroxyl groups in the substrate. Another example is the adsorption of p-nitrophenol from aqueous solution on alumina. The adsorbed solute is not completely desorbed by rinsing with water, and for this reason the retention forces are believed to be in part covalent.[35] The irreversible colorisation of anodised aluminium with mordant dyes has been attributed to covalent bond formation,[9] though there is now some reason to doubt this (Datye and Giles, private communication).

Ion Exchange. - Much attention has been paid in recent years to the adsorption of ionic solutes, particularly dyes, by inorganic and organic adsorbents (e.g., ref. 6,7,9,19,36). In most cases adsorption is believed to involve a process of ion exchange. Ion exchange may be regarded as a redistribution of ions between one environment and another. The ions keep their charges when adsorbed, no covalent bonds are made or broken, and the heat of reaction is very small. In many cases ion exchange has been characterised by an extremely rapid attainment of equilibrium. The surface coverage has been found to be complete, in most cases, and often much more solute than that required for a monolayer is found to have been adsorbed. This apparent "multilayer adsorption" has been attributed to the formation of micelles by face-to-face or side-by-side alignment of large flat or "surface-active" ions.

Walton[37] /...

Walton[37] has summarised cation exchange thus: "the strength of binding of cations depends upon their charge and radius, being greater the higher the charge and the smaller the radius in the hydrated condition; that is, the smaller the degree of hydration. The larger the pores of a cation exchanger, the more the environment in the exchanger resembles that of the solution, and the less difference there is between the adsorption of different cations". Anion exchange cannot be generalised so readily.

Many examples of ion-exchange adsorption have been reported. Technical alumina is alkaline in reaction owing to the presence of sodium carbonate impurity.[6] Cations are believed to be adsorbed by exchanging with surface sodium ions.[38] Pure alumina, however, also adsorbs cations,[39] probably by aluminium-hydrogen ion exchange. The adsorption affinity appears to rise with polarisability of the adsorbed ion, or of groups co-ordinated with it.[40,41] Alumina which has been acidified with hydrochloric acid contains partly ionised and partly covalently-bound chlorine.[28] Anionic dyes are adsorbed strongly by exchanging with the ionised chlorine.[6,42]

Silica has a negative zeta potential in water,[43] owing to ionisation of silicic acid groups formed at the surface, and thereby adsorbs cations strongly from aqueous solution (e.g., ref. 7 and other references quoted therein). Anionic dyes are also adsorbed, but to a much less extent[7] and it is believed that they are adsorbed by hydrogen bonding. Both anionic and cationic solutes are adsorbed from aqueous solution by commercial graphite.[36] Whereas anionic sulphonated azo dyes appear to be adsorbed from aqueous solution by physical forces, basic dyes are adsorbed by ion exchange of their cations with hydrogen ions in the electrical double layer of the graphite.

Ion /...

Ion exchange appears to play a less important part in the adsorption of organic ions on polyamide and protein fibres. As described previously physical forces appear to make a marked contribution to the ion-retention. Similarly, the adsorption of large anionic dye molecules from aqueous solution on cellulose, which has a negative zeta potential in water has been described. Cations, however, are believed to be adsorbed by exchanging with hydrogen ions from surface carboxyl groups, which are always present to some extent even in the purest forms of cellulose.

Other Factors Affecting Adsorption.

Physical Properties of the Adsorbent. - There are two classes of solids: crystalline and amorphous. Crystalline solids are typified by those which have reproducible properties, such as sharp melting points, and have a well-defined structure, with the constituent particles (atoms, ions or molecules) arranged in a rigid regular manner. Amorphous solids do not have reproducible properties and may be considered to be super-cooled liquids, for the constituent particles have considerable translational freedom and tend to be arranged in a random manner. There is, however, no sharp distinction, for crystalline solids are subject to thermal and mechanical distortions and distortions produced by the presence of impurities, whereas amorphous solids have localised regions of structural regularity. Most solids are predominantly crystalline.

Strains and distortions produced by thermal and mechanical damage have a pronounced effect on the surface properties of a solid. Corundum is a striking example.[28] After grinding the zeta potential is positive; ignition /...

ignition at 700° alters this value and indeed, ignition at above 1000°C. produces an allotrope with a negative zeta potential; grinding, however, restores a positive value.

Impurities, such as chemisorbed water, on the surface of an adsorbent may affect its adsorption properties. This can be illustrated by the following experiment performed by the author.[26,27].

Chromatographic alumina, as bought, has a considerable amount of firmly bound surface water. Roasting reduced the weight of a sample by 5.9%. If this loss is taken to be that of a monolayer of water molecules each occupying 8.2 $Å^2$ (the approximate cross-section, edge-on), it represents ca. 16 x 10^5 $cm.^2$ of extra surface per g. of powder, so that Grade I powder would on this assumption have 4.5 times the available active surface of Grade II. This figure agrees well with the observed 5.3-fold greater maximum adsorption of phenanthrene on Grade I than on Grade II powder. (An attempt was made to re-hydrate Grade I to Grade II powder by storing it under moist air until it had increased 5.9% in weight, but there were experimental difficulties in stopping the increase at the correct value. The product had only ca. 60% of the phenanthrene-adsorbing capacity of the original Grade II powder).

Since the constituent particles of a solid are rigidly held, the surface, unlike a liquid surface, will not contract spontaneously under the influence of the inter-particular attractive forces. For this reason the surface will not be smooth, but will contain protrusions, indentations and crevices. If the attractive forces are electrostatic, the adsorption potential is greater at the edges of a crystal than on a plane face[44] and still greater at a corner or at the tip of a protrusion, since fewer of the /...

the forces surrounding each particle are balanced there. If the forces are physical, the adsorption potential is greater in a hollow, or at the bottom of a crevice, than on a plane surface; the tip of a protrusion is least active.[45]

Many solids, particularly biological substrates, are highly porous and contain a three-dimensional internal network of channels and cavities. If these channels are of the same order of magnitude as the adsorbate particles a "molecular sieve" process may result, whereby large particles may penetrate more slowly than small ones, or not at all. Many such cases are known. For example, monosaccharides can be separated from polysaccharides by preferential diffusion of the former into dextran gels.[46] Only solutes the molecules of which are not larger than those of n-pentanol in the aliphatic series and anthracene or simple azobenzene derivatives in the aromatic series can penetrate the intermolecular pores of wool from non-aqueous solution.[12] In some cases, even the shape of the adsorbate particles is critical. For example, open-chain and branched-chain aliphatic hydrocarbons can be separated by preferential adsorption of the former on zeolite gels.[47]

Properties of the Interface. - Adsorption is a spontaneous process and hence the system must undergo a decrease in free energy ($-\Delta G$) on adsorption. This decrease in free energy may be considered as the adsorption affinity of the adsorbate. When an adsorbate particle leaves the bulk of the liquid phase and enters the diffuse layer at the interface it enters a region with a higher degree of order and thereby suffers restrictions on its freedom or, in other words, a decrease in entropy(ΔS). By considering the fundamental thermodynamic equation, $\Delta G = \Delta H - T(\Delta S)$, where ΔH is the heat evolved /...

evolved and T is the absolute temperature, it can be shown that this decrease in entropy reduces the adsorption affinity of the adsorbate and hence opposes adsorption.

Only a limited number of places at the interface are available to the components of the liquid phase and the amount of each component adsorbed is reduced by competition. This competition is affected by the dimensions and shapes of the adsorbate particles. A large particle with more than one active group may occupy several sites, which might otherwise have been taken by several smaller particles, or they may only block these sites without actually occupying them, e.g., by bridging crevices and pores in the solid surface.[48] In contrast, particles with several active groups distributed along their periphery may be adsorbed less than those with active groups at one end only, for the latter may be adsorbed with an end-on orientation and thereby cover less area than the former lying flat.

It must here be stressed that when the liquid phase is a solution, the solvent molecules, and not merely the solute particles, compete for a place at the interface. The experiments of Kipling et al. with binary mixtures of miscible liquids have thrown much light on this effect and it is clear that the proportion of solvent adsorbed varies from system to system. For example, from binary mixtures of ethanol and benzene, ethanol is preferentially adsorbed by α-alumina, but benzene is preferentially adsorbed by charcoal.[15] As would be expected, the ionic substrate, alumina, has a greater attraction for the polar compound, whereas charcoal, which is essentially a condensed aromatic system, has a greater attraction for benzene. It /...

It has also been observed by the author,[26,27] as already mentioned, that naphthalene is adsorbed by alumina from 2:2:4-trimethylpentane, but not from xylene, probably because of strong competition by the oxide for xylene. Nevertheless, when the liquid phase is a dilute solution, and, in particular, when the solute has low solubility, the proportion of solvent adsorbed appears to be negligible in many cases.[49] Fu, Hansen and Bartell[50] have shown that monolayers of butyric acid adsorbed on carbon from water contain solvent.

In the dyeing of wool by anionic dyes, where the adsorption is in part due to ion-exchange, the exhaustion of the dyebath is reduced by the presence of neutral inorganic salts.[51] The inorganic ions produced compete for the sites at the interface and, in effect, reduce the positive zeta potential of the fibre and hence the adsorption of the dye. Dyes with large aromatic residues, e.g., acid milling dyes, which experience stronger physical attraction from the wool are affected to a much less extent by the presence of these neutral salts.

Interaction between the Component Particles of the Liquid Phase. - The various types of interactions which occur between the component particles of the liquid phase have an important effect on adsorption. Solvation forces exert on dissolved solute particles a "viscous drag" which tends to reduce their ease of adsorption. In other words, it may be said that solutes of high solubility tend to be adsorbed less readily than those of low solubility, other considerations being equal. When only one solute is present maximum coverage of the surface may still be obtained, but a higher solute concentration will be required than that in a non-solvating solvent.

The /...

The effect of solubility on adsorption has been demonstrated by Tamamushi and Tamuki,[52] who showed that whereas the maximum adsorptions of duodecylammonium chloride (DAC), tetradecylammonium chloride (TAC) and hexadecylammonium chloride (HAC) on alumina are the same, the ease of adsorption decreases (i.e. the concentration required to give maximum adsorption increases) in the order HAC>TAC>DAC, and this is the order of increasing solubility.

When more than one solute is present the proportion of each solute adsorbed will be influenced by differences in their solubilities. Solutes of low solubility will tend to be adsorbed to a greater extent, other factors being equal.

In contrast, solutes such as long-chain electrolytes and other surface-active agents, the particles of which have a greater attraction for themselves than for water molecules, tend to aggregate into micelles in aqueous solution. The formation of large ionic micelles appears to favour adsorption when the adsorption forces are electrostatic. For example, there is much evidence to suggest that (a) long-chain electrolytes are adsorbed in the micellar form on carbon,[50,53] (b) sulphonated azo dyes are adsorbed as large anionic micelles on acidified alumina[6] for which they appear to have a high affinity and (c) basic dyes are adsorbed as cationic micelles on silica.[7] Nevertheless, the adsorption of anionic dyes on wool, which is in part due to van der Waals attraction, is favoured by conditions which reduce the aggregation of the dyes in solution, e.g., elevated temperatures and the presence of disaggregating agents.[51] In this case, however, /...

however, a permeability effect may be involved, whereby large micelles are excluded.

Temperature.- Adsorption is essentially an exothermic process and hence should be favoured by low temperatures. Indeed, in almost all reported cases adsorption is greater at room temperature than at elevated temperatures. If the heat of adsorption is low, however, then the effect of temperature is less marked. For example, in many cases where ion exchange is in operation, the adsorption appears to be the same at room temperature as at elevated temperatures. Further when the adsorption forces are weak, for example, physical attraction or weak hydrogen bonding, the effect of temperature is small.

Giles, Greczek and Nakhwa[54] have investigated a few anomalous cases where the adsorption is greater at elevated temperatures than at room temperature. This apparently endothermic effect appears to be the result of a high degree of aggregation of the solute molecules in dilute aqueous solution, for the effect of temperature on the adsorption is normal when a disaggregating solvent is used. The aggregates appear to be less readily adsorbed than the monodisperse form of the solute. The operation of the elevated temperatures breaks down these aggregates, increasing the proportion of the favoured monodisperse form and hence the adsorption. Nevertheless, the solute particles after adsorption appear to be in an aggregated form and it has been suggested that aggregation takes place at or near the adsorption sites, owing to the increased concentration and alignment of solute particles there.

BIOLOGICAL SUBSTRATES

The fundamental component in biology is the unit cell. It is the simplest unit that can be called living. Each cell may be considered to be a chemical factory making the material for its own reproduction or, if it has become very specialised, making material for use by other cells. Most living organisms consist of a very large number of cells which are varied in shape and characteristics and are often highly specialised for a particular purpose, and which are arranged in a highly complicated manner (i.e. they are multicellular). There are, however, some organisms which exist as single cells or as small groups of cells arranged in a comparatively simple manner. In this latter category there are many species of micro-organisms.

Living organisms may be divided into two main groups: plants and animals. There is no sharp distinction, but in general plants are capable of photosynthesizing nutrients under the influence of sunlight absorbed by the compound chlorophyll, whereas animals are not. Further, animals in general are able to move from place to place at will, whereas plants are not.

Micro-organisms are considered by many biologists to be a special intermediate group. There are three main types: algae, protozoa and fungi.

In this thesis attention is directed towards animal cells and the cells of micro-organisms.

Structure of the Unit Cell.[55] - The unit cell is represented diagrammatically in Fig.1. There are two distinct parts: the cell wall and the protoplasm. In multicellular organisms, however, many cells are enclosed within /...

within one cell wall and are separated from each other by a matrix which they produce themselves.

The cell wall in many types of cell is a rigid porous structure which protects the cell from mechanical damage and is responsible for its characteristic shape, which need not be spherical, e.g., human red blood cells (erythrocytes) have a peculiar deformed-disc shape, bacteria may be spherical (coccus), rod-shaped (bacillus) or spiral (spirillum, vibrio),[56] yeast cells are ovoids (see Fig.2). The sizes and shapes of cells vary widely not only between plants, animals, and micro-organisms, but between the cells in one organism.

The protoplasm is the part of the cell essential for life. It is the centre of metabolic and reproductive activity and consists of three parts: the nucleus, the cytoplasm and the cell membrane. The nucleus is the control centre of the cell and carries the hereditary characteristics. It has a highly complex structure which as yet has not been fully elucidated, but appears to consist largely of deoxyribonucleic acid and protein.

The cytoplasm, which is rich in ribonucleic acid and protein is a viscous fluid medium, the viscosity varying greatly with the type and age of the cell. It may be considered as a highly organised intricate network of fibrous protein molecules. Electronmicrospy has shown that in many animal cells the cytoplasm appears to contain a complicated network of parallel membranes on which are supported granular ribonucleoprotein particles called ribosomes.

Enveloping the cytoplasm is the cell membrane, which is an extremely thin /...

thin and delicate semi-permeable structure. It is the osmotic barrier and is responsible for the selective uptake of nutrients against the osmotic pressure gradient which exists between the interior of the cell and the external medium, and for the removal of waste matter. In some micro-organisms the osmotic pressure gradient may be 20-25 atmoshpheres.[57]

The uptake of nutrients, in many cases, appears to involve a facilitated transfer mechanism: a complex physico-chemical process whereby the nutrient molecules interact with a membrane component which serves as a carrier and transports them to the interior. This type of mechanism appears to involve a protein displaying a specificity of action (sometimes stereospecific).[58]

Although almost all biological cells have the type of structure and properties described above, there are many exceptions. Some types of cell appear to have more than one nucleus, whereas some, e.g., human red blood cells, have none at all. Almost all micro-organisms have, on the outer surfaces of the cell walls, accumulations of loosely-bound polymeric materials of high molecular weight and viscosity. In many types of bacteria these surface adherents are extended into well-defined capsules which afford additional protection to the cells. Some cells, e.g., nerve cells, are so highly specialised that, though they are living and increase in size with the growth of the animal, they are unable to reproduce themselves, and their destruction causes permanent damage.

The viruses are the smallest and perhaps the most remarkable group of living organisms. Although some of the larger types can be seen under the ordinary /...

ordinary light microscope, some are so small that they can be detected only with the aid of the ultraviolet or the electron microscope, whereby they are seen as small particles often of clearly defined geometrical shapes (see e.g., ref. 59,60). Viruses may be considered as giant molecules of nucleic acid and protein, and though in some cases they can be crystallised (e.g., the tobacco mosaic virus)[61] to give crystals containing millions of virus particles, yet they are living and can grow and reproduce themselves.

Chemical Constitution of Biological Substrates.

Biological substrates are composed of complex polymeric organic substances, the structures and properties of which are often extremely sensitive to small changes in environmental conditions, such as temperature, pH, etc. Nevertheless, in some cases, for example, cellulose, wool, and bacterial capsules, electronmicroscopy has shown that they have localised regions of crystallinity. All such complex polymeric substances are built up from one or more of five fundamental constituents: polypeptides, polysaccharides, nucleic acids, teichoic acids and lipids.

Polypeptides.[62,63,64] Polypeptides are naturally occurring compounds which consist of chains of amino-acid residues linked together by condensation of the amino group in one with the carboxyl group in its neighbour. In this way a linear chain can be formed. Twenty amino acids commonly occur in polypeptides and all have α-amino groups. Each, except the simplest, glycine, is optically active. In proteins, the largest and most important group of polypeptides, each is of the L-configuration. Since any /...

any amino acid can appear in a chain any number of times, there is a very large number of proteins.

Of the commonly-occurring amino acids in proteins, two, namely aspartic acid and glutamic acid, are strongly acidic in character. Thus proteins and other polypeptides in which they predominate have acidic properties. Three of the acids, namely arginine, histidine and lysine, are basic in character and those proteins in which they predominate, e.g., protamines and histones, have basic properties. The other fifteen amino acids are neutral.

Interchain bonding (hydrogen bonding, electrostatic bonding and covalent disulphide or cystine linkages) has a marked effect on protein structure and properties. A high degree of interchain bonding tends to favour the formation of large aggregates and reduce the solubility.

There are two main structural types of protein: fibrous proteins and globular proteins. Fibrous proteins, e.g., fibroin (silk), collagen (connective tissue), keratin (skin, hair, wool), are structural materials in nature and are insoluble in water. Collagen, however, on boiling is converted into the globular protein, gelatin, which is readily soluble. The chains of fibrous proteins (collagen to a much less extent) are stabilised by a high degree of inter-chain hydrogen bonding.

Globular proteins, e.g., albumins (e.g., gelatin), casein, blood plasma proteins (e.g., bovine plasma albumin, haemoglobin), histones and protamines, ribonuclease, are characterised by their solubility in water or aqueous solutions of acids, bases or salts. They consist of spherical clusters of folded /...

folded chains, stabilised by disulphide cross-linking.

Polypeptide chains, particularly those of fibrous proteins, are believed to have a conformation known as the α-helix. This helical conformation is stabilised by intrachain hydrogen bonds, formed such that 13-membered chelate rings result. The helix of an L-peptide winds anti-clockwise (to the left) from the N-terminal group; that of a D-peptide winds clockwise (to the right). The spherical particles of globular proteins are believed to consist partly of folded helices and partly of non-helical "random coils". The proportion of helical form can be estimated by several methods, e.g., optical rotation and deuterium-exchange measurements.

To sum up, it may be said that protein structure has three distinct levels: primary structure (amino-acid sequence), secondary structure (conformation) and tertiary structure (the manner in which the chains are folded, e.g., the spherical shapes of globular proteins).

Proteins normally have a large amount of water associated with them. Part of this water is firmly bound, whereas the remainder is held mechanically between the structural framework. This immobilised water can be frozen and has solvent properties. The bound water has no such properties.

Polysaccharides.[63,65,66] Polysaccharides occur in complex compounds called mucosubstances (e.g., mucoproteins and mucolipids), many of which are structural materials. They are polymers consisting of monosaccharides or their derivatives which are linked together according to a few simple rules. /...

rules. The following units are known to occur in natural polysaccharides: hexoses, viz. D-glucose, D-mannose, D-fructose, D-galactose, sometimes L-galactose and possibly D-idose or L-altrose; the pentoses, viz. D-xylose, L-arabinose and infrequently D-arabinose; modified monosaccharides, viz. D-glucosamine, D-galactosamine, D-glucuronic acid, D-galacturonic acid, D-mannuronic acid, L-fucose and L-rhamnose. In addition, derivatives containing acetyl and sulphate groups frequently occur.

The -OH group in the 1-position of a monosaccharide unit always participates in the linkage and may be condensed with any -OH group, other than that at the 1-position in the neighbouring unit. The chains thus formed are usually linear, with the same linkage pattern repeated throughout. Two-dimensional branching, however, sometimes occurs.

The repeating units of some well-known natural polysaccharides are shown schematically in Fig. 3A. Xylan, which is built from D-xylose units β-linked in the 1- and 4- positions, often occurs in nature with polyglucuronic acid, the name of which is self-explanatory. Both these compounds are often associated with cellulose, which is the most common structural polysaccharide in the cell walls of plants and consists of β-(1→4)-linked D-glucose units. Starch, the reserve carbohydrate in the majority of plants, consists of α-(1→4)-linked D-glucose residues. The repeating unit is in fact a residue of the disaccharide maltose.

Chitin is the polysaccharide which forms the hard shells of crustaceans and insects. It consists mainly of β-(1→4)-linked units of N-acetyl-D-glucosamine, with perhaps some unacetylated material. Hyaluronic acid is widely/...

widely distributed throughout the tissues of animals and humans and occurs in various micro-organisms. It consists of alternating units of N-acetyl-D-glucosamine and D-glucuronic acid linked β-(1→ 4). Glycogen is the major energy and carbohydrate reserve in the human and animal bodies and occurs in many micro-organisms. A large variety of forms of glycogen are known, but most have a highly branched structure comprising several hundred unit chains of ca. twelve α-(1→ 4)-linked D-glucopyranose residues with interchain links of α-(1→ 6)-type.

In mucosubstances of the polysaccharide-protein type the polysaccharide-proteinoid linkages probably involve the hydroxyl groups of the polysaccharide, particularly that in the 1-position of the monosaccharide residue, and -COOH, $-NH_2$ or -SH groups in the proteinoid.

Nucleic Acids.[63,67] Nucleic acids are high polymers consisting of linked units called nucleotides. A nucleotide is formed by the combination of one molecule of phosphoric acid with one molecule of a compound called a nucleoside, which consists of an organic base linked to the hydroxyl group in the 1- position of either of the pentoses, D-ribose or 2-deoxy-D-ribose. In most nucleotides the phosphoric acid molecule is condensed with the -OH group in the 2', 3' or 5' position of the D-ribose residue or the 3' or 5' position of the 2-deoxy-D-ribose residue in the nucleoside molecule.

In the nucleic acid chain, each nucleotide is linked to its neighbour by condensation of the phosphoric acid residue with either of the stated -OH groups which remain free in the pentose residue.

No nucleic acid contains both pentoses. The ribonucleic acids (RNA) contain /...

contain only D-ribose; the deoxyribonucleic acids (DNA) contain only 2-deoxy-D-ribose. In all known nucleic acids the base is either a derivative of pyrimidine or purine (examples of which are shown in Fig.3B) Their overall strongly acid character, however, may be attributed to the phosphoric acid residues.

Ribonucleic acids of 3 types have been isolated from cellular cytoplasm. They all conform to the same structural pattern and differ in minor degree in molecular weight or in the pattern of basic group sequence. Little information is as yet available about the conformation of the molecules.

Deoxyribonucleic acid occurs exclusively in the nuclei of cells. Much information about its structure and conformation has been deduced from the X-ray diffraction analyses of Watson and Crick.[68] Their conclusions are expressed in a model which shows two helical nucleotide strands that are identical and parallel, but which run in opposite directions. The strands, which are held together by base-base hydrogen bonds, are wound on a cylinder of diameter 20Å, such that the distance between neighbouring base-base bonds is 3.4 Å.

Nucleic acids never occur alone, but always in combination with a proteinoid compound in complexes called nucleoproteins. The proteinoid is strongly basic in character, since it is rich in arginine and/or lysine and contains only a limited number of neutral aminoacids and no sulphur. The mechanism of bonding between the nucleic acid and the proteinoid is probably salt formation, but it may also have some degree of covalent character.

Ribonucleoprotein /...

Ribonucleoprotein is the main constituent of cellular cytoplasm and is also found to some extent in the nucleus. Viruses have been shown to consist almost entirely of ribonucleoproteins containing extremely long ribonucleic acid chains.

Teichoic Acids.[69] - Teichoic acids are a group of natural polymers which have recently been discovered in the cell walls of some bacteria. They are polymers of either glycerol or ribitol phosphates in which units are joined together through phosphodiester linkages; in most cases carbohydrate residues are attached glycosidically to the polyol units and D-alanine residues are attached through labile ester linkages to hydroxyl groups. It is believed that teichoic acids are bound to the mucopeptide framework of the cell walls by hydrogen bonds and electrostatic linkages.

Lipids.[63] - Lipids are essentially complex esters consisting of higher fatty acids in combination with glycerol, sterols or higher aliphatic alcohols. There are two broad classes of simple (neutral)lipids: fats and waxes.

Most fats are esters of glycerol with 3-14 different saturated or unsaturated acids, all of which usually have an even number of carbon atoms. The most common saturated acids are the straight-chain variety, particularly the C_8- C_{18} (stearic) homologues. The most common unsaturated acids are derivatives of stearic acid, whereas unsaturated acids with less than 14 carbon atoms are unusual and those with less than 10 have never been found in nature in living materials.

Waxes /...

Waxes differ from fats in that glycerol is replaced by a sterol or by higher even-numbered aliphatic alcohols from C_{16} to C_{36}. These often occur in excess of the acids, which are also even-numbered and range from C_{24} to C_{36}.

Lipids from bacterial capsules have been found to have carbohydrates as the alcoholic components and to contain a range of unusual acids, e.g., methyl substituted stearic acid.

Many complex lipids have been found. The phospholipids are one particularly important group which, apart from the acid and alcoholic constituents, yield phosphoric acid and a quaternary organic base on hydrolysis. They have a strongly acid character which may be attributed to the phosphoric acid residues. It is believed that in any one phospholipid molecule more than one long-chain acid is incorporated and that the base is linked to the alcoholic component through the phosphoric acid residue.

PART I

The Adsorption of Non-Ionic Solutes on Protein Substrates.

INTRODUCTION

Part I of this thesis is concerned with the adsorption of non-ionic solutes on protein substrates. This study includes a series of experiments made to investigate interactions between model compounds in binary aqueous mixtures. Since all biological processes take place in aqueous media, it was considered to be essential for the present experiments to be performed in aqueous solution.

The "method of continuous variations" has been employed with refractive index as the independent variable.[70] Briefly, this technique is applied to a range of solutions of binary mixtures of solutes, A and B, of constant total molarity, but varying in relative proportions from 100% A + 0% B to 0% A + 100% B. The refractive index is chosen as the measured property of the solutions. Over small ranges this varies linearly with concentration, but if the two solutes form a complex, a "kink" (either a maximum or a minimum) appears in the curve, at a point shown both in theory and practice to correspond to the molecular ratio of the components in the complex. If more than one complex is formed, each gives its appropriate kink.

Several hundred systems of simple hydrogen-bonding solutes have already been examined, and the complex ratios found, or the reactivity of individual types of group, have in many cases been confirmed by other methods.[70-79]. There are numerous cross-checks in the data, e.g., the number of molecules of a solute B combined with a solute A agrees with the number of free bonding centres in A.

The method has also been used to detect intramolecular bonding, e.g., in diols, /...

in diols,[71,72] for which confirmation by infrared spectroscopy is available,[80] and by other investigators, using spectrophotometric procedure, to determine the combining ratios between metals and a chelating organic solute[81,82].

A recent comment on the method[80] is that large numbers of the systems used were examined in hydrogen-bonding solvents, e.g., ethanol, benzene, and especially water, and that more positive value could be placed on the method if the only possibility for hydrogen bonding were between the two solutes. The following section, Section 1, describes the application of this suggestion. It should, however, be emphasised that hydrogen bonding in aqueous solution is of the greatest importance in all biological systems and in many technical processes, and that one of the principal advantages of this method is that it is almost the only simple one that can be used to study hydrogen-bonding combining ratios in dilute aqueous solution. Thus, of nearly thirty methods described by Pimentel and McClellan[80] only about ten can be used with aqueous media, and few if any are as widely applicable as the present.

Section 1.

An Examination of the Reliability of the "Method of Continuous Variations" with Refractive Index as the Independent Variable for Detecting Intermolecular Complexes in Dilute Aqueous Solution.

The method of continuous variations with refractive index as the independent variable has been used to measure, in carbon tetrachloride solution, the combining ratios of a variety of pairs of solutes, chosen to represent the most important types of intermolecular bond, within the limits imposed by the small range of compounds that are sufficiently soluble in this solvent. The results are shown in Fig. 4 and 5 and Tables 1 and 2 which include the results with this solvent reported previously. All relevant comparisons with systems in water or other bonding solvents are also shown.

EXPERIMENTAL

The solutes were recrystallised or redistilled, and the solvent was of infrared "AnalaR" quality. A Bellingham and Stanley (Pulfrich) refractometer was used, with water circulation, by suction, from a cool thermostat bath, controlled to $\pm 0.01^{\circ}$ by a mercury-toluene regulator. An experienced operator's standard deviation is about 3.5 sec. This deviation was considerably reduced by taking the average of at least 5 readings on each solution. The data are given only in terms of instrument reading and not as absolute refractive index values (an increment of 1 min. of arc is equivalent to about 0.0001 unit of refractive index in the index range covered here).

The solutions in each series were aged for several hours in the bath before use, and were selected for test in a random manner, to avoid error due to possible progressive ageing.

RESULTS AND DISCUSSION

Comparisons between Results in Carbon Tetrachloride and Bonding Solvents. - In Table 1 the present results are set alongside others, in which the same type of bond is expected and which were made in bonding solvents. It will be seen that three conditions occur. (a) The bonding-ratio of each pair of groups is identical in carbon tetrachloride and in a bonding solvent; this is so for most cases. (b) A complex is formed in carbon tetrachloride but not in a bonding solvent; this applies only to aldehyde and ketone groups, in water which are unreactive, presumably being protected by preferential bonding to the solvent. (c) No complex is formed in carbon tetrachloride, though its formation would be expected. This applies to one case only (butyraldehyde-phenol). This was not further investigated, but there are two possible causes: hindrance by the bulky butyl group, or inactivation of the carbonyl-oxygen atom by its intra-molecular bonding in a 6-membered ring with hydrogen on the γ-carbon atom. Bonds of the latter type in carboxylic acids and ketones have been suggested by others (e.g., ref. 83,84,85).

Complexes with Hexachlorobenzene. - This compound was used, with phenol, to discover whether high-ratio complexes can be detected in carbon tetrachloride solution. A 1:6 complex was expected, in which each chloro-group is bonded to a phenol molecule. In fact, this complex is clearly evident (Fig.4G), but in addition a 1:9 complex also appears. The author suggests that the latter is formed when three extra phenol molecules each become sandwiched between a pair of those in the 1:6 complex (Fig.6).

There /...

There appears to be evidence that this type of structure can be formed between phenol and benzene also, in carbon tetrachloride. These solutes form both 1:2 and 2:1 complexes: presumably in one case the benzene molecules are sandwiched between two phenol molecules, and in the other, phenol is between benzene. These complexes were detected previously,[70] but the test has been repeated for confirmation (Fig.4E).

Mixtures not Forming Complexes. - In previous investigations in this laboratory about twenty systems in carbon tetrachloride were examined, and in nine of these (Table 2) no complex-formation was detected. In most of these nine the pairs of solutes would not be expected to form hydrogen-bond complexes. Systems indicated in Table 2 have now been re-examined at different temperatures, and the previous results confirmed. (A negative result at one temperature only is not conclusive evidence of non-bonding.[70])

CONCLUSION

The value of the method of continuous variations with refractive index as the independent variable for measuring complex ratios in either hydrogen-bonding or non-bonding solvents has thus been confirmed.

Section 2.

Interactions between a Variety of Carbohydrates or Simple Polar Non-Ionic Solutes and Proteins in Dilute Aqueous Solutions.

INTRODUCTION.

