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Transport Processes in the Formation of the Cerebrospinal Fluid

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Contents

I. Introduction

The cerebrospinal fluid (CSF) occupies the ventricles, canals, and spaces surrounding the central nervous system (CNS). In man the total volume of the CSF is 140 ml, and about 25 ml of this is contained within the ventricles of the brain (see Fig. 1). CSF production amounts to 600 ml/day, and it is agreed that about two-thirds of this originates in the ventricles from the choroid plexuses. These plexuses are richly vascularized epithelial tissues and in man weigh approximately 2 g. There are two plexuses in each of the two lateral ventricles, and one in both the IIIrd and IVth ventricle. In experimental animals 55–65% of the CSF formed within the

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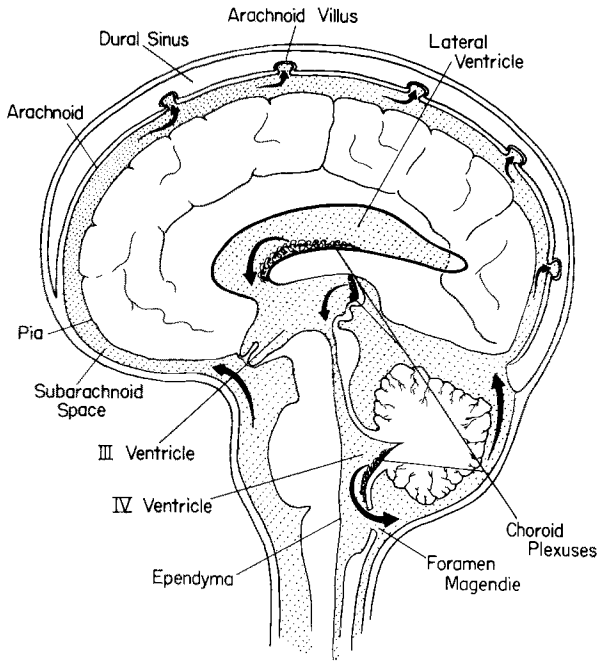

Fig. 1. A diagrammatic illustration of the human brain showing the relationship between the CSF and brain. Fluid secreted by the choroid plexuses in the lateral, IIIrd and IVth ventricles flows through the foramina into the cisterns and then over the surface of the brain. The CSF enventually reaches the blood in the dural sinus via the arachnoid villi. The CNS is enclosed by the arachnoid membrane and the dura (not shown in this diagram). A major function of the CSF is to provide physical support for the brain. (After *Milhorat,* 1972)

ventricles is produced in the IVth ventricle. CSF is secreted against a hydrostatic pressure of 15 cm H_2O, and once formed, the direction of bulk flow is from the lateral ventricles to the IVth ventricle where it passes via three exits (the foramina of Luschka and Magnedie) into the cisterns on the external surface of the brain. Fluid in the cisterns passes over the surface of the brain into the subarachnoid spaces, until it reaches the arachnoid villi, where it returns to the vascular system by way of the dural sinuses. The mechanism of CSF flow is unknown, but it is thought that pressure gradients brought about by fluid secretion, postural changes, and vascular pulsations are all involved. Ciliary activity contributes to the flow of CSF over the walls of the ventricles and canals, but the role of cilia in bulk CSF flow is probably insignificant.

CSF is separated from the neurones and glia of the CNS by the ependyma, which lines the ventricles and canals, and the pia, which covers the external surface of the brain and spinal cord. The choroid plexuses and

the arachnoid membrane separate the CSF from blood, and these two membranes form the so-called "blood-CSF barrier".

The composition of the bulk CSF closely resembles an ultrafiltrate of plasma (see Table 1). The protein concentration of CSF is less than 0.5%

Table 1. Composition of human CSF and plasma (in mM/kg H_2O)

	Plasma	CSF
Sodium	150	147
Potassium	4.6	2.9
Magnesium	3.2	4.5
Calcium	2.4	1.2
Chloride	99	113
Bicarbonate	27	23
Glucose	5.2	3.4
Glycine	0.22	0.02
pH	7.40	7.31
Osmolarity	289	289
Protein (mg/100 ml)	6800	28

These average estimates of CSF composition are taken from *Milhorat* (1972), *Dayson* (1967), and *Katzman* and *Pappius* (1973), and they represent values for lumbar CSF. There are regional differences in the composition of the CSF from the surface of the choroid plexuses to the arachnoid granulations.

of that in plasma, while the levels of the major cations and anions, the pH and osmolarity of the two fluids are quite similar. A major difference is that the concentrations of glucose and amino acids, e.g., glycine, are significantly lower in CSF than in plasma. A remarkable feature of the CSF is that its composition remains fairly constant in the face of wide fluctuations in plasma, e.g., CSF K remains between 2 and 4 mEq/liter even when plasma K is varied between 1 and 12 mEq/liter. Homeostatic mechanisms are known to maintain CSF K, pH, Mg, Ca, amino acids, catecholamines, organic acids and bases, and polyatomic anions concentrations at constant levels. The importance of these homeostatic mechanisms becomes clear in experiments where CSF composition is varied artificially (see *Winterstein*, 1961, and *Leusen*, 1972). Very small changes in CSF composition produce dramatic effects, e.g., respiratory ventilation rates increase four fold from the resting value upon lowering the CSF pH 0.05 U, and elevation of CSF glycine produces hypothermia, hypotension, and motor incoordination. These observations are not at all surprising in light of the fact that

the CSF is in free communication with the interstitial fluid of the brain parenchyma. Thus, changes in CSF composition produce changes in the fluids surrounding the neurones and synapses of the CNS, and this in turn influences the normal function of the brain. Obviously, it is important to identify the mechanisms responsible for controlling the chemical composition of the CSF in order to achieve a complete understanding of the operation of the CNS in health and disease.

II. Factors Influencing CSF Composition

The physiology of the CSF is complicated by the fact that the fluid comes into direct or indirect contact with many structures as it circulates through the ventricles and over the surface of the brain and spinal cord (see Fig. 1). Thus, the composition of the CSF in any region of the brain is directly related to the transport processes occurring at all the membranes exposed to the CSF. The relationships between the fluid compartments of the brain are represented diagrammatically in Figure 2.

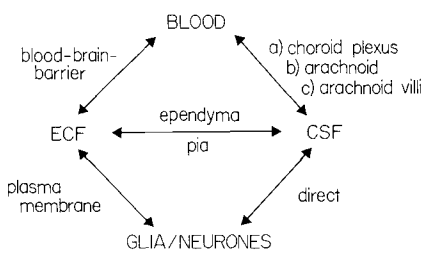

Fig. 2. A diagram summarizing the relations between the various fluid compartments in the CNS. The CSF communicates with the blood directly through the choroid plexuses, the arachnoid, and the arachnoid villi, and indirectly by way of the extracellular fluids of the brain parenchyma and the blood-brain barrier. The composition of the CSF is also influenced by the neurones and glia indirectly via the ECF and the ependyma and pia, and directly via those cellular elements that project into the CSF

CSF is in direct contact with blood across the choroid plexuses, arachnoid membrane, and the arachnoid villi. Fluid is actually secreted by each of the four choroid plexuses, and in addition, the choroidal epithelium is able to actively modify the composition of the ventricular CSF by transporting solutes back into blood. The arachnoid membrane also modifies the composition of the CSF as the fluid passes through the subarachnoid spaces and the arachnoid may actually secrete CSF. The CSF is in free communication with the extracellular fluid (ECF) of the brain parenchyma, and this in turn is in contact with neurones and glia, and with blood

through the "blood-brain barrier". There is mounting circumstantial evidence that the brain capillary endothelium is actually the "blood-brain barrier". Finally, there are some neurones and glia in direct contact with the CSF, particularly in the IIIrd ventricle, and these cells may exchange substances directly with the CSF.

This review is limited to a discussion of CSF production by the choroid plexuses and the regulation of CSF composition by the plexuses and the arachnoid membrane. Transport of substances across the "blood-brain barrier" has been reviewed recently (see *Partridge* and *Oldendorf,* 1977, and *Cserr* et al., 1975). Studies of the blood-brain barrier have been mainly limited to in vivo experiments on the extraction of solutes from blood by the brain (see *Rapoport,* 1976), but there have been preliminary reports on the use of isolated brain capillaries (*Sershen* and *Lajtha,* 1976; *Mrsulja* et al., 1976; and *Goldstein* et al., 1977). The exchange of solutes between the CSF and brain has also been reviewed (see *Cserr* et al., 1975), and it is concluded that there is free diffusion of ions and molecules between the CSF and ECF compartments. Nevertheless, specialized regions of the ependyma are involved in the secretion of hormones into the CSF, and this subject has been covered by *Rodriguez* (1976) and *Cserr* et al. (1975). Readers are referred to the following monographs and reviews for general coverage of the physiology and pathology of the CSF: *Davson* (1967), *Cserr* (1971), *Milhorat* (1972, 1976), *Katzman* and *Pappius* (1973), *Welch* (1975), *Netsky* and *Shungshotli* (1975), and *Rapoport* (1976).

III. CSF Secretion

A major proportion of the CSF secreted in the ventricles is produced by the choroid plexuses. In mammals there is one plexus in each of the four ventricles, but in amphibians they are only found in the IIIrd and IVth ventricles. Each plexus is a highly vascular tissue that projects into the ventricle, and during development they originate from the pia mater and blood vessels in subarachnoid space. Scanning electron micrographs of the ventricular surface of the choroideus rhombencephali are shown in Figure 3, and the histologic features of the same plexus are illustrated schematically in Figure 4. The surface is composed of highly complex, interlocking folds that are covered with a single layer of cuboidal epithelial cells. The epithelium rests upon a thin stroma of collagen fibers, fibroblasts, and blood vessels. The vascular elements include small arteries, arterioles, large venous sinuses, and capillaries. Blood flow to the plexuses amounts to about 3 ml/min/g, and this is about twice as high as the kidney (see *Csaky,* 1969). The capillaries are fenestrated unlike most other capillaries in the brain. It has been estimated that the epithelial cells account for 25–40% of the

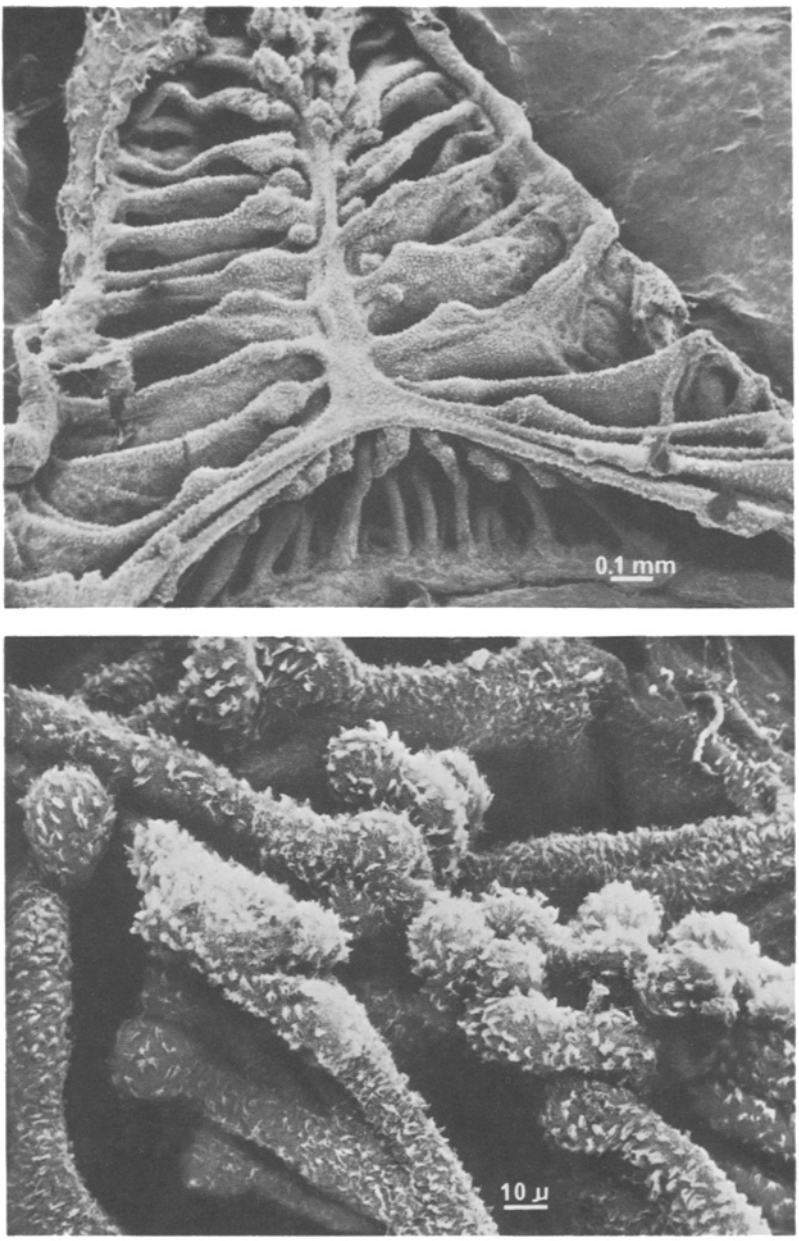

Fig. 3. Scanning electron micrographs of the ventricular surface of the posterior choroid plexus of the frog. Note that the structure becomes more complex toward the dorsal surface, and that the granular appearance of the low power micrograph is due to the clumps of cilia which project from each cell into the CSF. Taken from *Nelson* and *Wright* (1974)

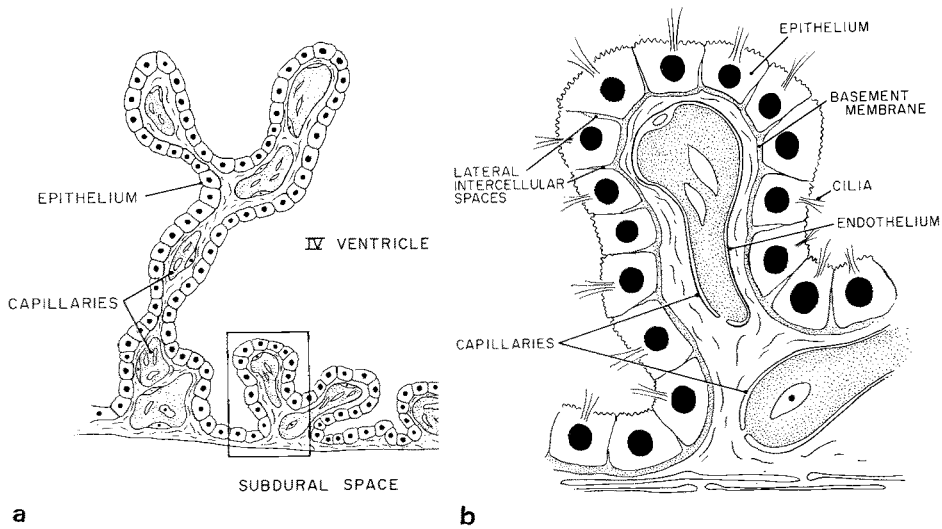

Fig. 4 a and b. Diagrams showing the major histologic features of the frog posterior choroid plexus. In the low power drawing the villi, which project into the CSF of the IVth ventricle, are covered with a single layer of cuboidal epithelial cells, and the core of these villi consists of a loose connective tissue containing numerous blood vessels. The higher power drawing shows an area of the plexus in more detail. It should be noted that the structure of the IVth ventricle choroid plexus in mammals is essentially identical to that in amphibians, but it is buried in the recesses of the traverse fissure of the cerebellum by the hind brain. (From *Wright,* 1972a)

total number of cells in the plexus, and that they occupy 65–95% of the total cell volume (*Quay,* 1966).

A transmission electron micrograph of the epithelium is shown in Figure 5. The cells are cuboidal in shape with a large, central nucleus and irregular, highly packed microvilli on the apical surface of the tissue. In many species, particularly during development, the epithelium is heavily ciliated. In the mature frog 20–40 cilia, each about 20 μ long, project from each cell into the CSF. These cilia beat at a frequency between 5 and 30 cps, and this ciliary motion promotes the flow of CSF over the surface of the epithelium (*Nelson* and *Wright,* 1974).

The cytoplasm contains numerous mitochondria and these appear to be concentrated at the apical end of the cell. Of the total number of mitochondria in the plexus, 80–95% are in the epithelium, and these account for the very high oxygen consumption of the epithelium (*Quay,* 1963; *Quay,* 1960; *Krebs* and *Rosenhagen,* 1931).

Each cell is joined to its neighbors by tight junctions, zonulae occludendes, at the apical surface. In this region the lateral faces of contigious epithelial cells fuse to form girdles around each cell. The junctions prohibit the passage of large molecules (proteins) between the lateral intercellular

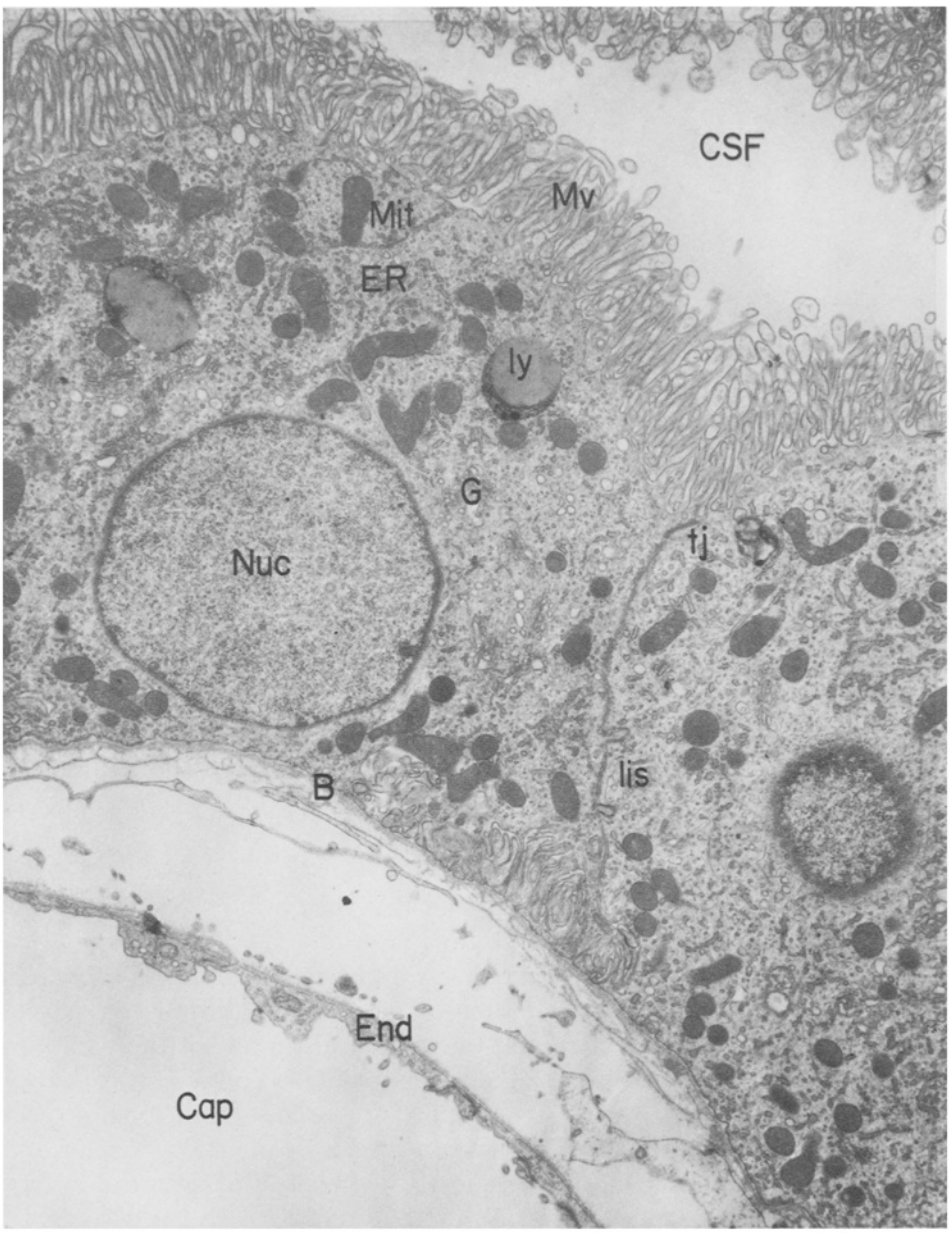

Fig. 5a. An electromicrograph of the rat choroid plexus (x 12,000). The cuboidal epithelial cells separate the CSF from the blood in the plexus capillaries (*Cap*). The cells rest upon a thin basement membrane (*B*) and are joined together at the apical surface by so-called tight junctions (*tj*) which form a girdle around the circumference of each cell. The lateral intercellular spaces (*lis*) separate each cell from the tight junctions to the basement membrane. The apical surface of the epithelium is composed of tightly packed, irregular microvilli (*Mv*), the large nucleus (*Nuc*) is centrally placed in the cytoplasm, and mitochondria (*Mit*) are more numerous near the apical membrane than other parts of the cell. The endoplasmic reticulum (*ER*) is well-developed and the Golgi apparatus (*G*) is in a supranuclear position. Lysosomes (*ly*) are also prominent in the cytoplasm. The endothelium (*End*) of the plexus capillaries is of the fenestrated type. (Taken with kind permission from *Peters* et al., 1976)

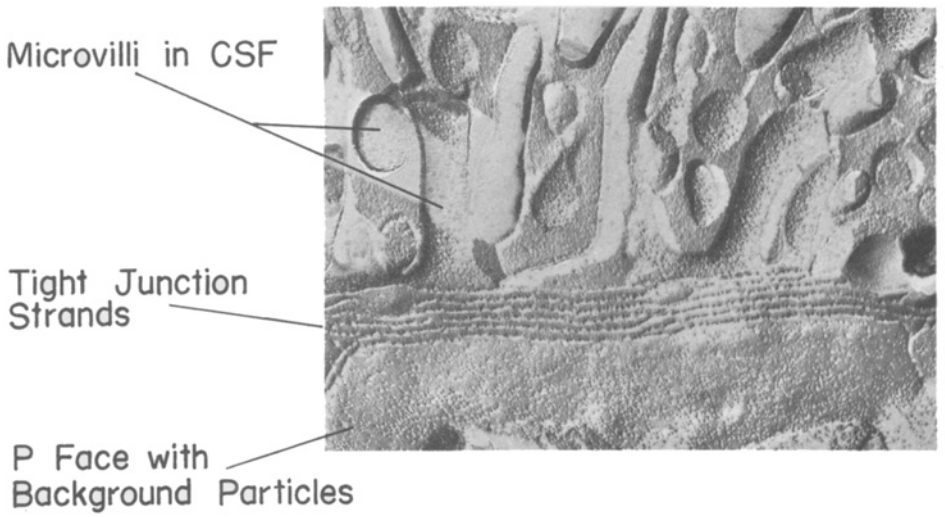

Fig. 5b. Freeze-fracture replica of a tight junction in the choroid plexus epithelium of the mouse IVth ventricle choroidal epithelium (x 47,000). The microvilli project into the CSF in the IVth ventricle, and the strands in the tight junction run parallel with the apical surface of the cell. The strands, or ridges, are composed of particles which are interrupted in places. The micrograph was kindly provided by Dr. *Milton W. Brightman*

spaces and the CSF, but there is substantial evidence to show that they are leaky to small ions (*Wright*, 1972a, 1974a; *Castel* et al., 1974; *Bouldin* and *Krigman,* 1975). Thus, the choroid plexus belongs to a group of epithelia with "leaky" so-called tight junctions; other members of the group include the gall bladder, intestine, and renal proximal tubule. In other epithelia, notably the frog skin and urinary bladder, junctions are tight even to small ions such as Na and Cl. Freeze-fracture studies of junctions in epithelia reveal a network of intramembraneous strands or fibrils (see Fig. 5b), and it has been suggested that the number of strands is correlated with the permeability of the junction. However, a survey of different epithelia under a variety of experimental conditions does not support this hypothesis (e.g., *Martinez-Palomo* and *Erlij,* 1975, and *Møllgard* et al., 1976). The physiologic implications of "leaky" tight junctions are considered below, and a more detailed account of the structure of the choroid plexus is given by *Peters* et al., 1976.

A. Methods of Study

There are four major approaches to the study of solute and water transport across the choroidal epithelium: 1) in vivo ventriculo-cisteral perfusion (see *Cserr,* 1971, and *Rapoport,* 1976, for details of this approach),

2) in vivo isolation of a choroid plexus (e.g., *Miner* and *Reed,* 1972), 3) extracorporeal perfusion of the isolated plexus (*Pollay* et al., 1972a, 1972b, 1973; *Segal* and *Pollay,* 1977), and 4) mounting the IVth ventricle plexus between Ussing type flux chambers (first introduced by *Patlak* et al., 1966). I have developed an in vitro preparation of the frog posterior choroid plexus, and this model system offers a number of advantages: 1) frogs provide convenient, simple in vitro preparations that are viable for many hours, e.g., active ion transport rates are stable for up to 8 h, and they tolerate a very wide range of experimental conditions that are not possible with mammalian in vivo or in vitro preparations, and 2) the ultrastructure of the plexus, the composition of the CSF, and the regulation of CSF composition in the frog are essentially identical to those in higher animals. When the posterior choroid plexus is mounted between flux chambers, it is possible to measure 1) unidirectional fluxes across the epithelium, 2) unidirectional fluxes across the apical surface of the epithelium, 3) the steady-state concentrations of solutes within the epithelium, 4) transcellular electric potentials and conductances, 5) intracellular electric potentials, and 6) water fluxes across the plexus (for experimental details see: *Wright,* 1972a, 1974a, and *Wright* et al., 1977). This in vitro approach has been largely responsible for the tremendous progress in our understanding of epithelial tissues such as the frog skin, urinary bladder, gall bladder, and intestine, and when applied to the choroid plexus offers unique insights into mechanisms of CSF secretion. This review provides a forum for the progress made with this model system over the last decade.

B. Passive Ion Permeation

With identical Ringer's solutions on each side of the frog choroid plexus, the transepithelial potential difference (p.d.) was less than 1 mV, and the electric resistance was 200 Ω cm^2 (*Wright,* 1972a). Similar results have been obtained for shark and mammalian plexuses (*Patlak* et al., 1966; *Wright,* 1972a; *Welch* and *Akari,* 1975; and *Eisenberg* and *Welch,* 1976). Passive ion permeability coefficients have been obtained from both diffusion potential and radioactive flux measurements. In the frog, the cation permeability sequence was P_{Ca} (1.32) > P_K (1.23) $\sim$ P_{Rb} (1.23) > P_{Cs} (1.02) $\sim$ P_{Na} (1) > P_{Li} (0.86), and the absolute sodium permeability coefficient was 5 x 10^{-6} cm s^{-1} (*Wright,* 1972a). The sodium conductance of the plexus was found to be sensitive to the drug 2,4,6-triaminopyrimidine (*Moreno,* 1975; *Eisenberg* and *Welch,* 1976; and unpublished observations). Similar results were obtained for the cat choroid plexus by *Welch* and *Akari* (1975). In the case of anions, the permeability sequence was P_I (1.39) > P_{Br} (1.29) > P_{SCN} (1.02) $\sim$ P_{Cl} (1) $\sim$ P_{HCO_3} (1) > P_{TcO_4} (0.54),

and the absolute chloride permeability coefficient was 8×10^{-6} cm/s^{-1} (*Wright,* 1974a). There was no evidence for chloride exchange diffusion across the plexus as judged by the fact that the Cl flux ratios were close to those predicted by the Ussing flux ratio equation (*Wright,* 1972a). The ion permeation sequences belong to those predicted by *Eisenman's* theory of ion selectivity (see *Diamond* and *Wright*, 1969; *Wright* and *Diamond,* 1977; *Wright,* 1977a).

There is considerable evidence that the major pathway for passive ion permeation across the plexus is via the extracellular shunt path, i.e., tight junctions and lateral intercellular spaces (*Wright,* 1972a, 1974a; *Castel* et al., 1974; and *Bouldin* and *Krigman,* 1975), and there is indirect evidence that diffusion along the lateral intercellular spaces may rate limit passive permeation under certain circumstances (see *Smulders* et al., 1972; *Bindslev* et al., 1974). In tight junctions, permeation was controlled by fixed charges with a pK of 4 and an isoelectric point of 3 (*Wright* and *Prather,* 1970). The mechanism of ion permeation across the tight junctions of the choroid plexus appears to be very similar to that in other leaky epithelia (see *Moreno* and *Diamond,* 1975).

C. Nonelectrolyte Permeability

The passive permeability of the choroid plexus to nonelectrolytes resembled that seen in other epithelia and in single cells (*Wright* and *Prather,* 1970; *Wright* and *Pietras,* 1974). In general, the permeability of a solute was directly proportional to the solute's oil:water partition coefficient, the higher the partition coefficient the higher the permeability coefficient (Table 2). Detailed analysis of permeability measurements on the plexus show that: 1) P's ranged from about $1 - 1000 \times 10^{-7}$ cm/s (Table 2), 2) the membrane lipids of the plexus were more "fluid" than those in other cells and tissues, and 3) small molecules, such as urea and water, permeated across the plexus more rapidly than expected from their oil: water partition coefficients. Rapid permeation of the small molecules may have been in part due to the presence of "pores", but it was in part due to high permeation of these substances through membrane lipids.

1. Water

The osmotic water permeability (L_p) of the frog choroid plexus was 2.2×10^{-3} cm/s (*Wright* et al., 1977), while that for the rabbit plexus was 4.0×10^{-3} cm/s (*Welch* et al., 1966). These values are similar to those obtained for the frog urinary bladder (see *Wright,* 1977b). The resistance to osmotic flow across the plexus increased when the lateral intercellular

Table 2. Permeability of frog choroid plexus to nonelectrolytes

Nonelectrolyte	K_{oil}	P (cm/s x 10^7)
Sucrose	1×10^{-6}	16
Mannitol	1.2×10^{-6}	21
Erythritol	3×10^{-5}	49 *
Glycerol	7×10^{-5}	69
Urea	1.5×10^{-4}	120
Water	7×10^{-4}	680
Acetamide	8.3×10^{-4}	125
1,2-propanediol	1.7×10^{-3}	98
1,4-butanediol	2.1×10^{-3}	96
Nicotinamide	5×10^{-3}	171 *
N-butyramide	1.7×10^{-2}	166
Iso-butyramide	1.4×10^{-2}	136
1,7-heptanediol	3.1×10^{-2}	283
Antipyrine	3.2×10^{-2}	242
Caffeine	3.3×10^{-2}	432

The P values are quoted as the means estimated from an average of 30 estimates. The standard errors of the means were less than 10% of the means, except for those two cases marked by asterisks where the errors were less than 20% of the means. All permeability coefficients were corrected for the presence of unstirred layers. The permeability coefficients were arranged according to the solute olive oil partion coefficients. (Taken from *Wright* and *Pietras*, 1974).

spaces collapsed, e.g., when 100 mM sucrose was added to the CSF the L_p dropped from 2.2×10^{-3} to 1.6×10^{-3} cm/s, and this was accompanied by an increase in the electric resistance of the tissue (see *Wright* et al., 1977). This showed that the lateral spaces are a common pathway for ion and water permeation across the choroid plexus, and that flow across the plexus decreased when the resistance of the spaces increased.

The diffusional water permeability coefficient for the frog choroid plexus was 0.07×10^{-3} cm/s (*Wright* and *Pietras*, 1974), and unpublished experiments showed that this was unaffected by the presence of the amphibian antidiuretic hormone arginine (vasotocin), phloretin, and PCMBS (p-chloromercuribenzenesulfonate).

2. Sugars

Sugars permeated across the frog choroid plexus by facilitated diffusion (*Prather* and *Wright*, 1970). Those sugars with a pyranose ring in the

D-glucose chair conformation were able to use the transport carrier, and the affinity of the sugar for the carrier was related to the number of hydroxyl groups on the sugar that were in the equatorial plane of the ring. The sugar with the most hydroxyl groups in this conformation, i.e., D-glucose, had the greatest affinity for the transport system. I have no evidence for active sugar transport either across the plexus or into the epithelium, even though there are reports of active sugar accumulation in mammalian plexuses (*Csaky* and *Rigor,* 1964; and *Segal* and *Pollay,* 1977). It is reasonable to conclude that the lower concentration of glucose in the CSF with respect to blood (Table 1) is due to the facilitated exchange of the sugar between blood and CSF and the high rate of glucose metabolism in the brain (see also *Rapoport,* 1976).

3. Amino Acids

The concentration of amino acids in the CSF is generally lower than in blood (Table 1), and it is generally accepted that amino acids are actively transported out of the CSF into blood (see *Rapoport,* 1976). It has long been maintained that the choroid plexuses were the sites of the active transport step, but this does not appear to be the case in the frog. Although the frog plexus, like mammalian plexuses, could accumulate neutral, basic, and acidic amino acids within the epithelium, there was no net transport of these solutes across the plexus (*Wright,* 1972b). It appears that the amino acids were unable to diffuse from the epithelium into the blood across the basolateral cell membrane. In the frog, it is the arachnoid membrane that is a more reasonable candidate for the active transport of amino acids from CSF to blood (*Wright,* 1974b).

The actual permeability of the frog choroid plexus to amino acids was quite low, i.e., 4×10^{-6} cm/s, and so it is the slow leak of amino acids into the CSF from blood combined with an active transport mechanism in the arachnoid and brain metabolism that accounts for the low concentration of amino acids in the CSF.

D. Active Ion Transport

To approach the question about the mechanism of CSF secretion across the choroid plexus, I first measured the unidirectional fluces of Na, K, Cl, and Ca in the absence of appreciable electrochemical potential gradients across the epithelium (*Wright,* 1972a). The results are summarized in Table 3, where it can be seen that there was a net transport of sodium from blood to CSF of 1 μmol/cm^2/h. Chloride was also transported in the same direction, but in this case the rate of transport was only about 60%

Table 3. Ion transport across the frog choroid plexus. Flux (in μmol cm^{-2} h^{-1})

	J_{sv}	J_{vs}	J_{net}
Sodium	2.9 ± 0.2 (22)	1.9 ± 0.2 (17)	+ 1.0
Chloride	2.7 ± 0.1 (36)	2.1 ± 0.1 (36)	+ 0.6
Potassium	0.09 ± 0.01 (6)	0.12 ± 0.01 (5)	− 0.03
Calcium	0.078 ± 0.015 (6)	0.042 ± 0.010 (7)	+ 0.036

All experiments were carried out in bicarbonate Ringer's solution gasses with 5% CO_2/ 95% O_2. The unidirectional fluxes were obtained from steady-state radioactive tracer fluxes, the errors are quoted as the standard error of the mean, and the number of experiments are given in parentheses. J_{vs} = unidirectional flux from the CSF to the serosal (vascular) side of the epithelium, and J_{sv} is the flux in the opposite direction. Positive net fluxes mean secretion into the CSF. The composition of the Ringer's in both the CSF and serosal compartments were identical, and the spontaneous p.d. across the plexus was less than 1 mV, i.e., the fluxes were measured in the absence of appreciable electrochemical potential gradients across the tissue. These results were taken from *Wright* (1972a, 1977).

of that observed for sodium. There is circumstantial evidence that bicarbonate transport from blood to CSF accounts for the difference between sodium and chloride secretion: 1) bicarbonate was the only additional ion present in the Ringer's solution to any significant amount, 2) sodium transport decreased about 50% when bicarbonate was omitted from the Ringer, 3) glycodiazine, a nonvolatile, lipid soluble buffer, mimicked the effect of bicarbonate on the sodium pump, and there was a net secretion of this buffer anion across the plexus (*Wright*, 1977c), and 4) the composition of the freshly secreted CSF was consistent with this hypothesis (*Wright* et al., 1977). There was also a net secretion of Ca across the plexus, but this amounted to only 0.036 μmol/cm^2/h. On the other hand, potassium was transported out of the CSF across the choroid plexus into the vascular compartment.

It would be anticipated from these results that the freshly secreted CSF should be largely a mixture of NaCl and $NaHCO_3$, and that the bicarbonate and calcium concentration should be higher than in plasma while the chloride and potassium should be lower than in plasma. Inspection of the results obtained by *Miner* and *Reed* (1972) on the composition of the CSF secreted by the cat choroid plexus in vivo shows that: 1) the Na, Ca, and Mg concentrations were slightly higher than those in an ultrafiltrate of plasma, and 2) the chloride and potassium concentrations were slightly lower than those in an ultrafiltrate of plasma.

E. Mechanisms of Ion Transport

1. Sodium

The approach first used with sodium was to test the effect of various pharmacologic agents on the unidirectional fluxes. The most potent inhibitor of net sodium transport across the plexus was the cardiac glycoside ouabain. At a concentration of 6.7×10^{-5} M this glycoside blocked active sodium secretion which was due to a reduction in the unidirectional sodium flux from blood to CSF (see Fig. 5, *Wright,* 1972a). Ouabain was only effective from the CSF side of the epithelium, and as sodium transport in other cells and tissues was only blocked by ouabain when it is the compartment to which sodium is pumped, this strongly indicated that the sodium pump sites were on the apical surface of the choroidal epithelium.

In view of the effect of bicarbonate on sodium secretion, it was of interest to test the effect of carbonic anhydrase inhibitors. Diamox failed to inhibit sodium transport in the frog, even though it reduced both sodium and CSF secretion in the isolated perfused sheep plexus (*Blount* et al., 1973). The explanation for the lack of effect in the frog is that an uncatalyzed rate of CO_2 hydration was sufficient to meet the demand for H^+ and HCO_3^- (*Maren, T.H.,* personal communication). A possible role of carbonic anhydrase in secretion will be discussed below. It is unlikely that a membrane HCO_3-ATPase is involved in HCO_3 transport across this epithelium, as we have been unable to locate the enzyme in the plasma membranes of the intestinal epithelium (*van Os* et al., 1977).

Amiloride inhibits CSF secretion in vivo (*Davson* and *Segal,* 1970), but at a concentration of 1×10^{-6} M, I was unable to produce an effect on Na secretion across the frog plexus (*Wright,* 1972a). However, at higher concentrations, amiloride caused a 25% reduction in the passive permeability of the plexus to sodium, and this is probably related to a TAP (2,4,6-triaminopyrimidine) effect on permeation through the tight junctions (unpublished observations). Neither spirolactone nor vasopressin produced any effect in frog or sheep isolated plexus (*Wright,* 1972a; *Segal* and *Pollay,* 1977).

2. Chloride

The mechanism of chloride transport across the frog choroid plexus was investigated by measuring the chloride flux ratios (i.e., J_{sv}/J_{vs}) when the p.d. across the plexus was clamped at voltages up to 40 mV and when NaCl on one side of the tissue was replaced iso-osmotically with mannitol. In both series of experiments, the measured flux ratios were close to those predicted by the Ussing flux ratio equation ($J_{sv}/J_{vs} = [Cl]_s/[Cl]_v \exp [FV/RT]$), and this strongly suggests that chloride was transported passively across the plexus.

These experiments indicate that active sodium transport is the primary process in the secretion of the CSF by the choroid plexus, and that the sodium pump is located on the apical membrane of the epithelium.