Simple monosaccharides and disaccharides are non-ionic compounds, the molecules of which are highly solvated in dilute aqueous solution. They exist in a tautomeric state in which the α- and β- pyranose (six-membered) ring structures are highly favoured, with only relatively small amounts of the furanose (five-membered) ring form and open chain form present. This applies particularly to aldose molecules; the favoured conformations of ketose molecules in aqueous solution is uncertain. Taylor and Rowlinson,[86] from a detailed study of the thermodynamic properties of glucose and sucrose solutions, have found that the solvation forces are apparently stronger or more abundant than the forces of attraction between water molecules themselves. From a study of molecular models, Arshid, Giles and Jain[72] have suggested that solvated water molecules on opposite sides of the ring form must be closer together at their nearest distance and must experience greater mutual attraction than in the open chain form in its most probable crumpled state. The surrounding water molecules are believed to stabilise the ring structure and to afford protection against weak interactions with other solutes.

Protein molecules in dilute aqueous solution[87] also exist in a highly solvated state. The water molecules compete for the -CO- and -NH- groups in the peptide chains and thereby reduce the stability of the intra-molecular hydrogen bonds and hence the proportion of the helical form present. Estimates by different physical methods of the proportion of helical form present /...

present in aqueous solutions of bovine serum albumin* (quoted in ref.87) are 47,37,46 and 56%. The remaining proportion is largely in the random-coil conformation.

The water molecules, however, increase the stability of the disulphide linkages, which play an important part in maintaining the three-dimensional tertiary structures. Interaction is believed to occur between these structures and the aggregates so formed may in some respects resemble a swollen hydrophilic gel, with a complicated network of internal cavities and channels.

For a considerable time much attention has been focussed on the permeability of red blood cell membranes to carbohydrate molecules. A facilitated transfer mechanism is believed to be involved. This mechanism first requires the formation of a complex between the sugar and some unidentified membrane component,[88] which is believed to be protein. Le Fevre and Marshall[89] have shown that the pyranose C1 conformation ("chair shape", designated by Reeves[90]) is essential in a monosaccharide if it is to react with the cell surface constituent involved in the transport mechanism.

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* Footnote. Centrifugation of unclotted blood separates the red blood cells from a supernatant liquid called blood plasma, which contains proteins, including albumins and fibrinogen. Centrifugation of clotted blood gives a supernatant fluid called blood serum. Serum differs from plasma in that it contains no fibrinogen. Bovine serum albumin is obtained from serum; bovine plasma albumin is obtained from plasma. They are essentially the same.

In an investigation performed in this laboratory, Bruce, Giles and Jain[91] examined dilute aqueous solutions containing mixtures of D-glucose, sucrose, or meso-inositol, with gelatin, edestin, casein, and models of proteins; they suggested that D-glucose and sucrose do not form hydrogen-bond complexes with proteins in dilute aqueous or aqueous alkaline solution, because of the protective effect of the solvated water atmosphere, and that there is a relation between this inability to form complexes, and the ease of transport of the carbohydrates across red blood cell membranes. It was also suggested that the interaction between the carbohydrate molecules and the active membrane component involved non-polar forces.

The present investigation was performed at the suggestion of Le Fèvre (private communication) to examine the possibility of "handedness" (stereospecificity) in the interaction of carbohydrates and proteins in dilute aqueous solution.

EXPERIMENTAL

Materials.

Gelatin was obtained from Ilford Limited and was of pure photographic quality, ash content 2-3%, moisture about 13.5%; samples of crystallised bovine plasma albumin were obtained from the Armour Pharmaceutical Co. Ltd. and stored at less than 5°C., sulphated ash content about 0.5%, moisture about 2-3%, varying from batch to batch (one batch used gave a 0.69% moisture analysis at time of release). Pure samples of some carbohydrates were obtained from commercial sources; small samples of L-glucose and L-galactose /...

L-galactose were obtained by courtesy of Dr. H.S. Isbell, Division of Chemistry, National Bureau of Standards, Washington 25, D.C.; other reagents were "AnalaR" substances or were purified by normal methods. Distilled water was used as solvent. (See Fig. 7 and 8 for carbohydrate formulae).

Carbohydrate molecules decompose on standing at pH values greater than about 8. When solutions of proteins are subjected to extremes of pH, changes in structure (primary, secondary and tertiary) and in physical properties may result.[64] These changes are known as denaturation and are accompanied by the loss of biological activity. For these reasons, the tests were made in neutral solution.

Denaturation of proteins in solution can also be caused by heating, prolonged exposure to light or excessive frothing. For this reason, the tests were made at room temperature or below, all solutions used in the tests were freshly prepared, experimental times were minimised and frothing and exposure to light were avoided as far as possible. Gelatin, which is stable to heat (like ribonuclease, see Part II, section 2), was dissolved in warm distilled water and bovine plasma albumin in cold distilled water. In each case the second solute was dissolved in the same respective liquid.

Methods.

The thermostatically controlled Pulfrich refractometer used in Section 1 was again used with sodium light.

The mixtures were left to stand overnight before being placed in the thermostat bath for at least three hours. Concentrations of the solutions of /...

of carbohydrates and proteins were arranged so that in 50:50 mixed solutions the number of carbohydrate molecules present corresponded approximately to the number of amino-acid residues. Values of the mean residue weights of bovine plasma albumin and gelatin, namely 118 and 101 respectively were obtained from ref. 64. The observed complex ratios of solution volumes (Tables 3 and 4, columns 8) were corrected to correspond to these values and for the moisture and ash contents of the proteins. The corrected results are given in Tables 3 and 4, columns 9. The complex ratio is the ratio of the number of molecules of carbohydrate to the number of protein amino-acid residues in the complex.

D-glucose (0.02M) and bovine plasma albumin (2g/l) were tested in water (Fig.10, system 13) in phosphate buffer solution at pH 7.17 (Fig.10, system 15) and in 0.1M sodium chloride solution (Fig.10, system 16). The complexes formed in each of these solvents respectively had identical molecular ratios. This appears to show that the observed refractive index changes are not influenced by changes in pH or ionic strength of the solutions.

Examination of dilute aqueous solutions of bovine plasma albumin, D-arabinose, L-sorbose, and D-glucose on a polarimeter showed that the specific rotation $[\alpha]_D^{20^\circ}$ remained constant on standing over long periods (in the latter case after a rapid drop to $+52.8^\circ$ due to mutarotation). It is therefore assumed that such solutions are stable under the conditions used /...

used and the protein and carbohydrate molecules do not decompose on standing. Solutions of D-arabinose in 1.0 N. sodium hydroxide showed a rapid decrease in specific rotation on standing, from -108° to ca. -70° in 2 hr., -52° in one day, -39° in two days and -22° in four days. This demonstrated that on account of possible decomposition, tests made under strongly alkaline conditions would be unreliable.

The molecular weight of L-sorbose was determined by Dr. A.C. Syme, by a cryoscopic method.

RESULTS AND DISCUSSION.

The results obtained are summarised in Tables 3, 4 and 5; the experimental readings are presented in Tables 6, 7 and 8; some typical graphs are shown in Fig. 9, 10, 11 and 12.

The author suggests that the interactions detected are caused by hydrogen-bonding, with the carbohydrate acting as the proton-donor. The reasons for this suggestion are as follows: proton-donors react readily with proteins both in solution (cf. meso-inositol, mannitol, phenol, Table 4, and other reported examples and in the vapour phase[12]; proton-acceptors do not appear to react, either in solution or in the vapour phase (c.f. dioxan, methyl ethyl ketone, Table 4, and other reported examples[92]; n.b., triethylamine, though a proton-acceptor, reacts with gelatin, Fig. 11, system 66, but it probably does so by an acid-base interaction[70,79]).

The critical test of this suggested bonding mechanism would be the determination of the heats of interaction. This, however, is not possible at /...

at present (a method suggested by the author's colleague, Dr. S.N. Nakhwa,[35] has been shown to be impracticable both by the author and by another colleague, J. Gallagher[93]).

The size and shape of the proton-donor molecule also appears to be a determining factor. Thus all the smallest proton-donor molecules, viz., phenol, mannitol, and all pentoses, react with all the proteins tested, but none of the largest molecules, i.e. the disaccharides reacts at all.

The behaviour of the intermediate sized molecules, i.e. the hexoses and meso-inositol, is interesting; interaction here seems to be specific for certain carbohydrate-protein combinations, and other combinations show no interaction; it is also stereo-specific, e.g., D-glucose reacts with bovine plasma albumin, but L-glucose does not; and with gelatin, the L-form, but not the D-form, reacts.

These facts suggest that some highly selective molecular sieve action is involved, and that the hexose molecules are approximately of the critical size, so that quite small changes in configuration of either the carbohydrate or the protein molecule are important in determining whether interaction occurs or not. If there is in fact a sieve action, then this implies that the carbohydrate molecules must penetrate into apertures in the three-dimensional protein aggregates which it is believed are present in solution.[87] The limiting size of carbohydrate which can thus penetrate is presumably determined by its dimensions in the water-solvated state. Although this associated water normally prevents the carbohydrate hydroxyl groups from hydrogen-bonding with other small polar molecules in aqueous solution, e.g., /...

e.g., phenols (c.f. Table 5, and ref. 72), yet in the present case they appear to form bonds with proteins. It may be that the protein molecules can "squeeze out" the solvated water by multipoint attachment.

No correlation between the molecular structure of the hexose molecules and their behaviour towards proteins in dilute aqueous solution can be observed at present, for although the pyranose ring form is favoured there is some uncertainty about its exact conformation (two "chair" forms and several "boat" and "skew" forms are possible, and the stability of each varies from hexose to hexose), and configuration (α-, β-, anomerism occurs and the stability of each anomer varies from hexose to hexose). Systems are further complicated by solvation and by the presence of small amounts of the furanose ring form and the open chain form.

Relevance of the Present Results to the Red Blood Cell Transfer Mechanism.- The present results show that the hypothesis proposed by Bruce, Giles and Jain,[91] must be modified, because (a) D-glucose does react with one of the proteins used (bovine plasma albumin), and (b) several of the carbohydrates found to react with protein do in fact readily traverse red blood cell membranes[89] (n.b. no disaccharide can traverse the membrane, and in the present results no disaccharide has been found to interact with protein). There is some evidence that the transport mechanism of carbohydrates across these membranes may, in fact, involve hydrogen-bonding with a component, perhaps protein, of the membrane. Thus, transport is retarded in the presence of phenolic compounds[94], more especially by polyhydric phenols /...

phenols with large hydrophobic residues, which presumably block the hydrogen-bonding sites in the protein.

It is of interest to note that Stein[95] has presented evidence which suggests that two molecules of carbohydrate react with one unit of the active carrier system and thereby appear to be transported in a dimeric form.

The monosaccharide-transfer system appears to be of wide biological significance and does not occur only in human red blood cells, but in many other species and tissues.[88] For example, only carbohydrates or related compounds which possess a D-pyranose structure, a methyl or substituted methyl group in the 5-position of this structure, and a hydroxyl group in the glucose configuration in the 2-position are transported across the walls of the hamster intestine[96].

Reactions of L-sorbose.- This ketose is anomalous; it is the only one tested that appears to react with a second solute in water. With phenol it forms a 4:1-complex, and with resorcinol, both a 4:1- and an 8:1- complex. The only explanation of these unexpected results seems to be that the carbohydrate associates into clusters of four or eight molecules, whereby the strength of the bonds with water is reduced sufficiently for phenols to react. Thus a group of eight sorbose molecules might be so arranged that one at each end can combine with phenol, thus giving the 4:1 combining ratio. On this hypothesis the 4:1-complex with resorcinol would also represent 8 sorbose molecules combined with two of resorcinol, one hydroxyl-group only being reactive in each resorcinol molecule; and the 8:1-complex would be explained /...

explained by assuming that the sorbose molecules in the cluster have enough freedom to orient in a position where the two reactive centres can each respectively combine with the two resorcinol hydroxyl-groups.

This hypothesis was tested by the determination of the molecular weight of L-sorbose in water. In about 0.03 M. solutions, replicate values were 1178, 1261, 1261, 1279, 1384 (not in order of testing). These represent about 7.1 molecules (i.e. 1277 ± 100), and it seems reasonable to suppose that there is an equilibrium between about 10% of monomeric molecules and about 90% of eight-fold aggregates. The monomeric form reacts with the protein, but like other carbohydrates in aqueous solutions, not with phenol.

CONCLUSION.

It may be concluded that the adsorption of large non-ionic solutes (such as monosaccharides and disaccharides) at biological surfaces depends largely on the physical characteristics of the solute molecules and of the substrate, and not merely on their chemical natures. The mechanism of bonding of polar non-ionic solutes has not been fully elucidated in the present case with proteins. The results, however, suggest that hydrogen bonding with the solute acting as the proton donor is involved, although van der Waals attraction may play some part.

PART II

The Adsorption of Ionic Solutes at Biological Surfaces.

INTRODUCTION

This part is concerned with the adsorption of ionic solutes at biological surfaces. Complete cells or sections of tissue, the chemical constitutions of which are not uniform throughout, are used as substrates in direct adsorption experiments. The solutes used are cationic and anionic dyes.

Much attention has been directed towards the staining of micro-organisms; this is discussed especially in Section 1. Although these organisms are not typical in the biological sense, the fact that many types tend to exist as single cells and hence give a biologically homogeneous substrate is convenient for the present experiments. Animal tissues, on the other hand, contain many types of cell and it is generally not possible to isolate the different types, at least not in bulk. A discussion of the complete field of biological staining processes is beyond the scope of the present thesis. An account, however, of the main aspects of the staining of micro-organisms is given in the introduction to Section 1. Section 2 demonstrates in detail one staining process on tissues and the type of technique used by the biologist to elucidate its mechanism. Indeed, almost all previous direct investigations of the mechanism of staining processes have been performed by the biologist, with little collaboration with the chemist, and hence the advancement of knowledge in what is essentially a branch of surface chemistry has been retarded.

The constituent parts of organisms are usually colourless and transparent and therefore cannot be distinguished from each other by using an optical microscope /...

microscope unless there are appreciable differencies in their refractive indices. (n.b. the electron microscope in effect depends on differences in density, and staining processes are unnecessary in conjunction with its use). Staining techniques were first used by Leeuwenhoek, the pioneer microscopist, as far back at 1719 in an attempt to overcome this difficulty, but he met with little success and it was not until the middle of last century that stains began to be successfully employed and their importance realised.

Staining processes have contributed greatly to the evolution of microscopic techniques and biology in general. They have helped in defining and classifying structural features and special characteristics of cells and tissues. A wide range of dyes is used in the staining of tissues; all micro-organisms readily take up cationic dyes from aqueous solution and consequently the stains used for these organisms are mostly selected dyes of this class. The dye uptake is influenced by the presence of impurities, some desirable, others undesirable; and in fact highly pure dyes may be unsuitable.[97] To obtain comparable and reproducible results, the dyes used must be manufactured according to certain recommendations and each batch must pass certain practical staining tests. In this country special qualities of dyes are supplied for use as stains. In the U.S.A. recommendations are made by the Biological Stain Commission, the official organ of which is "Stain Technology", and suitable batches of dye may be labelled "Commission Certified".[97]

Staining Methods.

Although most dyes are toxic to living cells, there are a few dyes which, when /...

when used at extremely low concentrations, stain cells but do not kill them (at least not immediately) and enable them to be investigated in the living state. This process is known as vital staining and it can be applied to the cells of micro-organisms. The practical difficulties involved, however, are extremely great and, as compared with the staining of dead ("fixed") cells, the technique has so far contributed little to microbiology. Part II of this thesis is concerned with the staining of dead cells.

Preparation of the Cells for Staining.- Dead cells are much more easily handled than living cells and after suitable treatment can be stored for long periods. Further, they can be stained by a wide variety of dyes. There are, however, a number of disadvantages. Firstly, certain structural changes are known to occur when the cell dies. Secondly, dead cells are extremely delicate and readily suffer damage from such causes as evaporation, changes in osmotic pressure, or internal enzyme attack (autolysis). These must all be guarded against and the actual method used for killing the cells and for preventing their decomposition is often important. The process is known as fixation and has been described at length elsewhere.[98] Fixation must perform the dual function of preserving the cells and rendering them immune to damage when subjected to subsequent treatments.

The choice of the method of fixation employed depends largely on the nature of the intended investigation. For routine staining purposes the application of heat is sufficient. Heating, however, produces coagulation of polymeric materials and may cause mechanical damage. Further, it does not prevent autolysis and preparations cannot be kept for longer than about 24 hours. /...

24 hours. Several coagulant and non-coagulant chemical substances, known as fixatives, are used for finer work. Aqueous preparations of formaldehyde are one of the most common types of fixative. These preparations are non-coagulant and particularly suitable when preparing for staining with basic dyes, because formaldehyde reacts with free amino groups in the side chains of polypeptides in the organism, thereby preventing them from becoming positively charged and thus repelling dye cations.

To investigate animal cells the part of the animal in which they occur is treated with a suitable fixative, embedded in paraffin wax, cut into sections a few microns thick and mounted on glass slides. Before staining the wax is removed by treatment with suitable solvents. Fixed micro-organisms, however, may be examined in smears containing many cells spread in a single layer, or as single cells cut into sections.[99] Section 1 is concerned with the staining of micro-organisms in smears; Section 2 is concerned with the staining of cells in sections of animal tissue.

Section 1

The Adsorption of Cationic Solutes : Mechanism of Adsorption of Cationic Dyes on Fixed Yeast Cells.

INTRODUCTION.

In this section a quantitative investigation of the adsorption of cationic dyes on dead yeast cells is carried out in an attempt to further elucidate the adsorption mechanism. The cells used are intact, as indeed they are in the smears used in the common staining techniques. The organism studied is brewer's yeast, a strain of Saccharomyces cerevesiae, chosen because (a) it is the micro-organism most readily available in bulk, (b) it is unicellular and (c) the cells are comparatively large and can be easily separated from suspensions by centrifuging.

Before proceeding further, however, it is necessary to discuss the staining techniques commonly used for yeast and bacterial cells and the nature of these cells.

Staining Techniques for Fixed Cells in Smears.[99]

There are four general types of staining technique commonly used. The following are examples representing each type, but there are many modifications, and various combinations of these individual techniques are often used to demonstrate particular cellular features.

Simple Staining. - The smear is treated with a suitable stain for a brief period (ca. 1 min.), then rinsed with water. The organisms are stained in contrast to the transparent background. Many stains can be used, e.g., dilute preparations of Crystal Violet, Methylene Blue, Safranine, Basic Fuchsine (usually as Carbol Fuchsine, an aqueous mixture of the dye and phenol), or various acid dyes. This technique is used to examine the general appearance of the organism.

An /...

An alternative to simple staining is sometimes employed, called negative staining. It is not however a true staining process, for after treatment with a preparation of the acid dye, Nigrosine, or indeed with a suspension of any black opaque solid the organisms appear colourless against an opaque background. In combination with other techniques it can be used to demonstrate particular cellular features.

Gram Staining. - The principle of this technique was first used in 1884 by Christian Gram, a Danish pathologist. The smear is treated with an aqueous preparation of Crystal Violet for about one minute, and the excess is rinsed off with water. A preparation of iodine is applied for about 1 minute and the smear is then rinsed with absolute alcohol or acetone for a short time (up to 30 sec.) and blotted dry. Finally a dilute solution of a suitable counterstain (usually red) is applied for about 30 seconds. On examination, the so-called Gram-positive organisms appear dark blue to violet, whereas the so-called Gram-negative organisms take the colour of the counterstain. Sharp differentiation does not always occur and uncertain results are classed as Gram-variable. There are many modifications of the original method; only the essential features are here described and it must be emphasised that the concentrations of all solutions and all times of reagent application or washing must be rigidly standardised in order to obtain reproducible results. The best primary stains are the basic triarylmethane dyes[100]; Methylene Blue, Rhodamine B and Safranine are unsuitable. The main requirement for a counterstain is that its colour is in contrast to that of the primary stain.

"Acid-Fast" /...

"Acid-Fast" Staining Techniques. - The smear may be treated with Carbol Fuchsine and kept hot for up to ca. 10 min., then rinsed with water. An alcoholic (ca. 3%) or aqueous (ca. 20%) solution of hydrochloric (sometimes sulphuric) acid is then applied until the colour is almost removed. The smear is then washed and coloured with a suitable counterstain, usually a dilute aqueous solution of Methylene Blue, and washed again. On examination, after drying, organisms which are classed acid-fast by this procedure appear red against a blue background; non-acid-fast organisms appear blue. There are several modifications of this technique. The above example is known as the Ziehl-Neelson procedure.

Nuclear Staining. - The smear is subjected to a modification of the so-called Feulgen reaction,[101] which is in general specific for deoxyribonucleic acid (DNA), ribonucleic acid (RNA) being unstained. In this technique, which is used for revealing the nuclei of yeasts and bacteria, most organisms are first subjected to hydrolysis in normal hydrochloric acid at 60°C. and are then treated with Schiff's reagent (a solution of Basic Fuchsine, the colour of which is suppressed by the presence of sulphurous acid). Various washing treatments follow and the persistence of a bluish-purple colour (not the reddish-purple colour of Basic Fuchsine itself) is a positive indication of the presence of DNA. In the case of yeasts and bacteria this coloration will render the DNA-rich nuclear materials clearly visible, the RNA-rich cytoplasm being only feebly stained.

General Physical Structure of Yeasts and Bacteria.[56,102,103]

Yeasts and many types of bacteria tend to exist as single cells (unicellular) or as small clusters of single cells (see Fig. 2 for their general appearance). The cells are not unidimensional. Yeast cells tend to be ovoids with an average diameter of the order of 5 to 6 microns and are in general larger than most bacterial cells, but smaller than most animal cells.

There are many other features which render yeast and bacterial cells distinct from animal cells. For example, they are less easily penetrated by solutes owing to the protective effect of the cell wall and of the accumulations of polymeric adherents on its surface. Further, the protoplasts of some yeasts and bacteria can be isolated under experimental conditions from the other cellular parts, but yet remain alive and ~~are~~ capable of reproducing.[104] This suggests that the function of the cell wall is largely mechanical.

The fine structure of the protoplasm of yeast and bacterial cells has not been fully explained and is still a highly controversial subject. It appears to vary considerably throughout the life of the cells. In contrast to typical animal cells, there is as yet little evidence to suggest the presence of membrane systems in the cytoplasm.[105] The presence of close-packed granules, which may correspond to the ribosome granules, has been established in bacterial cells.[105] The cell membrane can be revealed by several methods which include a staining technique using a dye of the Victoria Blue type or sometimes Crystal Violet.[106]

The /...

The cell walls of yeasts and bacteria tend to be rigid structures and account for up to 20% of the dried cell weight.[107,108,109] Those of bacteria vary in thickness from 100 to 200 Å., whereas yeast cell walls are much thicker,[110,111] from ca. 1600 to 2500 Å. Under the electron microscope isolated cell walls of Gram-positive bacteria appear to have a smooth surface, while fine structures can be distinguished in the walls of Gram-negative types, e.g., an apparent monolayer of large spherical macromolecules 100-140 Å. in diameter arranged in a hexagonal manner.[112] A rectangular arrangement of spherical macromolecules of diameter ca. 115 Å. has been observed, however, in the walls of a Gram-positive.[113] There is other evidence to suggest that spherical macromolecules may be more common structural elements of the cell walls of Gram-negative than of Gram-positive bacteria.[114]

On the outer surfaces of the walls, probably of all micro-organisms, there are accumulations of polymeric materials of high molecular weight and high viscosity. Electron microscopy has shown that in general these so-called surface adherents (see Fig. 1) are less electron-dense than the cell wall and the cytoplasm. In most micro-organisms the adherents are loosely bound into layers known as slime layers, with little suggestion of crystallinity, but in some bacteria and in a few yeasts the slime layers are extended into well defined and much more ordered structures known as capsules. These capsules have a low affinity for dyes and are revealed as unstained regions when certain dye staining processes are applied to the cells; some, however, are so thin that they can be detected /...

detected only by chemical and biological tests. Various types of capsule have been observed (e.g., ref. 115, 116).

Some Aspects of the Chemical Constitution of Yeasts and Bacteria.[105,117,118]

The development in recent years of techniques for the isolation of the various component parts of yeast cells and bacterial cells has enabled the chemical constitution of many of these organisms to be intensively investigated. The following is a brief account of the conclusions which seem to be the most relevant to the staining properties of the cells. The dye molecules first encounter the external layers of the cell, which are therefore described first.

Chemical Constitution of Cell Surface Adherents. - The surface adherents on bacterial cells have a highly complex structure which has not yet been fully elucidated. Indeed, their chemical constitution appears to depend somewhat on the external medium, although some of the adherents may have an intracellular origin. Capsular materials appear to consist largely of complex mixtures of polysaccharides and polypeptides which in a few cases are true proteins. Only a few yeasts are known to form capsules. In all cases, however, capsular materials, as already mentioned, have a low affinity for dyes and often can be shown up as unstained regions by a combination of the simple staining and negative staining techniques.

Chemical Constitution of Cell Walls [107,117] - It has been established that the cell walls of many Gram-positive micro-organisms consist largely of polypeptide-(amino-sugar)-polysaccharide complexes. These complexes are composed mainly of a polymeric substance containing only three or four /...

four principal amino-acids, together with acetyl glucosamine, acetyl muramic acid (acetyl derivatives of 3-O-∝- carboxyethyl hexosamine[119,120]) and perhaps galactosamine, various carbohydrates and their phosphate esters and teichoic acids. Moreover, these complexes appear to be present to a less extent in the cell walls of Gram-negative organisms, than in Gram-positive ones. Gram-negative organisms, however, have a much wider variety of amino acids than have Gram-positive ones, and in addition they contain appreciable amounts of lipids. The walls of Gram-positive organisms contain little or no lipid material. All micro-organisms have a proportion of their amino-acids in a readily extractable form, and the remainder are bound in the complex polypeptides. It has been suggested, however, that the presence of the readily extractable amino-acids may be more characteristic of Gram-positive organisms.[121]

The neutral amino-acid alanine and the acidic amino-acid glutamic acid appear to be common to the cell walls of all yeasts and bacteria, alanine being the predominant amino-acid in Gram-positive cells. Finally, a high proportion of the total amino-acid content is present as the D-isomer. This suggests that the polypeptides are not true proteins, for the amino-acid residues in true proteins have the L-configuration (see General Introduction).

The mechanical strength of the cell wall may be attributed to a mucopeptide framework to which the other constituents, such as teichoic acids, appear to be loosely bound.

Chemical /...

Chemical Constitution of Cell Membranes.[105] Several authors[57,122,123] have examined the isolated cell membranes of a few Gram-positive bacteria and have shown that the composition is quite different from that of the cell wall. The membrane is essentially a lipid-polysaccharide-protein complex, containing about 20-30% lipid and about 50% true protein (on hydrolysis it yields practically all the usual L-amino acids found in normal proteins). This high lipid content is particularly interesting, for as just stated the cell walls of Gram-positive organisms contain little or none at all. A number of enzymes (protein) have been detected in the membrane, but there is no conclusive evidence to suggest the presence of nucleic acids.

Chemical Constitution of the Inner Structure of the Cell.[105,124] - Chemically, the inner structure of yeast and bacterial cells is similar to that of animal cells. The cytoplasm is rich in protein and ribonucleo-protein, although it is not certain whether ribosome granules occur or not. The nucleus is largely chromatin.

Theories of Gram Staining (and Simple Staining).

In the early years of this century several theories were proposed in attempts to explain Gram differentiation. The two most important theories at that time were quite distinct, for one was based on a purely physical principle, while the other was essentially chemical.

The physical theory, proposed first [125,126], is based on the fact that disintegration of Gram-positive cells causes the loss of their Gram-positive character. According to this theory, a dye-iodine complex is formed /...

formed and the outward passage of this complex from Gram-positive cells is hindered by a mechanical permeability effect.

In the chemical theory, which was proposed some time later,[127] it was assumed that ionic groups in the cell protoplasm behave as if in solution and that the dye molecules are retained by electrostatic forces. It is suggested that there is a relationship between the isoelectric point of the protoplasm and its affinity for basic dyes. Thus Gram-positive organisms would have an isoelectric point at a lower pH value than Gram-negative organisms. This would explain why basic dyes appear to have a greater affinity for Gram-positive organisms, and why these are not easily decolorised by alcohol. This theory has been supported in recent years by the work of several authors,[128,129,130] who have concluded that Gram-positive organisms adsorb more dye than Gram-negative organisms, but this conclusion has been questioned[131]. It may be that the differences in interpretation are due to differences in experimental methods[132]. In any case a theory of this type cannot explain why a positive reaction only occurs when the cell is intact, i.e. why cell "integrity" is essential for the Gram reaction.

Today it is widely believed that a surface layer of material which confers Gram-positiveness is present in Gram-positive organisms, but not in Gram-negative organisms. This layer may be associated with the cell wall, for the isolated protoplasts of all organisms are Gram-negative. Although this belief is based on early work[133], it has only become generally /...

generally accepted in recent years and even today it is not certain how the surface layer is concerned in the staining reaction.

There is considerable evidence to suggest that the layer is composed largely of a complex containing RNA,[134,135,136] but this evidence is not universally accepted. Extraction of a material which is believed to be essentially RNA from Gram-positive organisms renders them Gram-negative. This RNA can be "replated" back on to the surfaces of the cells and it is then found that they have to a large extent regained their Gram-positive character. It has also been shown that addition of DNA to a typical Gram-negative organism can produce Gram-positiveness[137]. An alternative hypothesis is that a particular phosphoric ester fraction (probably teichoic acids) is responsible for Gram differentiation[138]; this view, however, has met with considerable opposition[139].

Nevertheless there is reasonable evidence suggesting that the presence of RNA is not essential for a positive Gram-reaction; some cells which do not contain RNA are Gram positive[98]; moreover, the cytoplasm of both Gram classes of organism is rich in RNA, hence if RNA is indeed involved in the differentiation, it must be located either on the outer surface of the cell membrane or in the cell wall. Yet chemical analyses of isolated cell walls and cell membranes have produced no satisfactory evidence to suggest the presence of nucleic acid, although it is possible that the methods used for their isolation also separate the cell walls from RNA.

Other Points of Interest. - There is evidence that the Gram staining properties /...

properties of bacteria can also be altered or reversed by various chemical treatments[127,133,140,141,142]. Treatment of Gram-negative organisms with alkalis or reducing agents will render them Gram-positive; treatment of Gram-positive organisms with acids or oxidising agents will render them Gram-negative. It has been proposed,[142] in an attempt to explain these observations, that reduction of disulphide (-S-S-, cystine) linkages to sulphydryl groups (-SH) converts Gram-negative organisms to a Gram-positive state, and vice versa, but this hypothesis has been criticised.[143]

It has also been observed that irradiation of Gram-positive bacteria with ultra-violet light causes the loss of Gram-positiveness[144]. This fact would suggest that the material responsible for the Gram-positiveness may be a macromolecular substance containing centres of unsaturation that suffer photolytic decomposition.

Site of the Gram Reaction. - It is convenient to consider that the Gram reaction takes place in a specific region of the cell. Photographic evidence[145] has shown that the Gram-positive staining region may occur below the outer part of the cell wall, since it appears to occupy a position within the region which is revealed by a particular process known as the Dyar cell wall stain[146,147]. Although the presence of acidic materials in the cell wall, particularly of Gram positive organisms, will cause primary stain, e.g., Crystal Violet, to be adsorbed thereon to some extent, solution containing unadsorbed dye will remain until the water rinsing treatment is applied. If rinsing is cut short or omitted altogether /...

altogether much of this excess dye solution will remain mechanically trapped in the cell wall, and the Gram positive region might then include the cell wall[148]. This fact could explain the reported observation that Gram differentiation is due to the formation of the dye-iodine complex actually in the cell walls of Gram-positive cells,[149] whereas, as just mentioned, the reaction site appears to be below the cell wall. It is now generally accepted that the site of the Gram-reaction is the inner part of the cell wall or perhaps the outer surface of the cell membrane and that this may be the location of the layer which confers Gram-positiveness.

Chemical Forces in the Dye Retention. - Stearn and Stearn[127] were perhaps the first to suggest that chemical forces are involved in the dye retention, but their work appears to have been treated with some scepticism. This was probably due to a misunderstanding of the true nature of adsorption from solution. There seems to be no reason why microbiological surfaces, despite their complexity, should react differently from other organic materials. Indeed, not only is there considerable evidence in favour of ion exchange at cell surfaces [150,151,152] but there is evidence which suggests that dye adsorption by both Gram-positive and Gram-negative organisms can be described by the normal Langmuir equation[153,154] applicable to adsorption at non-living surfaces. Several types of bacteria have been shown to bear a negative charge in water[152] and it is probable that the positively charged basic dye molecules or aggregates react with phosphatidic groups in the cell wall, nucleic acid or phospholipid /...

phospholipid constituents, with ionised carboxylic acid groups[155] in the amino-acid residues of the polypeptide complexes, and with ionised sulphur-containing groups present in the polysaccharide constituents.