3. The Electrochemical Potential Profile Across the Epithelium

Sodium and potassium were transported across the frog choroid plexus in the absence of external driving forces, i.e., there were no electrochemical potential gradients between the ventricular and serosal fluid compartments. Nevertheless, the electrochemical potential gradients between the external bathing solutions and the intracellular fluid compartment within the epithelium have important bearings on transepithelial transport. The intracellular sodium and potassium concentrations have been estimated from measurements of the total amounts of sodium, potassium, and water in the tissue, and the extracellular spaces (*Wright,* 1977d). The intracellular sodium and potassium concentrations were found to be 17 and 154 mEq/liter using this procedure, and these approached the extracellular concentrations when the tissue was incubated in ouabain. More satisfactory estimates await intracellular ion activity measurements using ion-specific microelectrodes. Preliminary results with K sensitive electrodes (*Zeuthen,* personal communication) indicated that the apparent intracellular K concentration was 120 mEq/liter and that ouabain reduced this to 3 mEq/liter within 2 hours. Measurements of the intracellular electric potential have also been made in both the frog (*Wright,* unpublished; and *Zeuthen,* personal communication) and the rabbit (*Welch* and *Sadler,* 1965). The intracellular compartment was negative with respect to the external solutions by -50 to -70 mV. The total electrochemical potential gradients between the external bathing media and the epithelium are therefore -120 mV for Na and $+50$ mV for K (i.e., $E_m + (RT/zF) \ln C_i/C_o$, where E_m is the membrane potential and C_i and C_o are the ion concentrations in the internal and external solutions respectively).

With regard to sodium transport across the plexus, these results suggest that Na enters the epithelium from the serosal compartment down its electrochemical potential gradient ($\sim$ 120 mV) across the basolateral membrane, and that it is subsequently pumped uphill out of the cell into the ventricle across the apical cell membrane. On the other hand, for potassium transport from ventricle to blood energy is necessary to move potassium into the cell from the ventricle, but once within the epithelium the potassium electrochemical gradient is favorable for the passive diffusion of potassium out across the basolateral membrane into the serosal compartment. In common with other cells, it is quite likely that sodium and potassium pumping at the apical cell membrane are linked through the ubiquitous ouabain-sensitive Na/K pump.

4. Interactions Between Ouabain and the Na/K Pump

As already indicated above, ouabain in the CFS blocked active sodium secretion across the choroid plexus, and this was due to a reduction in the unidirectional sodium flux from blood to CSF. The binding of ouabain to the epithelium was studied using ^{3}H-ouabain, and the location of the binding sites was determined by autoradiography (*Quinton* et al., 1973; *Wright,* 1977d).

Ouabain bound irreversibly and preferentially to the ventricular surface of the plexus. The maximum amount (5×10^{-11} mol/plexus) was bound at a concentration of 1×10^{-5} M, and half the maximal binding occurred at a ouabain concentration of 5×10^{-7} M. The half time for ouabain binding decreased with increasing ouabain concentration, and there was a good correlation between these binding half-times and the half-times for the action of ouabain on active ion fluxes across the plexus. Binding was specific as judged by: 1) the effects of cations on binding, and 2) competition between ouabain and other cardiac glycosides for the binding sites. Li, Na, Cs, and Mg all increased ouabain binding, but K and Rb effectively blocked it. These effects of monovalent and divalent ions are similar to that found for ouabain binding to both Na/K pumps and Na/K-ATPase (see *Glynn* and *Karlish,* 1975; *Schwartz* et al., 1975; and *Dahl* and *Hokin*, 1974). Cymarin and gitoxigen competed with ouabain for binding sites, and the degree of inhibition observed was consistent with the relative potencies of the three glycosides on Na/K-ATPases. These experiments lead to the conclusion that ouabain binds to Na/K pump sites on the plexus. Autoradiography of ^{3}H-binding shows that the pump sites are restricted to the apical membrane of the epithelium.

It should be mentioned that *Milhorat* et al. (1975) have been unable to visualize Na/K-ATPase on the apical membrane of the choroidal epithelium in a number of species including frog. These authors used the histochemical technique developed by *Ernst* for localizing ouabain-sensitive, potassium-dependent phosphatase activity in paraformaldehyde fixed tissues, and they found that this enzyme was located on the basolateral membrane of the cell. These results are difficult to reconcile with the physiology of the plexus, the specific binding of the ^{3}H-ouabain to the apical membrane of the frog plexus, and the cytologic studies of *Matano* and *Ishii* (1973). To resolve this dilemma, we have experiments in progress to study the distribution of enzymes between the apical and basolateral membranes of the choroidal epithelium using membrane isolation techniques (e.g., *Mircheff* and *Wright,* 1976; *van Os* et al., 1977; and *Walling* et al., 1977). Preliminary results with frog and rabbit plexuses show that Na/K-ATPase was largely confined to the apical cell membranes together with adenylate cyclase and alkaline phosphatase, and that guanylate cyclase

and glutamyl transpeptidase activities were found on basal lateral membranes. Preliminary characterization of the frog choroid plexus Na/K-ATPase suggest that it is identical to the enzyme in the cat choroid plexus (see *Wright,* 1977d).

5. Potassium Transport Across the Apical Plasma Membrane

The mechanism of potassium transport into the choroidal epithelium across the apical and basolateral membranes was investigated by measuring the unidirectional influx of ^{42}K (see *Wright,* 1977c, d). The entry of K across the apical membrane exhibited kinetics similar to that demonstrated for K transport into single cells (e.g., red blood cells and nerve) and K effects on Na/K-ATPases (see *Schwartz* et al., 1975). The kinetics deviated from that expected for the Michaelis Menten model in that plots of rate (J) vs. K concentration [K] were sigmoid rather than hyperbolic, and that plots of 1/J vs. 1/[K] were nonlinear. A more appropriate kinetic model is where there are multiple sites for the ligand on the pump, i.e.

$$J = \frac{J_{max}}{\left[1 + (K_m/[K])\right]^n}$$

where J_{max} is the maximum rate, K_m is the affinity constant, and n is the number of equivalent sites that must be occupied by potassium ions. Graphically, this equation may be displayed by plotting $[K]/J^{1/n}$ against [K], and estimates of J_{max} and K_m are obtained from the slope, $J_{max}^{-1/n}$, and the intercept on the horizontal axis respectively (see *Garay* and *Garrahan,* 1973). These plots are shown in Figure 6 for K transport across the apical membrane of the plexus.

In the case of the one-site model (n = 1, or the Michaelis Menten model), the data points at low K concentrations deviate upward from the regression line, but for the two-site model (n = 2) a straight line gives a better fit to all the data (r = 0.99 for n = 2, vs. r = 0.93 for n = 1). This suggests that in common with Na/K pumps and Na/K-ATPases, the choroid plexus K system has two equivalent binding sites: K_m and J_{max} for this two-site model are 0.3 mM and 8 μmol/cm^2/h respectively.

The K influx across the apical membrane was sensitive to ouabain, ethacrynic acid, sodium, phloretin, bicarbonate, cAMP, ATP, and theophylline. Ouabain inhibited by up to 80% with an inhibitor constant of 1×10^{-7} M (see Fig. 11, *Wright,* 1977d), and the time course of the inhibition correlated with the time course of ouabain binding. Ethacrynic acid inhibited by 70% at 1 mM, the combined effect of ouabain and this inhibi-

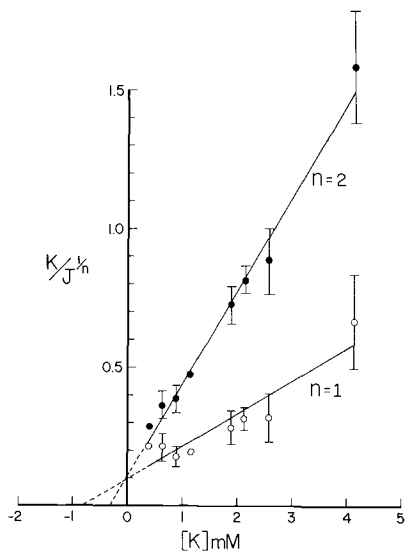

Fig. 6. The K influx across the apical plasma membrane of the frog choroid plexus (J) as a function of the external potassium concentration [K]. The K influx was obtained from ^{42}K fluxes at potassium concentrations between 0.25 and 4 mM. In these experiments the plexuses were depleted of K by incubating them overnight in K-free solutions at 2°C to ensure that the K concentration in the unstirred layers next to the epithelium was identical to that in the bulk solutions. The results are plotted as $[K]/J^{1/n}$ against [K], where n is the number of sites occupied by K on the pump. Regression lines are fitted to the data points for n = 1 and n = 2; the regression coefficients were 0.93 and 0.99 respectively. It can be seen that for n = 1 the data points at low [K] deviate upward from the regression line, but this is largely eliminated in the n = 2 plot. For further discussion see the text

tor was greater than either alone, and phloretin, another Na/K-ATPase inhibitor, also reduced the K flux by 80%. The presence of sodium was required to maintain the K influx, but I have no information of the relative effects of extracellular and intracellular sodium.

Theophylline, HCO_3, cAMP, and ATP all stimulated K transport. The most spectacular effect was brought about by HCO_3 (Fig. 7), where 25 mM HCO_3 caused a 100% increase in the ouabain-sensitive flux (*Wright,*

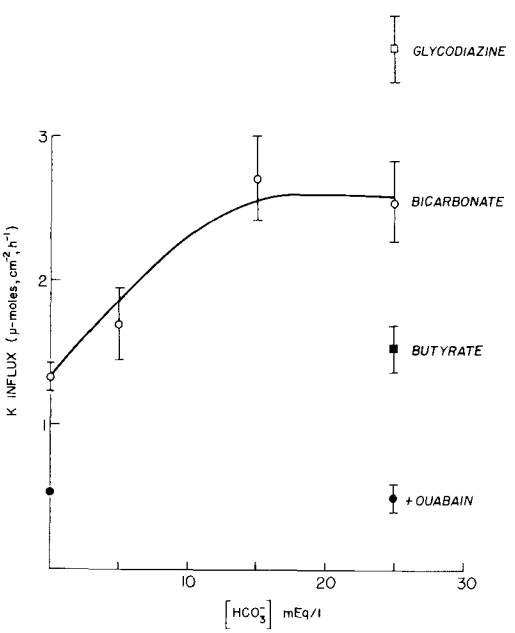

Fig. 7. Potassium transport across the apical plasma membrane of the frog choroid plexus as a function of the buffer composition. The K flux was obtained from the ^{42}K influx into the cell and the buffer composition of the Ringer was varied by partially replacing NaCl with $NaHCO_3$, Na glycodiazine, or Na butyrate. In all experiments the pH was maintained at 7.3. (From *Wright,* 1977)

1977c). Other lipid soluble buffers, e.g., glycodiazine, mimicked the effect of HCO_3, and it should be recalled that these buffer anions increased sodium transport across the epithelium. The increases brought about by ATP, cAMP, and theophylline were all about 20%, and these may be related to the cholera toxin-induced CSF secretion observed in dogs (*Epstein* et al., 1977). We also find that these agents increase the ciliary activity and sodium transport across the frog plexus (*Nelson* and *Wright,* 1974; and *Wright,* unpublished observations).

The ouabain-sensitive influx across the serosal surface of the plexus was only 7% of that across the apical plasma membrane. This finding agrees with observations on ouabain binding (*Quinton* et al., 1973), and indicates that the major site of K transport into the choroidal epithelium is at the apical plasma membrane.

There is good circumstantial evidence that the apical K pump is linked to the Na pump: 1) both pumps are in the same membrane, 2) ouabain inhibited both pumps from the ventricular side of the plexus, 3) K transport only occurred in the presence of sodium, 4) both K and Na pumps were stimulated by nucleotides, and 5) HCO_3 increased both Na and K transport approximately 100%.

F. Model for Ion Transport Across the Choroid Plexus

A simple model for the transport of Na and K across the frog choroid plexus is given in Figure 8. This shows Na entering the choroidal epithelium across the basolateral membrane in exchange for protons. Na entry is down the sodium electrochemical potential gradient ($\Delta\tilde{\mu}_{Na} \sim 120$ mV), and it is subsequently pumped out of the cell into the CSF against the electrochemical potential gradient by a Na/K exchange pump in the apical plasma membrane. Potassium is accumulated within the epithelium against its electrochemical potential gradient by Na/K pump, and the small net flux of this ion from CSF to plasma is accounted for by this pump and a leak of K down its gradient across the basolateral membrane.

The number of Na/K pump sites per cell may be calculated from the amount of ouabain bound to the apical surface of the plexus. The maximum amount bound, 5×10^{-11} mol, is obtained when sufficient ouabain is present in the CSF to cause maximum inhibition of the Na/K pump and the Na/K-ATPase (see *Wright,* 1977d). Assuming there are one or two ouabain molecules bound to each pump, there are about 3×10^6 pumps per cell. The turnover time of the pump is obtained from the number of sites and the maximal rate of K transport across the apical membrane (8 μmol/cm^2/h). Assuming that there are two K ions transported each cycle of the pump (see above), each pump turns over approximately 200 times per minute at 23°C.

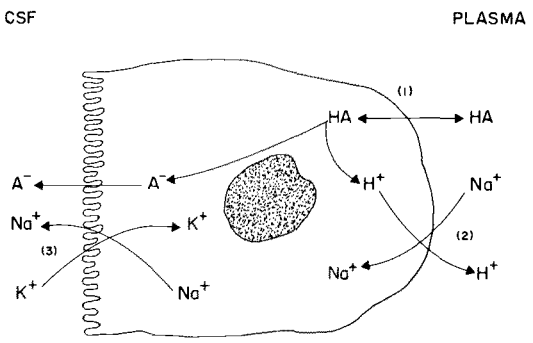

Fig. 8. A simple model for sodium transport across the frog choroid plexus. In this scheme, lipid-soluble buffers with pK values around 6, e.g., glycodiazine, enter the epithelium by nonionic diffusion and dissociate to provide an intracellular supply of protons and buffer anions (1). [In the case of HCO_3^-, the diffusion of CO_2 and the hydration of CO_2 play an additional important role in the supply of intracellular protons and anions.] At the basolateral cell membrane, protons are exchanged for Na in the plasma (2), and sodium is subsequently pumped out of the cell into the ventricle across the apical cell membrane by a Na/K exchange pump (3). The buffer anions and chloride follow sodium into the ventricle. (Taken from *Wright*, 1977c)

Clues about the specificity of the pump come from experiments on active anion transport across the plexus (*Wright*, 1974a). Active iodide transport from CSF to blood was eliminated in the absence of K and/or Na. Potassium was required in the CSF, and the relationship between iodide transport and [K] resembled that for K transport. Rb, and to a lesser extent Cs, could substitute for K, but the requirement for Na was absolute. The sequence for the K site, i.e., $K \sim Rb > Cs > Li$, is similar to that found for Na/K pumps and Na/K-ATPase in other cells and tissues (see *Dahl* and *Hokin*, 1974).

The relationship between the Na/K pump and buffer anions is indirect through the supply of intracellular protons. Sodium enters the epithelium across the basolateral membrane in exchange for H^+, and the intracellular supply of protons depends upon:

1) Metabolism. Production of CO_2 through metabolism and its subsequent hydration results in the intracellular formation of H^+ and HCO_3^-.

2) Entry of buffers from the extracellular fluids. Lipid soluble buffers, e.g., glycodiazine, permeate across plasma membranes by nonionic diffusion, and net inward transport occurs if there is a pH gradient between the two fluid compartments. When the intracellular pH is alkaline relative to the extracellular fluids, the dissociation of HA within the cell leads to a gradient of HA across the cell membrane.

3) CO_2 diffusion. CO_2 permeates across cell membranes very rapidly, and if the partial pressure of CO_2 in blood or CSF exceeds that in the cell

there will be net diffusion of the gas into the cell interior, where it is hydrated to form carbonic acid. The acid subsequently dissociates to produce H^+ and HCO_3^-. The protons are exchanged for Na in plasma and the buffer anions follow sodium into the CSF.

This scheme accounts for the relationship between HCO_3 and Na and K transport, the ability of glycodiazine to substitute for HCO_3, and the net transport of buffer anions into the CSF. The effect of carbonic anhydrase inhibitors on CSF secretion in mammals is explained if CO_2 hydration is the rate-limiting step in the intracellular supply of protons and HCO_3^-. In frogs this step is not rate limiting.

A major difficulty still remains in that we have to explain how Cl^- and HCO_3^- (buffer anions) transport is linked to the Na/K pump. In single cells there is evidence that the Na/K pump exchanges three sodium ions for two potassium ions across the plasma membrane, and the pump is electrogenic. This may also be the case on the plexus, because ouabain causes a very rapid fall in the membrane potential before there is any change in the intracellular potassium concentration (*Zeuthen,* personal communication). An electrogenic pump could then force anion movement to maintain electroneutrality. The p.d. across the choroid plexus, in vivo or in vitro, amounts to only a few millivolts, and ouabain reduced this further toward zero. Leaky junctions between the cell need to be invoked to explain the lack of a substantial transport p.d. across the plexus in the presence of an electrogenic pump on the apical cell membrane.

G. Fluid Secretion

Fluid secretion across the frog choroid plexus has been measured using two techniques. The first was to measure the rate of change in concentration of an impermeable marker in a known volume of fluid on the ventricular surface of the plexus. Ten microliters of fluid containing ^{14}C-dextran (mol wt 17,000) was placed on the surface of the plexus and 20 nl samples of the fluid were taken at 30 min intervals. Volume flow was calculated from the dilution of the isotope in the artificial CSF. The second method was to record the changes in volume of the fluid on each side of the plexus directly using a DIMEQ TE 200 transducer (Wayne Kerr, Bognor Regis, England). The instrument was calibrated to measure the change in volume by recording the changes in capacitances (distance) between capacitive probes and the surface of the saline on each side of the plexus. Volume changes as low as 1 nl can be recorded by this technique (see *Wiedner,* 1976; *Wright* et al., 1977).

The rate of secretion in bicarbonate saline was between 8 and 10 μl/cm^2/h at 23°C, and this corresponds to 0.07 μl/min/mg weight. Assuming

that the activation energy for transport is about 20 kcal/mol, the rate of secretion at 37°C would be 0.4 μl/min/mg. This estimate is close to that observed directly for the isolated choroid plexus of the cat (*Miner* and *Reed,* 1972), and higher than that measured indirectly for the perfused sheep plexus (*Pollay* et al., 1972).

Ouabain in the ventricular fluid abolished net secretion. In the frog 5×10^{-4} M ouabain reduced secretion by 7.6 ± 5 μl/cm^2/h in five experiments, and in the cat ouabain reduced CSF secretion in proportion to the inhibition of the plexus Na/K-ATPase (*Vates* et al., 1964). In the perfused sheep plexus, *Deane* and *Segal* (cited in *Segal* and *Pollay,* 1977) further observed that replacing Na in the vascular perfusate with Choline reduced CSF secretion 70%. These observations indicate that active sodium secretion is the primary event in the production of CSF by the choroid plexus.

The relationship between ion and water movement across the frog choroid plexus was studied by measuring the composition of the freshly secreted CSF under different experimental conditions (*Wright* et al., 1977, and in preparation). The experimental approach was to remove all the solution from the ventricular surface of the plexus, cover the surface with oil, and collect the newly secreted fluid from the tissue/oil interface at regular intervals of time. Microanalysis of the nascent CSF was carried out to obtain the Na, K, and Cl concentrations and the osmolarity of the fluid. The composition of the CSF obtained under control conditions is summarized in Table 4. This shows that the fluid was hypertonic, 236 vs. 205 mosmol/liter in the serosal bathing medium, and that this was primarily due to an increase in the Na concentration of the nascent CSF. The chloride concentration was virtually identical in the two fluids, but we observed an elevation in the CSF potassium.

Table 4. Composition of freshly secreted CSF (in mEq/liter)

	Saline	CSF
Na	110	125 ± 3 (4)
K	2	8 ± 1 (4)
Cl	83.5	83 ± 3 (4)
Mosmol/liter	205	236 ± 2 (4)

In this experiments the serosal side of the frog choroid plexus was exposed to a Ringer solution containing 25 mM Na glycodiazine and equilibrated with 100% O_2. The composition of the freshly secreted CSF was obtained from the ventricular surface of the plexus, and was analyzed for Na, K, Cl, and osmolarity as described in the text.

CSF collected from the surface of exposed choroid plexus in vivo was hypertonic to an ultrafiltrate of blood by 6–9 mosmol (*Davson* and *Purves,* 1954). Although there have been no recent measurements on mammals using isolated choroid plexuses, the ionic composition of CSF freshly secreted by the isolated choroid plexus has been studied by *Reed* and his collaborators (*Miner* and *Reed,* 1972; *Husted* and *Reed,* 1976). These authors found that the fluid Na, Ca, and Mg concentrations were slightly higher than an ultrafiltrate of serum, and that the K and Cl concentrations were lower than the ultrafiltrate. The sodium concentration was on average 6 mEq/liter higher in CSF, and, except for K, the data are in qualitative agreement in frog and cat. The reason for the high K concentration in frog CSF is obscure, particularly in view of the active transport of K from CSF into the epithelium. However, we do find that ouabain in the CSF greatly increased the K concentration in the newly formed CSF.

The relationship between the osmolarity of the secreted fluid and the osmolarity of the Ringer's solution bathing the vascular face of the plexus is shown in Figure 9. In these experiments, the osmolarity of the Ringer was varied between 110 and 290 mosmol/liter by varying the NaCl concentration. Over this range in osmolarity of the bathing solution, the nascent CSF remained hypertonic by 30 mosmol/liter. This result is in contrast to that observed for the rabbit gall bladder by *Diamond* (1964), where the transported fluid was isotonic to the bathing solution over a wide range of osmolarities.

In the gall bladder and other epithelia where the fluid transported is iso-osmotic with the bathing solutions, the osmotic forces necessary for

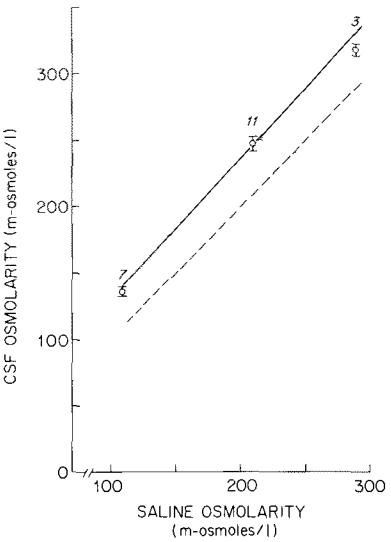

Fig. 9. The osmolarity of freshly secreted CSF as a function of the bathing solution osmolarity. The serosal (vascular) surface of the frog choroid plexus was exposed to saline containing different concentration of NaCl, and the osmolarity of the freshly secreted CSF was determined in the fluid collected from the ventricular surface of the plexus as described in the text. The solid line is the regression line for the data points and the broken line is the line of identity. Note that the CSF is hypertonic to the bathing solution by approximately 30 mosmol over the entire range of saline osmolarity

fluid transport must be generated within the epithelium. It is recognized through the work of *Diamond* and *Tormey* (1966) and *Tormey* and *Diamond* (1967), and *Kaye* et al. (1966) that the lateral intercellular spaces are the site of solute/solvent coupling in the gall bladder. Two models were proposed to explain the mechanism of water transport: one was the double membrane model first proposed by *Curran* and *Mackintosh* (1962) and adopted for the gall bladder by *Kaye* et al. (1966), and the second was the standing gradient model proposed for the gall bladder by *Diamond* and *Bossert* (1967). The starting point for both models was the assumption that sodium pumped into the lateral intercellular spaces increased the osmolarity of the fluid in these restricted extracellular spaces, and that water flowed across the epithelium in response to the local increase in osmotic pressure. The location of sodium pumps in absorbing epithelia such as the intestine (*Stirling*, 1972; *Mircheff* and *Wright,* 1976), and X-ray microanalysis of the composition of the fluid in the lateral intercellular spaces of the intestine (*Gupta* et al., 1978) suggest that the basic assumption is indeed correct. However, the actual mechanism by which the water movement is coupled to active salt transport is controversial (e.g., *Hill,* 1975a,b; *Sackin* and *Boulpaep,* 1975, and *Diamond,* 1977).

One issue in the debate over the mechanism of water transport is the magnitude of the osmotic water permeability of epithelial membranes (see *Wright,* 1977b). We have shown that the hydraulic conductivity of the gall bladder was seriously underestimated, by at least a factor of 10 and perhaps by as much as a factor of 30, and this has important implications about the nature of fluid transport, e.g., the original arguments (*Diamond,* 1964) used to reject the simple local osmosis theory proposed for the gall bladder are invalid.

Fluid transport across the choroid plexus is on one hand a simpler problem than in the gall bladder, but on the other hand is more complex. First, the CSF is hypertonic, due to active sodium secretion, and this is alone sufficient to generate osmotic water flow from plasma toward CSF. The actual magnitude of the osmotid flow (J_v) is given by

$$J_v = \sigma L_p \, RT\Delta C$$

where σ is the salt reflection coefficient, L_p the hydraulic conductivity of the plexus, and $RT\Delta C$ is the osmotic gradient between CSF and plasma. Whether or not this is sufficient to account for CSF secretion depends upon the value of the parameters used in the equation, and I have already indicated that there are serious questions about the actual magnitude of the L_p in epithelia.

Osmotic pressure gradients between the CSF and plasma will not account for secretion under experimental conditions where identical fluids

are placed on each side of the plexus (see *Wright* et al., 1977). The difficulty here is that the morphology of the choroidal epithelium corresponds to that expected for an epithelium designed to *absorb* fluid rather than secrete. A similar dilemma exists with the small intestine which can secrete fluid in the presence of cholera toxin. Although *Diamond* and *Bossert* (1968) suggested that the epithelia can work "backward", i.e., inward directed pumps on the lateral membranes could generate osmotic gradients between cell and lateral space by making the space hypotonic, in the choroid plexus the solute pumps are on the wrong membrane.

With pumps on the apical membrane, where is the local osmotic compartment that corresponds to the lateral intercellular spaces? One possibility is the interspaces between the microvilli, Figure 5a, and parameters can be chosen for both the local osmosis and standing gradient models to account for the composition of the newly formed CSF collected from the surface of the plexus (see *Wright* et al., 1977b). Another possibility suggested by *Rodriguez* (personal communication) is that the major local osmotic compartment is a "sequestered" ventricular space in the dorsal region of the plexus. In this region of the amphibian posterior plexus (see Fig. 3 and Plates XXIB and XXIIA, *Kemali* and *Braitenberg,* 1969), the CSF occupies interconnected cavernous spaces. These could serve as compartments where osmotic gradients are generated to pull water across the plexus. If this is indeed the case, ion gradients between the compartment and the bulk CSF are expected. This could be verified by the use of electron probe X-ray analysis of frozen sections of the plexus.

An alternative approach to the problem of coupling between salt and water transport across the plexus is a direct consequence of the presence of a large "unstirred layer" adjacent to the ventricular surface of the tissue. Despite the fact that the epithelium is ciliated, the plexus behaves as if there was an unstirred layer of 300 μ between the bulk of CSF and the apical membrane (*Wright* and *Prather,* 1970). This is probably a direct consequence of the complex anatomic arrangement seen in Figures 3 and 4. Active salt transport across the epithelium increases the osmotic pressure in this unstirred layer and this osmotic gradient could pull water across the plexus. The rate of volume flow (J_v) is related to the rate of active salt transport (M_o) and the thickness of the unstirred layer (δ) by the relation derived by *Dainty* and *House* (1966) for the frog skin, i.e.,

$$J_v = \frac{M_o L_p RT\delta}{D_s + C_s \delta L_p RT}$$

where L_p is the hydraulic conductivity of the plexus, C_s is the solute concentration in the bathing solution, D_s is the solute diffusion coefficient in the instirred layer, and δ is the thickness of the unstirred layer. Taking the

thickness of the unstirred layer as 300 μ and the rate of salt transport as 1 μmol/cm^2/h, a L_p of 110 x 10^{-3} cm/s is required to account for a volume flow of 10 μl/cm^2/h. This L_p is 50 times higher than the steady-state value reported above, but it is not unreasonable considering the problems of estimating this parameter in epithelia (*Wright,* 1977b).

H. Extrachoroidal Sources of CSF

Although it is generally agreed that 60–70% of the CSF is formed by the choroid plexuses, there is considerable debate over the source of the remainder (see *Welch,* 1975; and *Milhorat,* 1976). Some claim that extrachoroidal CSF is "secreted" across the ependyma while others favor secretion into the subarachnoid spaces. This fluid could be elaborated at the blood-brain barrier and then pass into the ventricles via the ependyma or into the subarachnoid spaces via the pia (see Fig. 2). Another possible source of extrachoroidal is the arachnoid membrane. The structure of the arachnoid membrane is illustrated in Figure 10.

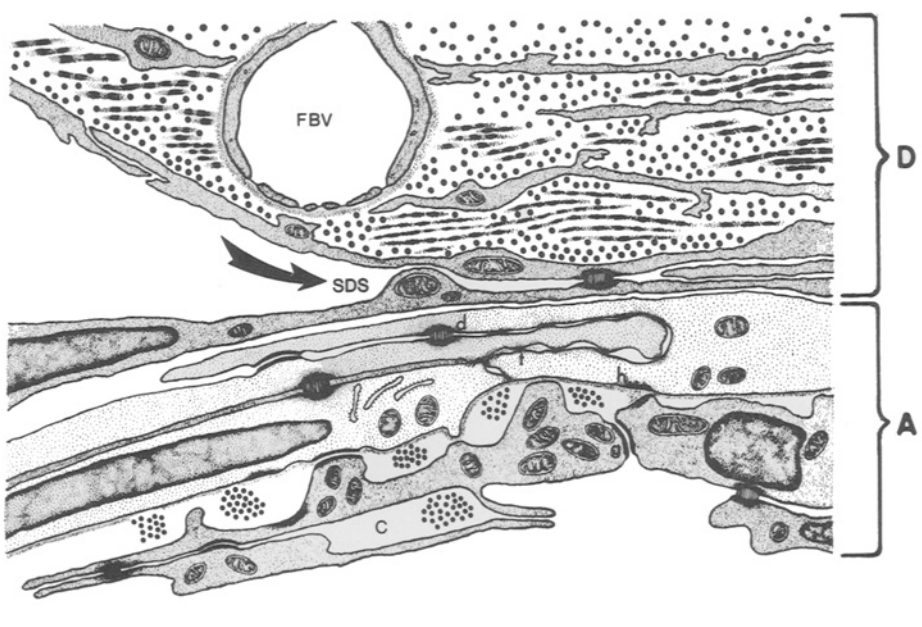

Fig. 10. A diagram showing the structure of the arachnoid membrane. The tissue is composed of a number of layers of thin cells connected to each other by desmosomes (*d* and *h*) and tight junctions (*t*). In frog there are up to 15 layers of cells, but in mammals there are usually far fewer layers. The subdural space (*SDS*) is normally quite narrow unless it is split during preparation of the tissue. The dura is composed chiefly of collagen bundles and a few elongated cells running parallel to the surface of the brain. CSF fills the subarachnoid spaces (*SAS*). (Modified from *Nabeshima* et al., 1975)

The arachnoid separates the CSF in the subarachnoid spaces (SAS) from the subdural space (SDS). The SDS marks the junction between the arachnoid and dura mater, but in situ it is often difficult to visualize. However, it should be noted that *Kerber* and *Newton* (1973) demonstrated that dural arteries give rise to a rich bed of capillaries at the interface between the two meninges. The arachnoid membrane itself is composed of a number of layers of thin cells connected to each other by desmosomes (*Perez-Gomez* et al., 1976; and *Nabeshima* et al., 1975). In mammals, at least, the cells of one layer close to the dura/arachnoid are joined together by tight junctions. The cells of the arachnoid contain the usual array of subcellular organelles, e.g., nucleus, mitochondria, and endoplasmic reticulum, but they are overshadowed by numerous fine microfilaments. These anatomic considerations alone lead to the conclusion that the arachnoid has all the prerequisites necessary to carry out transport functions.

To obtain clues about the role of the arachnoid in the physiology of the CSF, we have developed an in vitro preparation of the frog choroid arachnoid membrane. In this animal it is possible to separate the arachnoid from the dura and mount it in Ussing type flux chambers (*Wright,* 1974b; and *Perez-Gomez* et al., 1976). The frog arachnoid behaved as a diffusion barrier between the CSF and SDS. The electric resistance was about 2000 Ω/cm^2, an order of magnitude greater than the plexus, and it was quite impermeable to neutral amino acids.

There was an electric p.d. across the arachnoid, and in the steady state this ranges between 9 and 40 mM, CSF positive with respect to the SDS. The p.d. was abolished by 1) replacing the Na in the Ringer's solution with choline, and 2) the addition of ouabain to the CSF. The p.d. is probably due to active sodium transport from the SDS to the SAS, and estimates of the short circuit current suggest that the rate of sodium secretion was 0.4 $\mu mol/cm^2/h$, i.e., about one-half to one-third of the rate in the frog choroid plexus. In common with the plexus, the rate of sodium transport across the arachnoid, as judged by the short circuit current, increased with bicarbonate in the Ringer's solution. These experiments suggest that the arachnoid membrane is actively involved in the maintenance of the blood-CSF barrier and the secretion of CSF.

Additional evidence to support this view comes from experiments with amino acids and organic bases (*Wright,* 1974b; *Wright,* 1977e). In the case of amino acids, I found that glycine, L-proline, L-alanine, and α-AIB were actively transported across the arachnoid from CSF to blood by a ouabain-sensitive, sodium-dependent process. The kinetics of glycine transport were very similar to those described for the clearance of glycine from cat SAS (*Murray* and *Cutler,* 1970). Choline was also actively transported out of the CSF by the frog arachnoid membrane, but iodide, LSD, and paraaminohippuric acid were not. Further work is required to establish the significance of these transport processes in the secretion and regulation of the CSF in higher animals.

I. Control of CSF Secretion

Relatively little is known about this aspect of CSF physiology and this should provide a productive area for future research. In the intestine, it is well-established that fluid and electrolyte transport is related to levels of intracellular nucleotides such as cAMP and cGMP; elevation of cGMP stimulates absorption (*Brasitus* et al., 1976) and elevation of cAMP causes secretion (*Kimberg* et al., 1971). Furthermore, it is known that stimulation of intestinal α-adrenergic receptors elevates the concentration of cGMP in the gut, and cholera toxin and polypeptide hormones elevate the concentration of cAMP. In the rat duodenum, adenylate cyclase is restricted to the basolateral plasma membranes, whereas guanylate cyclase is found in both brush border and basolateral plasma membranes (*Walling* et al., 1977).

The choroid plexus is innvervated by both cholinergic and adrenergic fibers (*Edvinsson* et al., 1973, 1975), and sympathectomy and reserpine treatment both caused an increase in the activity of the plexus carbonic anhydrase. The actual relationship between carbonic anhydrase and salt and water transport is not quite clear, but, as outlined above, the hydration of CO_2 within the choroidal epithelium appears to be a rate-limiting step in sodium and water transport across mammalian preparations. Rat choroid plexus contains adenylate cyclase, and it has recently been observed that stimulation of β-adrenergic receptors elevates cAMP within the epithelium (*Rudman* et al., 1977). Although the effect of adrenergic drugs on CSF secretion is not established, cholera toxin, which elevates intestinal cAMP and promotes intestinal secretion (*Kimberg* et al., 1971), doubles the rate of CSF secretion in dogs (*Epstein* et al., 1977). Preliminary work in my own laboratory indicates that rabbit choroid plexus contains both adenylate and guanylate cyclases, and that adenylate and guanylate cyclases are on the apical and basolateral membranes respectively (*Walling* and *Wright,* unpublished observations). Theophylline and cAMP in the Ringer's solution stimulate the activity of the Na/K pump (see above) and ciliary activity in the frog choroid plexus (*Nelson* and *Wright,* 1974). These observations suggest that it would be profitable to study in more detail the interactions between nucleotide cyclases and transport across the choroid plexus.

Experiments designed to study the direct effect of hormones on Na and fluid secretion by the choroid plexus have produced negative results, e.g., in the frog and sheep isolated plexuses, vasopressin and aldosterone have no effect (*Wright,* 1972a; *Segal* and *Pollay,* 1977; and unpublished results).

Understanding the mechanisms controlling CSF secretion has important clinical applications. Pathologic conditions leading to cerebral odema

and elevated intracranial pressures are common, and methods for the management of the CSF under these conditions are largely empiric and the subject of debate. One example will suffice – hydrocephalus (see *Milhorat,* 1972). The incidence of this disease is approximately 1–3 in every 1000 live births, and in unoperated cases the mortality is close to 50% and only one-third of the survivors have IQ's greater than 75. Currently, the methods of choice in treatment of this disease are mechanical shunting procedures. These reduce mortality by one-half, and three-quarters of the survivors have IQ's greater than 75. Since currently available drugs, e.g., diamox and related compounds, only provide short-term relief, it is necessary to develop other drugs to replace surgical procedures for the treatment of the congenital disease. Such drugs might be important in the management of other disorders of the CSF.

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Rev. Physiol. Biochem. Pharmacol., Vol. 83

Optical Measurement of Membrane Potential

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I. Introduction

An optical measurement of membrane potential might be beneficial in circumstances where electrodes are difficult to use for reasons of cell size, complexity, or membrane topology. In this review we begin with a discussion of the evidence that an optical measurement is directly related to an electrode measurement. This is followed by a discussion of the mechanism(s) responsible for the optical signals and some difficulties associated with optical methods. We conclude with a summary of the attempted applications.

The possibility of using an optical method for measuring membrane potential was first suggested in 1968. Changes in light scattering and birefringence of axons that occur during the action potential were found (*Cohen* et al., 1968) and some components of these intrinsic signals were shown to depend upon the changes in membrane potential. Later that year *Tasaki* et al. (1968) discovered changes in the fluorescence of stained axons (extrinsic signals). Most of these fluorescence changes were subsequently shown to be potential dependent (*Cohen* et al., 1970; *Conti* and *Tasaki,* 1970; *Patrick* et al., 1971; *Davila* et al., 1974; *Cohen* et al., 1974; *Conti,* 1975; *Ross* et al., 1977) although there was some disagreement about this conclusion (*Conti* et al., 1971; *Tasaki* et al., 1972). The complicated changes in the absorption spectra of chloroplasts that result from illumination were first described many years earlier (e.g., *Witt* et al., 1956). Then, also in 1968, *Junge* and *Witt* suggested that these spectral shifts were the result of changes in membrane potential across internal membrane systems in the chloroplasts.

Although there are several applications where the optical signals first described in 1968 were large enough to be used for monitoring membrane potential (see Sect. IV), in others, much larger signals were required. Therefore, in 1971 a search was undertaken for dyes with larger signals (*Davila* et al., 1972; *Salzberg* et al., 1972; *Cohen,* 1973, p. 409). Very early on in this search we were joined by Dr. *A.S. Waggoner* and then by *C.H. Wang,* whose abilities as chemists greatly aided our efforts. Since then, the size of the signal in experiments on squid giant axons has increased

by two orders of magnitude (*Ross* et al., 1977) and these larger signals have facilitated some of the applications discussed in Section IV.

By now more than 1000 dyes have been tested for their response to changes in membrane potential in squid axons (*Cohen* et al., 1974; *Ross* et al., 1977; *L.B. Cohen, A. Grinvald, K. Kamino, S. Lesher* and *B.M. Salzberg*, unpublished observations; *I. Tasaki*, personal communication) and absorption or fluorescence signals were found with about half of these. Attempts have been made to use a number of these extrinsic signals as well as intrinsic signals to measure membrane potential in preparations as diverse as axons and chloroplasts and on time scales that range from 10^{-7} to 10^{3} s. It is likely that several different mechanisms will be required to account for all of the observed signals.

Changes in optical properties of cells and membranes have also been measured in an effort to study the structural basis of membrane activity. This topic has been reviewed elsewhere (*Radda*, 1971; *Cohen*, 1973; *Conti*, 1975; *Azzi*, 1975; *Levin*, 1976; *Tasaki* et al., 1976) and will not be discussed. Nor will we attempt to describe all of the changes in the optical properties of cells that occur as a result of activity; this review emphasizes those signals which are being used to monitor membrane potential. The application of optical methods to the measurement of interfacial or electrostatic surface potential (see *Fromherz*, 1973; *Fromherz* and *Masters*, 1974; *Haynes*, 1974; *Fortes* and *Hoffman*, 1974) was also considered to be outside the scope of this review.