The Role of Iodine in the Gram Reaction. - The importance of iodine in the Gram reaction appears to be less than might at first be thought.[156] Indeed, it has been shown that Gram-positive and Gram-negative bacteria can be distinguished without using iodine or any similar reagent.[100] The subsequent differentiation in alcohol, however, is then extremely difficult. Further, it has been shown that all the dyes which give Gram differentiation form precipitates with iodine.[100] It seems then that iodine merely serves to accentuate the differentiation, probably by forming some type of complex or precipitate with the adsorbed dye and thereby reducing its ease of removal in alcohol, especially since it has been shown that Gram-positive cells are less permeable to iodine in alcoholic solution than Gram-negative ones.[157] A study of reagents which can replace iodine in the Gram reaction, shows that each is a mild oxidising agent and can form a precipitate with Crystal Violet.[157] Nevertheless, some oxidising agents and some reagents which form precipitates with Crystal Violet are unsuitable for use in the Gram reaction, and this suggests that iodine must function not merely as an oxidising agent, nor merely as a precipitating agent, but as a combination of both.

Probable Mechanism of Gram Differentiation. - From studies of stained cells under the optical microscope it is generally believed that the cells are stained throughout by the primary stain. It must be stressed /...

stressed that this evidence is purely qualitative, for it is not possible to estimate the amount of dye adsorbed on the various cellular parts. Rinsing in water removes most of the unadsorbed dye. Treatment with iodine forms a complex, or stable dye-iodine aggregates, with the adsorbed dye and the outward passage of these aggregates is hindered in Gram-positive organisms more than it is in Gram-negative ones by the low permeability of the cell wall. The strongest evidence in favour of this cell wall permeability theory is the fact that cells must be intact to give a positive Gram reaction. The effect of the surface layer of material, which may contain RNA or teichoic acids and which is necessary for a positive Gram reaction may be, in part at least, to reduce the permeability of the cell wall of Gram-positive organisms. The permeability of the cell membrane appears to be of little significance, for isolated protoplasts are Gram-negative.

Aggregates of dye are involved in the marked retention effect that is the distinguishing mark of the Gram-positive reaction, and in the normal process these aggregates contain iodine. Iodine is not however essential, because some aggregating dyes (basic triaryl methane dyes) will also differentiate Gram-positive organisms in absence of iodine, though not so well.

Theories on the Acid-Fast Stain.

The Acid-fast stain was first used by Ehrlich in 1882 and is, in principle, somewhat similar to the Gram stain, although no complex-forming agent is involved. Acid-fast bacteria adsorb dyes much less readily /...

readily from dilute aqueous solution than other organisms, and the process requires an elevated temperature. When stained, however, they are extremely resistant to decolourisation even by such a drastic agent as alcoholic mineral acid. They are usually highly capsular, e.g., the tubercule organism which causes tuberculosis.

It was realised early this century that capsular materials are in some way involved in the staining process. Microscopic examination showed that a peculiar "beading" effect of the dye occurred and this could not be explained. One theory was that the Carbol Fuchsine is more soluble in the large proportion of fatty constituents, believed to be present, than in water and that this is responsible for the acid-fastness and the bead formation.[158] At the most, however, this can only be partially true, for it has been shown that cell integrity is essential for a positive result[125,159] and that beading is merely an artefact which depends on the staining procedure.[160,161] Hence it would appear that the result of the acid-fast staining technique depends largely on the permeability of the surface layers of the organism, those of acid-fast organisms being the less permeable.[125,162]

Basic Fuchsine is applied at an elevated temperature in the presence of phenol (Carbol Fuchsine) and under these conditions the dye molecules will be to a large extent disaggregated. The large proportion of mono-disperse molecules thus formed and the operation of the higher temperature will increase the amount and the rate of dye penetration, respectively. Further it is probable that the adsorbed dye will be reaggregated at or near the active sites in the organism

in /...

in a manner similar to the behaviour of other dyes on other organic surfaces and this reaggregation may be responsible, in part at least, for the subsequent difficulty of elution, if indeed the cell wall permeability is a critical differentiating factor, as seems to be the case. Finally, it is generally true to say that Gram-positive organisms may be acid-fast, while Gram-negative organisms are probably never acid-fast[56] and this in itself is strong support for the theory involving cell wall permeability.

Theory of Nuclear Staining.

The Feulgen reaction has been the subject of intensive research and its mechanism is well known. The mechanism of the nuclear staining of micro-organisms has been clearly described elsewhere[163]; the following is a brief summary. Treatment of the organism with hydrochloric acid at 60°C. serves two purposes; (a) it partially hydrolyses the nucleo-proteins of underlying structures, releasing the aldehydic groups of the pentose sugar constituent; (b) it completely hydrolyses stainable material in the outer layers and thereby reduces their masking effect. Subsequent treatment with Schiff's reagent produces a bluish-purple coloration caused by interaction with the free aldehydic groups.

The Adsorption Isotherm.

Much qualitative and quantitative information can be obtained about an adsorption system by determining the adsorption isotherm. This is a graphical representation of the adsorption process at a fixed temperature and may be regarded as a plot of the distribution of solute /...

solute between the interface and the liquid phase as a function of the concentration of solute in solution. In this thesis the adsorption per unit weight of adsorbent is expressed as a function of the concentration of adsorbate remaining in solution.

A very large number of adsorption isotherms have been reported in the literature. Several types have been observed and there have been a number of attempts to produce a satisfactory general classification. Possibly the first attempt was made in 1922 by Ostwald and Izaguirre[164] who were concerned with adsorption from binary mixtures and dilute solutions. Brunauer[165] later defined five types of isotherm that are observed in vapour phase adsorption.

Recently, Giles and his co-workers[166,167] have proposed a system of classification of solution adsorption isotherms which aids the diagnosis of adsorption mechanisms and also the measurement of specific surface areas of solids. All isotherms are divided into four main classes according to the initial slope, and for each class there are described sub-groups based on the shapes of the upper parts of the curves. The four main classes (see Fig. 13) are named the S, L (i.e. "Langmuir" type), H ("high affinity") and C ("constant partition") isotherms. The L-curves are the best known; indeed, the L2 curve occurs in probably the majority of cases of adsorption from dilute solution and few cases of the other types appear to have been previously recorded.

The Initial Slope. - The initial slope of the adsorption isotherm depends /...

depends on the rate of change of site availability with increase in the solute adsorbed. As the amount of solute adsorbed increases there will usually be progressively less sites available at the interface, and the adsorption in the normal case tends to decrease. This corresponds to the L isotherm and the upper portions of the S and H isotherms. In the initial part of the S isotherm, however, the opposite condition applies and the amount of solute adsorbed increases with increase in the number of sites occupied. It is believed that this is due to side-by-side interaction between adsorbed solute molecules. In the C isotherm the site availability remains constant until saturation adsorption is reached.

The S Isotherm. - The S isotherm is obtained in systems where (a) the solute particle is monofunctional (i.e. has a fairly large hydrophobic residue and a marked localisation of attractive forces over a short section of its periphery), (b) has moderate interaction with other solute particles and (c) meets with strong competition from the solvent. It is believed that the adsorbed particles are oriented vertically to the plane of the interface and, as already stated, are stacked face-to-face.

The author has observed S-shaped isotherms for the adsorption of condensed-ring polynuclear aromatic hydrocarbons on chromatographic alumina from xylene and 2:2:4-trimethylpentane[26,27] (Fig.14).

The L Isotherm. - The L curve is obtained when the solute molecules are oriented flatwise on the adsorbent (i.e. parallel to the plane of the /...

the interface), e.g., resorcinol from water on chromatographic alumina[6], or when there is no strong competition for the surface. It is also given by monofunctional ionic solutes which appear to have such strong interparticular attraction that they are adsorbed in a micellar form, e.g., cationic dyes from water on silica[7].

The H Isotherm. - The H isotherm may be regarded as a special case of the L isotherm in which the solute has such a high affinity for the adsorbent that it is completely withdrawn (or almost so) from dilute solutions. The initial part of the isotherm is almost vertical and, in extreme cases, the isotherm is a horizontal line starting at a positive value on the vertical axis. It has often been observed for the adsorption of large units such as large ionic micelles or polymeric molecules, e.g., sulphonated azo dyes from water on chromatographic alumina,[6] but has been obtained in some cases where the adsorbed species appears to be single ions which exchange with others of much lower affinity for the adsorbent.

The C Isotherm. - The C isotherm is characterised by the constant partition of solute between the interface and the solution right up to the saturation value, where an abrupt change to the horizontal plateau occurs, e.g., water from n-butanol on wool.[12] It is obtained when the following conditions are satisfied: (a) when the substrate is porous, but has regions with differing degrees of crystallinity such that many of the molecules are flexible and they can therefore more readily be forced apart; (b) when the solute has a much higher affinity for the adsorbent than the solvent has. The C isotherm resembles the type of curve /...

curve obtained for the partition of solute between two immiscible solvents, and indeed the substrate appears to act as an immiscible liquid.

"First Degree" Saturation Adsorption. - All isotherms, unless experimental difficulties do not permit (sub-group 1), have either a plateau or an inflection, which indicates that all the available sites at the interface have been occupied. One or other of the following situations may prevail. (a) A complete close-packed monolayer of adsorbed solute particles may be formed (this occurs usually with non-ionic solutes on polar and non-polar solids, e.g., phenols on alumina, silica and graphite[6,7,36]); it has been suggested that polar solvents are included in monolayers on polar solids and that non-polar solvents are included in monolayers on non-polar solids. (b) A layer of close-packed or isolated ionic micelles may be formed (see ref. 168; also, it can be seen from electronmicrographs that some direct cotton dyes on cellulose exist as minute particles[169]); the exact nature of the micelles is not known. (c) The solute may be adsorbed as isolated clusters of molecules; for example the author has observed that condensed-ring polynuclear aromatic hydrocarbons are adsorbed as isolated clusters on the surface of chromatographic alumina[26,27] (see Fig. 15).

Significance of the Plateau or Inflection. - A long flat plateau indicates that saturation adsorption has been attained; the adsorbed solute particles present a second surface which repels the solute particles remaining in solution and hence prevent further adsorption. A short plateau followed by a rise in the adsorption with increasing concentration /...

concentration of solute indicates that the adsorbed particles present a second surface which has almost the same attraction for the particles in solution as the original surface. A second plateau may be reached if experimental conditions will permit (sub-groups 3 and 4).

In some cases, however, a maximum (and not a plateau) may be observed and the adsorption falls off with further increase in the concentration (sub-group mx). This is believed to be due to association of the solute molecules or ions in solution with increase in concentration, whereby the solute-solute interaction may exceed the solute-solid interaction and the equilibrium is influenced in favour of desorption. In some cases a minimum may be obtained at higher solute concentrations. The exact reason for this is not clear; possibly it is caused by changes in the nature of the solute aggregates in solutes, as the concentration rises.

Application of the Isotherm Classification System. - The above system of classification of solution adsorption isotherms enables information about the nature of the adsorption mechanism and the state and orientation of the adsorbed molecules to be deduced from the shape of the isotherms. The substrates so far investigated have been homogeneous. There seems, however, to be no reason why the same principles should not, with due care, be applicable to heterogeneous substrates, such as biological cells.

Present Work.

In the present work the adsorption of basic dyes on dead yeast cells /...

cells is investigated. It is well known that basic dyes are strongly adsorbed by the cells of dead micro-organisms and there is evidence to suggest that the mechanism of retention is ion-exchange.[150,151,152,155] It has also been suggested that the adsorption is of the Langmuir type,[153,154] (cf. ref. 152).

EXPERIMENTAL.

Purification of dyes. - The dyes (formulae shown in Fig. 16 and 17, cf. ref. 170) Victoria Blue RN, Victoria Pure Blue BO and Rhodamine 3B, were purified by leaching diluent-free "batch" grade samples with hot 10% hydrochloric acid, filtering the hot liquors and allowing the dyes to crystallise from them. The crystallisation was repeated at least once and the crystals were dried first at 40-50°C. and then in a vacuum desiccator over potassium hydroxide overnight (high temperature drying was considered undesirable). Victoria Blue BN and Victoria Pure Blue BO could not be purified satisfactorily, because of their low solubility in the acid solution; Rhodamine 3B formed an unfilterable gel with ethanol (probably the best alternative solvent; recrystallisation from hydrochloric acid was considered to be undesirable on account of possible hydrolysis of the $-CO_2C_2H_5$ group), untreated batch grade samples were therefore used. The purity of the dyes was determined by micro-analysis for C,H, and N; by flame analysis for Na (a rough measure of salt impurity) (Evans Electroselenium Ltd., flame spectrophotometer), and by potentiometric titration of Cl^- ion with silver nitrate /...

nitrate in neutral solution in the case of Victoria Pure Blue BO and Victoria Blue BN. All the dyes had only trace amounts of Na; Rhodamine B had no detectable amount (confirmed by ashing; 0.04% ash).

Purity of Dye Samples Used. - The results of the dye analyses are given in Table 9. The purity values of the dyes used were: Crystal Violet (C.I.^{170}42555), 88%; Ethyl Violet (C.I. 42600, 92%; Magenta P (C.I. 42510), 87%; Malachite Green (C.I. 42000), 89%; Methylene Blue BP (C.I. 52015), 85%; Rhodamine B (C.I. 45120), 95%; Rhodamine 3B (C.I. 45175), 92%; Safranine (C.I. 50240), 87%; Victoria Blue BN (C.I. 44045), 94%; Victoria Pure Blue BO (C.I. 42595), 94.5%. The elementary analyses (Table 9) suggested that the impurity present in the recrystallised samples was mainly water.

Preparation of Dye Solutions. - Distilled or demineralised water was used in all experiments. All the dyes were dissolved in the cold, except Victoria Blue BN and Victoria Pure Blue BO, which were dissolved with careful warming. In all cases the solution concentrations were corrected for purity of the samples. All dye solutions were prepared fresh immediately before use and exposure to light was minimised.

Pretreatment of Glassware for Dye Solutions. - Basic dyes are strongly adsorbed by glass. To minimise this effect all glassware to be used in contact with dye solutions was steeped before use for at least one hour (usually overnight) in a solution (1-2g.l.$^{-1}$) of a cationic surface-active agent, cetyl trimethylammonium bromide, which is preferentially adsorbed. The glass surfaces were then thoroughly rinsed with water before use.

Analysis of Dye Solutions. - The dye solutions were analysed at the long wave-length absorption peak on a Unicam SP 600 spectrophotometer before and after the adsorption tests.

Tests of Stability of Basic Dyes at Elevated Temperatures. - Some basic dyes in solution are susceptible to decomposition on standing at elevated temperatures (greater than ca. 50°C.) It was found by observing the optical densities of the present dye solutions before and after rotation in sealed glass tubes at 50°C. (highest temperature used in the adsorption tests) in a thermostatically controlled water bath, that no significant change occurred over a period of two hours. It was concluded that no significant decomposition has occurred.

Yeast. - This was a sample of brewer's yeast, a strain of Saccharomyces cerevesiae, obtained from the Distillers Company Ltd. The yeast was fixed and stored in a 4% formaldehyde solution, such that the yeast to liquid ratio was ca. 1:4 by weight. Preliminary tests showed that the adsorption characteristics of basic dyes on this substrate stored for 18 months were the same as on freshly fixed yeast.

A few preliminary control tests were performed using unfixed yeast cells (the live proportion of the cells being killed during the experiment, by the dyes) and on yeast cells fixed by boiling a concentrated aqueous suspension for 20 minutes (Table 10, Fig.18 and 19). The shapes of the isotherms obtained for the adsorption of the dyes on these substrates were essentially the same as on the formalin-fixed cells. This was taken to confirm that these are real characteristics of the yeast, /...

yeast, and are unaffected by the treatment given.

For all the experiments a small sample of yeast was removed from storage, washed three times and suspended in distilled or demineralised water.

Standardisation of Yeast Suspension. - 5 ml. of a yeast suspension were pipetted into a silica crucible previously dried to constant weight. The sample was carefully evaporated to dryness at 120°C. The dry weight of yeast and hence the concentration of the suspension was then found. The experiment was performed in triplicate. A series of dilute suspensions was prepared from the original and their optical densities measured at a suitable wavelength (5000 A., arbitarily chosen). A calibration graph (Fig. 20) of optical density against yeast suspension concentration (dry weight basis) was then drawn for use in subsequent experiments.

Adsorption Procedure. - Preliminary rate tests showed that in all cases the adsorption is extremely rapid (Fig.21). For determining adsorption isotherms, a series of standard dye solutions was prepared and 5 ml. samples of each were mixed with 5 ml. samples of a standard yeast suspension in oven-dried test-tubes. The tubes were sealed in a Bunsen flame and rotated mechanically at ca. 35 r.p.m. in a thermostatically controlled water bath for two hours. The tubes were then removed, broken, and the contents centrifuged. The supernatant solution was decanted off for analysis.

Effect of pH of the Test Solutions. - Preliminary tests using buffer solutions (see e.g. Table 10 and Fig. 19) showed clearly that with basic dyes the adsorption on yeast varies with the pH of the test mixture: the adsorption is greater at high pH values than low. In the present tests, however, no pH control was used because this is the practice in normal staining techniques, and any results obtained in the presence of buffers would not be comparable with normal tests. Moreover the presence of buffer salts would probably affect the degree of aggregation of the dyes.

Basic dyes give slightly acid solutions owing to hydrolysis. The final pH of the test mixture, corresponding to the point on the isotherm for each dye at which the maximum adsorption is attained, was measured and all values were within the range 4.42 to 5.10 (except for Rhodamine B, which gave a value of 3.63). There appears to be no correlation between the amount of each dye adsorbed and the final pH of the test mixture.

Effect of Liquid : Solid Ratio on Adsorption on Yeast. - The amount of dye adsorbed by a given weight of yeast rises with the liquid : solid ratio (see Table 10 and Fig. 22, c.f. Fig.18 and 19). This is unexpected, because the amount adsorbed should in theory depend only on the equilibrium concentration of solution and not on its volume. The same anomaly has been observed in the adsorption of anionic dyes by anodic alumina films, and in that case it is apparently due to an "etching" effect. The anodic alumina has a very slight solubility in water /...

water and when it dissolves, the surface is increased by a minute degree of roughening. In the present case it can be postulated that a similar process occurs, whereby some very low solubility constituent of the yeast cell is leached out.

Adsorption Experiments on Other Substrates. - A few adsorption tests were made on other substrates, viz. silk and wool (cleaned and scoured fabrics, wool, s.s.a., 56.5m.^{2}g.$^{-1}$ by p-nitrophenol from water; silk, not determined) graphite (Acheson Colloids Ltd., 0.28% ash, s.s.a., 125 m.^{2}g.$^{-1}$), chromatographic alumina (M. and B., s.s.a., 4.8m.^{2}g.$^{-1}$) and deoxyribonucleic acid. In the latter case, 5ml. portions of a 4% aqueous solution of the sodium salt of DNA (L. Light and Co.) were acidified with 0.1 ml. of 50% v/v hydrochloric acid. The free deoxyribonucleic acid was thus precipitated out; the final pH of the suspension was 2.5. To each 5ml. portion thus treated, a 5ml. portion of standard dye solution was added and the mixtures were rotated mechanically for 24 hr. in the thermostat bath at 20°C. The supernatant liquor was separated by centrifuging and analysed spectrophotometrically.

RESULTS AND DISCUSSION.

Adsorption Isotherms for Yeast.

The results on yeast are shown in Tables 10 and 11 and in Fig. 18, 19, 21, 22 and 23. The adsorption is extremely rapid and clearly depends on (a) the concentration of the dye solution (particularly at low concentrations), (b) the concentration of the yeast suspension (c.f. ref.153) and (c) the temperature.

When /...

When examined under the microscope (magnification 1000x) the cells appeared to be stained throughout. Nevertheless, with dilute solutions of dye, or with those dyes which give low adsorption, the cell walls and nuclei could be distinguished from the less-strongly stained cytoplasmic regions. It was not possible to estimate quantitatively the relative intensity of staining of each part, but it would appear that the cell walls and nuclei have the greater affinity for the dye.

Adsorption isotherms at elevated and at room temperatures have been determined for several of the dyes used, over a range of yeast suspension concentrations (Table 10, Fig.18 and 19). For each dye the isotherm shape is essentially the same under all the conditions used. For comparison purposes, isotherms for all dyes have been determined at a fixed suspension concentration (0.246 ± 0.006 g. dry wt. $l.^{-1}$) of yeast in the test mixture (Fig.23).

Crystal Violet, Magenta P, Malachite Green, Methylene Blue BP, Rhodamine 3B and Safranine give isotherms of type L2 (as classified in ref. 167), which is characteristic of the adsorption of monodisperse molecules, oriented flatwise on the surface, or of ionic micelles. Victoria Pure Blue BO and Victoria Blue BN give isotherms of type H2. This type of isotherm is often obtained in adsorption of large ionic micelles.

All these observations, including those of the rapidity of adsorption, suggest that the adsorption mechanism on yeast is largely micellar ion-exchange. Most of the isotherms (Fig.23) have long flat portions ("plateaux"), /...

("plateaux"), which indicate that saturation of the surface has occurred, and which are also observed in other cases of ionic micellar adsorption.[7,167] Some isotherms (e.g. Fig.23C) have a slight maximum (sub-group mx), indicative of marked aggregation of the dye in solution.

Adsorption of the Rhodamines on Yeast. - Rhodamine B on yeast gives isotherms of type S2 (Fig. 18B and Fig.23H), characteristic of the adsorption of monodisperse molecules or ions, with an end-on or edge-on orientation.[167] Unlike the molecules of the other dyes used, the Rhodamine B molecule (Fig.17) contains a carboxyl group, and will therefore be to some extent zwitterionic. Electrophoresis on paper at pH 7.1 (phosphate buffer), however, has verified its cationic nature. The carboxyl group will be repelled by the basophilic substrate, thereby reducing the affinity of the dye for the surface and causing the cation to approach with an end-on orientation.

The isotherm has a well-defined plateau suggesting that a complete monolayer of ions is formed and that there is no build-up of a second layer. This is surprising, for the adsorbed layer would be expected to present a surface of free carboxyl groups, on which a second layer of cations could form. It must be assumed that these groups are involved in the formation of hydrogen bonds (R_3N .... HOOC-R) with neighbouring adsorbed dye cations, all stacked edgewise to the surface (Fig. 24).

This hypothesis was tested and confirmed by repeating the experiment with the closely related dye Rhodamine 3B which is fully esterified (Fig.17) and has no hydrogen free for inter-ionic bonding. It was postulated /...

postulated that, since the above conditions would not occur with this dye, the dye cations would preferentially be adsorbed flat on the surface, and an L-type isotherm would result.[167] In fact Rhodamine 3B does give this isotherm (Fig.23G). (The author's colleague, Mr. I.A. Easton has observed that Rhodamine B also gives an S isotherm from water on chromatographic alumina, whereas Rhodamine 3B gives an L isotherm).

Specific Surface Area Measurement. - The behaviour of Rhodamine B has enabled the specific surface area (internal plus external) of the yeast cells to be estimated by simple calculation from the maximum amount of this dye adsorbed and the dimensions of the dye ion, measured by Stuart-type models (edge-on cross-sectional areas, 124Å^2), (cf. ref.167, 171). The value for the total surface obtained thus is 65.7m.^{2}g.$^{-1}$ (Table 11). This may be compared with the value of 1.5m.^{2}g.$^{-1}$ for the external surface area calculated assuming that the yeast cells are smooth spheres of average diameter 4/u. with a density near that of water. (the author's colleage, Mr. I.A. Easton has observed that the s.s.a. of chromatographic alumina by Rhodamine B adsorption is 1.3 x that by p-nitrophenol).

The Aggregation of Basic Dyes on Adsorption. - Values of the total specific surface area have been calculated from the data for the adsorption of the other basic dyes (Table 11, Fig.23). Since each ion has one positive charge distributed by resonance over several peripheral sites, it is assumed, for purposes of calculation only, that the ions are oriented flat on adsorption. In all cases except Malachite Green the /...

the area values are substantially greater than the value calculated from Rhodamine B adsorption, and undoubtedly this is the result of adsorption of cationic dye micelles(note ref. 170 a).

The ratio of the calculated specific surface area to that obtained from Rhodamine B adsorption gives a coverage factor[7], which may be regarded as a measure of the degree of aggregation. It is significant that with all the dyes used giving L or H isotherms, except Malachite Green and Rhodamine 3B, this coverage factor increases with the size and weight of the dye cation (Fig.25). The degree of aggregation will depend on the short-range attraction forces between the dye molecules. These are reasonably flat and have large projected areas such that the close approach of several points in the molecules may occur simultaneously, hence they may be expected to aggregate readily.

It is interesting to note that those dyes which have the highest coverage factor give H2 type isotherms (i.e. they start at a positive value on the vertical axis). This form of isotherm is in fact often characteristic of the adsorption of large ionic micelles.[167]

Malachite Green and Rhodamine 3B are exceptions to the above generalisation. They are also the only two (apart from Rhodamine B, discussed above) which have unsymmetrical cations, and this fact must in some way at present unknown, reduce their tendency to be adsorbed as large cationic micelles. Malachite Green appears to be adsorbed in the monodisperse form, since it gives a coverage factor of about 1 (Table 11).

Effect of Temperature. - There is much evidence to suggest that cationic dyes form aggregates in aqueous solution. Although it is widely believed that aggregation is brought about by van der Waals attraction, there is some evidence to suggest that hydrogen bonding may be responsible.[171a] Isotherms for several of the dyes used have been determined at two temperatures (e.g., Fig.23, cf. Fig.22).= In each case the adsorption is anomalous in being greater at high than at low temperature. This type of anomalous adsorption has been observed with some inorganic substrates and appears to be due to aggregation of the dyes in solution.[54]

It is well known that high temperatures favour the disaggregation of dye molecules in solution[51] and it was suggested[54] that the dye molecules are reaggregated on adsorption. This seems reasonable since it is probable that the adsorbed micelles have different structures from the micelles in solution. In the present case, the smaller size of the monodisperse dye cations will enable them to pass more rapidly than micelles through the porous structure of the cells. This would account for the rapid and complete staining, but the high coverage factors show that the species adsorbed must be an ionic micelle. It is therefore suggested that aggregation of the dye cations to form ionic micelles must occur at the moment of close approach to the adsorption sites. This hypothesis agrees with previous observations[167,54]. No doubt the aggregation is favoured by the loss of solvated water from the dye cations and their increased concentration at the surface.

Relevance of the Present Results to the Gram-Stain Mechanism. - It is generally accepted that in the Gram-stain technique a dye-iodine complex is formed within the cells. Differentiation between Gram-positive and Gram-negative cells then occurs largely because the outward passage of this complex, on elution with alcohol, is retarded more in Gram-positive cells than in Gram-negative cells[125,126,157]. It has been suggested as a result of a series of qualitative tests using unpurified dyes that the basic triarylmethane dyes, particularly Crystal Violet, are best for differentiation, whereas Rhodamine B gives no differentiation owing to its feeble staining characteristics.[100] Even without the use of iodine or any similar agent, however, differentiation is possible though difficult.

The author now tentatively proposes that the intensity of staining of a basic dye is greater, the greater its degree of aggregation at the surface. In the present work, in which purified dyes were used, the degree of aggregation appears to depend on the size of the dye molecule, provided that the peripheral positive sites are symmetrically arranged. The feeble staining by Rhodamine B can be explained by its anomalous adsorption behaviour.

Rhodamine B Isotherms on other Substrates and their Relation to Mechanisms of Adsorption.

The unusual behaviour of the Rhodamine dyes could, it was hoped, be used to throw light on the nature of the adsorption sites in the yeast cell. For this purpose isotherms have been determined at room temperature /...

temperature for the adsorption of Rhodamine B on various other substrates (Fig.26). On wool and silk, isotherms of type L2 have been observed (Fig.26, A,B,C) by the author's colleague, Mr. A.H. Tolia, and on graphite (Fig.26D) the author has observed one of type H2. The adsorption on wool (Fig.26A) and graphite corresponds to a flatwise monolayer of dye molecules (area of Rhodamine B molecule flat, ca.230$\mathring{A}^2$). On chromatographic alumina, however, an S-type isotherm (Fig.26E) has been observed by the author's colleage, Mr. I.A. Easton. This suggests a vertical orientation of the dye molecules (area, 124$\mathring{A}^2$), and the adsorption corresponds to a monolayer so oriented.

The author concludes that on substrates with a high proportion of hydrocarbon residues, e.g., protein fibres, or with condensed aromatic systems, e.g., graphite, adsorption is largely due to van der Waals attraction between non-polar surfaces and the aromatic ring system of the dye ions, which favours flatwise orientation (Fig.27). On alumina, which has an ionic surface and no hydrocarbon residues or aromatic system, ion-ion attraction is predominant and the basic centres of the dye molecule are preferentially attracted. Hence the vertical orientation occurs (cf. Fig.24)

According to the foregoing argument the adsorption sites in yeast cannot be protein, but must be some other highly ionised material, possibly substances containing phosphatidic groups, e.g., nucleic acids or teichoic acids. To check this hypothesis, Rhodamine B was adsorbed on DNA; the isotherm is of S-type (Fig.26F) as on yeast, and quite unlike /...

unlike that obtained with the protein fibres. (Isotherms for wool were determined using both unacidified dye solutions, for comparison with the yeast experiment, and solutions acidified to pH 2.5, for comparison with the DNA experiments. There is no difference in isotherm type, though as expected, adsorption is less from acid solution, because of the increased positive charge on the fibres).

The Relationship between the Coverage Factor and the Size of the Dye Cations.

In the above account it was evident that there is a relationship between simple functions of the coverage factors of cationic dyes adsorbed on yeast and the size of these dye cations in which the positive peripheral sites are symmetrically placed. The exact relationship, however, was uncertain. The author's colleague, Mr. I.A. Easton, who has investigated the adsorption of a similar series of cationic dyes on chromatographic alumina (s.s.a. by p-nitrophenol adsorption from water, 4.8 $m.^2 g.^{-1}$) has observed a linear relationship between the logarithm of the coverage factor and the logarithm of the cationic weight. In fact, the same relationship applies to the adsorption on yeast and also on graphite[36] (s.s.a. determined from data obtained by measuring the average superficial area of the particles by the optical microscope and their average thickness by the electron microscope), and indeed all three plots can be superimposed (Fig.28). This suggests that the aggregation of cationic dyes on adsorption is largely a property of the dye /...

dye cations alone and that the influence of the substrate is small.

A similar trend can be observed in data reported for the adsorption of cationic dyes on several types of silica[7]. The numerical values of the coverage factors on these substrates, however, are much higher than expected, for the specific surface area in each case has been measured by the less satisfactory air-permeability method, which gives much lower area values than p-nitrophenol adsorption.[171]

CONCLUSION.

The mechanism of adsorption of cationic dyes on dead yeast cells, which are chemically heterogeneous, resembles that on the simple inorganic substrates, chromatographic alumina, silica and graphite. This conclusion is drawn from the following facts: (a) most of the dyes are adsorbed in a micellar form; (b) the number of cations in each adsorbed micelle appears to increase with increase in the size of the dye cations, with few exceptions, on all four substrates. Indeed there is some evidence to suggest that the micelle formation is independent of the nature of the substrate.

The dyes appear to interact with nucleic acids or related constituents of the yeast cells, but not with proteinoids.

SECTION 2.

Adsorption of Anionic Solutes : Adsorption of Anionic Dyes on the Nuclei of the Spermatogenetic Cells of the Mouse.

INTRODUCTION.

This section is concerned with the adsorption of anionic (acid) dyes on sections of animal tissue, which may be regarded as heterogeneous substrates. The techniques used are qualitative and, since the solutes are coloured, adsorption can be estimated visually with the aid of the optical microscope (it is usually sufficient merely to observe whether an object is strongly stained or feebly stained). Much information about the adsorption can be obtained by subjecting the substrate to various chemical treatments and observing the changes produced in the adsorption characteristics. Further information can be obtained by allowing more than one solute to compete for the adsorption sites; control experiments, however, are essential.