There has been an unfortunate tendency for each user of the same dye to give it a different name. Therefore, we have included Roman or Arabic numbers in parentheses following the names of dyes; these numbers refer to Figure 1 or to the tables and appendices of *Cohen* et al. (1974) and *Ross* et al. (1977) where either a structure or a chemical name is given.

Our own research has been directed towards the use of optical signals to monitor activity in central nervous systems and our experiments have been limited to intrinsic signals and to fast extrinsic signals. However, we shall attempt to review the entire field. The review of the literature was completed in March 1977. Optical methods for measuring membrane potential were reviewed earlier by *Rottenberg* (1975) and *Waggoner* (1976).

II. Characteristics of the Optical Signals

A. Classification, Relation to Membrane Potential, and Time Courses

We have classified the optical signals into three categories: (1) fast signals, (2) redistribution signals, and (3) intrinsic signals. We hope that this classification will facilitate discussion of the mechanisms and difficulties involved.

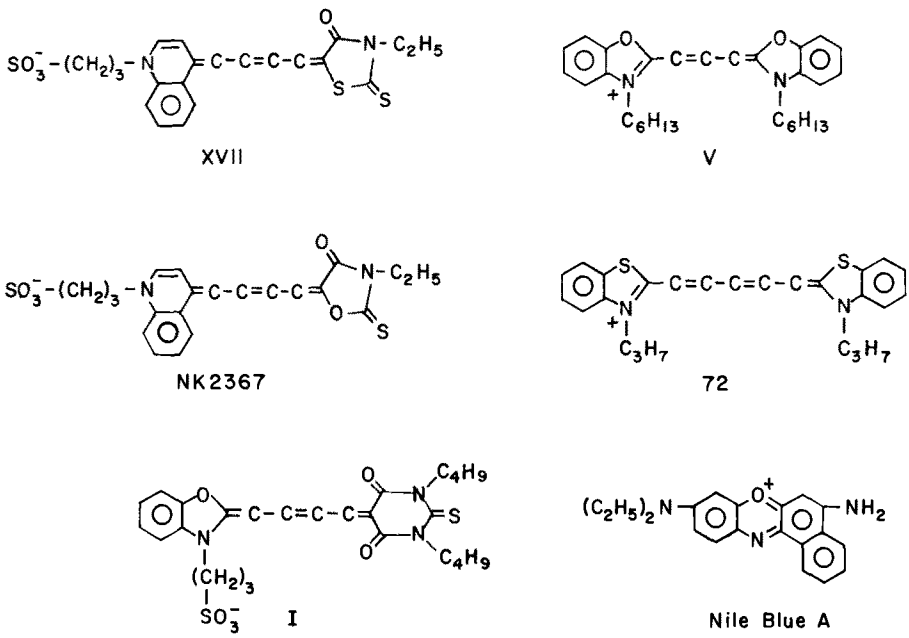

Fig. 1. Structures of several dyes which have been used to monitor membrane potential. Dye XVII was the dye used in the experiments illustrated in Figures 2, 3, 4A, and 7; dye NK 2367 was used in the experiments illustrated in Figures 8 and 9; dye I was used in the experiment illustrated in Figures 7 and 12; dye V was used in the experiment illustrated in Figure 5; dye 72 was used in the experiment illustrated in Figure 6; and Nile blue A was used in the experiments illustrated in Figures 10 and 11. Even though they have been frequently used, dyes I and V are no longer recommended

Although each of the three kinds of signals will be discussed primarily with respect to one preparation we think that it should, in principle, be possible to measure all three kinds of signals on any preparation.

In some preparations the evidence that an optical measurement is related to an electrode determination of potential comes from a direct comparison of the two. In other preparations indirect arguments must suffice. By way of example three different preparations with progressively less direct evidence will be discussed: (1) giant axons (fast signals), (2) red blood cells (redistribution signals), and (3) photosynthetic systems (intrinsic signals).

1. Fast Signals – Squid Axons

Figure 2 illustrates the results of a measurement of light absorption during an action potential in a squid axon stained with a merocyanine-rhodanine dye (dye XVII, Fig. 1). The dotted trace is the light intensity (750 nm)

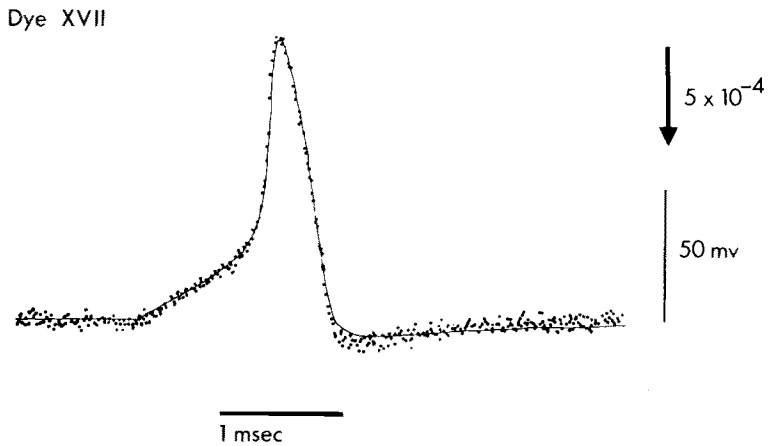

Fig. 2. Changes in absorption (*dots*) of a giant axon stained with dye XVII during a membrane action potential (*smooth trace*) recorded simultaneously. The change in absorption and the action potential had the same time course. In this figure and in Figures 3, 8, and 9 the direction of the *arrow* adjacent to the optical trace indicates the direction of an increase in absorption; the size of the *arrow* represents the stated value of a change in absorption, ΔA, in a single sweep divided by the resting absorption due to the dye, A_r. Incident light of 750 nm was used; 32 sweeps were averaged. The response time constant of the light measuring system was 5 μs. (*Ross* et al., 1977)

transmitted by the axon, whereas the smooth curve is the potential measured between internal and external electrodes. The axon membrane was space clamped, and the internal potential-sensing electrode was in the illuminated region of the axon, so that the optical and electric measurements were made at the same time and place. Because the two measurements had very similar time courses, it seems likely that the absorption signal is related to the changes in membrane potential that occur during the action potential and not to the ionic currents that flow or the membrane permeability increases that occur at the time of the action potential. Additional evidence for potential dependence can be obtained from voltage-clamp experiments such as the one illustrated in Figure 3. The top trace is the absorption signal; clearly, it has a time course similar to that of the membrane potential (middle trace) and distinctly different from that of the ionic currents (bottom trace).

While inspection of Figure 3 might suggest that signals with a time course similar to the currents or permeability are less than 5% of the total signal, in fact a conclusion this strong is unwarranted because the result in Figure 3 was obtained with a rather arbitrary amount of compensation for the resistance in series with the axon membrane (*Hodgkin* et al., 1952). Although the compensation used in this experiment implies a series resistance within the range of reported values, we did not measure the series

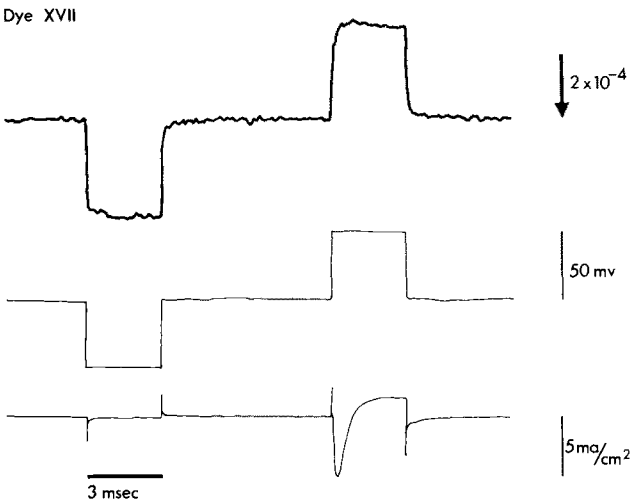

Fig. 3. Changes in absorption of a giant axon stained with dye XVII (*top trace*) during hyperpolarizing and depolarizing potential steps (*middle trace*). The *bottom trace* is the current density. The absorption changes had the same shape as the potential changes and were insensitive to the large currents and conductance changes that occured during the depolarizing step. The holding potential was the resting potential, and hyperpolarization is represented downward; inward currents are downward. Incident light of 750 nm was used; 128 sweeps were averaged; the time constant of the light measuring system was 20 μs. (*Ross* et al., 1977)

resistance independently to determine the amount of compensation. If the dye signal also followed membrane potential in a properly compensated axon, then the optical signal would, in at least one respect, be a better indicator of membrane potential than electrodes even in squid axons, since the dye would be measuring membrane potential and not the sum of membrane potential plus potential drop in the series resistance. Voltage-clamp experiments with and without compensation are illustrated in Figure 8 of *Davila* et al. (1974).

If an experiment with four potential steps is carried out, and the size of the absorption change versus the size of the potential step is plotted, then the result shown in Figure 4A is obtained. The absorption change was linearly related to membrane potential in squid axons over the range ± 100 mV from the resting potential. In subsequent experiments with larger steps carried out with dyes XVII and NK 2367, the absorption signals were found to be linearly related to membrane potential over the range − 130 to + 200 mV from the resting potential (*S. Lesher, K. Kamino, A. Grinvald, B.M. Salzberg* and *L.B. Cohen,* unpublished results). While a simple linear relationship between membrane potential and optical signal was found for many dyes, in some cases, two straight lines provided a better fit to the experimental results (*Cohen* et al., 1974; *Ross* et al., 1977).

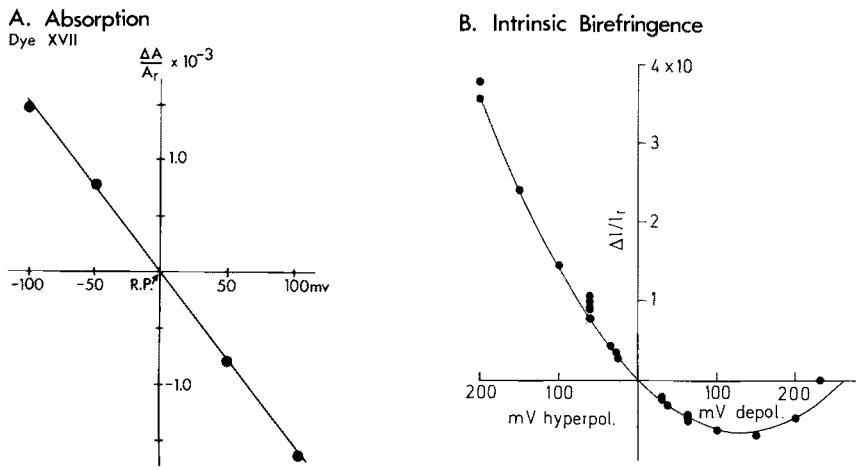

Fig. 4. (A) Change in dye XVII absorption as a function of membrane potential in a squid axon. The absorption of dye XVII was linearly related to membrane potential over the range tested. The duration of the voltage clamp steps was 1 ms. (*Ross* et al., 1977). (B) The relationship between membrane potential and the retardation change in a squid axon. The birefringence signal was proportional to potential squared (*Cohen* et al., 1971). In both A and B the origin of the abscissa was the resting potential

The rise time of the absorption change found with the merocyanine-rhodanine was too short to measure with our apparatus. At temperatures between 13° and 23° C the absorption change lagged behind the change in membrane potential by less than 10 μs. Since the dye is charged, its distribution across the axon membrane would be expected to change when membrane potential changed, if it were membrane permeant. However, because of the very large ratio of volume to membrane area, such a redistribution process should occur many orders of magnitude more slowly; therefore another sort of mechanism must be sought to explain this absorption change. Because the mechanism(s) responsible for this kind of extrinsic absorption signal has (have) not been identified, for want of a better name, we will call it a "fast signal" to distinguish it from the slower redistribution signals described next and the intrinsic signals discussed subsequently.

Almost all of the potential-dependent optical signals measured on giant axons had fast components (fast signals); some had additional components that were often much slower. For example, at the end of a half-second step the dihexyloxacarbocyanine (dye V) fluorescence was still changing (Fig. 6 of *Cohen* et al., 1974). Because our voltage-clamp apparatus was not reliable for longer steps, it was not possible to determine the time course of such slow components. However, slow components are found with the same dyes that exhibit redistribution signals (see below) and it is possible that they have the same origins.

One might conclude from Figure 4A that an optical measurement of membrane potential in a squid axon using dye XVII would have an accuracy of a few mV. In fact, such accuracy is presently attainable only in the very restricted circumstance that a calibration curve like the one in Figure 4A has been obtained on the same axon with only a short time (few minutes) intervening. With dye XVII the signal size/mV changed by 20% over a period of 10–30 minutes, and the signal varied (*Ross* et al., 1977) from axon to axon by a factor of up to two. With other dyes the drift with time on a single axon and the variation from axon to axon were considerably larger (*Cohen* et al., 1974). In addition, as a function of concentration, a few dyes exhibited signals that differed not only in magnitude and time course, but also in direction (*Cohen* et al., 1974). Furthermore, experiments with the same dyes on different preparations (see Sect. II, B, 1) suggest that there are sometimes substantial specie differences in the relative signal sizes. Clearly, if the magnitude of the potential change is of interest, it is desirable to have a calibration for each use of a fast signal. However, a calibration does not seem to be necessary if information about the time course of a potential change is all that is desired.

2. Redistribution Signals – Red Blood Cells

After we had tested about 150 dyes on squid axons, Dr. *J.F. Hoffman* suggested that the same probes might be used to measure membrane potential in red blood cells, so we gave *Hoffman* and *P.C. Laris* samples of our ten best dyes. They quickly found (*Hoffman* and *Laris,* 1974) that addition of valinomycin to red cells incubated with two cyanine dyes (dyes V and 72) in Ringer's solution led to an easily measured decrease in fluorescence. The untreated red cell membrane is more permeable to chloride than to sodium or potassium and measurement of the chloride distribution suggests a membrane potential of -10 mV. Addition of valinomycin increases potassium permeability and, in red cells in normal Ringer's solution, the ratio of internal to external potassium is large; therefore the membrane potential would become more negative. If the fluorescence change found after addition of valinomycin was dependent on membrane potential, the size of the signal should depend on the concentration of potassium in the Ringer's solution. The results of such experiment are shown in Figure 5 (from *Hoffman* and *Laris,* 1974) for red cells with an internal potassium concentration of 152 mM. Although the expected result is obtained, the change in fluorescence is not linearly related to the log of the external potassium concentration. A linear result would be expected on the two assumptions that fluorescence was linearly related to potential and that the potassium permeability was much larger than the permeability for any other ion. The solid curve in Figure 5 was computed from a Goldman equation with the ratio of potassium and chloride permeabilities taken as 3.

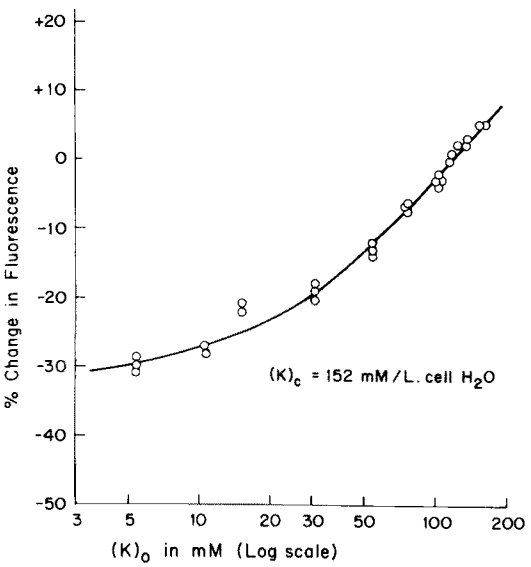

Fig. 5. The percent change in fluorescence of red blood cells incubated with dihexyloxacarbocyanine (dye V) following the addition of valinomycin is plotted versus external potassium. The internal potassium concentration was 152 mM. When the potassium permeability was increased and the external potassium concentration was low, there was a large decrease in fluorescence. (*Hoffman* and *Laris*, 1974)

To support the conclusion that the fluorescence signals in red cells were related to membrane potential, *Hoffman* and *Laris* then altered the Ringer's solution in two additional ways and in both cases decreases in fluorescence were found when the Ringer's solution was altered in a manner expected to cause a hyperpolarization. Further evidence that the fluorescence changes were related to changes in potential came from a measurement of the ratios of internal to external potassium and chloride at the external potassium concentration at which valinomycin resulted in no fluorescence change (115 mM in Fig. 5). Under these conditions the ratio of external to internal potassium should be equal to the ratio of internal to external chloride, and this was the result obtained (within 5%) using dipropylthiadicarbocyanine (dye 72). Using the potassium ratio (or the chloride ratio) at this external potassium, the membrane potential could be estimated in human and *Amphiuma* red cells and the estimation for *Amphiuma* cells (– 19 mV) could be compared with results of microelectrode measurements (– 15 mV) (*Hoffman* and *Lassen*, 1971; *Lassen*, 1972). Thus, all of these results were consistent with the hypothesis that the fluorescence changes in red blood cells were somehow monitoring membrane potential.

Considerable information has been obtained about the mechanisms responsible for the changes in fluorescence in red cells. *Sims, Waggoner, Wang,* and *Hoffman* (1974) showed that hyperpolarization led to an increased association of dyes with cells and that the fluorescence of this dye was quenched relative to dye in the solution. In addition to other possibilities, they suggested that the dye might be permeant and that the change

in cell-associated dye was actually a reequilibration of the positively charged dye across the membrane in response to changes in membrane potential. *Szabo* (1974) and *Waggoner* et al. (1975, 1977) showed that artificial lipid membranes did have a relatively high permeability for the dye. Then *Hladky* and *Rink* (1976a) provided direct evidence that addition of valinomycin does lead to a redistribution of the dye across the red cell membrane. On the assumption that the dye was in equilibrium they concluded that the relation between fluorescence and potential was non-linear; see Figure 6 (*Hladky* and *Rink*, 1976; see also *Freedman* and *Hoffman*, 1977). Indeed this was the expected result since a 30 mV hyperpolarization removes a substantial fraction of the dye from the extracellular medium so that an additional hyperpolarization has a smaller effect.

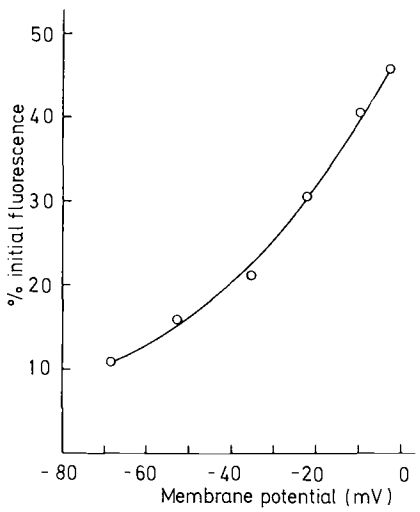

Fig. 6. A plot of the fluorescence of a suspension of red cells incubated with dye 72 against membrane potential. This relationship was also nonlinear. The membrane potentials were calculated from the dye distribution. (*Hladky* and *Rink*, 1976)

The rate of reequilibration following a change in potential would depend upon the dye permeability and the surface-to-volume ratio; other factors such as internal binding sites for the dye would prolong the time for reequilibration. In a series of thiadicarbocyanine dyes, *Sims* et al. (1974) found half times ranging from less than 2 to 30 s and similar values were obtained by *Hladky* and *Rink* (1976a). We classify fluorescence or absorption changes which depend upon reequilibration of a probe molecule across a membrane as "redistribution signals".

Although the results presented above suggest that these redistribution signals are potential dependent, their relatively slow time course allows the possibility that the redistribution signal does not depend directly on membrane potential but upon some other event that results from the

change in potential. One possibility is that a change in potential is followed by a redistribution of protons and that the change in internal pH alters the binding of dye to hemoglobin which actually leads to the changes in fluorescence. This possibility is discussed below (Sect. III, D, 2).

The sign of the redistribution signals for some oxa- and thia-carbocyanine dyes are concentration dependent (*Hoffman* and *Laris,* 1974; *Sims* et al., 1974; *Pick* and *Avron,* 1976; *Hladky* and *Rink,* 1976a). For example, in liposomes incubated in low concentrations of dye, addition of valinomycin leads to an increase in fluorescence while at higher dye concentrations a decrease is found. This kind of result emphasizes the importance of obtaining the most direct calibration possible before using a redistribution signal experimentally.

3. Intrinsic Signals – Photosynthetic Systems

Intrinsic optical changes as indicators of membrane potential have the obvious advantage that there can be no associated pharmacologic effects. Although intrinsic signals are smaller than some extrinsic signals, they have been used in situations where the membrane area is large or the experimental sensitivity is high.

When *Junge* and *Witt* (1968) first suggested that light-induced absorption changes in chloroplasts were the result of a change in potential across internal (thylakoid disc) membranes, the evidence was indirect. Clearly, the organelles are too small to allow transmembrane potential to be measured with microelectrodes. Later experiments did provide indirect evidence to support the hypothesis of potential dependence. In 1972, *Fowler* and *Kok* (1974) found, following illumination, that small, transient potential changes could be measured between bulk electrodes in a suspension of chloroplasts if the electrodes were separated in the direction of the light path. *Fowler* and *Kok* (1974) and *Witt* and *Zickler* (1973) suggested that the electric signals resulted from asymmetric changes of membrane potential in the individual organelles due to the fact that the side of the organelle facing the light source received slightly more light than the side away from the source. These electric events were very short lived ($\sim$ 10 μs) as would be expected if the membrane potential asymmetry was discharged by passive potential spread around the thylakoid disk. To make the electric signal last longer *Witt* and *Zickler* (1973) reduced the rate of passive discharge (due to ion fluxes) by a thousandfold increase in viscosity of the aqueous medium. To reduce the duration of the optical signal, they added ionophores to increase the membrane permeability. Under these conditions both the electrically measured potential and the light-induced absorption signal decayed with a time constant of about 1 ms. Thus, a special situation could be found where the optical and bulk electrode measure-

ments had similar time courses, supporting the hypothesis that the optical signal was measuring potential.

In another photosynthesizing preparation, chromatophores from the bacterium, *Rhodopseudomonas spheroides, Jackson* and *Crofts* (1969) compared the absorption changes induced by light with the absorption change induced by diffusion potentials generated by altering ion concentrations after the addition of ionophores. Because the spectral changes were relatively complex, the fact that both kinds of procedures resulted in essentially the same shifts supports the hypothesis that the signals resulting from the light flash are caused by the same events that result from changing the ion gradients. *Jackson* and *Crofts* (1971) found that the absorption change was linearly related to the logarithm of potassium concentration over the range 10^{-4}–10^{-1} M. On the assumption that the only relevant effect of changing ionic gradients is to change membrane potential, this result suggests that absorption was linearly related to membrane potential over a range of 170 mV. While both experiments provide preliminary evidence that the absorption signals result from changes in transmembrane potential, others (*Chance* et al., 1974; *Rottenberg,* 1975) have suggested that the signals originate from changes in potential that do not occur across membranes (local potentials).

The light-induced absorption signal in chromatophores occurs very rapidly, with $t_{1/2} < 100$ ns (*Jackson* and *Crofts,* 1971; *Wolf* et al., 1969). Other intrinsic signals that have been used to monitor membrane potential follow potential more slowly. The intrinsic birefringence change in axons has a fast component (20 μs) as well as slower components ($\sim$ 50 ms) (*Cohen* et al., 1971; *Watanabe* and *Terakawa,* 1976a). There is, in addition, a very slow component of the birefringence change that disappears with perfusion and presumably has axoplasmic origins (*Watanabe* and *Terakawa,* 1976a,b). The potential-dependent intrinsic light-scattering changes had time constants of 20 μs and 1 ms (*Cohen* et al., 1972). Neither the fast birefringence nor the light scattering changes were linearly related to membrane potential; they depended on the square of potential. The potential dependence of the birefringence signal is shown in Figure 4B. Although it is possible to assume that a birefringence signal will vary pseudolinearly with potential for small changes in potential, the fact that birefringence is actually related to the square of the potential introduces ambiguity when it is used in situations where an electrode calibration is not possible.

B. Signal Size

The size of an optical signal can be measured in two different ways. The first is to divide the detected change in intensity by the resting level to give a fractional change and the second expresses the size of the signal in relation to the noise in the measurement to give a signal-to-noise ratio.

1. Fractional Change

Fast Signals. Since the change in intensity is divided by the resting intensity, differences in the efficiency of different experimental setups are automatically compensated. If a given dye were to bind at the same membrane locations in different preparations, and if the concentration bathing the membrane were the same, then the fractional change/mV should be the same from preparation to preparation. The length of vertical arrows to the right of the optical traces in Figures 2 and 3 represent the stated value of the change in absorption divided by the resting absorption for the merocyanine-rhodanine dye (dye XVII, Fig. 1). Thus during a 100 mV action potential there is a $\Delta A/Ar$ of 10^{-3} or a change of 0.001%/mV. A change of 0.0003%/mV was found in leech neurons. However, since it is likely that the dye binds to many sites in addition to the neuron membrane, these values underestimate the fractional change of membrane-bound dye. If we assume that 10% of the dye is bound to excitable membrane, then the fractional change for membrane-bound dye is on the order of 0.01%/mV. Using dye XVII and NK 2367, *Ross* and *Reichart* (1977) found substantial species differences in signal size and in the spectrum of the signals. In the vertebrate preparations they tested, the signals were smaller by an order of magnitude. This suggests that the squid axon is not an ideal preparation for screening dyes for use in vertebrate systems. Using dye I, fractional changes between 0.005%/mV and 0.25%/mV have been obtained (*Landowne,* 1974; *Vergara* and *Bezanilla,* 1976; *Oetliker* et al., 1975; *Salama* and *Morad,* 1976; *Kamino* et al., 1978).

Redistribution Signals. The fractional changes associated with redistribution signals have been somewhat larger than the fractional changes found with fast signals. From Figure 10 of *Hoffman* and *Laris* (1974) a change of 0.5%/mV in red cells can be calculated (in this experiment the external pH was adjusted in an attempt to keep internal pH constant when potential was changed). From other experiments, values of 0.7–1.0% were obtained (*Hoffman* and *Laris,* 1974). *Hladky* and *Rink* (1976) obtained a value of 0.8%/mV for potentials between – 30 and 0 mV (see Fig. 6). *Pick* and *Avron* (1976) compared thiocyanate distributions with fluorescence and estimated 0.14%/mV in *Rhodospirillum rubrum* chromatophores and *Renthal* and *Lanyi* (1976) found 0.35%/mV in envelope vesicles of *Halobacterium halobium.* However, using the same concentrations of dye and red cells, *Freedman* and *Hoffman* (1977) obtained a value of 8.0%/mV, an order or magnitude larger. [This very large value may be partially explained by an influence of internal pH on dye binding to hemoglobin (see Sect. III, D, 2)]. In addition, the size and direction of redistribution signals depend on dye concentration and the ratio of dye concentration to cell number.

Intrinsic Signals. The fractional changes in the intrinsic signals have been small. For the light-induced absorption changes in chloroplasts it was 0.001%/mV (*Emrich* and al., 1969); for the fast birefringence change and the light scattering change it was 0.00001%/mV during an action potential using total axon birefringence or light scattering as the resting level (*Cohen* et al., 1970; *Cohen* et al., 1972). If these axon signals were expressed as fractional changes in membrane optical properties, however, they would be several orders of magnitude larger.

2. Signal-to-Noise Ratio of Fast Signals

The combination of larger fractional signals, slower measurements, and large membrane areas has generally resulted in redistribution signals that are so large that noise can be ignored. However, in the case of fast signals, noise in the measurements is often troublesome and considerable attention has been given both to reducing the noise and to increasing the signal size. An earlier summary of signal-to-noise ratios in fast signals can be found in *Cohen* et al. (1969).

a) **Noise.** There are two kinds of noise in light measurements that cause difficulty, *viz.*, irreducible noise inherent in the measurement of light (shot noise) (*Braddick,* 1961; *Levi,* 1968) and extraneous noise resulting from vibrations, disturbances in the optical path, instability of the light source, and dark noise in the measuring system.

The shot noise arises from the statistical nature of photon emssion and detection. Thus, fluctuations in the number of photons emitted per unit time will occur and if a light source emits an average 10^{14} photons/ms, the root-mean-square (RMS) deviation in the number emitted is the square root of this number or 10^{7} photons/ms. Collection inefficiencies in the lamp housing condenser lenses and the presence of filters or monochromators in the light path might typically reduce the light reaching the preparation to 10^{10} photons/ms. If the light collecting system has high efficiency (e.g., in an absorption measurement) then $\sim 10^{10}$ photons/ms will reach the photodetector and if the photodetector has a quantum efficiency of 1, then 10^{10} electrons/ms will be measured. The RMS shot noise will be 10^{5} electrons/ms and thus the relative noise is 10^{-5}. Since any reductions in efficiency will increase the relative noise, other advantages that may be obtained by using inefficient devices must be weighed against the disadvantage of a reduced signal-to-noise ratio. For instance, one might consider using a photomultiplier (quantum efficiency, $< 0.01-0.3$) in place of a silicon photodiode (quantum efficiency, 0.8) because the photomultiplier has a lower dark noise than the photodiode. This will be advantageous only if the dark noise is so much larger than the shot noise that a reduction in

dark noise more than compensates for the increase in relative shot noise. Alternatively, one might consider that a monochromator is more convenient and has better wavelength resolution than interference filters. However, the greater efficiency of filters means that the use of a monochromator will, in most instances, reduce the signal-to-noise ratio. Light sources having higher luminous fluxes will increase the signal-to-noise ratio in shot-limited situations. For example, using a quartz-halogen, tungsten-filament lamp in place of ordinary tungsten-filament lamps improved the signal-to-noise ratio. Further increases in intensity can be obtained with arc lamps (e.g., mercury or mercury-xenon) or a laser, but, with these sources, there are relatively large fluctuations in light output and the advantage of reduced relative shot noise is offset by difficulties in stabilizing the light source and in establishing differential (split-beam) optics to reduce the effect of the remaining fluctuations.

Reducing extraneous noise seems to be mainly a matter of persistence and attention to detail. Interference from line frequency noise (60 Hz and harmonics) is easy to eliminate with shielding and the use of battery power supplies. A number of precautions that seem to reduce noise from vibrations and other optical disturbances are described in *Salzberg* et al. (1977). With presently available detectors and amplifiers it is not difficult to keep the dark noise and amplifier noise smaller than the shot noise. Because the noise is dependent on so many properties of the apparatus, the signal-to-noise ratio for a given dye will, in general, not be constant from apparatus to apparatus even if the fractional change is identical.

b) Signal Size. The search for larger signals has been, to some degree, successful. In 1968 the largest signal-to-noise ratio obtained during a single action potential in a squid giant axon was about 0.6. Now the largest signal-to-noise ratio on the same apparatus is about 90, an increase by a factor of 150. Although Figure 1 illustrates the structures of several dyes which have been used to monitor membrane potential, some of these are no longer recommended. Dye I has too much photodynamic damage for many applications and dye V had more pharmacologic effects in red cells than did dye 72. Dyes which give relatively large signals with squid axons (Table 2 in *Ross* et al., 1977) have absorption maxima which range from 570 to 840 nm, a range broad enough so that it should be possible to use dyes in preparations with naturally occurring pigments without interference.

Unfortunately, ours are the only laboratories that are searching for larger fast signals. Although we have now tried 875 dyes (*Cohen* et al., 1974; *Ross* et al., 1977; *L.B. Cohen, A Grinvald, K. Kamino, S. Lesher,* and *B.M. Salzberg,* unpublished observations), our approaches and efforts have been limited. From 1971 to 1973 we measured only extrinsic fluorescence and since then mainly fluorescence and absorption although a few

dyes have been tested for birefringence. This leaves a large number of intrinsic and extrinsic phenomena that have not been explored, including circular dichroism, optical rotation, infrared absorption and dichroism, linear and circular polarization of fluorescence, slow fluorescence, phosphorescence, and energy transfer. Our attempts to find better signals have consisted mainly of testing commercially available compounds and then having analogues of the most sensitive of these synthesized. Most of the syntheses have been carried out by Drs. *C.H. Wang* and *A.S. Waggoner* although recently Nippon Kankoh-Shikiso Kenkyusho Co., Ltd. have also synthesized dyes at our request. However, our efforts have centered around photosensitizing dyes; we have ignored most other types. A number of applications in Section IV would undoubtedly be simplified, and others, not yet attempted, would become feasible if larger signals were available.

Our progress in finding larger signals has been reasonably steady; the largest signal has increased by a factor of between 1.5 and 5 each year since 1971. The fact that the increase in signal size has been fairly constant indicates that even larger signals may be found. However, one estimate of the maximum possible signal size (*Waggoner* and *Grinvald,* 1977) suggests that it will be difficult to do very much better.

C. Mechanism

A combination of experiments on biologic and artificial systems has been carried out in an effort to understand the mechanism(s) responsible for the optical signals that monitor membrane potential. Although these experiments have been relatively successful in explaining the resistribution signals, the fast signals and the intrinsic signals are not well understood.

1. Fast Signals

The spectra of the potential-dependent signals may provide clues about their origins. If the change in absorption in a squid axon stained with a merocyanine dye (dye I) is measured as a function of wavelength, the result obtained is shown in Figure 7A. This spectrum has a relatively complicated shape but it can be synthesized rather accurately from the difference spectra of monomers and dimers obtained from spectral studies of dye bound to lipid vesicles (*Waggoner* and *Grinvald,* 1977). Although the extraction of monomer and dimer spectra involves some assumptions, the ability to resynthesize the spectra in Figure 7A encouraged us to think that a potential-dependent, monomer-dimer equilibrium would explain the signals found with this dye (*Ross* et al., 1974, 1977). However, it has

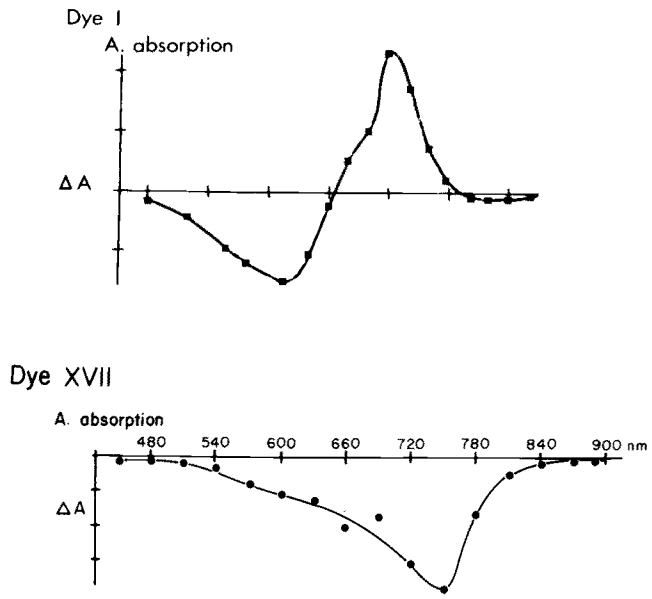

Fig. 7. The wavelength dependence of the absorption changes of dye I and dye XVII in experiments on squid axons. The absorption change found with dye I seemed to be consistent with a potential-dependent monomer-dimer equilibrium, whereas the curve found with dye XVII was qualitatively different. (*Ross* et al., 1977)

been difficult to explain the results of three subsequent experiments (*L.B. Cohen, A. Grinvald, K. Kamino* and *B.M. Salzberg,* unpublished results) with this simple hypothesis. First, the spectrum was found to depend on the concentration of the dye in the staining solution. If a lower concentration was used, the crossover point (near 550 mm in Fig. 7A) was shifted toward longer wavelengths. (In fact, this result argues against any two-state mechamism, not only the specific monomer-dimer mechanism that was originally proposed.) Second, the emission spectrum in fluorescence measurements was strikingly dependent on the concentration of dye in the staining solution. Third, the shape of the excitation spectrum depended on the emission wavelength that was used for the measurements. None of these findings could be explained by a simple potential-dependent shift in a monomer-dimer equilibrium where the monomers were highly fluorescent and the dimers weakly or non-fluorescent. Two additonal mechanisms, dye rotation and energy transfer from monomer to dimer have been suggested to explain these and other features of signals found with this dye (*Dragsten* and *Webb,* 1977; *Ross* et al., 1977). Another result that was not easily reconciled with a monomer-dimer hypothesis was the fact that the absorption changes of some membrane-bound dyes were in the same direction at all wavelengths (for example, dye XVII,

Fig. 7B), a result that is inconsistent with a simple wavelength shift in an absorption spectrum.

The mechanism(s) responsible for the fluorescence changes found with aminonaphthalenesulfonates has been investigated in some detail (*Tasaki* et al., 1973; *Conti* et al., 1974; *Carbone* et al., 1974; *Carbone* et al. 1975; *Conti,* 1975). Even though the fractional changes in fluorescence with this class of dye are only ten times smaller than those found with dye I, no changes in absorption have been detected (*H.V. Davila, B.M. Salzberg,* and *L.B. Cohen,* unpublished observations), indicating yet another mechanism. *Conti* (1975) suggests that three different phenomena are sufficient to account for all the results obtained with aminonaphthalenesulfonates: (1) a change in dye environment to account for shifts in the emission spectrum, (2) a change in dye binding to account for slow components and, (3) dye rotation to account for the sign of several signals.

Because there are a large number of possible mechanisms that could give rise to fluorescence and absorption changes, and since it has been necessary to invoke several mechanisms to explain the signals with any one or any small group of dyes, it may not be easy to provide a complete explanation for any signal. It seems likely that further investigations will confirm the suggestion that several different mechanisms give rise to fast optical signals and a more detailed classification of this kind of signal will be required.

2. Redistribution Signals

The results discussed in Section II, A, 2 provided convincing evidence that the redistribution signals somehow resulted from a reequilibration of the dye across the membrane. If membrane potential shifts in a hyperpolarizing direction, then the internal concentration of a permeant cationic probe will increase. Indeed, in experiments on lipid vesicles, red cells, synaptosomes, chromatophores, *Halobacterium* vesicles, and ascites cells, this increased internal concentration resulted in an decrease in fluorescence. *Sims* et al. (1974) described changes in dye absorption spectra that accompany the decrease in fluorescence in lipid vesicles, and in terms of earlier spectroscopic assignments for cyanine dyes (*West* and *Pierce,* 1965), these absorption shifts were attributed to dye aggregation resulting from the increased internal concentration. Aggregates are known to be much less fluorescent than monomers. Similar spectral shifts have been described for Ehrlich ascites cells (*J.C. Freedman, P. Laris* and *R.M. Johnstone,* personal communication). In both cases the new absorption bands were on the long-wavelength side of the monomer absorption suggesting that the aggregate was of higher order than dimer. However, in red blood cells, *Hladky* and *Rink* (1976), *Tsien* and *Hladky* (1978), and *Freedman* and

Hoffman (1977) found the major new absorption band on the short-wavelength side, suggesting the formation of dimers as internal dye concentration increased. Indeed, *Hladky, Rink,* and *Tsien* present spectroscopic evidence that the dimers are bound to hemoglobin. Thus, in both situations, increased internal concentrations led to aggregation and quenching of fluorescence, but the nature of the aggregate depended upon the internal composition.