The Testis of the Mouse.[55]

Structure. - The organ used in the present investigation is the testis of the mouse, a cross-section of which (as seen under the low power microscope) is represented diagrammatically in Fig.29A. It consists essentially of a large number of convoluted capillaries (the convoluted seminiferous tubules) enclosed in a thick envelope called the tunica albuginea. This envelope is composed of a material known to the biologist as "connective tissue", which consists mainly of the protein, collagen. The wall of each tubule, the tunica propria, is thinner than the tunica albuginea, but is also composed of connective tissue. The spermatogenetic cells, which are packed around the inner surface of the tunica propria (see Fig.29B for a cross-section of a tubule as seen under /...

under the high power microscope), are produced in the process of spermatogenesis, the final product of which, spermatozoa, accumulates at the centre of the tubule.

The initial stage of spermatogenesis is the production of spherical cells called spermatogonia just inside the tunica propria; they are difficult to distinguish from the other cells under the microscope.

The spermatogonia undergo binary fission to produce large spherical cells known as the primary spermatocytes and each of these in turn divides to give two secondary spermatocytes. The secondary spermatocytes, however, divide almost at once to give young spermatids and therefore can rarely been seen; they are not shown in Fig.29. The "young" spermatids thus produced are spherical cells which do not divide, but undergo profound changes, gradually elongate and develop into the final product, spermatozoa. Spermatids at the stage when their nuclei have lost their spherical shape and are just beginning to assume their final form will be referred to as "adolescent spermatids"; the term "late spermatids" will be applied to those about to become spermatozoa. Each spermatozoön resembles a tadpole for it has an oval shaped "head" (largely chromatin) and a long "tail" (mainly protein); these characteristics can easily be seen even under the low power microscope.

Staining Properties. - A recent investigation of the staining of sections of the testis of mouse, fixed in Clarke's (Carnoy's) fluid (3:1 v/v mixture of absolute ethanol and glacial acetic acid), by anionic and cationic dyes,[172] has enabled certain parts of the organ to be /...

be characterised as typically acidophil (i.e., they appear to have a positive zeta potential in water in that anionic dyes, but not cationic dyes, are strongly adsorbed). These parts are for example, the cytoplasm of the interstitial cells (see Fig.29B) the tunicae propriae of the seminiferous tubules (collagen) and to a lesser extent the tails of the spermatozoa. Other parts are typically basiphil (i.e., they appear to have a negative zeta potential in water in that cationic, but not anionic dyes, are strongly adsorbed). These parts are the chromatin of the spermatogenetic cells, above all the primary spermatocytes, and of the adolescent spermatids. (The nuclei of the late spermatids and of ripe spermatozoa are acidophil; this may be attributed to the high proportion of the diamino acid arginine present in the protamine-rich proteinoid constituent of the chromatin of these nuclei).

The anionic dyes, Methyl Blue and Aniline Blue, however, behave in an unexpected manner; for although they stain the characteristically acidophil parts, they have been found to stain strongly the characteristically basiphil chromatin of the spermatogenetic cells. The purpose of the present investigation is to examine this anomalous behaviour.

EXPERIMENTAL.

Electrophoresis Experiments.

The horizontal paper electrophoresis apparatus of the Shandon Scientific Co. was used. A phosphate buffer solution was used to give pH 7.1, and phosphate/citric acid buffer solutions to give pH 3.0 and 2.2. The dyes were dissolved at 0.005 M.

Preparation and Pretreatment of Sections of Testis of Mouse.

Pieces of the testis of the mouse were fixed in Clarke's fluid and embedded in paraffin wax. 7μ sections of this material were used as substrate in most of the staining experiments. The wax was removed by immersion in xylene and the sections were brought to water before staining or subjection to one or more of the following pretreatments.

Extraction of DNA (and Some of the RNA). - DNA and some of the RNA were extracted from the sections by incubation at 50°C. for 30 hr. in a 5% (aq.) solution of trichloroacetic acid. The sections were then washed in running water.

Complete Extraction of Nucleic Acids. - The sections were first subjected to the previous treatment. The remaining RNA was extracted by incubation at 60°C. for 3 hr. in a preparation of ribonuclease.[173] The sections were then washed in running water.

Destruction of Acidophilia. - Acidophilia was destroyed by deamination in van Slyke's reagent for 7 hr. at room temperature.[174].

Deactivation of Basiphilia. - Basiphilia was deactivated by the incubation of the sections for 3 hr. at 37°C. in a 0.5% (aq.) solution of cetyl trimethylammonium bromide, a strongly cationic surface-active agent; sections were then quickly rinsed in distilled water. This substance is strongly adsorbed by basiphil objects and will reduce the uptake of basic dyes thereon by competition. The effectiveness of this treatment was demonstrated by staining treated and untreated sections with a 0.5% (aq.) solution of basic fuchsine for ½ min. and then dehydrating in isobutanol, as /...

as detailed below. Comparison showed that the intensity of staining on the treated sections was clearly less than on the untreated ones. The elevated temperature is necessary to reduce aggregation of the reagent and increase the rate of penetration of the monodisperse form.

Control Experiments.

All the experiments performed on sections subjected to hot water treatments were carefully controlled by corresponding experiments performed on sections which had been subjected to the same temperatures for the same times in distilled water.

Preparation and Pretreatment of Other Substrates.

To preserve glycogen, pieces of the liver of a mouse that had been fed on carrot were fixed in Rossman's fluid and embedded in paraffin; 7 μ sections were used. Sections of the pancreas of the mouse, treated in the same way, were also used in certain experiments.

Sections (15 μ) of nitrocellulose (collodion), not containing any tissue, were also used as substrates. They were stored in 70% alcohol and rinsed in distilled water before use. Cellulose (filter paper) was used without previous treatment.

Dyes.[97]

The dyes used were commercial samples of biological staining grades. Those used in the principal experiment were the typical acid dyes Eosin WS (yellow shade) and Xylidine Ponceau (both from B.D.H.), the acid milling dyes Methyl Blue and Aniline Blue WS (both from G. Gurr), and also two dyes with some of the characteristics of acid milling dyes, namely /...

namely Indigo-Carmine (of unspecified purity) and Nigrosine WS (G. Gurr). A few experiments were also performed with the typical acid dyes, Acid Fuchsine (B.D.H.), Fast Green FCF (G. Gurr), and Orange G (B.D.H.). The direct cotton dyes Chlorazol Black E (G. Gurr) and Congo Red (B.D.H.), and Vital New Red (B.D.H.), which dyes cellulose strongly, were also used, as well as Azo-Carmine B (G. Gurr).

Staining Solutions.

In all cases the dyes were dissolved in distilled water. The principal experiments were performed with equimolar binary mixtures in which each dye was at a concentration of 0.005 M. Parallel series of experiments were performed with solutions of each dye alone at a concentration of 0.005 M. Preliminary staining experiments showed that Orange G stained extremely feebly, and a higher concentration was necessary. In all other tests the dye solutions used were at a concentration of 0.005 M., unless otherwise stated.

Staining Procedure.

The same staining procedure was used in all the experiments, unless otherwise stated. (1) Stain sections for 5 min.; (2) blot quickly to remove excess dye solution; (3) dehydrate by immersion in 3 consecutive baths of isbutanol, 5 sec. in the first (with agitation), 1 min. in each of the second and third; (4) transfer to xylene and mount in Canada balsam for examination.

RESULTS AND DISCUSSION.

Electrophoresis Experiments.

The following dyes were found to be anionic at pH 7.1: Methyl Blue, Aniline /...

Aniline Blue, Indigo-Carmine, Nigrosine, Eosin, Orange G, Xylidine Ponceau, Azo-Carmine B. The following were tested also at pH 3 and 2.2 and found to be anionic: Methyl Blue, Aniline Blue, Indigo-Carmine, Nigrosine, Orange G.

At pH 7.1, 3.0, and 2.2, Methyl Blue and Aniline Blue differed from the other dyes in that they obviously reacted with the paper and formed a continuous band from the starting line to the front. The other dyes formed narrower bands which moved across the paper with little widening. Further, Methyl Blue and Aniline Blue after an initial rapid movement towards the anode soon became stationary, while the other dyes continued moving with only a slight decrease in rate of travel with time.

Methyl Blue and Aniline Blue are known to react more strongly with cellulose under acidic conditions than at neutrality (see later).

Staining Experiments: Introductory Statement.

It may be helpful to the reader to state in advance that the acid milling dyes, Methyl Blue and Aniline Blue, showed identical staining properties; Indigo-Carmine behaved as a typical acid dye; and Nigrosine behaved in a manner intermediate between the acid milling and typical acid dyes.

Staining of Sections of Testis with an Equimolar Mixture of Methyl Blue (or Aniline Blue) and Eosin.

Untreated Sections. - When Methyl Blue (or Aniline Blue) and Eosin are competing for the substrate in an equimolar mixture, the characteristically acidophil parts are strongly stained, the contribution of each dye varying slightly from part to part. The outer part of the tunica albuginea /...

albuginea is stained more by the Methyl Blue, while the inner is clearly stained by the Eosin alone. (It will be remembered that the parts of a tissue that are immediately exposed to the action of Clarke's fluid often stain somewhat differently from the internal parts.) The rims of the red blood-corpuscles and the residual cytoplasm cast off from the spermatozoa are stained by both dyes, though Eosin makes the greater contribution to the blood-corpuscles. The characteristically basiphil chromatin of the spermatogenetic cells, particularly of the primary spermatocytes, is strongly stained, largely by the Methyl Blue (or Aniline Blue), though the Eosin obviously makes some contribution. The acidophil chromatin of the ripe spermatozoa and late spermatids is brilliantly stained by the Eosin alone, Methyl Blue (or Aniline Blue) making little or no contribution; von Ebner's granules are brilliantly stained in the same way.

Methyl Blue (or Aniline Blue) when used alone stains strongly the characteristically acidophil parts, particularly the tunicae propriae of the seminiferous tubules. The basiphil chromatin of the spermatogenetic cells, particularly the primary spermatocytes, is strongly stained, but von Ebner's granules and the chromatin of the late spermatids and spermatozoa are only feebly stained. The connective tissue is very feebly stained blue and the rims of the red blood-corpuscles are lightly stained. Otherwise the staining characteristics are the same as in the mixture.

When Eosin is used alone, the characteristically acidophil parts, the basiphil chromatin of the spermatogenetic cells (particularly the primary spermatocytes), and the chromatin of the late spermatids and spermatozoa /...

spermatozoa are quite strongly stained. Von Ebner's granules are clearly visible and the tunica albuginea is stained moderately throughout. Otherwise the staining characteristics are the same as in the mixture.

Those parts which are stained preferentially by Eosin would appear to have a low permeability, so that the large Methyl Blue (or Aniline Blue) molecules are excluded.

Sections from Which the DNA and Some RNA has been Extracted. - By comparison with the control experiment it is clear that there is only one significant change in the overall staining pattern, though the intensity is slightly reduced, and the strongly stained tunicae propriae of the seminiferous tubules are much more distinct. The nuclei of the spermatogenetic cells, particularly the primary spermatocytes, are stained preferentially by Eosin and only to a very small extent by Methyl Blue (or Aniline Blue). Thus it is clear that the removal of the DNA constituent of the chromatin of these cells, along with some of the RNA, greatly reduces the intensity of staining by Methyl Blue (or Aniline Blue). This observation is confirmed by a corresponding result when Methyl Blue (or Aniline Blue) is used alone. When Eosin is used alone, comparison with the control experiment shows that the intensity of staining of the nuclei of the primary spermatocytes is slightly increased by this treatment. Von Ebner's granules and the nuclei of the late spermatids and spermatozoa are still brilliantly stained by the Eosin, while Methyl Blue (or Aniline Blue) make little or no contribution.

Sections from Which all the DNA and RNA has been Extracted. - By comparison /...

comparison with the control experiment it can be seen clearly that the overall intensity of staining is reduced, even slightly more than in the previous case, and that the strongly stained tunicae propriae of the seminiferous tubules are even more distinct. The nuclei of the spermatogenetic cells are scarcely stained at all by the Methyl Blue (or Aniline Blue), but are strongly stained by the Eosin. With Methyl Blue (or Aniline Blue) alone they are only lightly stained, but when Eosin is used alone, comparison with the control experiment shows a definite increase in the intensity of staining. Von Ebner's granules and the chromatin of the late spermatids and spermatozoa are still brilliantly stained by Eosin. It seems clear, then, that both DNA and RNA are responsible for the staining of the chromatin of the spermatogenetic cells by Methyl Blue and Aniline Blue and that the nucleic acids hinder the staining by Eosin.

Sections Subjected to van Slyke's Deamination. - By comparison with the stained untreated sections it is clear that destruction of acidophilia by treatment with van Slyke's reagent has the effect of reducing the overall intensity of staining until it is feeble. The chromatin of the primary spermatocytes, however, is still quite strongly stained, almost entirely by Methyl Blue (or Aniline Blue), Eosin making only a small contribution. The tunicae propriae of the seminiferous tubules and the outer rim of the tunica albuginea can still be distinguished and appear to be stained largely by Methyl Blue (or Aniline Blue). The most strikingly demonstrated parts, however, are the nuclei /...

nuclei of the late spermatids and spermatozoa, which are still strongly stained by Eosin. This must be due largely to the fact that the guanidine amino-group in the diamino-acid, arginine, which is present in large amounts in the protamine-rich protein constituent of the chromatin of these cells, is not destroyed, for with few exceptions van Slyke's deamination is specific for α-amino groups.[175]

Staining with Methyl Blue (or Aniline Blue) alone and with Eosin alone confirms these results.

Sections Pretreated with Cetyl Trimethylammonium Bromide. - The overall intensity of staining is extremely strong and the general result is unsatisfactory for most cytological purposes. Nevertheless, the nuclei of the spermatogenetic cells, particularly the primary spermatocytes, appear as feebly stained discs against a very strongly stained background. This unusual appearance can be seen when Methyl Blue (or Aniline Blue) is used alone and suggests that the cetyl trimethylammonium bromide, which can be considered as a colourless basic dye, reacts with the same constituent of the chromatin as the blue dye. A similar effect, however, can be seen when the staining is by Eosin alone. This observation suggests that the technique may to some extent reduce the permeability of the chromatin and that the results obtained are inconclusive.

The observations described above provide strong evidence that Methyl Blue and Aniline Blue react with the nucleic acid constituents of the chromatin of the spermatogenetic cells, particularly the primary spermatocytes, /...

spermatocytes, and that Eosin reacts with the protein constituent of the chromatin of these cells, as Azo-Carmine B is known to do.[98]

Staining of Sections of Testis with an Equimolar Mixture of Nigrosine and Xylidine Ponceau.

The staining pattern obtained on untreated sections is similar to that of the mixture of Methyl Blue (or Aniline Blue) and Eosin, although the overall intensity of staining is considerably less. The characteristically acidophil parts are quite strongly stained by both dyes to varying extents while the characteristically basiphil chromatin of the spermatogenetic cells, particularly the primary spermatocytes, is quite strongly stained, largely by Nigrosine, and the chromatin of the late spermatids and spermatozoa is quite strongly stained by Xylidine Ponceau.

Extraction of the nucleic acid constituents reduces the intensity of staining of the nuclei of the spermatogenetic cells in a manner similar to that already described. In this case, however, the control experiments show that the reduction is due (in part at least) merely to hot water treatment, and this suggests that the effect of the nucleic acids in the chromatin is much less significant. Further, when Nigrosine is used alone, the results obtained do not appear to correspond to its behaviour in the mixture with Xylidine Ponceau. The reason for this is not clear. It can only be concluded that Nigrosine may share to some extent with Methyl Blue and Aniline Blue the ability to react with the chromatin of the spermatogenetic cells.

The nuclei of the late spermatids and spermatozoa are still strongly /...

strongly stained by the Xylidine Ponceau even after deamination. Xylidine Ponceau thus resembles Eosin. This is confirmed when Xylidine Ponceau is used alone. In general, however, Xylidine Ponceau stains rather feebly compared with Eosin and appears to be less able to stain the chromatin of the spermatogenetic cells.

Staining of Sections of Testis with an Equimolar Mixture of Indigo-Carmine and Xylidine Ponceau.

When used alone, Indigo-Carmine behaves as a typical acid dye and shows no particular ability to stain the chromatin of the spermatogenetic cells in untreated sections. The overall intensity of staining is quite strong. Nevertheless, when competing in an equimolar mixture with the rather feebly staining Xylidine Ponceau, its action is to a large extent suppressed and the overall intensity of staining is rather feeble, with an obvious preference of the tissue-constituents for the Xylidine Ponceau.

In fact, with this mixture, no area appears to be stained by one dye in preference to the other. This applies even to the chromatin of the late spermatids and spermatozoa, which is quite strongly stained and remains so after removal of the nucleic acids or after destruction of the acidophilia by van Slyke's reagent. The removal of the nucleic acids, however, allows Indigo-Carmine, when used alone, to stain the nuclei of the spermatogenetic cells to some extent.

Staining Experiments on Sections of Pancreas.

In the previous experiments on sections of testis it would appear that /...

that Methyl Blue and Aniline Blue can react with both the DNA and the RNA constituents of the chromatin of the spermatogenetic cells. Now RNA is supposed to be stained preferentially by basic dyes of low cationic weight,[176] and therefore it is doubly surprising to find that it is stained by the anionic dye, Methyl Blue, which has an anionic weight of 745, and by the closely related Aniline Blue. The cytoplasm of the exocrine cells of the pancreas is rich in RNA and thus it was considered to be of interest to examine these cells after staining them with an equimolar mixture of Methyl Blue and Eosin.

It was observed that the chromatin of the cells was stained only by the Methyl Blue. The parts of the cells that are rich in RNA were stained only by the Eosin. Methyl Blue when used alone does not stain the RNA-rich parts in some cells and stains them only slightly in others. Eosin when used alone behaves in the same way as it does in the mixture, staining strongly the parts of the cells that are rich in RNA, but staining the chromatin only very feebly.

The significance of these results is not clear. It may be that RNA exists in a less compact form in chromatin than in the ribosomes of the cytoplasm.

Mechanism of the Staining of Nucleic Acids by Methyl Blue and Aniline Blue.

The Apparently Basic Character of Methyl Blue and Aniline Blue. - Methyl Blue, Aniline Blue, and perhaps Nigrosine appear to act as basic dyes in their interaction with nucleic acids. In addition, when sections that /...

that have been stained with an equimolar mixture of Methyl Blue and Eosin are rinsed for 3½ hr. in running water at about pH7, the intensity of staining by Methyl Blue, particularly of the chromatin of the spermatogenetic cells, is diminished only to a small extent, while only the edges of the sections and the rims of the red blood-corpuscles retain traces of Eosin. When a similarly stained section is rinsed for 5 min. in 70% alcohol, Methyl Blue is almost completely removed, while Eosin remains to some extent, particularly in the chromatin of the late spermatids and spermatozoa, and in connective tissue and the rims of the red blood-corpuscles. A high resistance to extraction in water at neutrality and ease of extraction in aqueous ethanol or in acid solution are properties typical of basic dyes, while the opposite effect, as displayed by Eosin, is typical of many acid dyes.[98] The high resistance of Methyl Blue to extraction in water and its ease of extraction in 70% alcohol suggests that it may be reacting as a basic dye.

The most likely source of the basic character in Methyl Blue and Aniline Blue under acid conditions is the positively charged nitrogen atom in their molecules (Fig.30). Yet the electrophoretic experiments (see earlier) show that these dyes are anionic at pH 2.2 and pH 3.0. In addition, Acid Fuchsine and Fast Green FCF, which also have a positively charged nitrogen atom in their molecules (Fig.30), and, like Methyl Blue and Aniline Blue, are triarylmethane dyes, behave as typical acid dyes and show no particular ability to stain the chromatin of the spermatogenetic cells in sections of testis.

These facts, taken together, suggest strongly that Methyl Blue, Aniline /...

Aniline Blue, and perhaps Nigrosine do not behave as basic dyes towards nucleic acids and that the mechanism of their retention is not ionic in character.

The Staining of Other Acidic Substrates by Methyl Blue and Aniline Blue. - It is well known that Methyl Blue and Aniline Blue, unlike typical acid dyes, can be used as direct dyes for cellulose (cotton). This is a very slightly acidic substance, yet direct cotton dyes are acid dyes, differing from typical acid dyes in that they have large, flat molecules with a high degree of conjugation (i.e. a large number of loosely bound electrons; ~~see appendix~~), e.g., Congo Red (Fig.30). In fact, it has been shown that the thermodynamic affinity of dyes for cellulose increases linearly with the logarithm of the number of bonds in the longest conjugated chain.[33,177] The mechanism of interaction is not yet fully understood, but is thought to be van der Waals attraction, although there is some evidence to suggest that hydrogen bonds are formed between the hydroxyl groups in the cellulose and the π electrons in the dye molecules.[178]

Methyl Blue, Aniline Blue, and the direct cotton dyes, Chlorazol Black E, Congo Red, and Vital New Red stain nitrocellulose (collodion) strongly. This substance is much more acidic in character than cellulose and has staining properties which closely resemble those of the nucleic acids.[98] It can therefore be used as a model for the nucleic acids in comparing their staining properties with those of cellulose.

Experiments /...

Experiments were carried out on sections of nitrocellulose with a mixture of Methyl Blue and Xylidine Ponceau. Xylidine Ponceau was preferred to Eosin, because the latter is insoluble in acid buffer solutions. When the sections were dyed in the mixture for 5 min. the nitrocellulose was stained only by the blue dye, and the stain was fast to washing for 3½ hr. in running tap-water at about pH7. When used alone, Xylidine Ponceau produces only a feeble coloration. At pH 3.0 the intensity of staining by Methyl Blue is less than at pH 7.1. When the experiment was repeated with cellulose (filter paper), Methyl Blue again stained preferentially, but the intensity was greater at pH 3.0 than at pH 7.1

In a further attempt to correlate the staining properties of cellulose and nucleic acids, sections of testis were stained with solutions of the direct cotton dyes, Chlorazol Black E (about 0.005 M), Congo Red (0.005 M), and Vital New Red (about 0.001 M) for periods of 5 min. and 15 min. Vital New Red stained strongly the chromatin of the spermatogenetic cells, especially after 15 min., while Chlorazol Black E and Congo Red behaved as typical acid dyes and clearly had no ability to stain chromatin.

These results show that the staining properties of cellulose, nitrocellulose, and nucleic acids are not exactly analogous and that a high degree of conjugation is not the only requirement for interaction with nucleic acids. Yet is is surely involved, for it is the only significant difference between Methyl Blue and Acid Fuchsine or Fast Green FCF. The chief /...

chief difference between Methyl Blue, Aniline Blue, or even Nigrosine and the direct cotton dyes is largely one of molecular shape. The dyes in question have reasonably compact molecules with several alternative long conjugated chains, while the direct cotton dyes are azo dyes with long, thin molecules and only one possible long conjugated chain. Long, thin molecules are ideal for interacting with cellulose chains, in that they can orient themselves parallel to the chains. It seems unlikely, however, that such molecules would be suited for interaction with nucleic acid helices.

Experiments on Sections of Liver. - The cytoplasm of liver cells is rich in glycogen, which in constitution resembles cellulose, and a few experiments were performed to discover whether the staining properties of these two substrates are similar.

When an equimolar mixture of Methyl Blue and Eosin is used, the chromatin is stained distinctly by the Methyl Blue, but the glycogen-rich cytoplasm is lightly stained by the Eosin. When Methyl Blue is used alone, the chromatin is quite strongly stained, but the cytoplasm is only feebly stained. When Eosin is used alone, the chromatin and the cytoplasm, particularly the latter, are lightly stained.

Further tests showed that the direct cotton dyes, Chlorazol Black E (about 0.005 M), Congo Red (0.005 M), and Vital New Red (about 0.001 M) stain the cytoplasm only very feebly, if at all.

It would appear, then, that glycogen and cellulose, although closely related, have completely different staining properties, for those dyes which /...

which stain cellulose strongly appear to have little or no affinity for glycogen. Yet it may be that this apparent difference is due to possible low permeability of glycogen in the form in which it exists in liver cells. It is not surprising, then, that the staining properties of cellulose, nitrocellulose, and nucleic acids do not appear to be exactly analogous.

Aggregation of Milling Dyes. - Milling dyes are known to have a higher degree of aggregation in solution than other acid dyes;[51,179,180] This must be a result of higher intermolecular attraction. A high intermolecular attraction might favour the interaction of Methyl Blue and Aniline Blue with nucleic acids, for molecules adsorbed on each side of a nucleic acid chain could attract each other through the chain.

CONCLUSION.

Methyl Blue and Aniline Blue, although anionic dyes, interact with the negatively charged nucleic acids. The interaction is non-ionic and may be a donation of π electrons from the long conjugated chains of the dye molecules to electron-deficient sites in the nucleic acid helices. Thus it would appear that anionic solutes may be retained by forces other than ionic, and in this respect the action may resemble the adsorption of anionic dyes on wool or cellulose.

TABLES

TABLE 1. Comparison of results of complex-detection tests in carbon tetrachloride with those in bonding solvents.

Type of bond	Solutes a	Solutes b	Solv.*	Total mol. concn.	Temp. (°C.)	Mol. Ratio of Complex.	Fig No
Alk-OH + Ar-OH	Butan-1-ol	Phenol	CCl_4	0.1	15.85	1:1	4A
	Several alcohols		T,D,W			1:1	
-CHO + Alk-OH	Acetaldehyde	Ethanol	CCl_4	0.1	20	1:1	
	Benzaldehyde		CCl_4	0.1	20	1:1	
	D-Glucose		W[72]	0.25	20	0	
	Propionaldehyde		CCl_4[72]	0.2	17.5	1:1	
			W[72]	0.2	17.5	0	
-CHO + Ar-OH	n-Butyraldehyde	Phenol	CCl_4	0.1	13.08	0	4B
					17.00	0	4B
	D-Glucose		W[72]	0.25	20	0	
	Propionaldehyde		CCl_4[72]	0.1	17	1:1(?)	
			EG	0.2	18	1:1	

TABLE 1. contd.

Type of bond	Solutes a	Solutes b	Solv.*	Total mol. concn.	Temp. (°C.)	Mol. Ratio of Complex.	Fig. No.
			W	0.2	17.5	0	
-CHO + N<	Acetaldehyde	Diethylamine	W^{72}	0.25	20	1:1	
	n-Butyraldehyde	Triethylamine	CCl_4	0.1	13.08	1:1	4C
	Formaldehyde		W^{72}	0.2	20	1:1	
	D-Glucose		W^{72}	0.25	20	1:1	
	Propionaldehyde	Dimethylformamide	CCl_4^{72}	0.2	18	1:1	
			W^{72}	0.2	18	1:1	
	Terephthaldehyde	Triethylamine	T^{72}	0.2	18	1:2	
	Terephthaldehyde		W^{72}	0.1	17	1:2	
>C=O + Alk-OH	Acetone	Methanol	C^{72}	0.1	20	1:2	
			D^{72}	0.1	20	1:2	
>C=O + Ar-OH	Acetone	Phenol	W^{72}	0.1	20	0	
	Di-isobutyl ketone		D^{72}	0.25	20	1:2	

TABLE 1. contd.

Type of bond	Solutes a	Solutes b	Solv.*	Total mol. concn.	Temp. (°C.)	Mol. Ratio of Complex	Fig No
	Ethyl methyl ketone		CCl_4	0.1	14.83	1:1, 1:2	4
Ar-H + ArOH	Benzene	Phenol	CCl_4	0.1	17.00	2:1, 1:2	4
Ar-Cl + Ar-OH	Chlorobenzene	Phenol	CCl_4	0.1	14.85	1:1	4
	"		EW[79]	0.1	20	1:1	
	Hexachlorobenzene		CCl_4	0.05	12.08	1:6, 1:9	4
-C(=O)-N< + ArOH	Dimethylformamide	Phenol	CCl_4[71]	0.1	22	1:1, 1:2	
			B[71]	0.25		1:1†	
			W[71]	0.25	19.35	1:1	
-N=N- + ArOH	Azobenzene	Benzyl alcohol	CCl_4[71]	0.1	20	1:1	
		Phenol	B[71]	0.25	19	1:1, 1:2	
$-CO_2R$ + Ar-OH	Ethyl acetate	Phenol	CCl_4[72]	0.1	13.11	1:1	
		1-Naphthol-5-sulphonic acid	W[72]	0.04	24	1:1(?)	

~~-CO~~

TABLE 1. contd.

Type of bond	Solutes a	Solutes b	Solv.*	Total mol. concn.	Temp. (°C.)	Mol. Ratio of Complex	Fig. No.
$-CO_2R$ + Ar-Cl	Ethyl acetate	Chlorobenzene	CCl_4	0.1	14.85	1:1	4H
			EW[79]	0.1	14	1:1	
	Phenyl acetate		B[79]	0.1	21	1:1	
			EW[79]	0.25	14	1:1	

* Solvents and references (where no reference is given, the test is by the author): B-benzene; D-dioxan; EW - ethanol-water (1:1 v/v); T - toluene; W - distilled water. † By dielectric constant measurement.

TABLE 2 Systems giving no complex in carbon tetrachloride

Solutes		Total mol concn. (and ref.)*	Temp.(°C)	Fig.No.
a	b			
Acetone	Benzoquinone	0.05[70]	20	
2-Acetylpyridine	Di-isobutyl ketone	0.1[72]	21	
Azobenzene	Pyridine	0.05[83,84,85]	17	
Benzene	Ethylene dichloride	0.05[70]	20	
Benzoquinone	Di-isobutyl ketone	0.05[70]	20	
Dimethylformamide	Diphenylamine	0.05	13.33	5A
		0.05[72]	17	
Naphthalene	2-Naphthol	0.05	13.33	5B
		0.1(?)[70]	20(?)	
Phenol	Toluene	0.1	17.10	5C
		0.1[70]	20(?)	
Pyridine	Triethylamine	0.1[70]	20	

* Where no reference is given, the test is by the author.

TABLE 3. Interactions of Carbohydrates and Proteins.