3. Intrinsic Signals

Junge and *Witt* (1968) and *Schmidt* et al. (1971) claimed that the light-induced absorption changes in chloroplasts are electrochromic, i.e., due to a direct effect of the electric field on chlorophyll, carotenoid, and other chromophores. If these changes were electrochromic then one would expect them to exhibit the following properties: (1) The absorption changes should follow changes in the electric field very rapidly (time constants in the nanosecond range), (2) the field-induced spectral change should be a linear combination of the electrochromic shifts measured on the individual pigments. In addition, since the signals are small, the field-induced spectral change should have the shape of the derivative of the resting absorption. The first requirement may be satisfied in both chloroplasts (*Wolff* et al., 1969) and in *Rhodospeudomonas* chromatophores (*Jackson* and *Crofts,* 1971). In both systems the absorption changes occur in less than 100 ns after a flash. However, there is no way to be certain that transmembrane potential is changing this rapidly and, furthermore, this result does not distinguish among possible mechanisms since a number of other mechanisms (*Cohen,* 1973; *Conti,* 1975) might also have very rapid time courses. The second expectation will be difficult to fulfill without knowing the precise membrane localizations of the different chromophores in the chloroplasts. Thus, when *Schmidt* et al. (1971) tried to reconstruct the absorption signal in chloroplasts from the electrochromism of three constituents, they were only partially successful (some peaks were missing, others were displaced by 10–20 nm), but even this success was achieved only after a selection of molar ratios that was substantially different from that occurring naturally. In *Rhodopseudomonas* chromatophores the reconstruction was somewhat more successful (*Schmidt* et al., 1972). An additional difficulty with the suggestion that the absorption changes are electrochromic in origin is the finding that the signals are linearly related to potential (*Jackson* and *Crofts,* 1969) while electrochromic effects are expected to have components that are proportional to the square of the potential. This has been explained (*Schmidt* et al., 1971) on the assumption that there is a permanent potential gradient of 2×10^6 V/cm due to fixed charges. This hypothetical permanent gradient is quite large. There-

fore, although electrochromism may indeed be the mechanism responsible for the absorption changes, we feel that the evidence is not yet compelling. These (and additional) arguments against the electrochromic nature of the carotenoid signal have been put forward by *Chance* et al. (1974).

III. Difficulties

A. Pharmacologic Effects

Fast Signals. Two kinds of pharmacologic effects are possible: general toxicity and effects on specific membrane functions. Our own experience with fast signals on axons follows the universal rule that if you add enough of a foreign substance, it will be toxic. We would guess that toxicity was detected with a third of the dyes we have tried. Fortunately, the question of interest is whether the dyes are toxic at the concentrations necessary to give large signals; in general, they are not. Thus, at the concentrations necessary to give the signals reported in Table 2 of *Ross* et al. (1977) the sodium, potassium, and leakage currents in squid axons were unaffected in size or time course. The difference between the concentration needed to give a relatively large signal and the concentration where toxicity is evident will differ from dye to dye. Although we have no quantitative data, we think that the merocyanine (dye I) was more toxic than the merocyanine-rhodanine (dye XVII) or merocyanine-oxazolone (NK 2367) on squid axons and the same conclusion has been suggested by experiments on striated frog muscle (*S.M. Baylor, W.K. Chandler, S. Nakajima* and *H. Oetliker,* personal communication). We did not discover any toxic effects of the merocyanine-rhodanine or the merocyanine-oxazolone in experiments on leech segmental ganglia or supraesophageal ganglia from the barnacle (*Salzberg* et al., 1977; *Grinvald* and *Cohen,* 1977), although *D. Livengood* (personal communication) did find that the merocyanine-oxazolone dye had toxic effects in the cardiac ganglion of the lobster. If toxic effects are a problem in a given preparation, this factor could be evaluated in screening experiments and less toxic dyes might be found.

Redistribution Signals. Although *Hoffman* and *Laris* (1974) found that the dihexyloxacarbocyanine dye (dye V) altered sodium and potassium fluxes in red cells, a different cyanine, dipropylthiadicarbocyanine (dye 72), was found to have no such effects (*Sims* et al., 1974). However, specific effects of this dye and an analogue (dye 71) on the calcium-dependent potassium fluxes have been reported by *Simons* (1976). In addition, *Kinnally* and *Tedeschi* (1977) reported that an oxonol (dye XXI) inhibited succinate oxidation and that dye 72 inhibited β-hydroxybutyrate oxidation in mito-

chondria. Also, *Avron* and *Pick* (1976) reported that high concentrations of dipentyloxacarbocyanine (64) uncoupled *Rhodospirillum rubrum* chromatophores. As might be expected from charged dyes which bind to membranes, the addition of cyanine and merocyanine dyes to bilayers changes their surface potential (*Krasne,* 1977; and personal communication).

Since a redistribution dye must be permeant in order to function as a membrane-potential probe, it is expected that its addition to cells will increase membrane conductance. These effects are discussed below (Sect. IV, 1).

By now, dyes have been tried in a large number of preparations and in some instances pharmacologic effects are found. As might be anticipated from their permeance and access to the inside of cells, this seems to be especially true of dyes which give redistribution signals.

B. Photodynamic Damage

Damage to axons caused by illumination in the presence of dye and oxygen (*Arvanitaki* and *Chalazonitis,* 1961; *Pooler,* 1972) is found with all of the dyes illustrated in Figure 1 (*Cohen* et al., 1974; *Ross* et al., 1977). But, just as with pharmacologic effects, it is possible to find conditions where the damage is negligible. In squid axon experiments the amount of damage differed by a factor of 100 between the most phototoxic dye (dye I) and the least (dye XVII). With dye XVII the peak inward current was reduced by 50% in 1300 s as compared with 10 s for dye I. The photodynamic damage with the merocyanine-oxazolone dye (NK 2367) was also minimal and in experiments on barnacle ganglia, none was detected (*Salzberg* et al., 1977). *Hoffman* and *Laris* (1974) and *Doughty* and *Dodd* (1976) also found no photodynamic damage in their experiments on red blood cells and *Paramecia.* Even with dye I there are situations where photodynamic damage was not detected. *Salama* and *Morad* (1976) measured large fluorescence changes in heart muscle but report no damage. However, in later experiments with increased staining using a Langendorf preparation, damage was found after 15 min of illumination (*G. Salama* and *M. Morad,* personal communication). It is likely that much of the difference in amount of damage with dye I in squid axon and heart muscle is due to the fact that the incident intensity in the squid experiments was 100 mW/cm^2 (*Ross* et al., 1977) while the incident intensity in the heart experiments was only 0.1–1.0 mW/cm^2 (*G. Salama* and *M. Morad,* personal communication), although other factors such as density of staining are probably involved. In any case, photodynamic damage does not seem to be an obstacle to the use of extrinsic signals for measuring membrane potential.

C. Interactions of Reagents and Dyes

In some experiments where optical methods are used to monitor potential, the potential changes are initiated by passing current between electrodes. In this situation it is easy to control for stimulus artifacts. However, in other kinds of experiments, various agents are added to suspensions of cells, organelles, or vesicles and these agents may interact directly with the dye or directly with a dye-membrane complex. Several cases of direct interactions of agent and dye are known (Table 1). Although this list suggests caution, it is clear from the results in Section IV that there are many instances where dyes and agents do not interact. Controls for interactions between agents and dye-membrane complexes were first done by *Hoffman* and *Laris* (1974) and seem to be generally useful.

Table 1. Dyes and agents which interact

Dye	Agent	Reference
Dipentyloxacarbocyanine (dye 64)	Perchlorate	*Pick* and *Avron* (1976)
	Dinitrophenol	*Pick* and *Avron* (1976)
	Tetraphenylboron	*Pick* and *Avron* (1976)
	Salicylate	*Hoffman* and *Laris* (1974)
	Phloretin	*Hoffman* and *Laris* (1974)
Merocyanine (dye I)	Valinomycin	*J.C. Freedman* and *J.F. Hoffman* (personal communication)
	Gramicidin	*J.C. Freedman* and *J.F. Hoffman* (personal communication)
Merocyanine-rhodanine (dye XVII)	Cyanide	*A. Naparstek* and *C.L. Slayman* (personal communication)
	Azide	*A. Naparstek* and *C.L. Slayman* (personal communication)

D. Problems in Interpretation of Redistribution Signals

There are at least two kinds of difficulties that might be expected to occur with the use of redistribution signals. The first results from the fact that a redistribution dye must be permeant in order to function and the addition of a permeant molecule on one side of the membrane may itself alter the membrane potential or affect physiologic changes in potential. The second kind of difficulty arises from the relatively slow time course of the signals.

If it takes seconds or minutes for an optical signal to develop in response to a step change in potential, then it is possible that some of the signal is actually a response to some secondary effect of the potential change rather than the change in potential itself.

1. Dye Alters Resting Permeability

In chromatophores from *Rhodospirillum rubrum, Pick* and *Avron* (1976) reported that dipentyloxacarbocyanine (64) reduced photophosphorylation by 10% and increased hydrogen ion uptake by 30%. They suggested that the increased hydrogen ion uptake resulted from an increased membrane conductance and that these effects led to an underestimation of the changes in potential. *Krasne* (1977) reported that some cyanine dyes increased anion permeability in bilayers by forming channels. *Hladky* and *Rink* (1976) estimated the permeability of dipropylthiadicarbocyanine (dye 72) in red blood cells and obtained a value that was 10^4 larger than that for chloride (*Tosteson* et al., 1973; *Knauf* et al., 1977). Even though the dye concentration is quite low compared with other ions, this raises the possibility that the dye itself may participate in determining the membrane potential. However, this effect is probably small in *Amphiuma* red cells since potentials estimated from fluorescence were similar to those obtained with electrodes (*Hoffman* and *Laris,* 1974). Thus it would seem that only in membranes with relatively low resting conductances will the dye's permeance have detectable effects at the dye concentrations used thus far.

2. Dye Responds to Something Other Than Potential

A change in membrane potential may have many effects. For example, a change in potential will alter the equilibrium concentrations of protons and an altered internal pH might affect fluorescence. In intact red cells this is possible since pH has dramatic effects on the dimerization of dipropylthiadicarbocyanine (dye 72) on hemoglobin (*Hladky* and *Rink,* 1976a; *Tsien* and *Hladky,* 1978). If such an effect were responsible for the fluorescence changes, then the change in internal pH must have a time course similar to or more rapid than the change in fluorescence and thus the permeability of hydrogen must be similar to that of the dye and this is, in fact, the situation (*Hladky* and *Rink,* 1976a; *Knauf* et al., 1977; *Tosteson* et al., 1973). Therefore it is critical to know whether there is a change in internal pH when valinomycin is added to red cells. On this point the evidence is contradictory. Although *Hladky* and *Rink* (1976a) did not report a change in pH, *Kaplan* and *Passow* (1974) and *Callahan* and *Hoffman* (personal communication) did. These inferences about

changes in internal pH were made from measurements of external pH using hydrogen ion electrodes. The pH-dependent spectral changes of methemoglobin (*Drabkin* and *Singer,* 1939) could also be used to monitor internal pH, but we are not aware of attempts to resolve the dispute with this method. Thus, it is not yet certain whether the valinomycin-induced changes in fluorescence (and dye uptake) are simply the result of a change in membrane potential or are confounded to some extent by altered internal pH. If internal pH were affected, then a standard curve prepared using valinomycin would not be applicable to situations where there was no change in internal pH.

Callahan and *Hoffman* (1976) used dipropylthiadicarbocyanine (dye 72) in a measurement of erythrocyte responses to imposed transmembrane pH gradients. Hyperpolarizations of 15–20 mV were induced by an increase in extracellular pH from 7.1 to 9.0, and an equivalent depolarization resulted from a lowering of external pH from 7.1 to 6.2. However, the interpretation of the fluorescence signals in terms of membrane potential is ambiguous since any change in internal pH would alter the fluorescence of the dye or dye-protein complex independently of any change in membrane potential, and the interpretation of *Callahan* and *Hoffman* has been challenged on these grounds (*Hladky* and *Rink,* 1976b).

A change in internal pH is clearly not the only possible effect of a change in potential that could obscure the use of redistribution signals. Certainly the large variations in sensitivity, from 0.4%/mV to 8.0%/mV in different kinds of experiments on red cells (Sect. II, C, 1) suggest that effects other than changes in potential may be important.

IV. Applications of Optical Methods for Measuring Membrane Potential

A. Monitoring of Neuron Activity

Shortly after the discovery of optical signals that occurred during the action potential, *David Gilbert* called our attention to the possibility that such signals could be used to monitor activity in central nervous systems. However, the signals available at that time were quite small; thousands of sweeps had to be averaged, even in giant axons, and many more would have been required to measure a signal in an individual neuron. Following the discovery of the merocyanine dye (dye I) (*Salzberg* et al., 1972; *Davila* et al., 1973) it was used to monitor activity in individual neurons of a segmental ganglion from the leech (*Salzberg* et al., 1973). The signal-to-noise ratio was large enough that signal averaging was not necessary. An optical method was attractive because of the possibility of monitoring activity in many neurons both simultaneously and individually. We have recently

succeeded in monitoring activity in 14 cells simultaneously (see below) and hope to assemble an apparatus that will be able to monitor 50–100 neurons. If this succeeds, then one might consider even larger numbers. When the number of monitored neurons approaches the total number present in a portion of a nervous system, it should be possible to provide a reasonably detailed analysis of the neuronal basis of very simple behaviors.

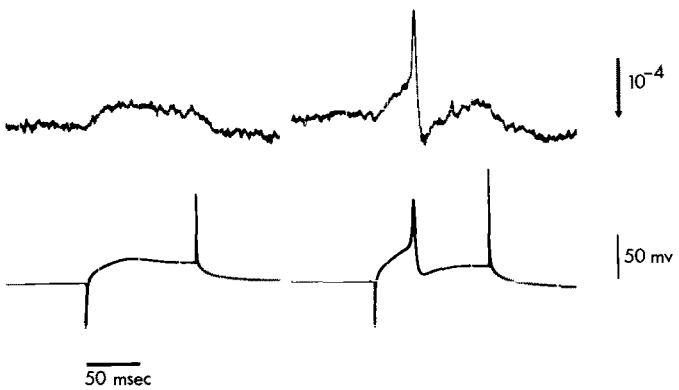

Fig. 8. Changes in light absorption (*top traces*) during subthreshold and suprathreshold depolarizations of a neuron from a supraesophageal ganglion that was stained with NK 2367. Simultaneous microelectrode recordings are shown in the *bottom traces*. These were single trials; signal averaging was not used. For both subthreshold and suprathreshold depolarizations the absorption changes have the same shape as the changes in membrane potential. [In experiments on tissue-cultured cells from the rat superior cervical ganglion the largest signals were found between 660–690 nm, not at 720 nm (*W.N. Ross* and *L. Reichardt*, 1977)]. The response-time constant of the light measuring system was about 600 μs. (*Salzberg* et al., 1977)

The results illustrated in Figure 8, comparing an optical and electric recording of membrane potential, indicate that an optical measurement also monitors membrane potential in barnacle neurons. This experiment was carried out on a supraesophageal ganglion from the barnacle using the merocyanine-oxazolone dye (NK 2367) (*Salzberg* et al., 1977; *Grinvald* et al., 1977). On the left a subthreshold depolarization results from passing current across the neuron membrane and on the right a slightly larger current results in an action potential. In both cases the optical and electrode recordings are essentially identical although the optical record is noisier (because of shot noise) and a careful inspection would show that it is slightly slower because the high-frequency response of the light measuring system was limited to reduce noise. On the other hand, there is ambiguity in the electric recording because of subjectivity in the setting of the bridge balance. The signal-to-noise ratio in the optical traces is large

enough so that we hoped to be able to monitor relatively large synaptic potentials optically and this was realized (Fig. 5 of *Salzberg* et al., 1977).

In the experiment illustrated in Figure 8 an enlarged image of the ganglion was formed with a microscope objective and then the light at the image of an individual neuron was conducted to a photodetector by a light guide having a diameter about the same as that of the image of the soma. The soma had been impaled with a microelectrode. Control experiments showed that the signal from the impaled neuron could be detected in the light forming its image but not from adjacent regions of the image plane.

It was not difficult to position 14 such light-guide-photodiode combinations over the images of 14 individual neurons. We recorded spontaneous activity in four 500 ms trials and then selected for each neuron the trial with the most activity. The results are illustrated in Figure 9. A photograph of the ganglion is on the right and the lines from the optical traces indicate the approximate position of the tips of the light guides. Most of the action potentials in Figure 9 result from spontaneous activity that was increased by reducing the concentration of divalent cations in the bathing solution.

In the traces in Figure 9 both the signal-to-noise ratio and the fractional change vary from neuron to neuron. Some of this variation may represent a real variation in action-potential size. In addition, improperly positioned light guides will yield signals that are misleadingly small. While it is likely that these two factors are important in explaining the variation, others may also be involved.

Chance et al. (1976) have reported preliminary attempts to use the merocyanine dye (dye I) to monitor activity in a mammalian central nervous system. They measured the light intensity from a relatively large area and used a relatively slow optical recording system so that there was no possibility of recording from individual axons or cell bodies. Since it is possible that optical recording might ultimately be quite useful in the study of vertebrate central nervous systems, additional efforts in this direction seem desirable.

B. Membrane Systems in Muscle

1. Skeletal Muscle

In skeletal muscle, a number of steps are known to occur during the latent period between the rise of a surface-membrane action potential and the development of tension by the contractile filaments. These steps include the spread of the action potential down the transverse tubules (T-system)

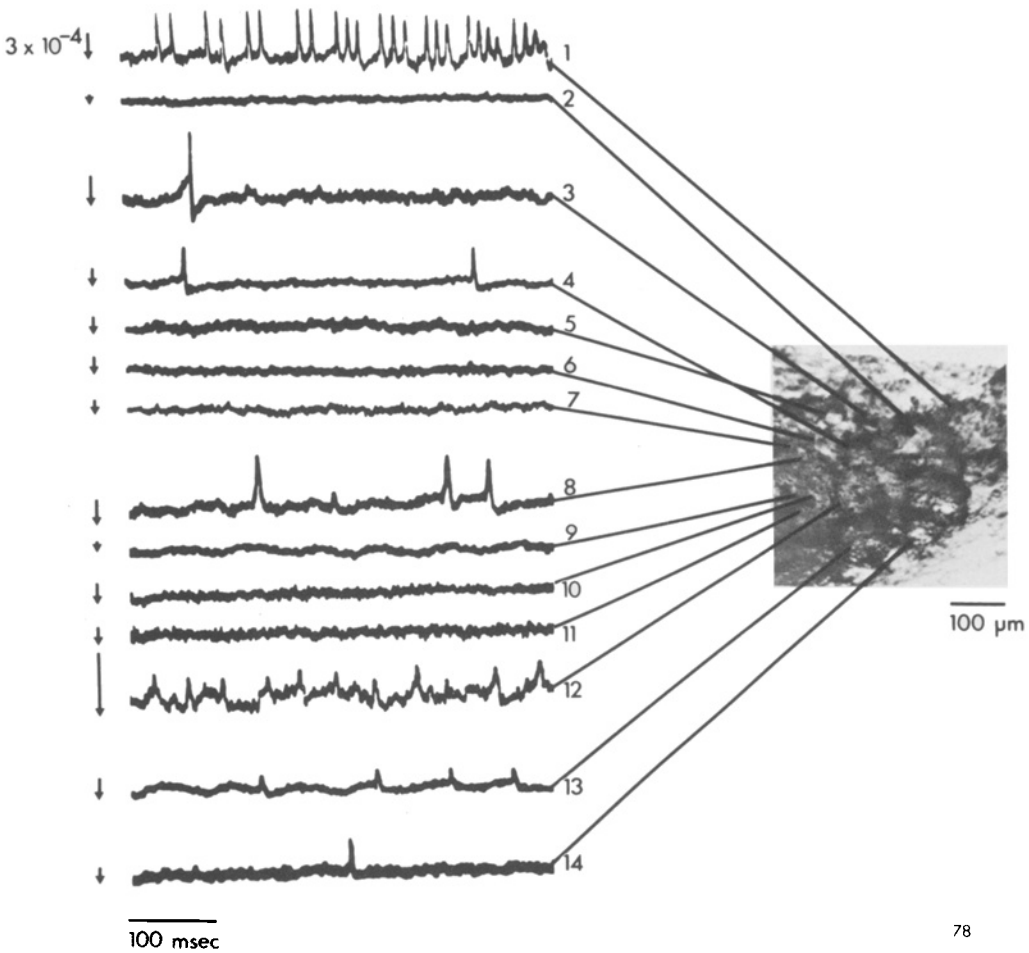

Fig. 9. Simultaneous optical monitoring of 14 neurons in a supraesophageal ganglion stained with NK 2367. This was a composite record from an experiment in which the amount of spontaneous activity was relatively large. Not all neurons were spontaneously active at the same time so four trials measuring the output of 14 detectors were recorded and for each detector the trial with the most activity was selected. All four trials were carried out in a barnacle saline with 1/10th the normal concentration of calcium and magnesium. The light measuring systems had response-time constants of about 600 μs. (*Salzberg* et al., 1977)

(*Constantin,* 1970), the release of ionized calcium from stores contained in the sarcoplasmic reticulum (SR) (*Jöbsis* and *O'Connor,* 1966), and the binding of calcium to troponin which removes inhibition on actin-myosin interaction (*Ebashi* et al., 1969). The release of calcium by the SR may be triggered by, or associated with, a change in trans-SR membrane potential. Thus, at least two membrane systems, the T-system and the SR, topologically inaccessible to microelectrode penetration, play essential roles in

excitation-contraction coupling. Several groups have now applied intrinsic and extrinsic optical methods to the problem of measuring membrane-potential changes in the surface membrane and T-system as well as the SR.