System No.	a	concn. (M.)	b	concn. (g/l.)	Sol-vent*	Temp. (°C.)	Observed ratio of complex, a:b, by soln. vol. ratio	Calc. mol. ratio of complex, a:b**
	Pentoses							
(1)	D-Arabinose	0.05	Gelatin	5.3	W	15.40	(9:1),1:1,3:7	(10:1),1:1,1:2
(2)	L-Arabinose	0.05	"	5.3	"	"	(9:1),1:1,3:7	(10:1),1:1,1:2
(3)		0.02	Bovine Plasma Albumin	2.0	"	14.70	4:1,1:4	5:1,1:3
(4)	D-Xylose	0.05	Gelatin	5.3	"	15.40	3:2,1:4	2:1,1:3
(5)		0.02	Bovine Plasma Albumin	2.0	"	12.68	3:2,2:3	2:1,1:1
(6)	L-Xylose	0.05	Gelatin	5.3	"	15.40	3:2,1:4	2:1,1:3
(7)		0.02	Bovine Plasma Albumin	2.0	"	11.08	3:2,2:3	2:1,1:1
(8)	D-Ribose	0.05	Gelatin	5.3	"	18.42	1:1	1:1
(9)		0.02	Bovine Plasma Albumin	2	"	18.45	7:3,3:7	3:1,1:2
(10)		0.04	" "	4	"	17.00	7:3,3:7	3:1,1:2
	Hexoses							
(11)	D-Glucose	0.1	Gelatin	10.6	"	21.20	0	
(12)		0.05	"	5.3	"	12.85	0	
(13)		0.02	Bovine Plasma Albumin	2.0	"	14.58	7:3,3:7	3:1,1:2
(14)		0.02	" "	2.0	"	14.60	7:3,3:7	3:1,1:2
(15)		0.02	" "	2.0	B7	13.97	7:3,3:7	3:1,1:2
(16)		0.02	" "	2.0	0.1M.NaCl	13.57	7:3,3:7	3:1,1:2
		0.01	Casein	1.25	B9		0(ref.91)	
(17)	L-Glucose	0.02	Gelatin	2.17	W	12.85	+	
(18)		0.02	"	2.17	"	14.58	+	
(19)		0.02	Bovine Plasma Albumin	2.0	"	14.60	0	
(20)		0.02	" "	2.0	"	18.70	0	

TABLE 3. (Continued)

System No.	a	concn. (M.)	b	concn. (g/l.)	Sol-vent*	Temp. (°C.)	Observed ratio of complex, a:b, by soln. vol. ratio	Calc. mol. ratio of complex, a:b*
	Hexoses (cont.)							
(21)	D-Galactose	0.05	Gelatin	5.3	W	12.83	3:2	2:1
(22)		0.02	Bovine Plasma Albumin	2.0	"	18.40	7:3,1:1	3:1,1:1
(23)		0.01	" "	1.0	"	18.40	7:3,1:1	3:1,1:1
(24)	L-Galactose	0.02	Gelatin	2.12	"	12.83	0	
(25)		0.02	Bovine Plasma Albumin	2.0	"	18.70	0	
(26)	D-Mannose	0.05	Gelatin	5.3	"	17.00	0	
(27)		0.05	"	5.3	"	18.42	0	
(28)		0.02	Bovine Plasma Albumin	2.0	"	18.42	3:2,2:3	2:1,1:1
(29)	D-Fructose	0.05	Gelatin	5.3	"	17.45	0	
(30)		0.05	"	5.3	"	15.53	0	
(31)		0.02	Bovine Plasma Albumin	2.0	"	18.70	0	
(32)	L-Sorbose	0.05	Gelatin	5.3	"	18.45	0	
(33)		0.05	"	5.3	"	12.60	0	
(34)		0.02	Bovine Plasma Albumin	2.0	"	18.45	2:3,1:4	1:1,1:3
(35)		0.02	" "	2.0	"	14.58	2:3,1:4	1:1,1:3
(36)		0.04	" "	4.0	"	17.00	3:2,1:1,1:4	2:1,1:1,1:
	Disaccharides							
(37)	D-Cellobiose	0.05	Gelatin	5.3	"	15.45	0	
	Sucrose	0.1	Casein	1.25	B9	20	0 (ref.91)	
(38)	β-Lactose	0.05	Gelatin	5.3	W	12.60	0	
(39)		0.05	"	5.3	"	16.25	0	
(40)		0.02	Bovine Plasma	2.0	"	14.80	0	
(41)		0.02	" Albumin	2.0	"	12.83	0	
(42)	Maltose	0.05	Gelatin	5.3	"	14.82	0	
(43)		0.05	"	5.3	"	16.50	0	
(44)		0.02	Bovine Plasma Albumin	2.0	"	16.43	0	
(45)		0.02	" "	2.0	"	14.50	0	

TABLE 3. (Continued)

System No.	a	concn. (M.)	b	concn. (g/l.)	Sol-vent*	Temp. (°C.)	Observed ratio of complex, a:b, by soln. vol. ratio	Calc. mol. ratio comple a:b**
	Some other results							
(46)	D-Glucose	0.02	Bovine Plasma Albumin	2.0	W	14.58	7:3,3:7	3:1,1:
		0.0085	Edestin	1.07	1.0N NaOH		0(ref.91)	
		0.01	Casein	1.25	B9		0(ref.91)	
(47)	L-Glucose	0.0085	Edestin	1.07	1.0N NaOH	13.47	4:1	
(48)	D-Galactose	0.0085	"	1.07	1.0N NaOH	13.47	7:3,3:7	
(49)	L-Galactose	0.0085	"	1.07	"	13.47	0	
(50)	D-mannose	0.02	Bovine Plasma Albumin	2.0	W	?	27:23,2:3	3:2,1:

* W = water; B7 and B9 = buffer solutions at pH 7.17 and 9.0, respectively.

** This is the ratio of the number of molecules of carbohydrate to protein amino-acid residues in the complex, calculated using the mean residue weights, namely 101 (gelatin) and 118 (bovine plasma albumin) obtained from reference 64, and corrected for the ash and moisture contents of the protein (the weights of protein in column 5 are uncorrected).

† Definite complex formed, but observed volume ratio uncertain, lying between 3:2 and 2:3 (corrected value of mol. ratio** limits is 2:1 and 4:5). Insufficient reagent was available for confirmation.

TABLE 4 Reactions of proteins with hydrogen-bonding agents

System No.	a	concn. (M)	b	concn. (g/l.)	Solvent	Temp. (°C.)	Observed ratio of complex, a:b, by soln. vol.ratio.	Calc. Mol. ratio comple a:b.
	H-donors							
	meso-Inositol	0.1	Gelatin	10.6	W	20	1:4(ref.91)	1:3
(51)		0.02	Bovine Plasma Albumin	2.0	"	17.45	3:2	2:1
		0.01	Casein	1.25	B9	20	0 (ref.91)	
(52)	Mannitol	0.05	Gelatin	5.3	W	14.58	2:3	4:5
(53)		0.02	Bovine Plasma Albumin	2.0	"	14.58	3:2,1:4	2:1,1:
(54)	Phenol	0.05	Gelatin	5.3	"	12.60	1:1	1:1
(55)		0.02	Bovine Plasma Albumin	2.0	"	11.40	1:1	1:1
(56)	Propionaldehyde	0.02	" " "	2.0	"	13.38	0	
(57)		0.02	" " "	2.0	"	16.60	0	
	H-acceptors							
(58)	Methyl ethyl ketone	0.05	Gelatin	5.3	"	13.20	0	
(59)		0.05	"	5.3	"	17.10	0	
(60)		0.02	Bovine Plasma Albumin	2.0	"	14.58	0	
(61)		0.02	" " "	2.0	"	11.03	0	
(62)	Dioxan	0.05	Gelatin	5.3	"	13.20	0	
(63)		0.05	"	5.3	"	17.10	0	
(64)		0.02	Bovine Plasma Albumin	2.0	"	14.58	0	
(65)		0.02	" " "	2.0	"	11.03	0	
(66)	Triethylamine	0.05	Gelatin	5.3	"	16.65	1:1	1:1

TABLE 5 Reactions of carbohydrates with phenol and other simple molecules in water.

System No.	a	concn. (M.)	b	concn. (M.)	Temp. (°C.)	Mol.ratio of comple a:b	
	D-Glucose	0.125	Phenol	0.125	20.35	0	(ref.72)
(67)	L-Glucose	0.02	"	0.02	12.83(?)	0	
(68)	D-Galactose	0.05	"	0.05	16.55(?)	0	
(69)		0.05	"	0.05	12.58		
	D-Fructose	0.25	"	0.25	21.00	0	(ref.72)
(70)		0.05	"	0.05	12.32	0	
(71)	L-Sorbose	0.1	"	0.1	18.80	4:1	
(72)		0.1	"	0.1	16.50	4:1	
(73)		0.05	"	0.05	16.55	4:1	
(74)		0.05	"	0.05	12.58	4:1	
(75)		0.05	"	0.05	11.87	4:1	
(76)		0.04	Resorcinol	0.04	15.30	8:1, 4:1	
(77)		0.05	n-Butanol	0.05	15.38	0	
(78)		0.05	"	0.05	11.32	0	
(79)		0.05	Triethylamine	0.05	17.10	0	
(80)		0.05	"	0.05	14.60	0	
(81)		0.05	N-Methylacetamide	0.05	11.00	0	
(82)		0.05	"	0.05	16.32	0	
(83)	D-Arabinose	0.05	Phenol	0.05	12.85	0	
(84)	D-Xylose	0.05	"	0.05	12.85	0	
(85)	Ethyl Methyl ketone	0.05	"	0.05	15.52	0	
(86)		0.05	"	0.05	12.32	0	

COMPOSITION OF MIXTURE (%)		INSTRUMENT READING (min.)								
a	b	(1)*	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
100	0	24.83	27.45	6.00	23.80	5.38	33.85	8.45	6.90	6.95
96	5	24.80	27.22		23.72		33.75			
90	10	24.73	26.98	6.00	23.58	5.25	33.68	8.52	6.80	6.83
80	20	24.57	27.00	6.00	23.50	5.15	33.53	8.67	6.72	6.73
70	30	24.37	26.90	5.83	23.38	5.08	33.37	8.77	6.62	6.62
60	40	24.28	26.88	5.65	23.18	5.00	33.28	8.87	6.53	6.55
50	50	24.07	26.70	5.52	23.20	5.00	33.10	8.77		6.48
40	60	24.03	26.53	5.35	23.15	5.00	33.05	8.63	6.39	6.83
30	70	24.02	26.32	5.20	23.18	4.92	32.92	8.53	6.32	6.35
20	80	23.85	26.32	5.03	23.17	4.83	32.83	8.38	6.27	6.25
10	90	23.75	26.37	4.93	22.97	4.75	32.80	8.32	6.22	6.15
0	100	23.53	26.38	4.83	22.70	4.67	32.82	8.23	6.13	6.08

a	b	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)
100	0	7.41	10.53	14.07	12.00	12.00	7.67		7.58	12.25
95	5		10.30							
90	10	7.17	10.03	13.78	11.83	11.83	7.67		7.52	12.12
85	15		9.68							
80	20	6.97	9.53	13.35	11.67	11.67	7.68		7.38	12.00
75	25		9.26							
70	30	6.88	8.98	12.98	11.50	11.50	7.67		7.37	12.00
65	35		8.67							
60	40	6.57	8.28	12.72	11.50	11.52	7.50		7.30	
50	50	6.37	7.98	12.37	11.50	11.50	7.43		7.17	
40	60	6.25	7.45	12.08	11.50	11.52	7.27		7.12	
30	70	6.10	6.92	11.80	11.50		7.13		6.93	11.47
20	80	5.90	6.42	11.65	11.33	11.33	7.07		6.67	11.12
10	90	5.72	5.90	11.20	11.17	11.17	7.00		6.67	10.88
0	100	5.53	5.38	10.97	11.00	11.00	6.93		6.55	10.75

a	b	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)	(27)
100	0	12.17	5.98	9.00	7.07	9.38	5.32	5.62	8.68	8.33
90	10	12.07	5.83	8.67	7.03	9.30	5.08	5.50	8.40	
80	20	11.97	5.80	8.32	6.96	9.25	4.97	5.38	8.20	7.83
70	30	11.87	5.68	7.93	6.91	9.20	4.93	5.28	7.95	
65	35				6.86					
60	40	11.75	5.55	7.67	6.68	9.18	4.72	5.17	7.73	7.38
50	50	11.67	5.43	7.63	6.47	9.25	4.63	5.05	7.43	7.12
40	60	11.55	5.32	7.28	6.38		4.42	4.95	7.32 7.28	6.83
30	70	11.47	5.22	7.03	6.30	9.07	4.43	4.83	7.93	6.65
20	80	11.37	5.13	6.82	6.25	8.95	4.25	4.72	6.77	6.45
10	90	11.27	5.02	6.67		8.87	3.92	4.62	6.43	6.15
0	100	11.18	4.83	6.50	6.16	8.75	3.97	4.50	6.23	5.95

* System number as given in Table 3, column 1.

TABLE 6. Refractometer "drum" readings for systems reported in Table 3.

COMPOSITION OF MIXTURE (%)		INSTRUMENT READING (min.)								
a	b	(28)	(29)	(30)	(31)	(32)	(33)	(34)	(35)	(36)
100	0	7·13	8·00	8 79	5·71	11·36	12·00	7·07	5·62	10·88
90	10	7·02	7 83	8·55	5·58	11·03	11·75	6·98	5·50	10·60
80	20	6·87	7·70	8·33	5·50	10 82	11·50	6·90	5·37	10·37
70	30	6·78	7·65	8·15	5·39	10·67	11·25	6·78	5·27	10·17
60	40	6·65	7·43	7·98	5·27	10·40	11·00	6·68	5·12	9·90
50	50	6·60	7·35	7 77	5·19	10·25	11 73	6·55	5·03	9·73
40	60	6·53	7·18	7·53	5·07	10·10	11·50	6·46	4·87	9·62
30	70	6·45	7·00	7·32	4·98	9·83	11·25	6·32	4·82	9·38
20	80	6·32	6 85	7·17	4·87	9·63	11·03	6·20	4·78	9·08
10	90	6·23	6·72	6·92	4 73	9·47	9·77	6·15	4·62	8·99
0	100	6·12	6 63	6·73	4·68	9·30	9·50	6·12	4·50	8·77

a	b	(37)	(38)	(39)	(40)	(41)	(42)	(43)	(44)	(45)
100	0	40·48	7·67	13·00	9·52	10·50	9·68	8·50	8·32	9·67
90	10	39·20	6 67	12·17	9·03	9·92	9·30	8·08	8·00	9·33
80	20	38·35	5·67	11·33	8·53	9·33	8·96	7·80	7·67	9·00
70	30	37·47	4·85	10·50	8·00	8·75	8·60	7·45	7·35	8·67
60	40	36·47	3·92	9·67	7·50	8·17	8·25	7·13	7·00	8·33
50	50	35·82	2·92	8·83	7·05	7·57	7·85	6·80	6·67	8·00
40	60	34·90	2·00	8·00	6·50	7·00	7·53	6·42	6·33	7·67
30	70	33·95	1·03	7·00	6·00	6·43	7·13	6·08	6·00	7·33
20	80	33·17	14·97	6·32	5·50	5·83	6·80	5·63	5·67	7·00
10	90	32·35	14·00	5·50	5·00	5·25	6·42	5·40	5·33	6·68
0	100	31·25	13·25	4·67	4·50	4·67	6·08	4·95	5·00	6·33

a	b	(46)	(47)	(48)	(49)	(50)
100	0	11·17	12·58	12·55	12·48	4·05
90	10	11·12	12·52	12·55	12·52	
80	20	10·88	12·48	12·55	12·52	3·87
70	30	10·85	12·53	12·65	12·52	3·75
60	40	10·70	12·55	12·62	12·57	3·65
53·5	46·5					3·58
50	50	10·70	12·62	12·68	12·57	3·52
40	60	10·67	12·63	12·73	12·60	3·35
30	70		12·70	12·80	12·60	3·25
20	80	10·52	12·72	12·73	12·63	3·17
10	90		12·77	12·68	12·63	
0	100	10·23	12·80	12·55	12·65	2·98

TABLE 6 (continued).

COMPOSITION OF MIXTURE (%)		INSTRUMENT READING (min.)								
a	b	(51)*	(52)	(53)	(54)	(55)	(56)	(57)	(58)	(59)
100	0	2·63	10·00	8·00	0·27	9·75	4·27	4·00	8·67	9·33
90	10	2·82	9·83	7·90	0·15	9·38		4·03	8·97	9·63
80	20	3·00	9·67	7·80	0·03	9·18	4·67	3·97	9·25	9·90
70	30	3·20	9·50	7·72	14·95	8·89	4·87		9·55	10·20
60	40	3·33	9·33	7·62	14·83	8·58	5·07	3·83	9·83	10·50
50	50	3·52	9·17	7·47	14·77	8·35			10·13	10·78
40	60	3·68	9·00	7·33	14·70	8·23	5·47	3·70	10·42	11·07
30	70	3·78	8·67	7·23	14·65	8·12			10·70	11·37
20	80	3·95	8·33	7·08	14·63	8·00	5·87	3·58	11·00	11·67
10	90	4·08	8·00	7·00	14·58	7·87			11·28	11·95
0	100	4·23	7·67	6·97	14·55	7·73	6·25	3·43	11·58	12·25

a	b	(60)	(61)	(62)	(63)	(64)	(65)	(66)
100	0	3·52	7·00	9·17	9·75	3·67	7·50	11·78
90	10	3·67	7·15	9·42	10·00	3·75	7·58	11·75
80	20	3·78	7·30	9·67	10·23	3·83	7·67	11·75
70	30	3·90	7·45	9·92	10·50	3·92	7·73	11·75
60	40	4·03	7·60	10·17	10·75	4·00	7·83	11·75
50	50	4·15	7·75	10·42	11·00	4·08	7·92	11·75
40	60	4·28	7·90	10·67	11·23	4·17	8·00	11·87
30	70	4·37	8·05	10·92	11·50	4·25	8·08	12·00
20	80	4·50	8·20	11·17	11·75	4·33	8·17	12·17
10	90	4·62	8·35	11·42	12·00	4·42	8·25	12·32
0	100	4·73	8·50	11·65	12·25	4·50	8·33	12·50

* System number as given in Table 4, column 1.

TABLE 7. Refractometer "drum" readings for systems reported in Table 4.

COMPOSITION OF MIXTURE (%)		INSTRUMENT READING (min.)								
a	b	(67)*	(68)	(69)	(70)	(71)	(72)	(73)	(74)	(75)
100	0	3·58	7·90	9·50	8·82	5·27	11·67	7·42	9·33	9·12
95	5									7·07
90	10	3·52	7·67	9·25	8·58	4·92	11·33	7·42	9·17	8·96
85	15									8·72
80	20	3·40	7·40	9·02	8·30	4·50	11·00	7·42	9·00	8·85
70	30		7·17	8·75	8·07	3·97	10·33?	7·17	8·73	8·65
60	40		6·92	8·50	7·82	3·72	10·33	6·92	8·50	8·43
50	50		6·68	8·27	7·58	3·42	9·98	6·67	8·25	8·20
40	60	2·92	6·42	8·00	7·32	3·13	9·65	6·42	8·00	7·92
30	70	2·82	6·17	7·73	7·07	3·72	9·32	6·17	7·75	
25	75	2·75								
20	80		5·92	7·52	6·82	2·48	9·00	5·93	7·50	7·58
10	90		5·57	7·23	6·59	1·98	8·67	5·68	7·27	
5	95	2·57								
0	100	2·55	5·42	7·00	6·32	1·77	8·30	5·43	7·00	7·17

a	b	(76)	(77)	(78)	(79)	(80)	(81)	(82)	(83)	(84)
100	0	3·48	4·92	6·50	0·83	2·07	9·67	7·70	12·70	12·60
95	5	3·57								
90	10	3·70	4·42	6·00	0·53	1·70	9·17	6·50	12·60	12·42
85	15	3·62								
80	20	3·40	3·92	5·50	0·23	1·47	8·67	6·02	12·53	12·50
70	30	3·32	3·42	5·00	14·88	1·02	8·17	5·48	12·50	12·45
60	40	3·23	2·92	4·50	14·58	0·70	7·67	5·00	12·42	12·45
50	50	3·15	2·42	3·98	14·22	0·28	7·17	4·50	12·42	12·35
40	60	3·08	1·92	3·50	13·80	14·92	6·67	4·00	12·30	12·38
30	70	2·98	1·42	3·00	13·47	14·57	6·17	3·50	12·25	12·33
20	80	2·90	0·92	2·52	13·20	14·28	5·67	3·00	12·27	12·35
15	85	2·90								
10	90	2·90	0·42	2·00	12·88	13·90	5·17	2·50	12·12	12·18
5	95	2·80								
0	100	2·75	14·92	1·52	12·53	13·68	4·67	2·00	12·02	12·18

a	b	(85)	(86)
100	0	1·73	2·98
90	10	1·93	3·35
80	20	2·20	3·72
70	30	2·58	4·13
60	40	2·70	4·42
50	50	3·28	4·72
40	60	3·63	5·15
30	70	4·00	5·52
20	80	4·43	5·80
10	90	4·72	6·13
0	100	5·28	6·65

* System number as given in Table 5, column 1.

TABLE 8. Refractometer "drum" readings for systems reported in Table 5.

TABLE 9. Elementary analyses of dyes used.

Dye	Element. Analyses* (%)					Purity (%)
	C	H	N†	Cl	Na	
Crystal Violet**	66.36(73.58)	7.60(7.41)	7.73?(10.30)		0.24	88
Ethyl Violet	69.36(75.63)	7.85(8.60)	7.80(8.54			92
Magenta P	61.78(71.08)	6.44(5.97)			0.08	87
Malachite Green	66.96(75.68)	6.36(6.91)	6.08?(7.68)		0.01	89
Methylene Blue BP	51.14(60.07)	7.17(5.67)	10.20?(13.14)		0.12	85
Rhodamine B	67.00(70.21)	6.10(6.51)	5.51(5.85)		0††	95
Rhodamine 3B	65.89(71.05)	6.62(6.96)	4.97(5.53)			92
Safranine	59.27(68.44)	6.46(5.46)	14.20(15.97)		0.21	87
Victoria Blue BN	75.98(78.65)	7.64(6.37)	7.78‡(8.30)	6.38(7.01)	0.02	94
Victoria Pure Blue BO	72.43(77.43)	7.31(7.84)	7.78(8.17)		0.31	94.5

* Theoretical values given in brackets

† Many nitrogen values were found to be poorly reproducible (shown queried); this is believed to have been due to practical difficulties encountered at that period in the micro-analytical laboratory. Carbon and hydrogen values all showed good reproducibility.

** 88% pure by titanous chloride titration.

†† Confirmed by ashing, 0.04% ash.

‡ By macro-scale Kjeldahl method (performed by P.N. Mehta)

TABLE 10 Preliminary experiments with yeast cells subjected to various treatments, and at various suspension concentrations.

	Yeast Suspn.				Isotherm	Max.
Dye	Yeast Treatment*	Conc.(g/l.)	Solvent**	Temp.(°C.)	Type	Adsn.†
Crystal Violet	F1	0.110	W	22	L2	420
	F1	0.440	"	39	L2	283
	F1	0.384	"	50	L2	335
	F1	0.083	B9	20	L1	400
	U	0.160	W	19	L2	370
	U	0.279	"	16	L2	288
	U	0.158	"	40	L2	364
	B	0.241	"	20	L2	230
	B	0.343	"	50	L2	230
Methylene Blue BP	F2	0.312	"	17	L2	168
	F1	0.184	"	21	L2	308
	F2	0.222	"	49	L2	272
	F1	0.208	"	43	L2	312
	F1	0.213	B9	23	L2	534
Safranine	F1	0.324	W	18	L2	194
	B	0.391	"	17	L2	133
	B	0.475	"	49	L2	171
Rhodamine B	F1	0.151	"	14	S1	70 ††
Victoria Pure Blue BO	U	0.175	"	19	H2	478
	U	0.208	"	40	H2	483

* F1, Formalin-fixed yeast cells (sample 1)
F2, " " " " (" 2)
U, Untreated yeast cells
B, Boiled yeast cells (20 mins.)

** W, Water
B9, Buffer solution at PH9

† Corrected for dye purity

†† Maximum adsorption not reached.

TABLE 11. Adsorption data for cationic dyes on yeast.

Dye	Class of Isotherm	Probable Orientation	Max. Equil. Adsorption* (m.mole/Kg.)	Area of dye cation** ($Å^2$)	Calc. Specific Surface Area ($m.^2g.^{-1}$)	Coverage Factor † (C.F.)	Cationic Weight
Crystal Violet	L2	Aggregated	264	224	356	5.41	372
Ethyl Violet	L2	"	316	266	509	7.70	456
Magenta P	L2	"	163	168	165	2.51	302
Malachite Green	L2	Flat	61	181	66.5	1.01	329
Methylene Blue BP	L2	Aggregated	153	120	111	1.69	284
Rhodamine B	S2	Edge-on	88	124	65.7	1.00	-
Rhodamine 3B	L2	Flat	ca.130	184	144	2.19	592
Safranine	L2	Aggregated	176	148	157	2.39	316
Victoria Blue BN	H2	"	300	251	453	6.89	470
Victoria Pure Blue BO	H2	"	368	268	595	9.04	478

* Corrected for purity of dyes

** Area of smallest enclosing rectangle (in most probable orientation).

† i.e. ratio of maximum amount of dye adsorbed to calculated monolayer capacity (for stated probable orientation).

FIGURES

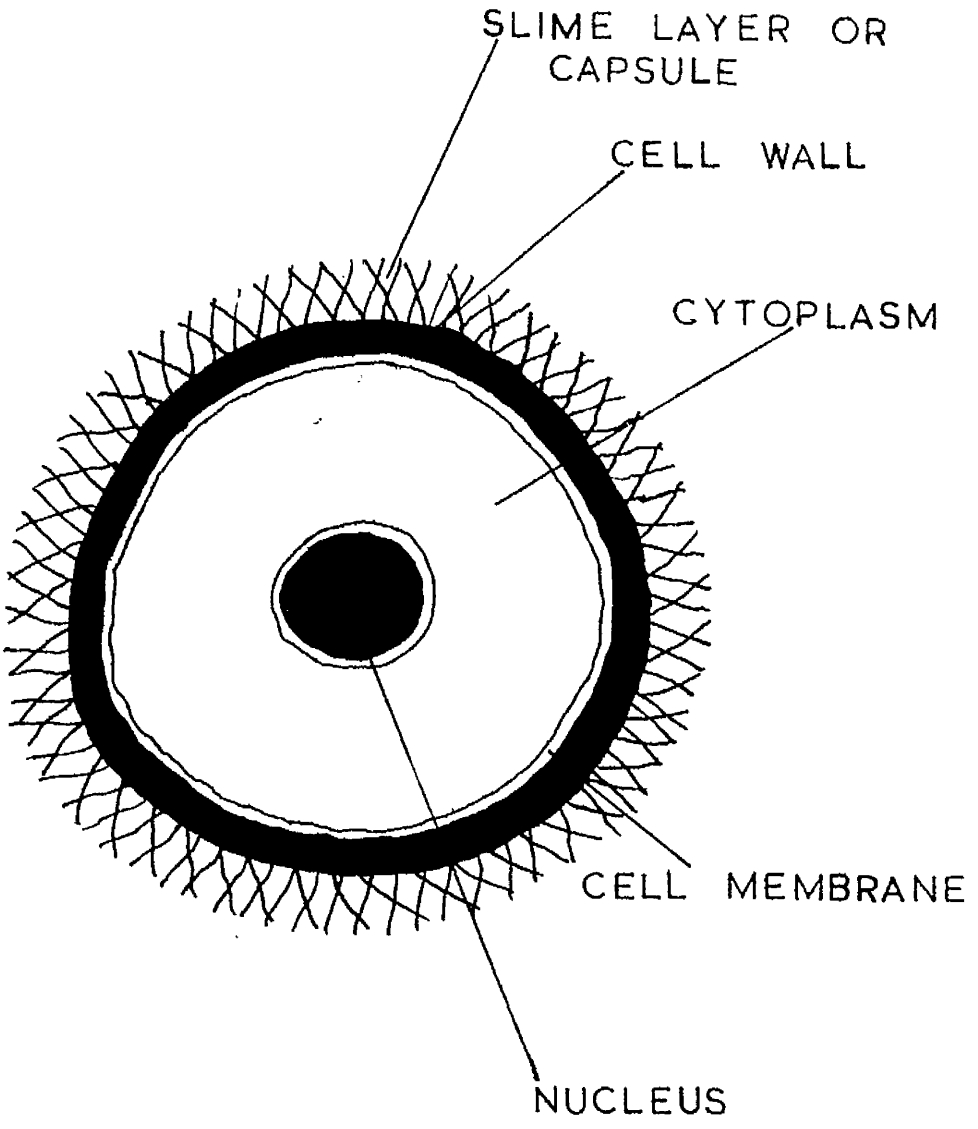


Fig. 1. Diagrammatic representation of the biological cell (cross-sectional view, showing the slime layer or capsule found on the cells of micro-organisms).

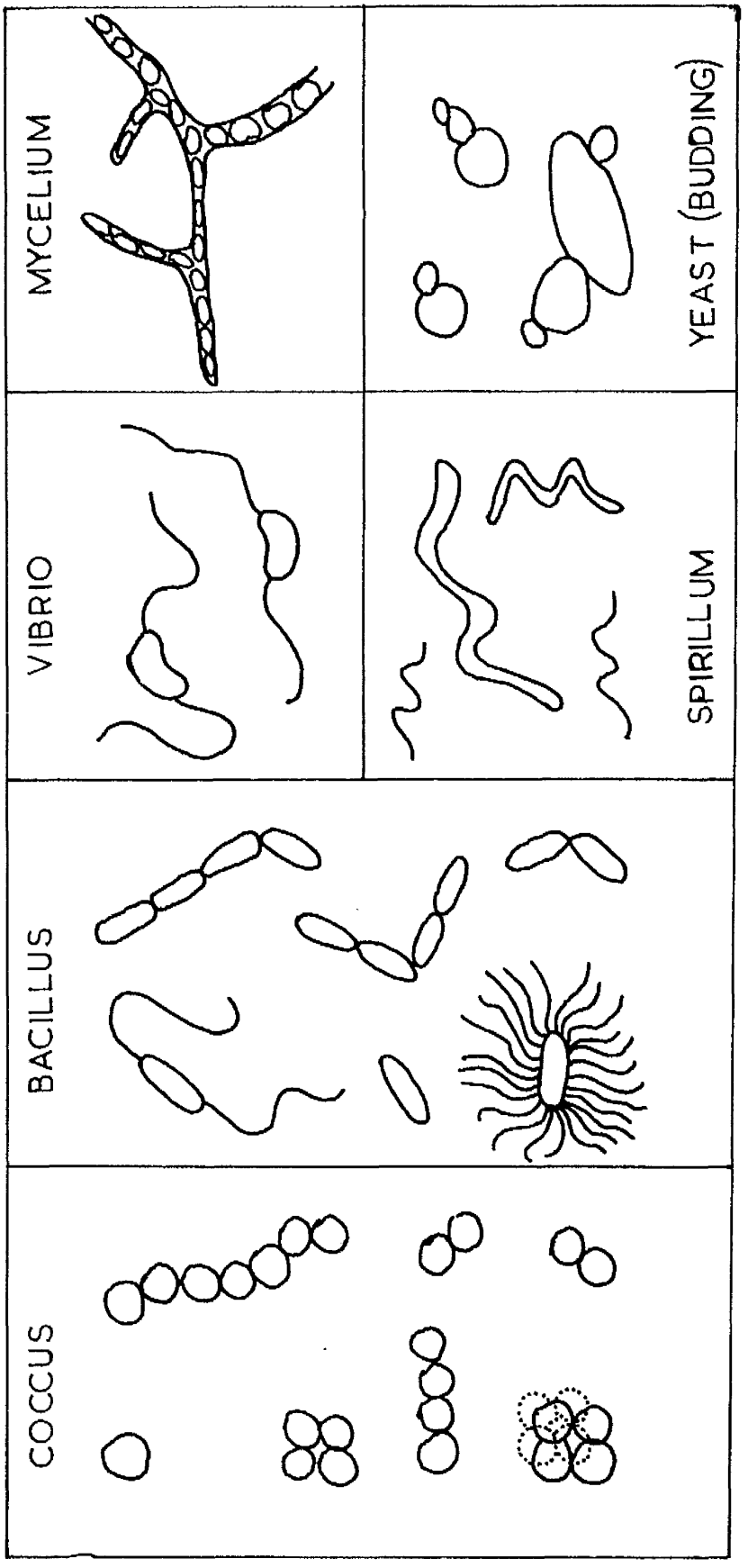
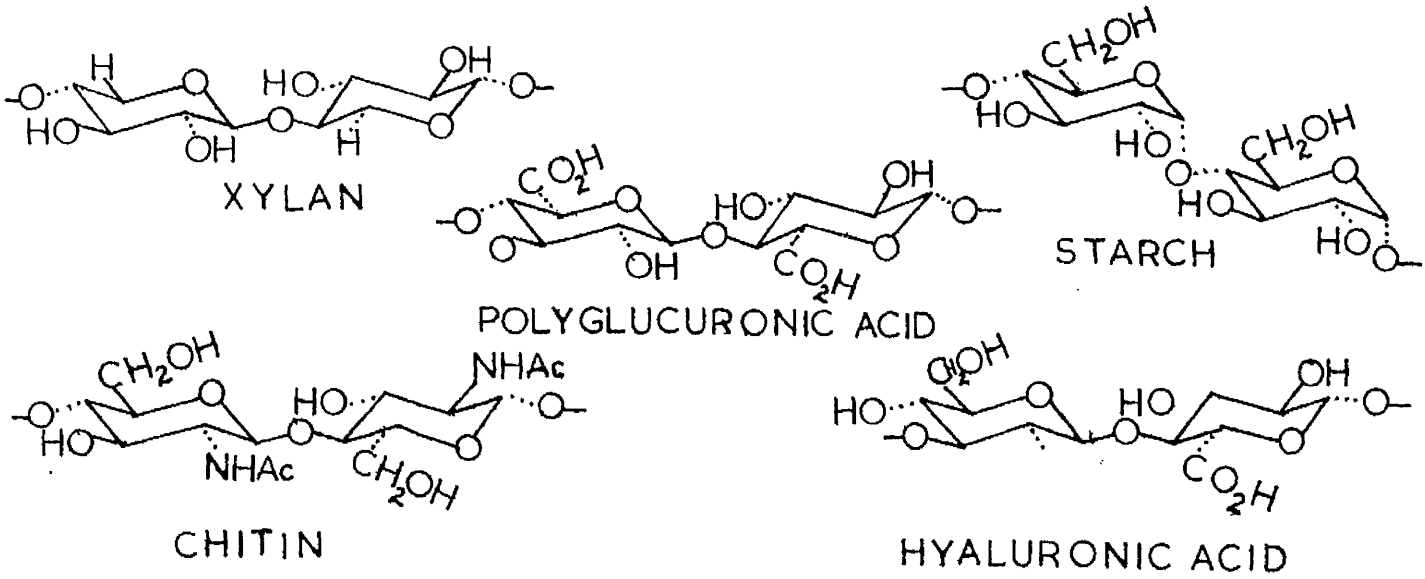
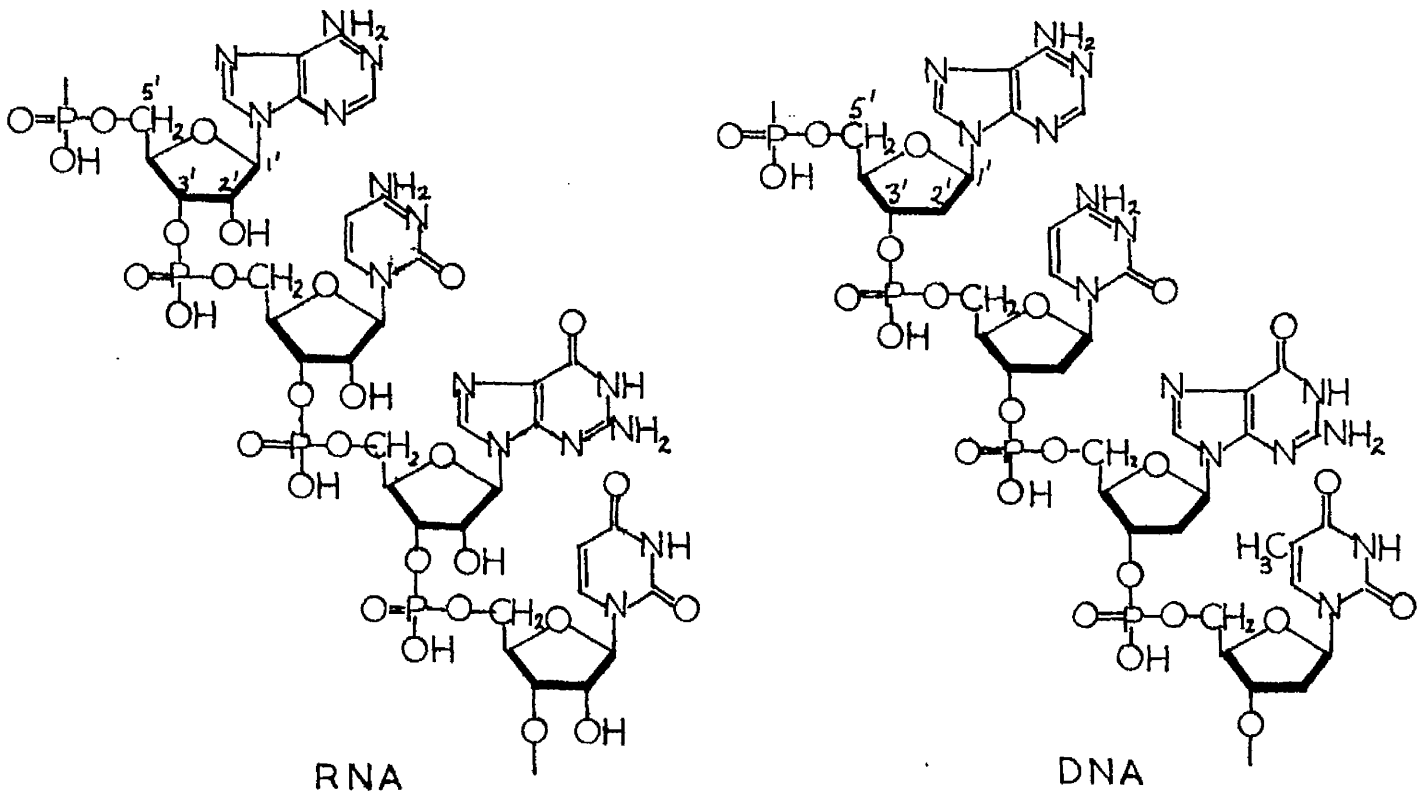


Fig. 2. The shapes and general appearance of the common types of bacterial and yeast cells.



A



B

Fig. 3. A Some typical repeating units from common naturally-occuring polysaccharides.
B Typical repeating units from nucleic acids.

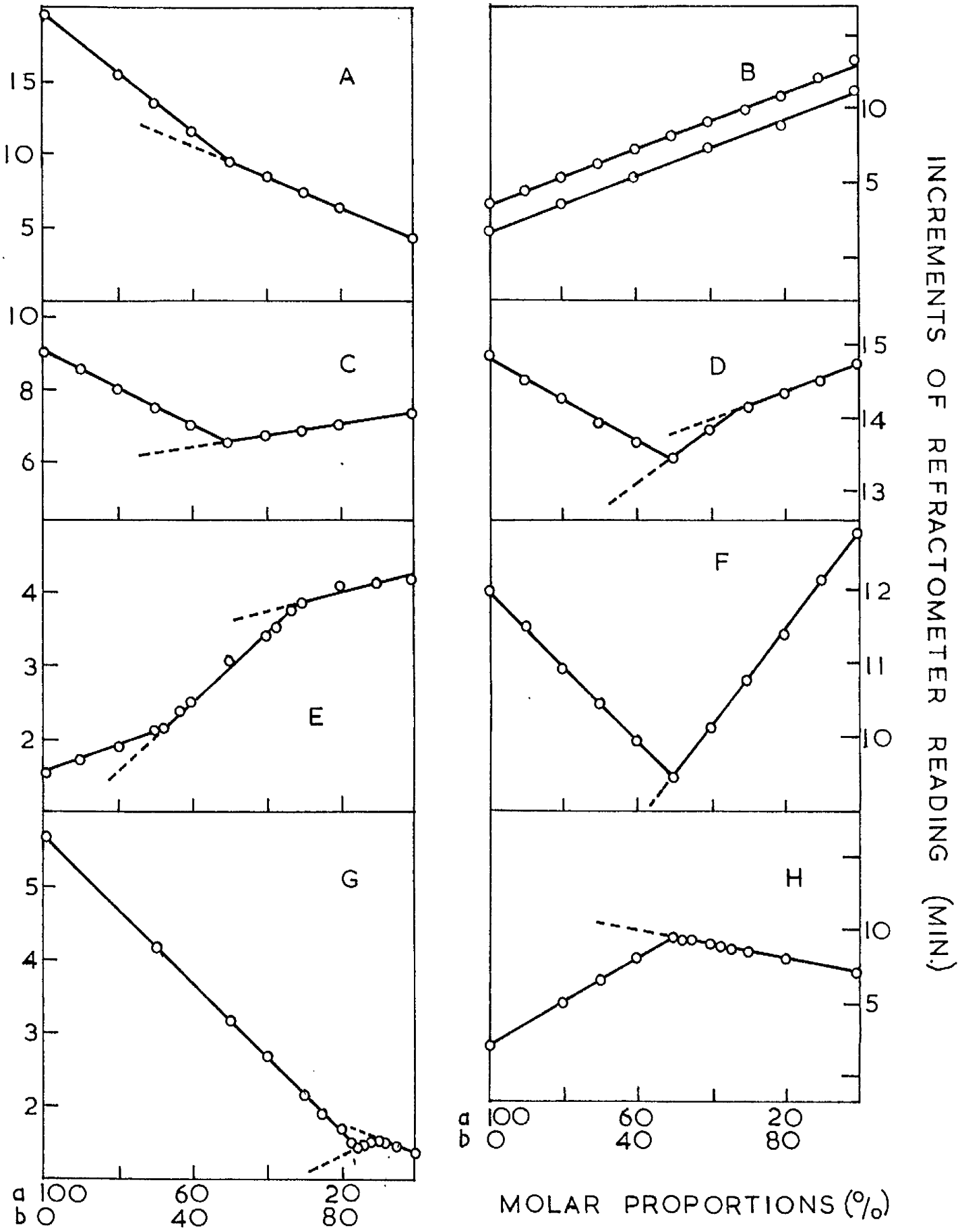


Fig. 4. Relation between refractive index (as instrument reading) and ratio of components in carbon tetrachloride solution.

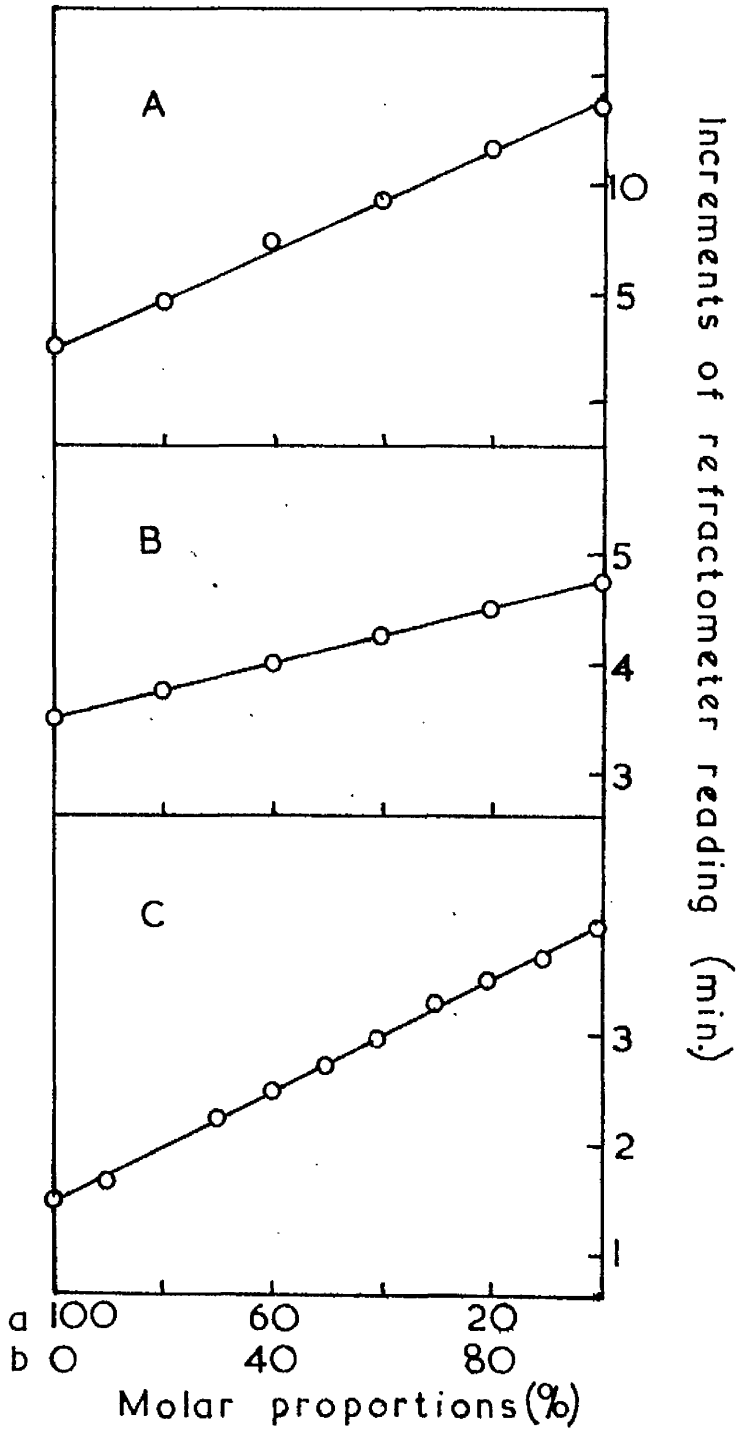


Fig. 5. Relation between refractive index (as instrument reading) and ratio of components in carbon tetrachloride solution.

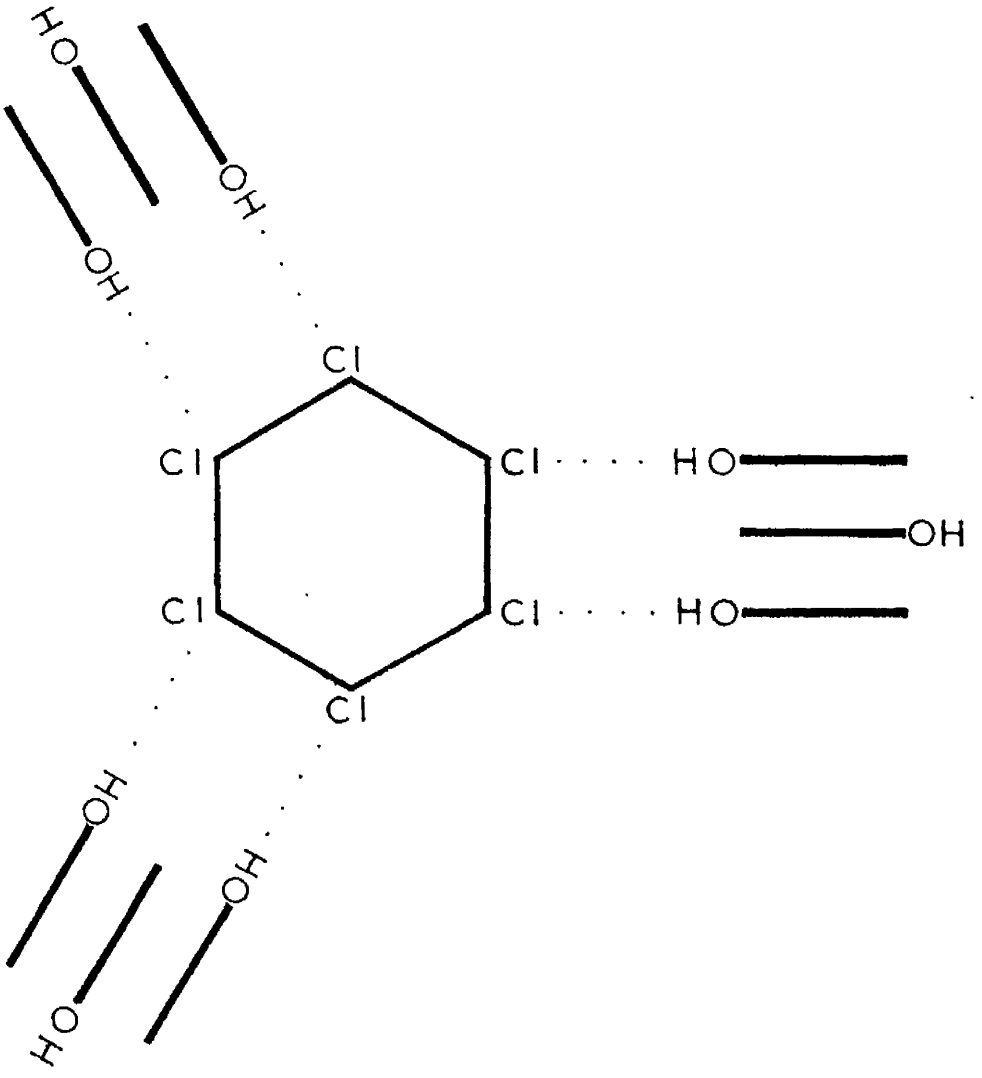


Fig. 6. Diagrammatic representation of the 1:9 complex formed between hexachlorobenzene and phenol.

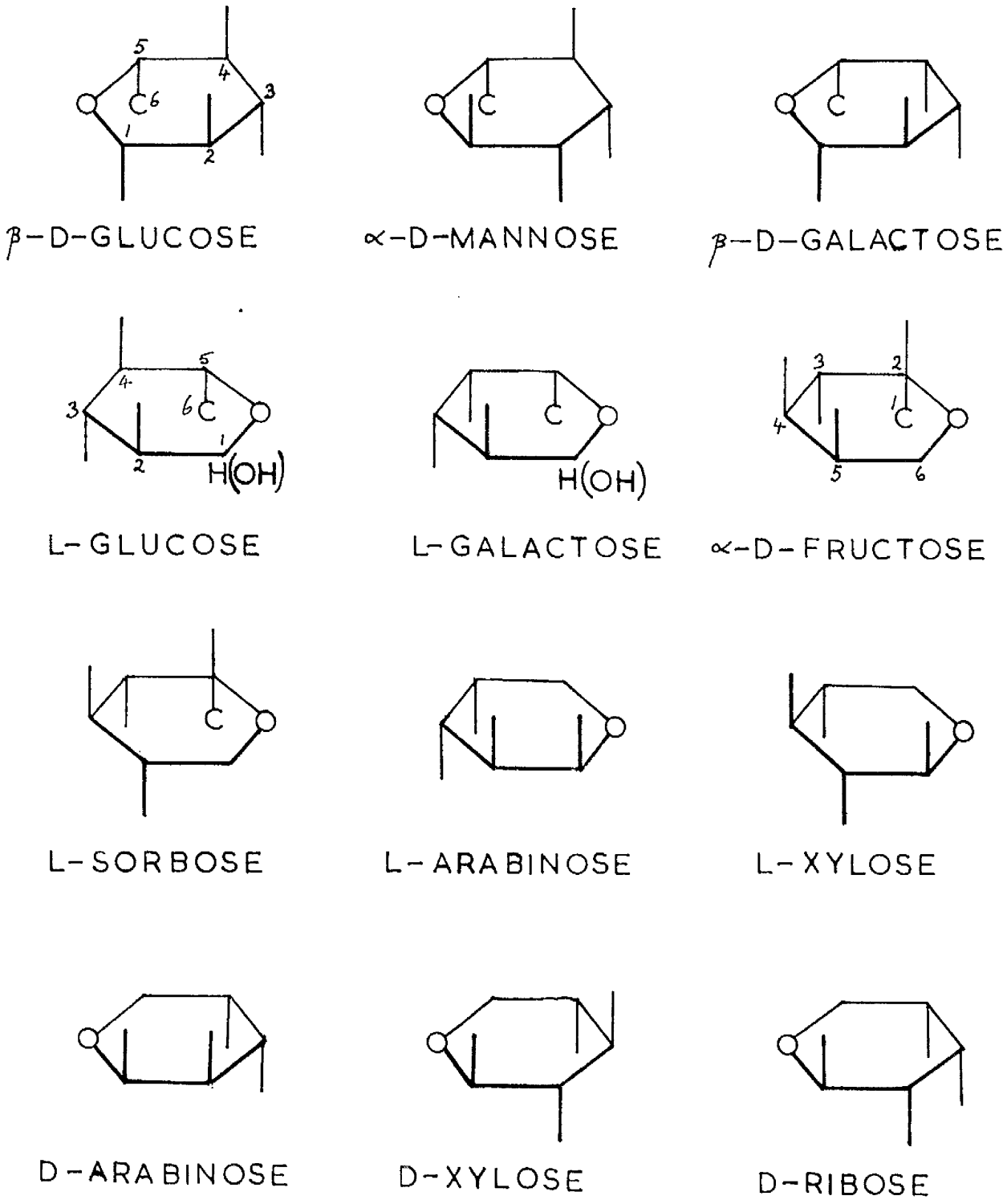
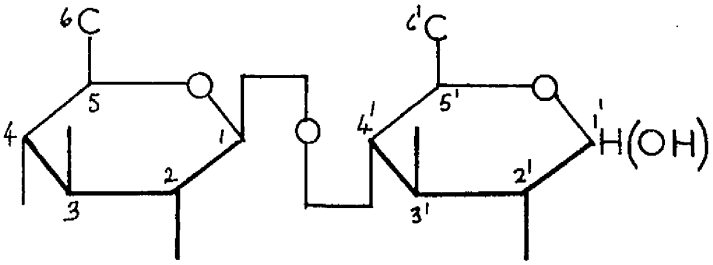
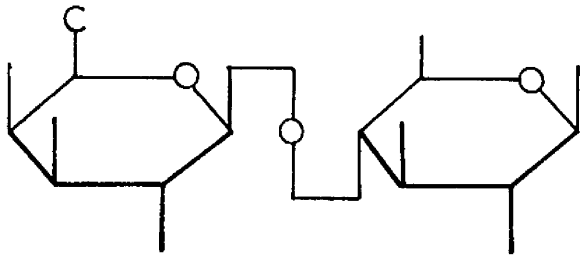


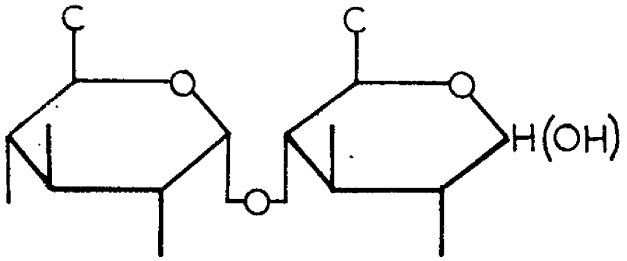
Fig. 7. Skeleton formulae for the monosaccharides used. The positions of the hydroxyl groups and the pendant $-CH_2OH$ group in the pyranose ring form are shown. ∝-,β-anomerism occurs at the 1 position in aldoses and at the 2 position in ketoses.



CELLOBIOSE

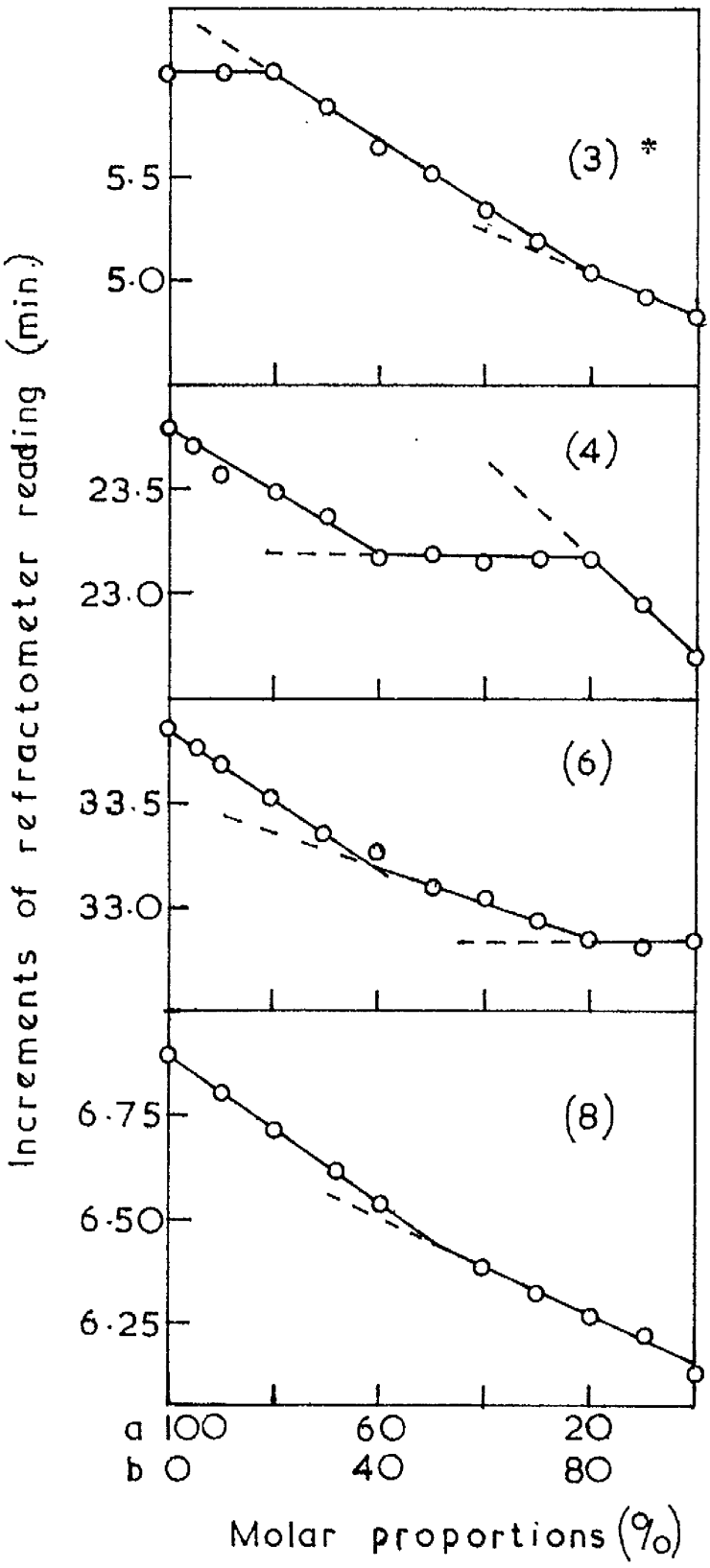


β-LACTOSE



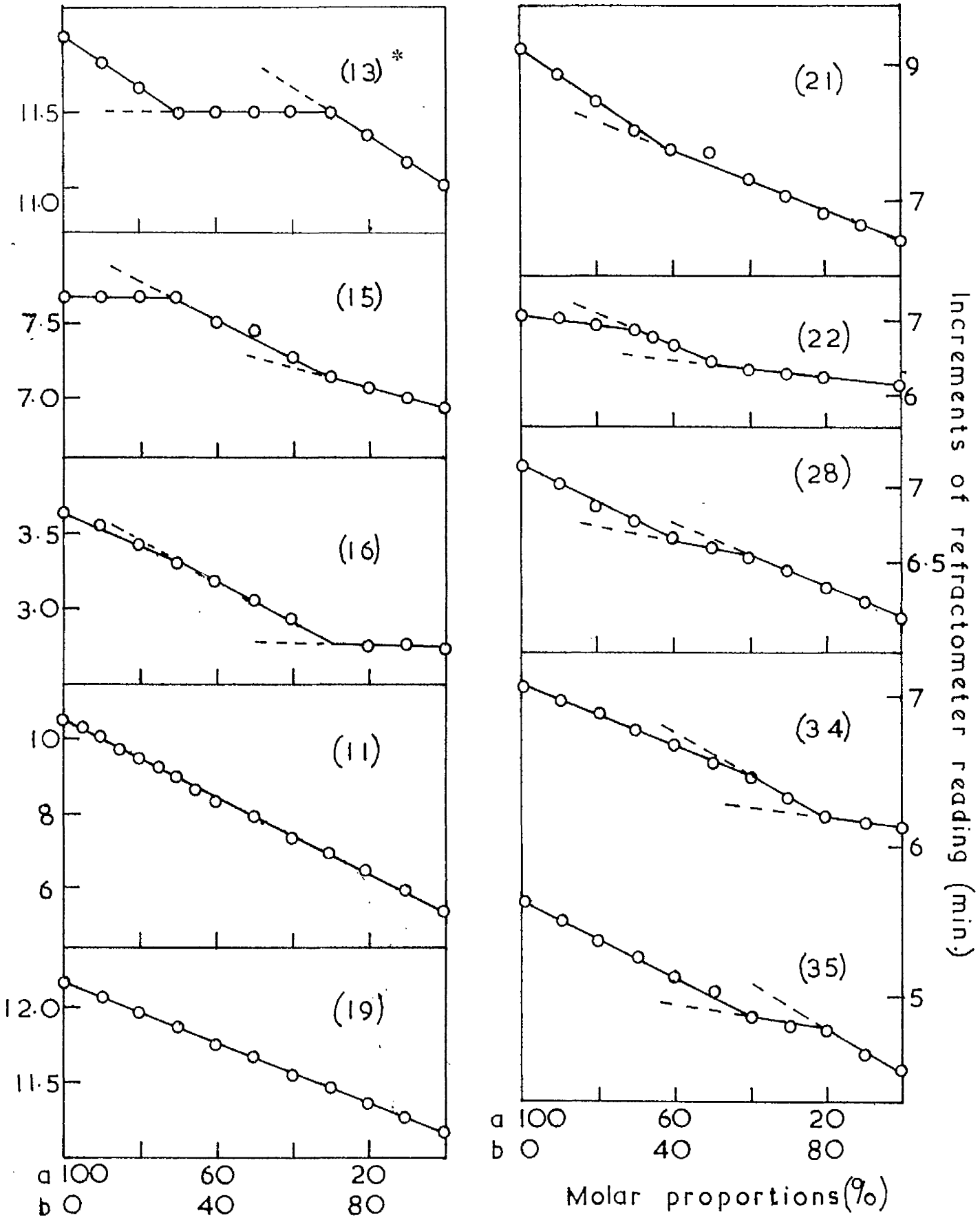
MALTOSE

Fig. 8. Skeleton formulae for the disaccharides used. The positions of the hydroxyl groups and the pendant CH_2OH groups are shown.



* system number as given in Table 3.

Fig. 9. Some typical graphs for pentose-protein mixtures.



*System number as given in Table 3.

Fig. 10. Some typical graphs for binary hexose-protein mixtures.

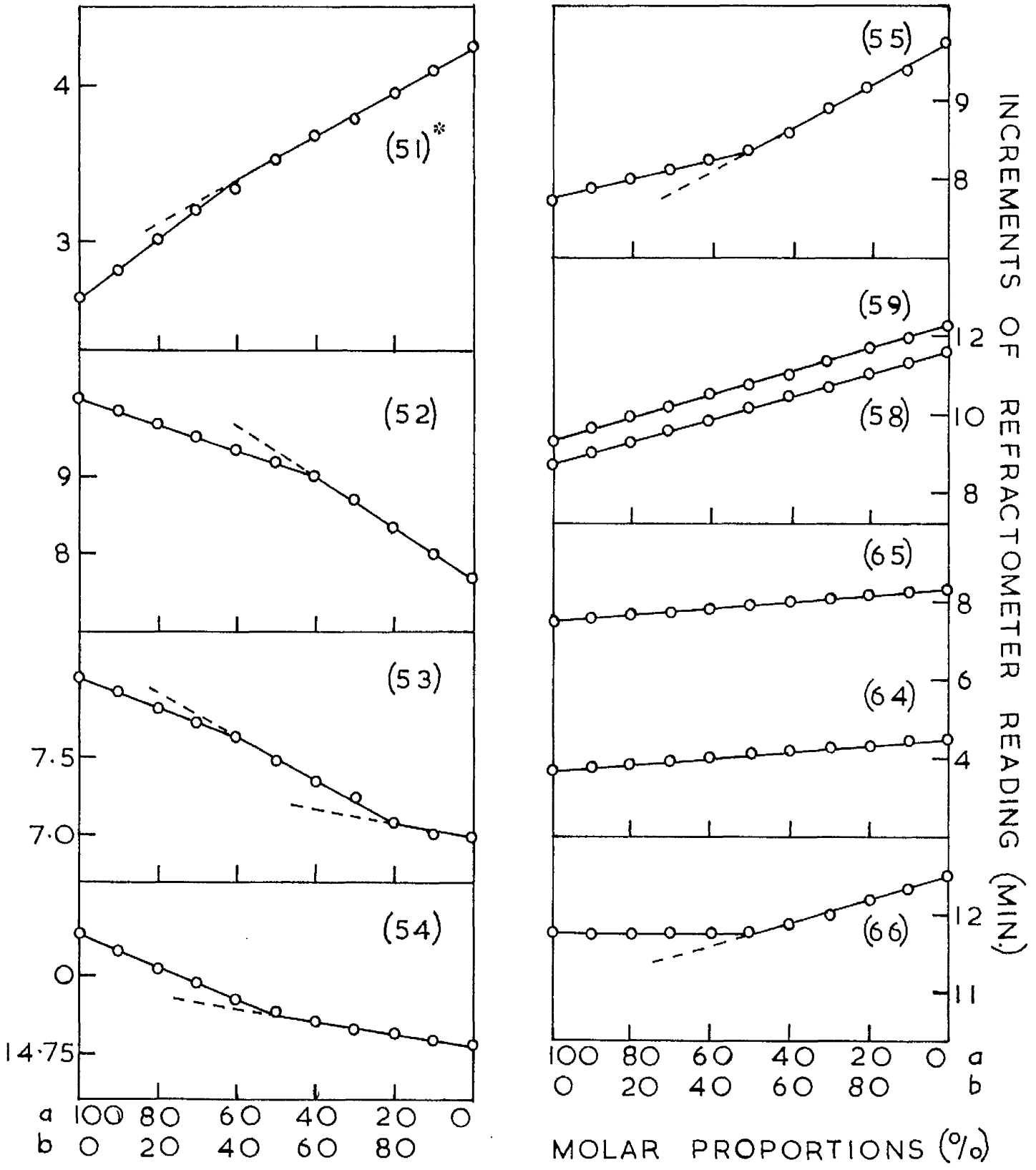


Fig. 11. Some typical graphs for binary mixtures of simple hydrogen-bonding agents and proteins.

* System number as given in Table 4.

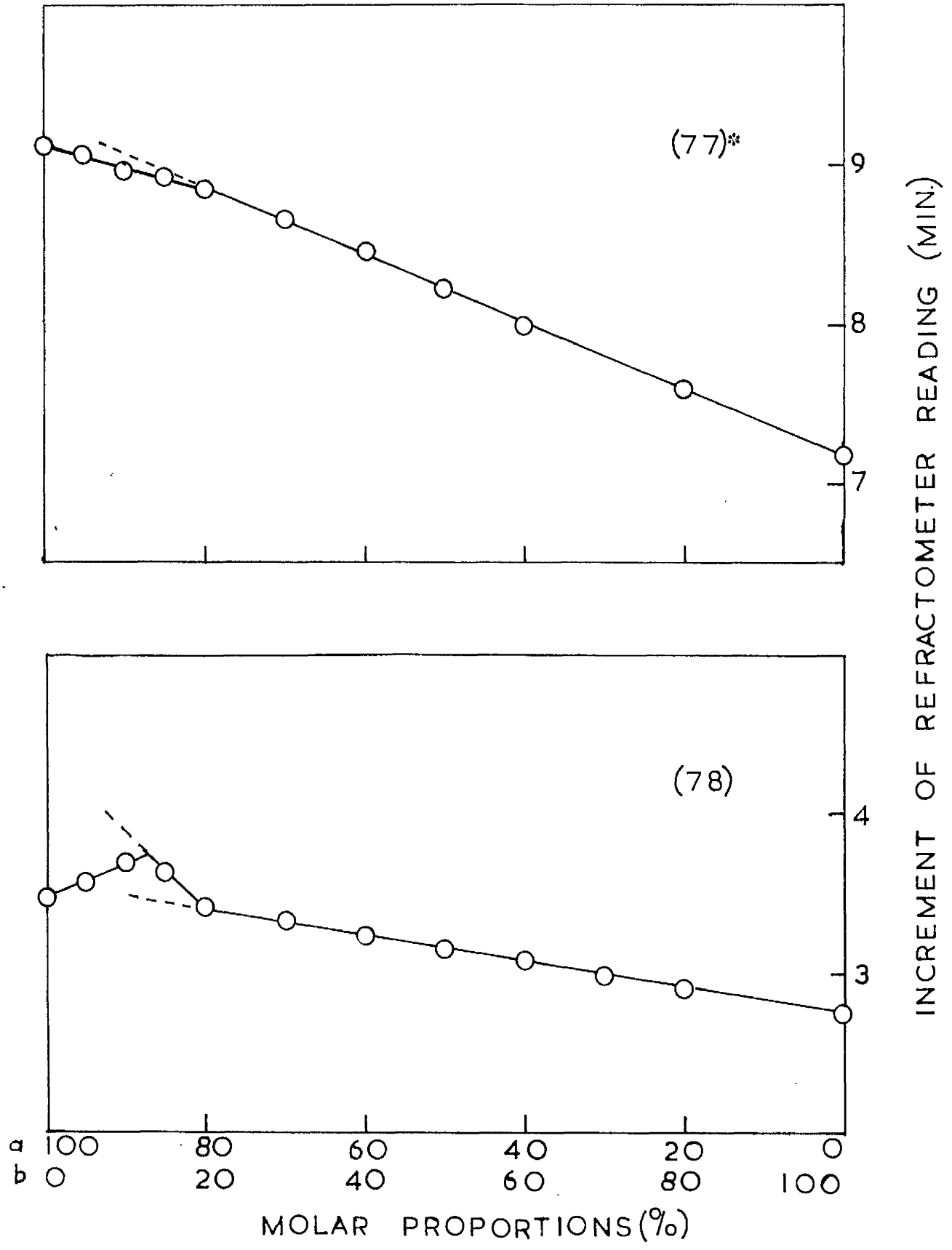
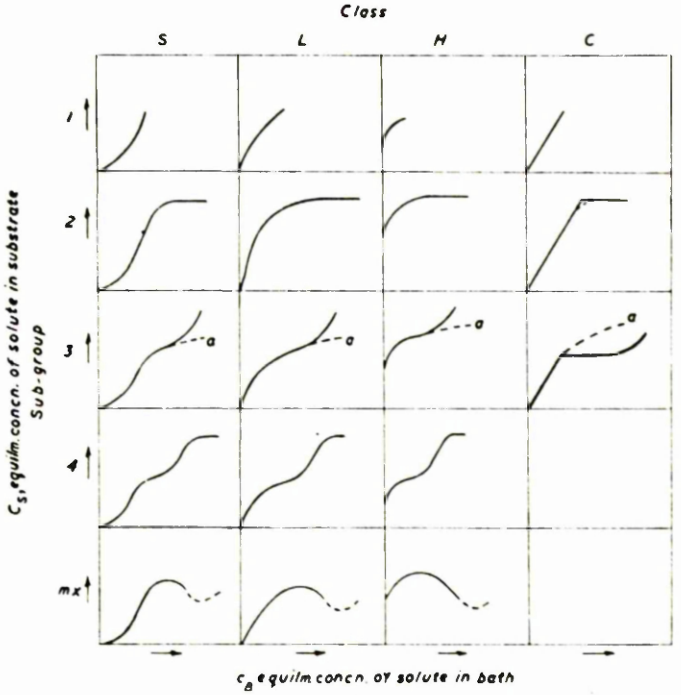


Fig. 12. Graphs for the interactions of L-sorbose with phenol (top) and resorcinol (bottom).

* System number as given in Table 3.

FIG. 13. *System of isotherm classification.*



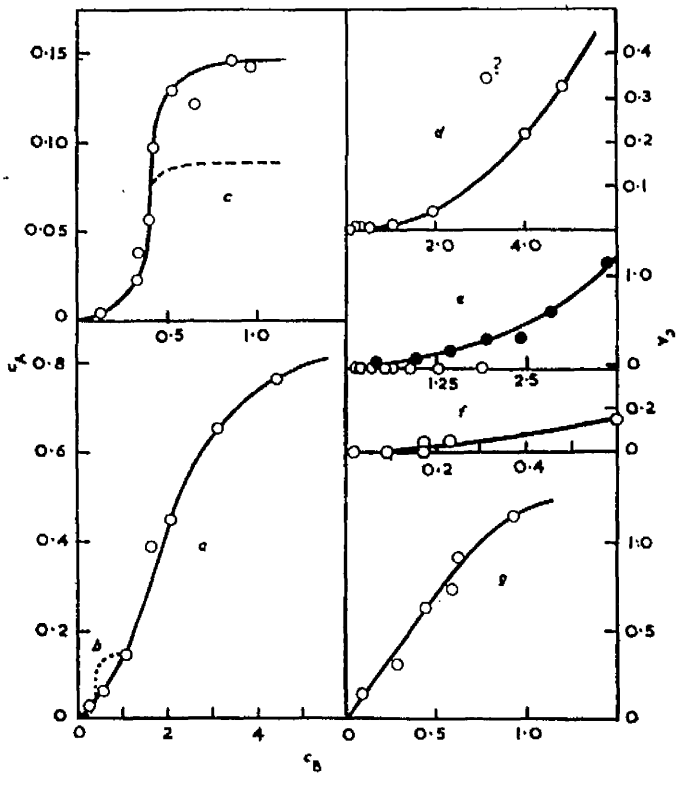
Ostwald and de Izaguirre's two types of curve for adsorption from dilute solution are classified as *L*2 and *C*1 here, and their several other curves for adsorption from concentrated liquid binary mixtures would come under the present sub group *mx*, with one exception (*C*1, with *negative* slope).

Brunauer's five types of vapour-phase (physical) adsorption isotherm [illegible] are defined as *H*2, *L*3, S1, *L*4, and S2 under the present system.

The present nomenclature is slightly different from that originally given.

The system can be readily adapted to describe curves with additional variations not shown above, *e.g.*, the isotherm for phenol adsorption on graphite [illegible] has two inflections followed by a further rise and would here be defined as *L*5 and curves with two successive maxima [illegible] as *mxmx*.

FIG. 14. *Adsorption isotherms of aromatic hydrocarbons, etc., in xylene (except where indicated otherwise) on chromatographic alumina at 20°.*



c_A is the equilibrium concentration on the alumina (mmole/kg.) and c_B that in the bath (mmole/l.).

(*a*) *Phenanthrene (Grade* I *alumina).* (*b*, *c* *) *Phenanthrene (Grade* II *alumina); broken line shows isotherm from recycled Grade* II *(see Experimental section).* (*d*) *Pyrene (Grade* II*).* (*e*) *Naphthalene (Grade* II*) from 2,2,4-trimethylpentane; open circles show result of test with xylene solvent.* (*f*) **2,3-***Benzanthracene (Grade* II*).* (*g*) *Oxidation product of phenanthrene (Grade* II*).*

* Curve *c* is repeated as *b* for comparison with *a*.

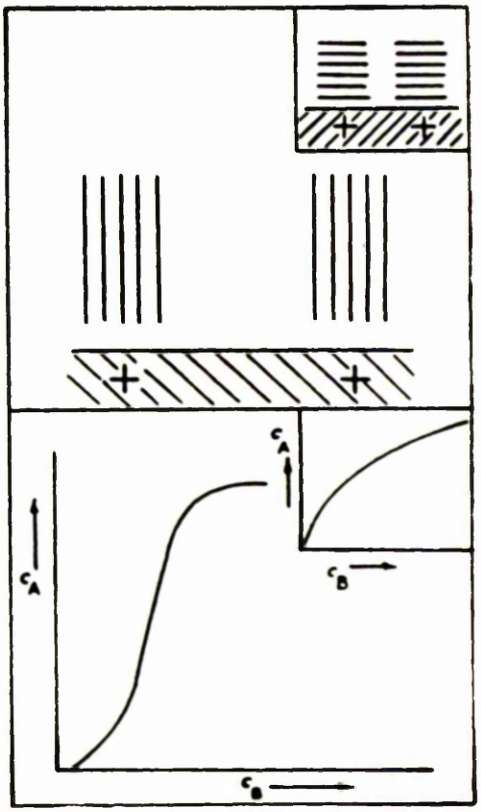


FIG. 13. *Adsorption mechanism of aromatic hydrocarbons on alumina.*

Top: *Showing suggested stacking of isolated clusters of planar aromatic molecules (end-on) at* Al^+ *atoms exposed at the surface.*

Bottom: *Adsorption isotherm obtained, characteristic of end-on adsorption*

Insets: *Alternative stacking (not favoured) and corresponding isotherm.*

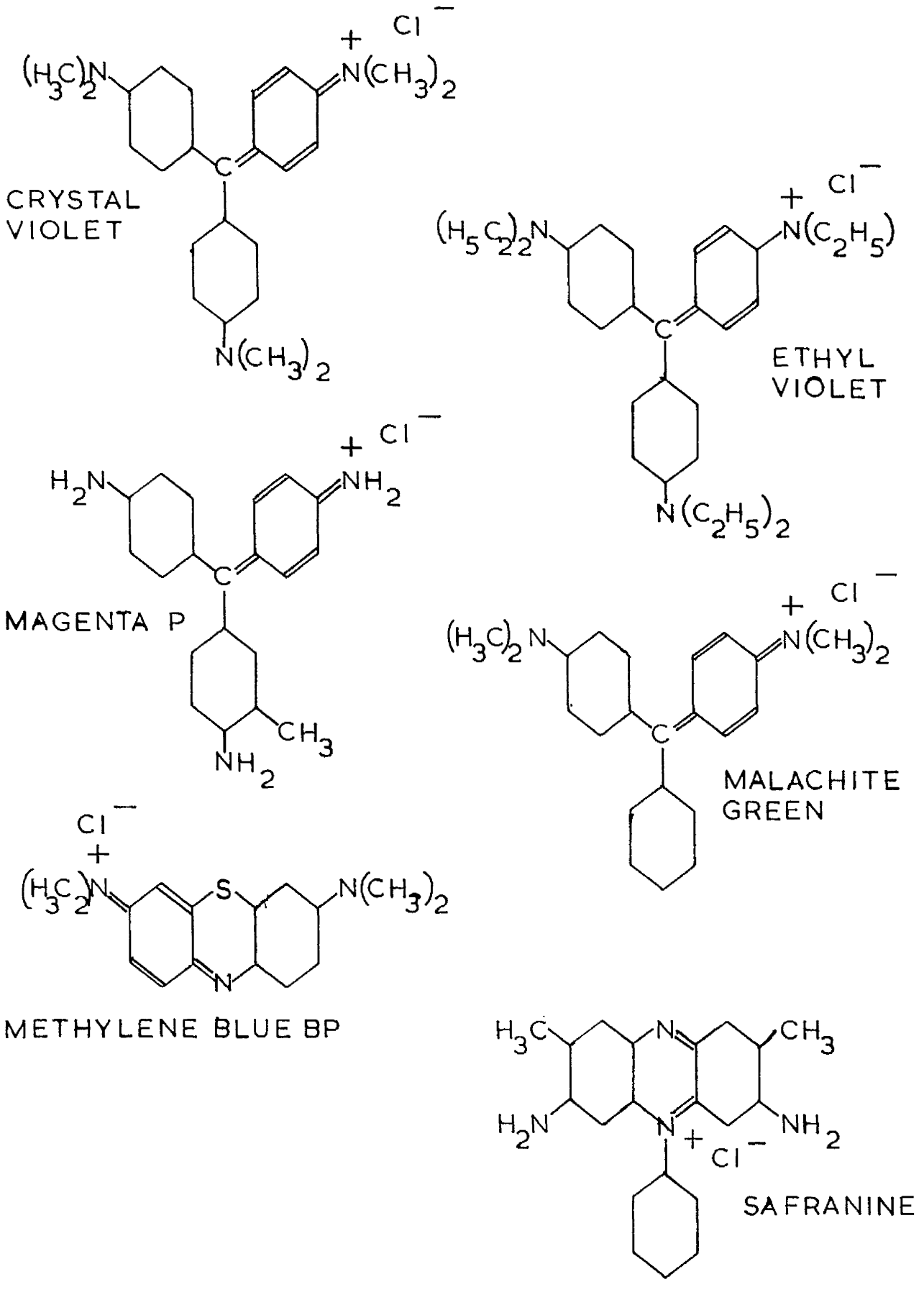


Fig. 16. Structural formulae for the cationic dyes used.

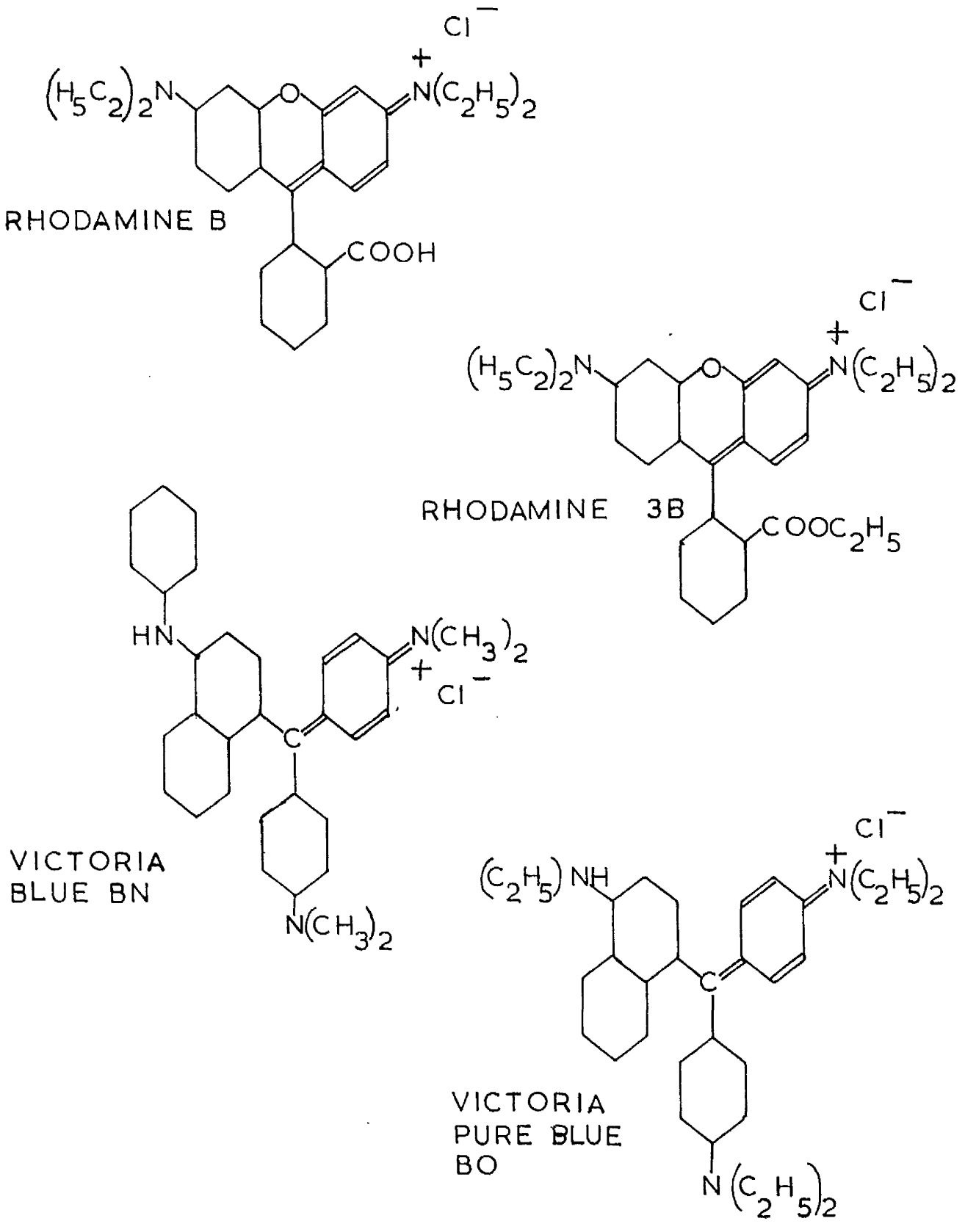


Fig. 17. Structural formulae for the cationic dyes used.

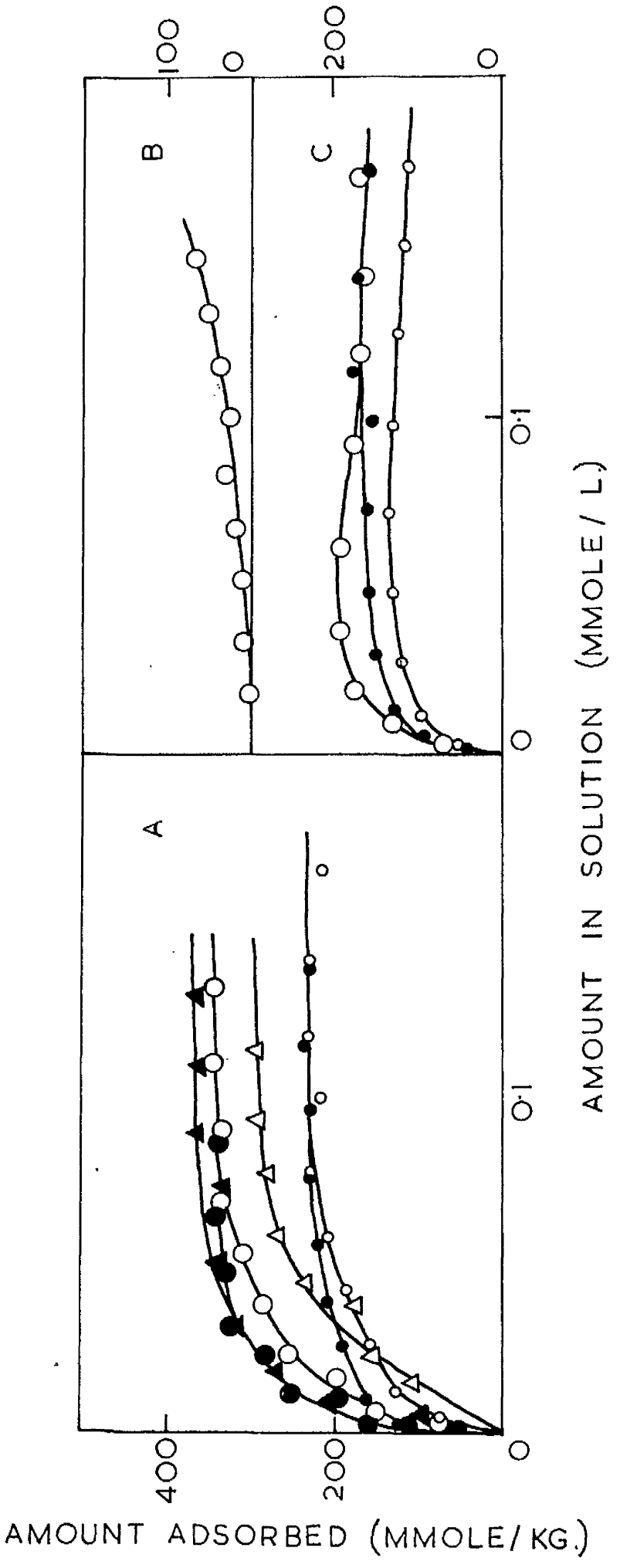


Fig. 18. Details given overleaf.

Legend to Fig.18. Equilibrium isotherms for the adsorption, under a variety of conditions, of cationic dyes on yeast cells subjected to various treatments.

A CRYSTAL VIOLET

Formalin-fixed cells (sample 1): ○ 19°C., suspension concentration = 0.252 g/l.; ● 50°C., suspension concentration = 0.384 g/l.

Boiled cells: ○ 20°C., suspension concentration = 0.241 g/l.; ● 50°C., suspension concentration = 0.343 g/l.

Untreated cells: △ 16°C., suspension concentration = 0.279 g/l.; ▲ 40°C., suspension concentration = 0.158 g/l.

B RHODAMINE B

Formalin-fixed cells (sample 1): ○ 14°C., suspension concentration = 0.151 g/l.

C SAFRANINE

Formalin-fixed cells (sample 1): ○ 18°C., suspension concentration = 0.374 g/l.

Boiled cells: ○ 17°C., suspension concentration = 0.391 g/l.; ● 49°C., suspension concentration = 0.527 g/l.

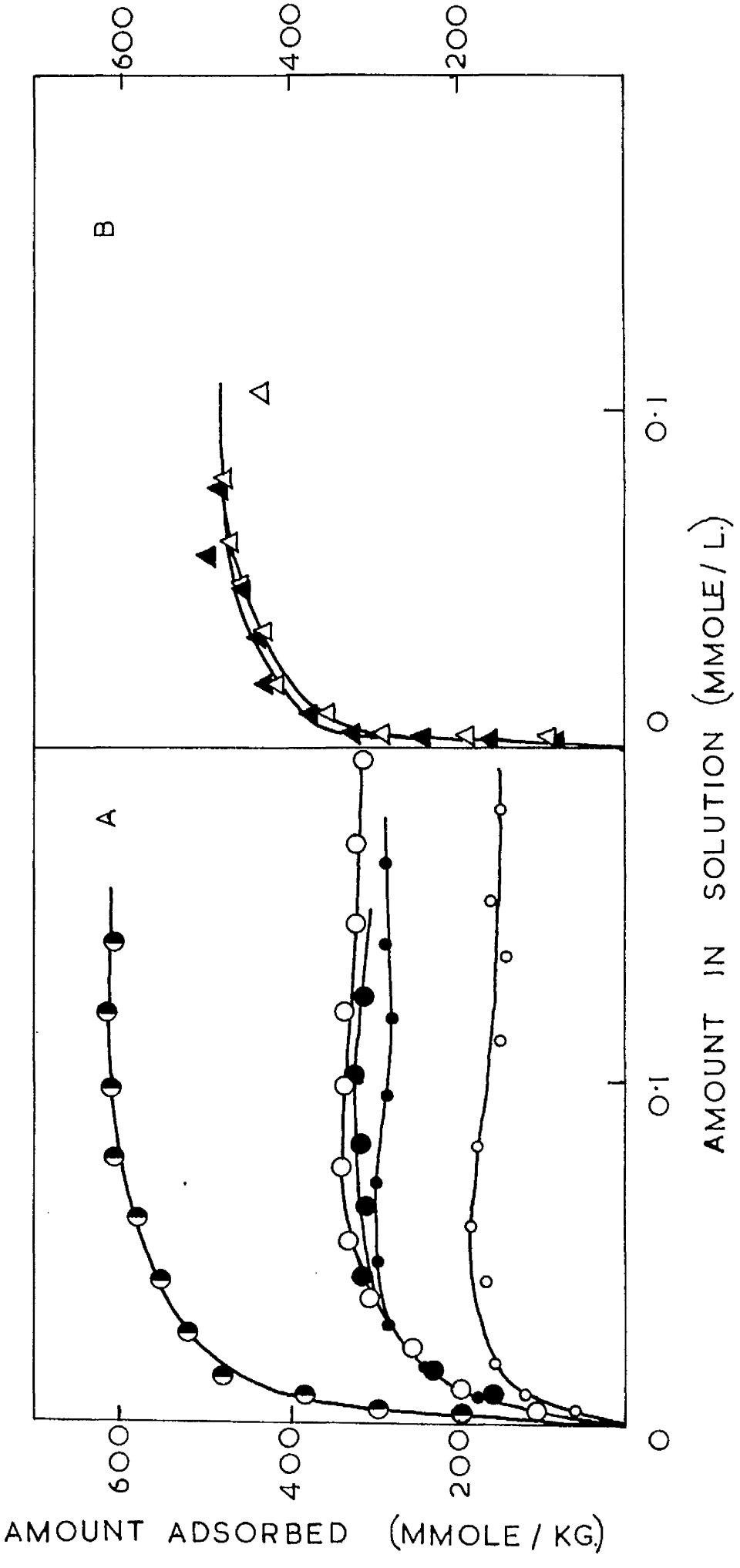


Fig. 19. Details given overleaf.

Legend to Fig.19. Equilibrium isotherms for the adsorption under a variety of conditions of cationic dyes on yeast cells subjected to various treatments.

A METHYLENE BLUE BP

Formalin-fixed cells (sample 1): O 21°C., suspension concentration = 0.184 g/l.; ● 43°C., suspension concentration = 0.208 g/l.; b 23°C., suspension concentration = 0.213 g/l.; buffered at pH9.

Formalin-fixed cells (sample 2); o 17°C., suspension concentration = 0.312 g/l.; ● 49°C., suspension concentration = 0.222 g/l.

B VICTORIA PURE BLUE BO

Untreated cells: Δ 19°C., suspension concentration = 0.275 g/l.; ▲ 40°C., suspension concentration = 0.208 g/l.

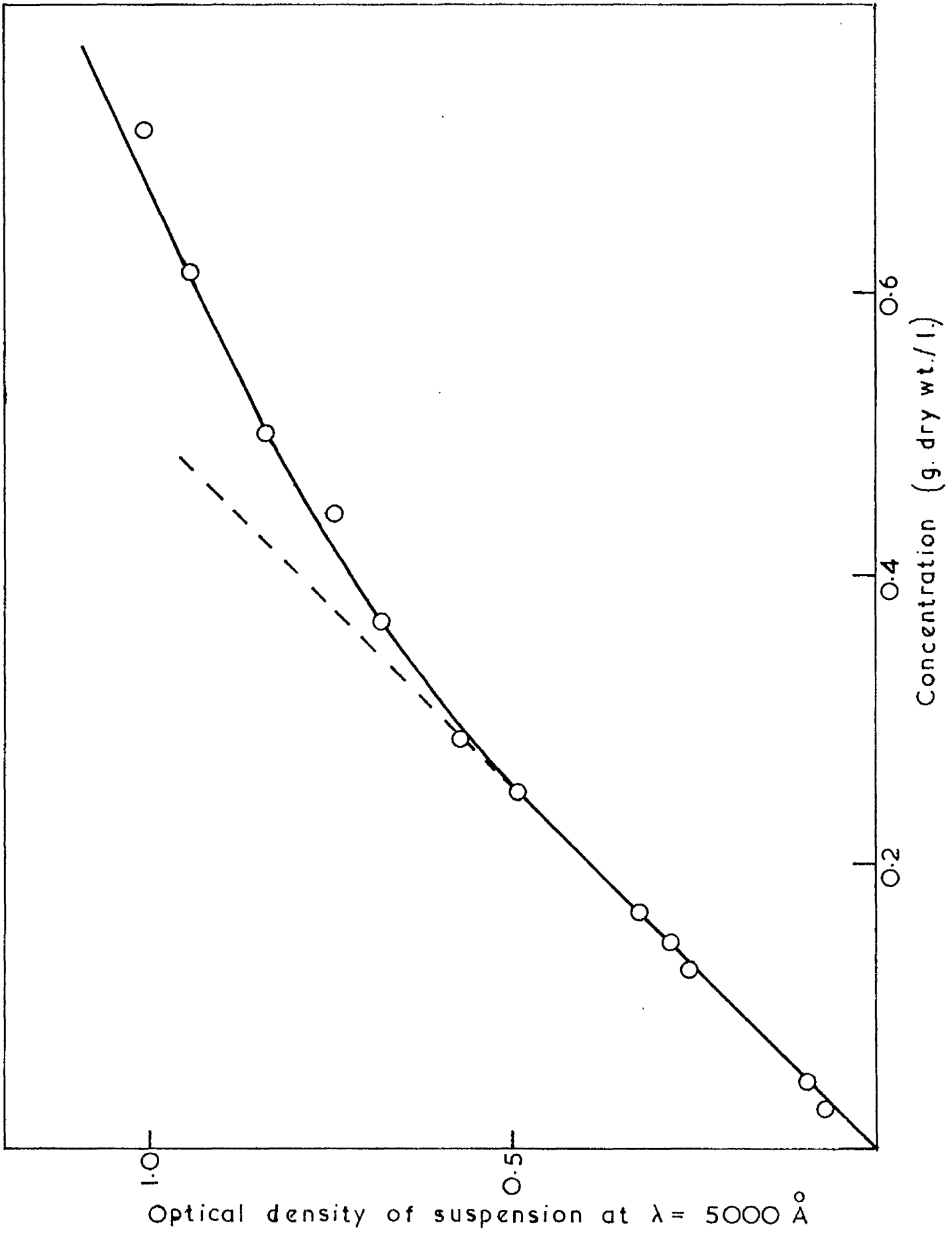


Fig. 20. Calibration graph for yeast suspensions.

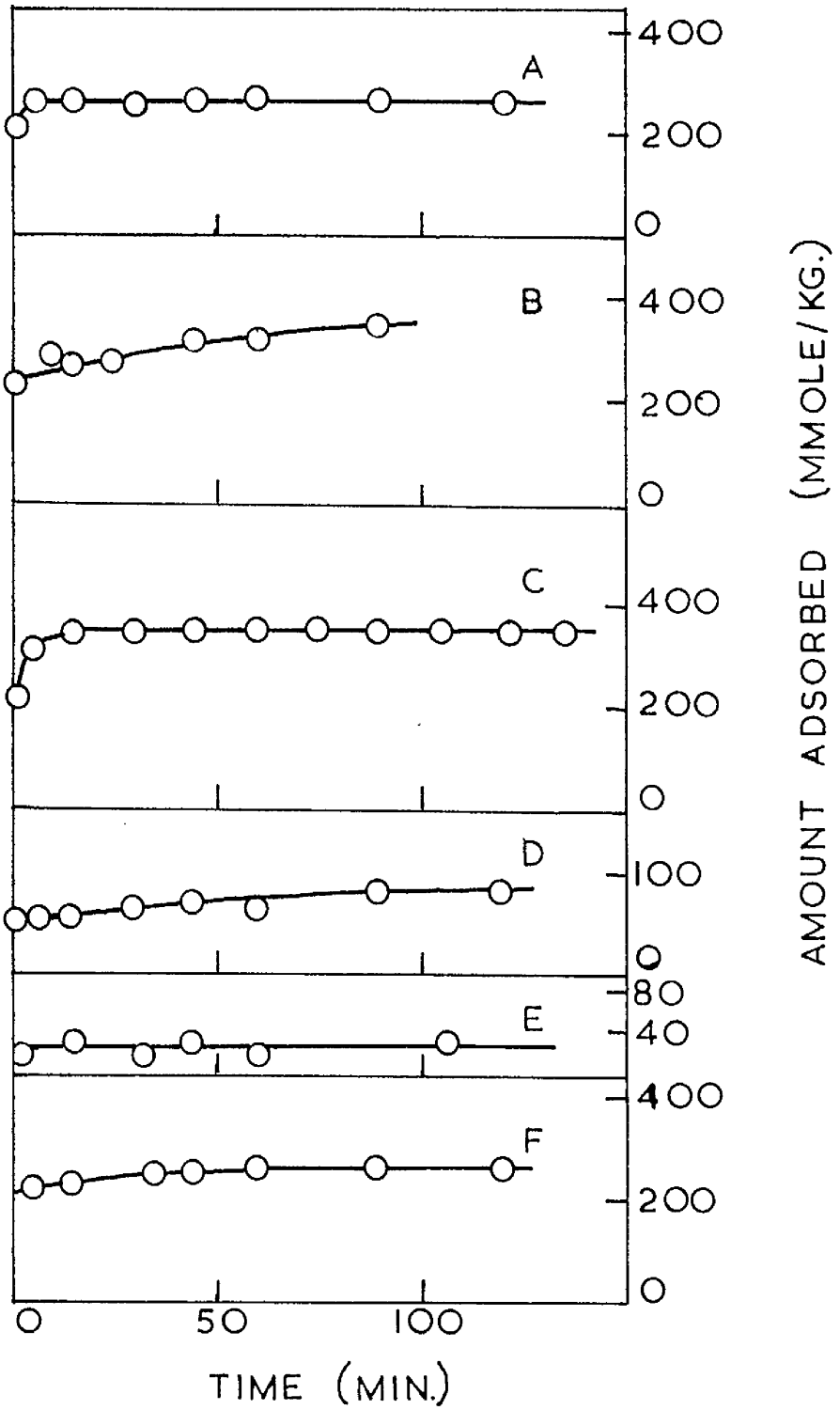


Fig. 21. Typical rate curves for the adsorption of cationic dyes on formalin-fixed yeast cells. A Victoria Pure Blue BO at 18°C.; B Methylene Blue BP at 18.5°C.; C Methylene Blue BP from a solution buffered at pH 9 at 21°C.; D Malachite Green at 17°C.; E Rhodamine B at 19°C.; F Safranine at 20°C.

N.b.: the yeast suspension concentrations used varied from 0.065 g.$l.^{-1}$ for B to 0.34 g.$l.^{-1}$ for C.

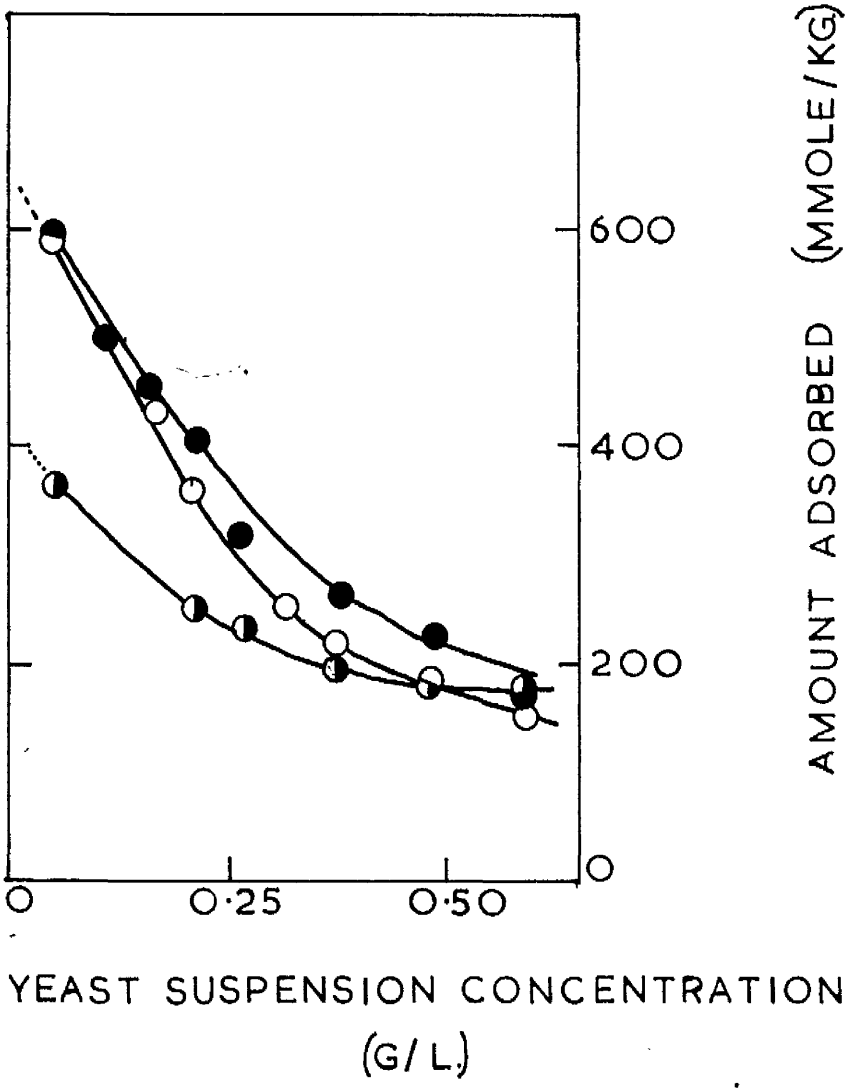


Fig. 22. Curves showing dependence of adsorption of cationic dyes upon yeast suspension concentration. ● Crystal Violet, 0.108 mmole/l., 53°C., ○ 20°C.; ◑ Safranine, 0.124 mmole/l., 20°C. Time, 2 hr. Formalin-fixed cells, sample 2.

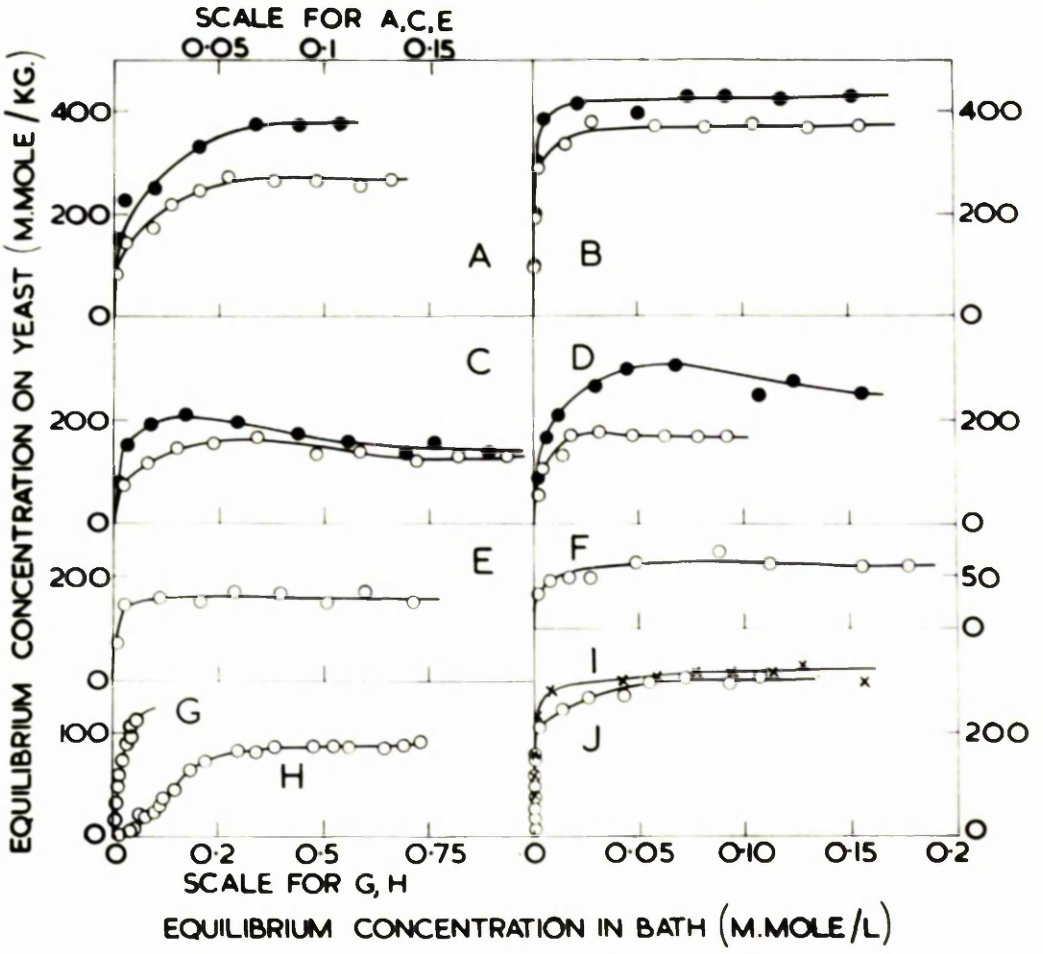


Fig.23. Equilibrium adsorption isotherms for cationic dyes on formalin-fixed yeast cells in suspension at a concentration of 0.246 ± 0.006 g. dry weight. litre^{-1} in the test mixture.

and 20± 0.5°C.; 50± 1°C.

A, Crystal Violet; B, Victoria Pure Blue BO;
C, Methylene Blue BP; D, Safranine; E, Magenta P;
F, Malachite Green; G, Rhodamine 3B;
H, Rhodamine B; I, Ethyl Violet; J, Victoria Blue BN.

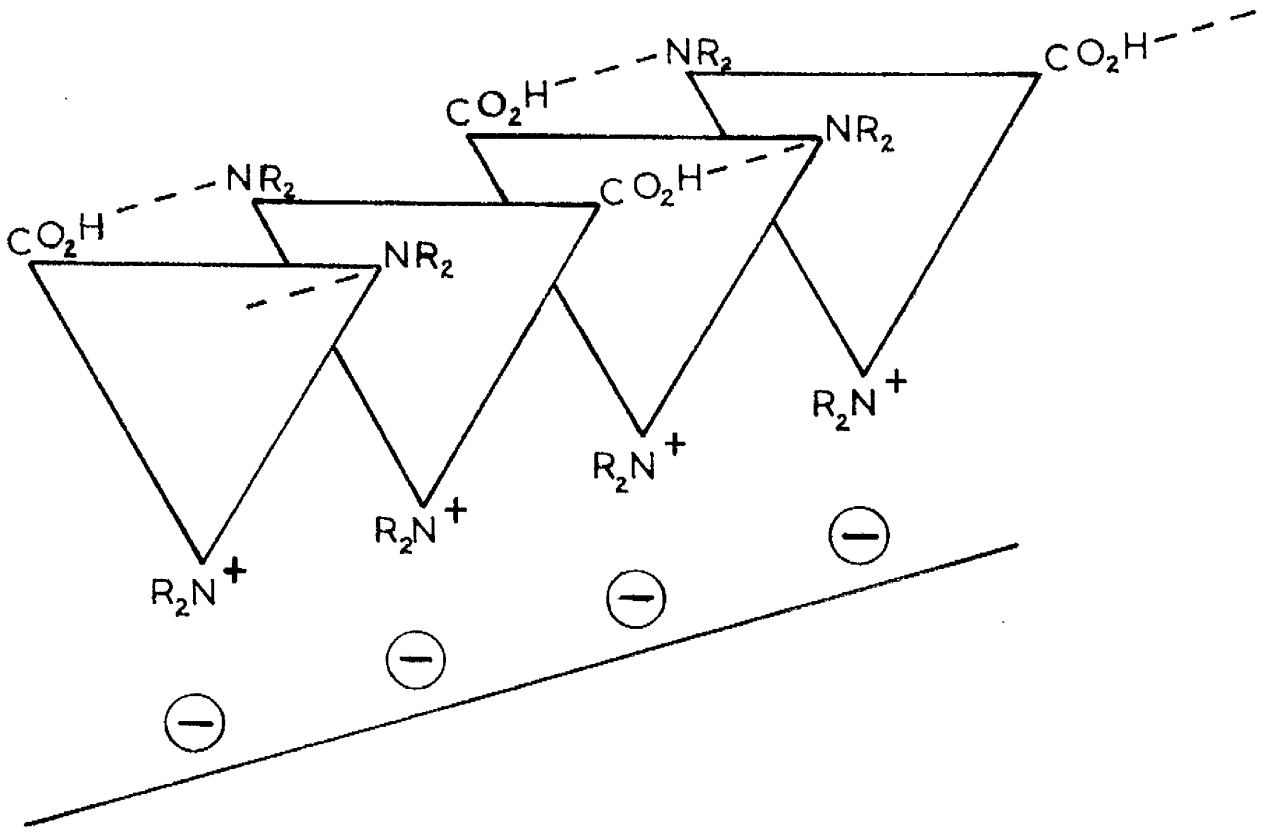


Fig. 24. Suggested orientation of Rhodamine B cations at anionic surfaces. Each carboxyl group is bonded to an amino-group in the adjacent cation. If only one such bond between each pair of ions is involved, there will be a chain-like structure in the monolayer, as shown. An alternative arrangement, however, would involve stacked dimers, in which the two cations oriented as shown here, are joined by two bonds, one at each end.

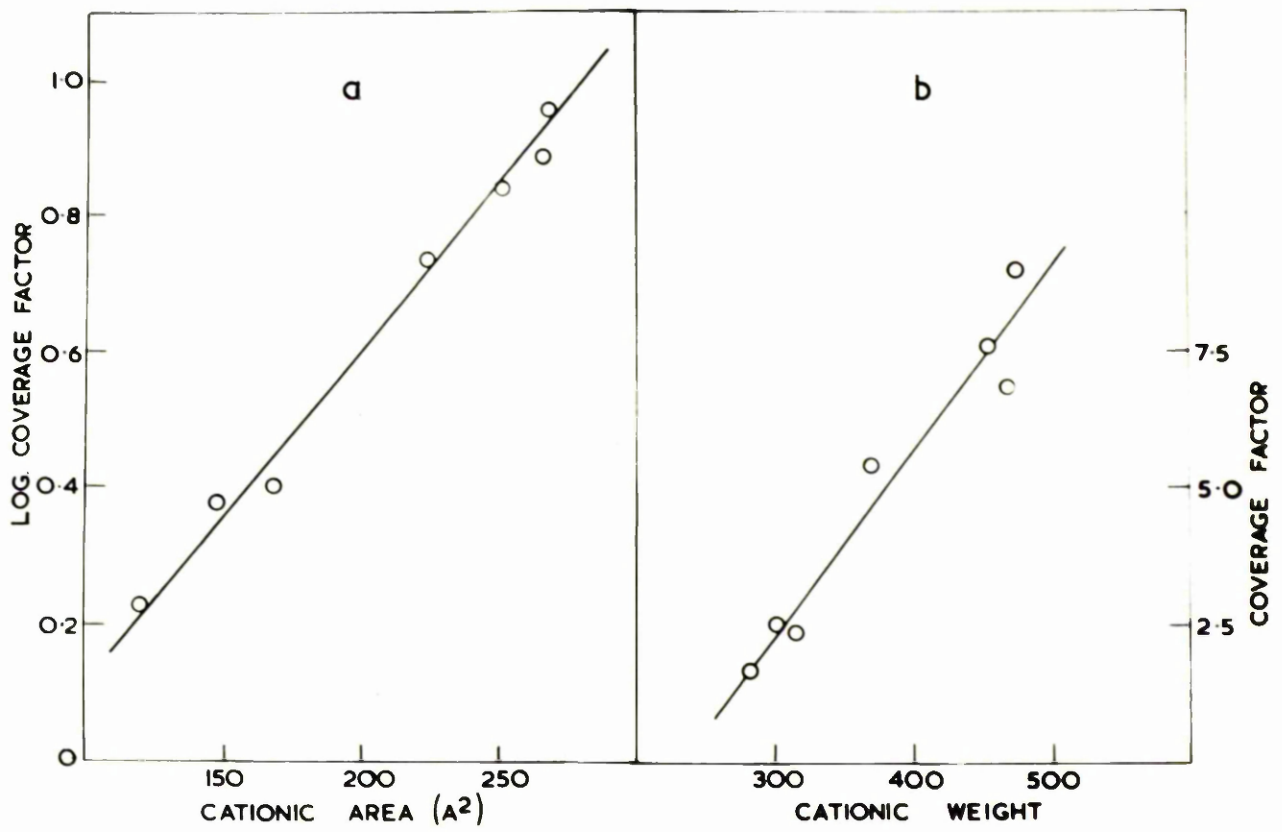


Fig. 25. Linear relationship between (a) log. coverage factor and projected area of dye cation (flat); and (b) coverage factor and dye cationic weight.

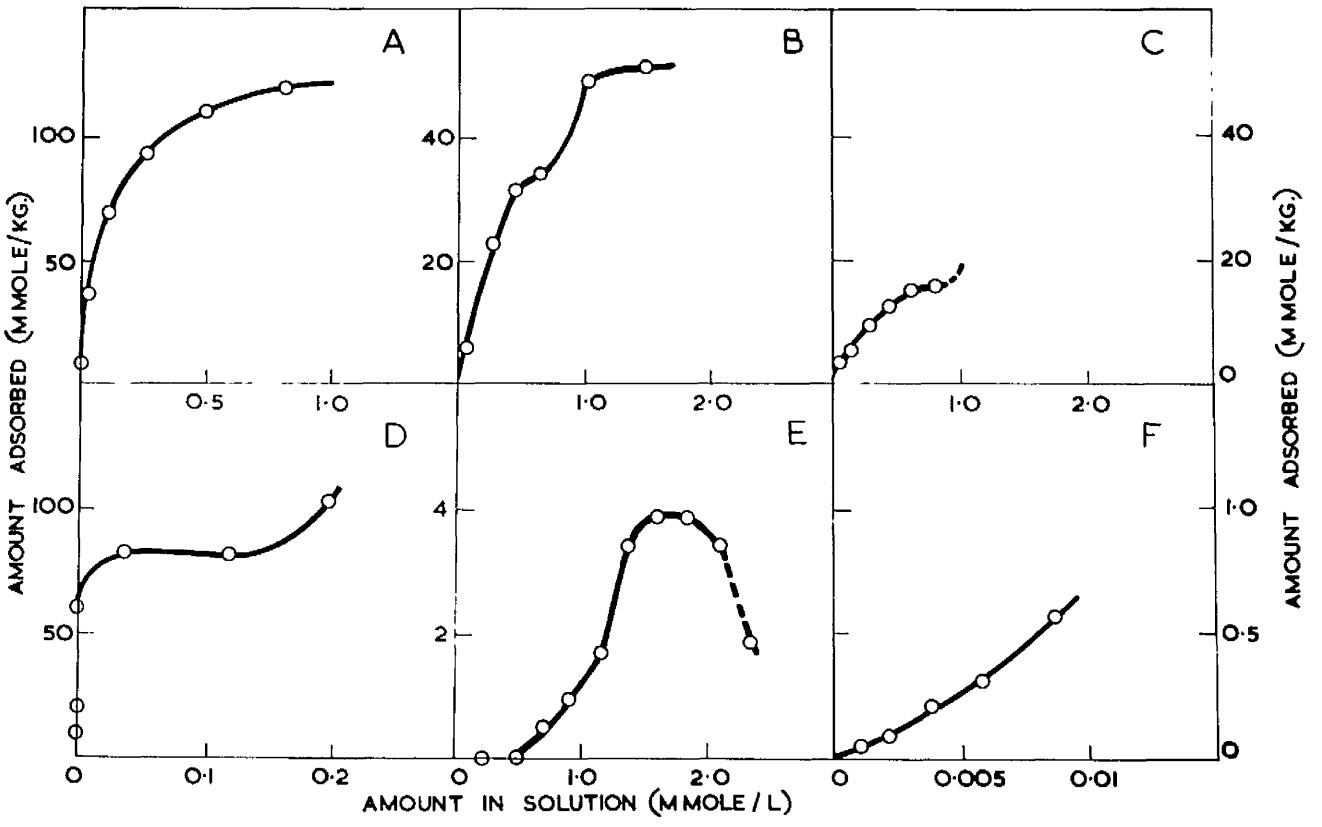


Fig.26. Equilibrium adsorption isotherms for Rhodamine B on various substrates (at room temperature).

A, Silk; B, Wool (unacidified); C, Wool (initial bath pH 2.5); D, Graphite; E, Chromatographic alumina; F, DNA (initial bath pH 2.5; weight of substrate refers to that of initial sodium salt).

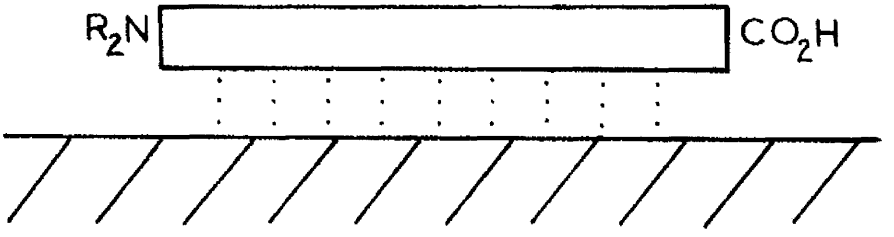


Fig. 27. Suggested orientation of Rhodamine B cations on graphite or protein surfaces. Here the surface attracts preferentially the aromatic nuclei of the dye cations, and thus there is flatwise orientation.

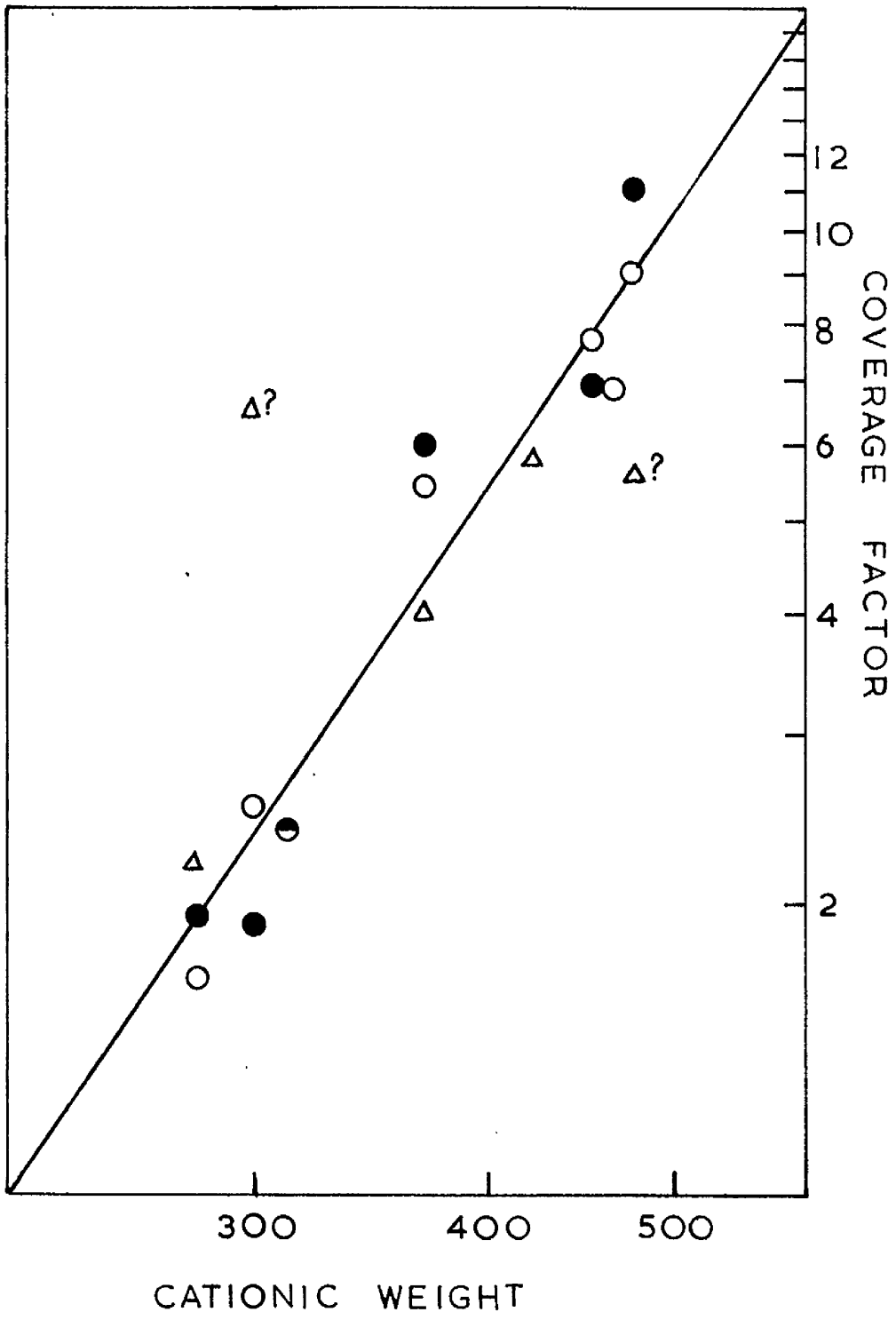
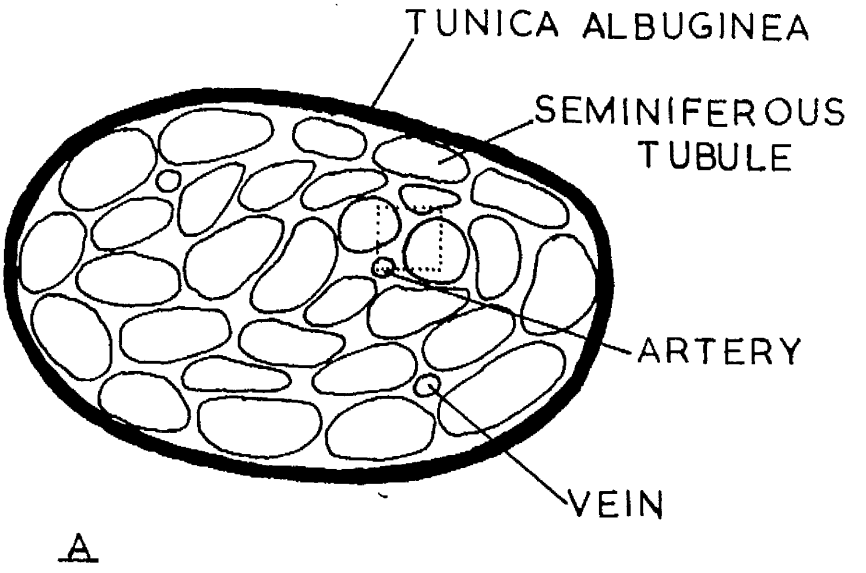
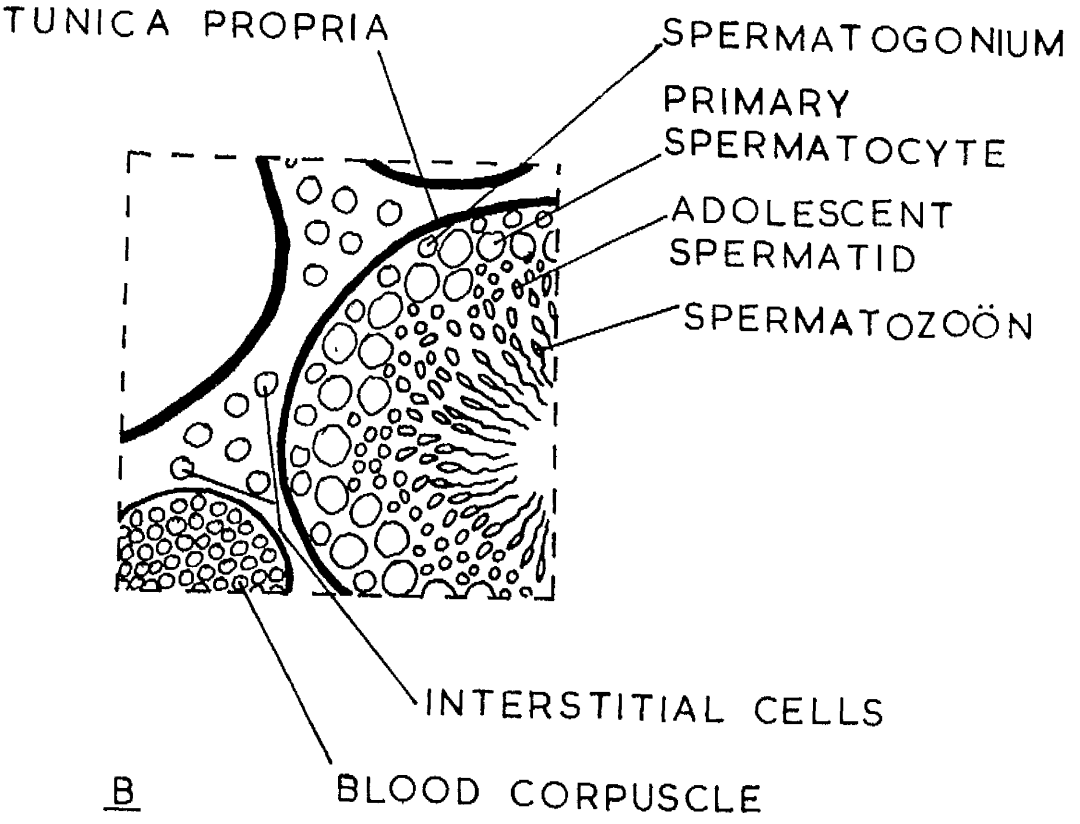


Fig. 28. Linear relationship between log. coverage factor and log. cationic weight of dyes adsorbed on three chemically distinct substrates. ○ Yeast; ● Chromatographic Alumina; Δ Graphite.



A



B

Fig. 29. The testis of the mouse in cross-section: A as seen under the low-power microscope; B the dotted square in A as seen under the high-power microscope.

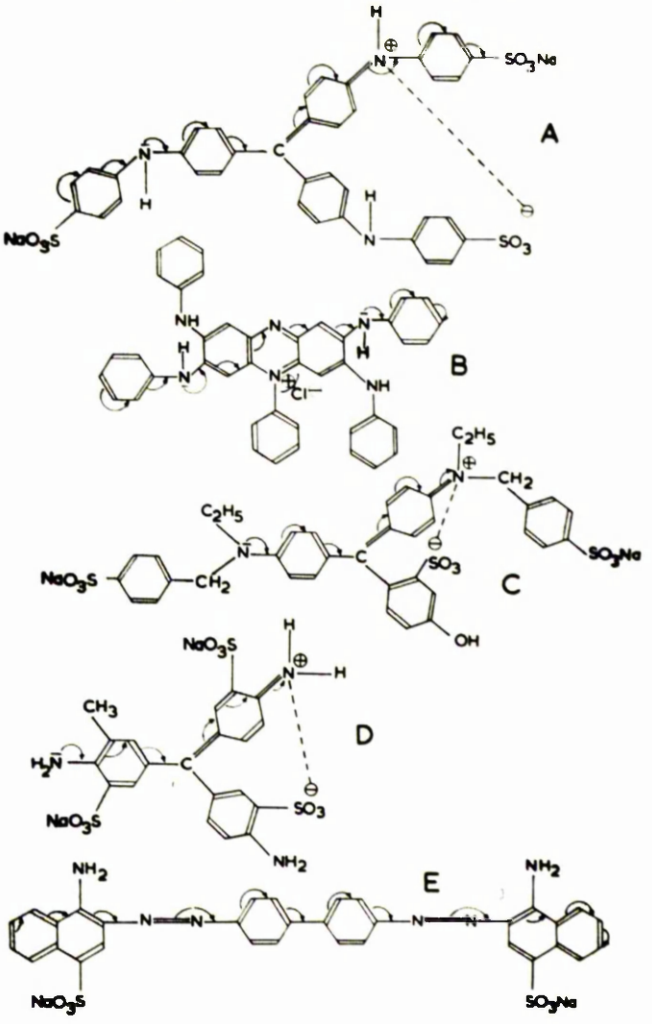


Fig. 30. Structural formulae for typical constituent molecules of some of the anionic dyes used. The longest possible conjugated chains are indicated by the curved arrows. A Methyl Blue; B the sulphonation product of this is a typical constituent of Nigrosine; C Fast Green FCF; D Acid Fuchsine; E Congo Red.

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