D.K. Hill (1949, 1953) and *Barry* and *Carnay* (1969; *Carnay* and *Barry,* 1969) first measured the transient and complex changes in light transmission, scattering, birefringence, and fluorescence (Pyronin B, dye 29) from skeletal muscle. Using stretch, hypertonic Ringers, and detubulation with glycerol to interrupt excitation-contraction coupling, *Barry* and *Carnay* (1969) divided the light scattering signal into three components: a fast component approximately coincident with the muscle action potential, a second, slower component, which occurred at about the time of latency relaxation, and a third, the slowest component, which was associated with contraction. Subsequent researchers have obtained results consistent with this general separation into three components. The slowest component may reflect changes in the optical properties of muscle that occur during contraction, or it may be an artifact of movement. We will not discuss this component further. The second component, which occurs at about the time of latency relaxation, may, as has been suggested recently, arise from changes in the SR membrane potential (*Bezanilla* and *Horowicz,* 1975; *Baylor* and *Oetliker,* 1975). For convenience we will call it an SR signal even though the evidence linking the signal to the SR membrane potential is not yet compelling. The earliest signal has a time course similar to that of the electrically recorded action potential and we will refer to this as the surface and T-system signal.

a) Surface Membrane and T-System Signal. Because *Barry* and *Carnay* used a penetrating dye, measured light from a bundle of about 50 fibers rather than from single fibers, but did not use intracellular recording, it was not possible to determine the precise relationship of their early signals to the action potential of the surface membrane. *Landowne* (1974) measured changes in fluorescence from snake muscle stained with merocyanine 540 (dye I). Since dye I was found to be relatively impermeant in squid axons (*Cohen* et al., 1974) and in liposomes (*Waggoner* et al., 1975), it is assumed that any signals in fibers stained from the outside with this dye originate from surface and T-system and not from SR membranes. *Landowne* found that glycerol-shock detubulation resulted in muscles from which no fluorescence change could be detected, and suggested that the fluorescence changes were associated with potential changes in the T-system membrane. However, these experiments were also performed on bundles and without simultaneous potential measurement. Subsequently, measurements of birefringence (*Baylor* and *Oetliker,* 1975. 1977c), merocyanine (dye I) fluorescence (*Vergara* and *Bezanilla,* 1976), and absorption (*Nakajima* et al., 1976) have been made with simultaneous measure-

ments of the intracellularly recorded action potential. *Nakajima* et al. (1976) and *Vergara* and *Bezanilla* (1976) concluded that the dye I signal was slower than the surface action potential. Since the dye I signal in squid axons is known to follow changes in membrane potential with very little delay (30 μs or less), it was concluded that a considerable fraction of the dye I signal in muscle must arise in the T-system membranes where membrane potential was presumed to change more slowly than at the surface. This conclusion was supported by further detubulation (*Eisenberg* et al., 1971) experiments. *Vergara* and *Bezanilla* (1976) found that detubulation decreased the amplitude of the signal by a factor of ten, increased the rate of decay of the signal, and increased the propagation velocity by 38%. In addition, they carried out fluorescence measurements of the membrane time constants using long pulses applied uniformly to TTX-treated bundles. The results, 7 ms on detubulated fibers as compared with 18 ms in intact bundles, were consistent with the interpretation that most of the dye I fluorescence signal originates with T-system membrane and that glycerol detubulation removes the tubular component of the signal along with the T-system membrane capacity. Although arguments are made suggesting that these effects could not result from damage during detubulation, the conclusions would be more certain if the experiment were repeated on single fibers.

While there was general agreement that the dye I signal was slower than the surface action potential, *Baylor* and *Oetliker* (1975, 1977c) found a birefringence signal in strongly hypertonic Ringer's with a time course almost identical to that of the intracellularly recorded surface action potential. They suggested that the birefringence signal from the T-system might be greatly reduced if approximately equal amounts of the T-system membranes were oriented parallel and perpendicular to the longitudinal axis of the muscle, as the morphologic data of *Mobley* and *Eisenberg* (1975) indicated.

These preliminary experiments with the fast signal certainly suggest that the potential spread in the T-system could be studied in considerable detail if a better dye (e.g., dye XVII) were used in experiments on voltage-clamped single fibers.

b) Sarcoplasmic-Reticulum Signal. The early reports (*Hill,* 1949, 1953; *Carnay* and *Barry,* 1969) of slower optical signals that occur at about the time of latency relaxation have now been confirmed in several laboratories and four such signals have been studied in some detail. A birefringence signal has been investigated by *Baylor* and *Oetliker* (1975, 1977a,b); a Nile blue A (dye 24) fluorescence signal by *Bezanilla* and *Horowicz* (1975) and *Vergara* and *Bezanilla* (1977); a dimethylindodicarbocyanine (dye XIX) fluorescence signal has been studied by *Oetliker* et al. (1975);

and a transparency (probably light-scattering) signal has been studied by *Kovacs* and *Schneider* (1977). Both Nile blue A and the indodicarbocyanine dye are membrane permeant (*H.V. Davila, A. Grinvald, W.N. Ross, B.M. Salzberg,* and *L.B. Cohen,* unpublished observations) and thus might be expected to monitor potential changes in both internal and external muscle membranes. Since the surface area of the SR membrane is 10–15 times greater than that of the surface plus T-system, the signal from a permeant dye might be dominated by changes in SR membrane potential if these were of comparable magnitude.

There are, however, unresolved ambiguities in the interpretation of the birefringence, indodicarbocyanine, and Nile blue A signals in muscle. In squid axons both the Nile blue A and indodicarbocyanine signals were linearly related to membrane potential when 1-ms steps were used (*Cohen* et al., 1974; *Ross* et al., 1977). However, the axon results do not demonstrate linearity of the signals in muscle membrane nor do they imply that linearity would obtain for longer steps. In addition, the birefringence signal in axons was proportional to the potential squared (see Fig. 4B). If this result holds for SR membrane, then the conversion of birefringence to potential will depend upon the potential across the SR in the resting state; and, since this potential is unknown, the interpretation of a SR birefringence signal will be ambiguous. Finally, both the indodicarbocyanine and the birefringence signal in squid axons had fast and slow components. If this is also the case in SR membrane, the time course of these signals will not have the same time course as a potential change. (However, since the surface-to-volume ratio in SR is much larger than in axons or red blood cells, a redistribution signal from the indodicarbocyanine dye signal might be more rapid than expected from axon or red cell experiments.) Since it is not easy to evaluate the importance of these difficulties, they have generally been ignored.

Birefringence. Baylor and *Oetliker* (1975), studying birefringence changes in single muscle fibers from the frog, found a three component signal. In normal Ringer's, the magnitude of the second component was about 150 times larger than that of the first component, which was thought to originate with the depolarization of the surface membrane. The second component was dependent on tonicity; with increased tonicity peak size and rate of rise declined and time to peak increased. In experiments with polarizing currents and TTX to block regenerative depolarization of surface and T-system membrane, these authors concluded that this signal is produced by some mechanism that is strongly regulated by the potential across the surface and T-system membranes. On the assumption that the second component results directly from a change in membrane potential, only the SR has sufficient area to account for the size of the

signal. The time required for this signal to peak, 2–5 ms after the surface action potential, was consistent with the suggestion that the second component reflects a change in SR membrane potential associated with the release of Ca^{2+}. In full-length papers, *Baylor* and *Oetliker* (1977a,b) presented results suggesting that the second component is not a manifestation of mechanical activity, although its magnitude is correlated with the magnitude of the tension response in a variety of conditions. The second of these papers presents detailed evidence that the intensity changes actually result from changes in optical retardation (birefringence times pathlength).

Nile Blue A Fluorescence. At about the same time, *Bezanilla* and *Horowicz* (1975) reported transient increases in fluorescence emission from frog semitendinosus muscles stained with Nile blue A (dye 24). Figure 10, from *Bezanilla* and *Horowicz* (1975), illustrates the Nile blue A fluorescence change (middle trace) together with intracellularly recorded action potential (top trace) and tension (bottom trace). The signal begins during the falling phase of the action potential and precedes tension development. *Bezanilla* and *Horowicz* suggested that the extrinsic fluorescence change was monitoring some event in the excitation-contraction-coupling sequence subsequent to the depolarization of the surface and T-system membranes and prior to the appearance of ionized calcium in the myoplasm. In particular, they concluded that the fluorescence responded to changes in the transmembrane potential of the sarcoplasmic reticulum.

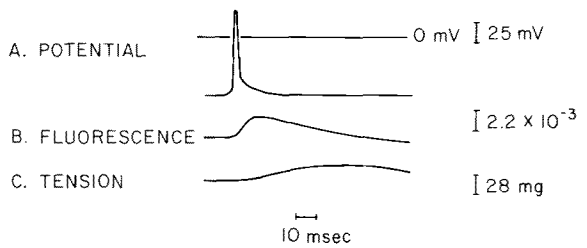

Fig. 10. Comparison of the time course of the action potential recorded intracellularly (A), the Nile blue A fluorescence (B) and the mechanical response (C), from a bundle of semitendinosus frog fibers. Voltage, fluorescence, and tension calibrations are as shown. Time calibration 10 ms. $T = 24^{\circ}C$, (*Bezanilla* and *Horowicz*, 1975)

Using an ingenious method of achieving uniform depolarizations of long lengths of muscle with triangular-shaped fluid electrodes (*Taylor*, 1952, 1953), *Bezanilla* and *Horowicz* also demonstrated a strongly nonlinear relation between the surface membrane potential and the fluorescence response, including the absence of any decrease in fluorescence on

hyperpolarization, This was taken as evidence for an origin of the signal with a process subsequent to the depolarization of surface plus T-system membrane. The effect of two agents known to affect E-C coupling was studied. D_2O depresses E-C coupling, and its replacement of water greatly reduced the fluorescence signal. By contrast, nitrate replacement of chloride in the Ringer's solution, a procedure that enhances E-C coupling, resulted in an 35% potentiation of the optical response. The effects of D_2O and nitrate substitutions indicated that the process being monitored optically was subsequent to that affected by nitrate and coincident with, or subsequent to, that depressed by D_2O. *Bezanilla* and *Horowicz* also examined the fluorescence response to brief trains of repetitive stimulation at different frequencies. The optical responses to successive action potentials were not additive, and, in fact, the response to a train of high-frequency (50 Hz) action potentials was similar to that obtained with depolarizing pulses using fluid electrodes. Each stimulus gave rise to a small increase in fluorescence, with the peaks declining to a constant plateau as the train was maintained. The falling phase at the end of the train had the same time constant as that at the end of a single stimulus. Assuming that the optical signal is monitoring membrane-potential changes in the sarcoplasmic reticulum, the maximum change in potential during a long tetanus is the same as that following a single action potential, and the effect of the tetanus is to prolong the change in membrane potential at the level of the sarcoplasmic reticulum. *Vergara* and *Bezanilla* (1977) have reexamined fluorescence signals obtained with Nile blue A, using cut single muscle fibers under voltage-clamp control. The changes detected were similar to the optical signals observed in whole muscles (*Bezanilla* and *Horowicz*, 1975).

Comparisons of the Four Signals. In general, the results concerning the physiologic properties of the birefringence and the Nile blue A fluorescence measurements were consistent. Observations on conduction velocity and effects of D_2O and nitrate replacement were similar for both signals.

All four optical changes tentatively associated with the sarcoplasmic reticulum had roughly similar time courses, are definitely delayed with respect to the action potential, and clearly precede the development of positive tension (see Fig. 10 for the Nile blue A fluorescence change). However, the time courses of the Nile blue A fluorescence change and the birefringence change, for example, compared by *Oetliker* et al. (1975), although similar, are hardly superimposable. *Kovács* and *Schneider* (1977) state that their transparency change has a time course similar to that of the Nile blue A fluorescence change although precise comparison is not possible because of differences in experimental conditions. In addition, the indodicarbocyanine fluorescence change had a time course that was

similar to that of the birefringence signal (*S.M. Baylor* and *W.K. Chandler*, personal admission). Although the time courses are thus reasonably similar, there are differences the origins of which are not presently understood. Some of these may reflect differences in experimental procedure; others may, when explained, reveal new information about the events being monitored.

An important property, referred to earlier and common to all four signals, is their strikingly nonlinear dependence on surface membrane potential. Hyperpolarizations and small depolarizations lead to very small or undetectable signals, whereas larger depolarizations into the range of potential where contraction is activated lead to large signals. Figure 11 shows a plot of signal size versus potential for the light scattering (open circles), birefringence (triangles) and Nile blue A fluorescence (filled squares) changes. The voltage dependence of the three signals are both similar and dramatic. The threshold for each of the signals is close to the contractile threshold (*Hodgkin* and *Horowicz*, 1960), and, as is apparent from a comparison with the contractile activation data of *Hodgkin* and *Horowicz* (1960) (filled circles), the steepness is close to that of the voltage-tension relation. The differences in steepness may be attributed to the duration of the depolarization (*F. Bezanilla* and *C. Caputo*, personal communication); *Hodgkin* and *Horowicz* used KCl contractures; the optical studies used step depolarizations of less than 0.1 s duration.

Bezanilla and *Horowicz* (1975), *Baylor* and *Oetliker* (1977a), and *Kovacs* and *Schneider* (1977) have considered possible origins of the optical signals and have presented arguments against latency relaxation,

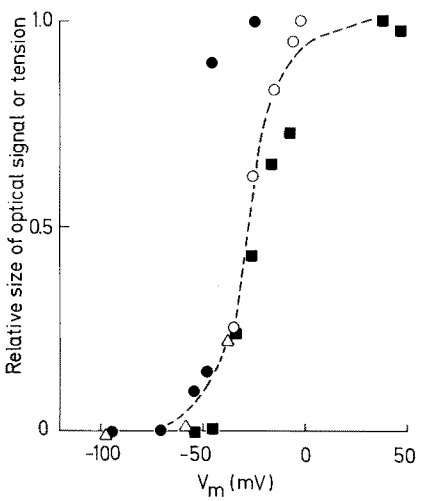

Fig. 11. Size of the transparency signal (*open circles*), the birefringence signal (*open triangles*), and the Nile blue A fluorescence signal (*closed squares*) as a function of surface membrane potential. These are compared with fraction of peak tension developed at these potentials (*closed circles*) taken from the data of *Hodgkin* and *Horowicz* (1960). The optical signals all have a similar voltage dependence to that exhibited by tension. The ordinate scale gives the response, optical or mechanical, as a fraction of the maximum elicited. All three optical records obtained in TTX Ringer's; mechanical record in choline-substituted Ringer's. (*Kovácz* and *Schneider*, 1977; *Baylor* and *Oetliker*, 1975; *J. Vergara* and *F. Bezanilla*, personal communication; *Hodgkin* and *Horowicz*, 1960, fiber G)

a signal from the T-system, triadic junction (*Franzini-Armstrong,* 1970), or myoplasm. These authors think it is likely that the signals do originate from the sarcoplasmic reticulum. On this evidence and using reasonable geometric assumptions (*Peachey,* 1965), *Baylor* and *Oetliker* (1975) estimated that there is a change in SR membrane potential of about 135 mV. While *Kovács* and *Schneider* (1977) suggested that their transparency signal was related to changes in volume or refractive index of the SR, it is possible that the transparency signal is also monitoring SR membrane potential since light scattering changes dependent on membrane potential occur in squid axons (*Cohen* et al., 1972). However, another possible origin of all the signals, changes in the contractile proteins, has not been ruled out. Conformational changes in contractile proteins preceding tension development or conformational changes in regulator proteins (e.g., troponin-tropomyosin complex) might account for the birefringence and transparency signals and they could alter the binding (or other properties) of Nile blue A and dimethylindodicarbocyanine in such a way as to mimic the optical changes usually associated with alterations in membrane potential. In this regard, *Scordilis* et al. (1975) have provided evidence that changes in fixed charge on muscle proteins can affect the optical properties of another cyanine dye (dye V) associated with the muscle proteins. Also, electrostatic effects on the binding of dyes to membranes have often been noted (*Fortes* and *Hoffman,* 1974; *Haynes,* 1974, 1977; *Kragh-Hansen* and *Moller,* 1973a,b, 1974; *Fromherz* and *Masters,* 1974).

We do not know of a critical experiment that would conclusively determine whether the four signals are monitoring SR membrane potential or, rather, some events involving contractile proteins. However, experiments in which calcium binding to troponin is blocked immunologically, or actin-myosin interaction is blocked pharmacologically, might help to clarify this point. Another kind of experiment that might help resolve the question is the use of several other extrinsic probes of membrane potential that have quite different structures and charges. For example, if a negatively charged dye such as merocyanine-oxazolone (NK 2367) were microinjected or allowed to diffuse into the inside of a muscle fiber and if it gave the same signal as Nile blue A and dimethylindodicarbocyanine, this would be additional support for the hypothesis of a membrane-potential origin of the signal. A second experiment that might be useful in this regard is a simultaneous determination of the time course of the change in myoplasmic ionized calcium using Arsenazo III (*Brown* et al., 1975) or aequorin. If a detailed comparison of the time courses of the change in calcium and the change in dye signal were carried out, additional information about the origin(s) of the optical signals might be obtained.

2. Cardiac Muscle

Since the membrane area per unit volume in cardiac muscle is very large and many cells are synchronously active, optical recording of transmembrane electric activity should be facilitated. *Illanes* and *von Muralt* (1975) reported a dye I fluorescence signal from frog atrial septum related to excitation that was not eliminated by the calcium blocking agent D-600. However, the relation of the signal to the cardiac action potential could not be assessed from their abstract. At about the same time *Salama* and *Morad* (1976) demonstrated that dye I could be used to monitor electric activity in a variety of cardiac muscle cells; they subsequently found (personal communication) that dyes XVII and NK 2367 could also be used. They have monitored fluorescence, absorption, and extrinsic scattering (see *Ross* et al., 1977) changes in cardiac muscle preparations in which motion was suppressed by prolonged perfusion in Ca^{2+}-free Ringer's. Using a 2-mm diameter incident light beam, and simultaneous electric recording from a microelectrode impaling a single cell within the illuminated region, they obtained virtually noise-free fluorescence signals having the characteristic shape of cardiac ventricular action potentials, with fast upstroke, prominent plateau, and slow repolarization. Although the rate of rise of the upstroke of the optical signal (40 ms) was slower than the rate of rise of the electric recording (15 ms), this was attributed to the conduction delay as the action potential invaded the 2-mm spot. A variety of physiologic and pharmacologic interventions (increased frequency, cooling, epinephrine, Ni^{2+}, and Mn^{2+}) produced changes in the optical recording which were similar to those observed with intracellular electrodes in unstained preparations. *Salama* and *Morad* (1977) have also used the fluorescence signal of dye I to evaluate the spatial homogeneity of potential control in a sucrose-gap voltage clamp of cardiac muscle. In addition, they have used the extrinsic scattering changes of merocyanine-rhodanine (dye XVII) and merocyanine-oxazolone (NK 2367) to provide the voltage-sensing signal in their voltage clamp (photon clamp) (*G. Salama* and *M. Morad,* personal communication). We think that optical measurement of membrane potential in cardiac muscle holds considerable promise for improving the understanding of conduction anomalies in diseased heart. Rapid scanning techniques or two-dimensional detector arrays might be used to define the propagation pathway(s) for the cardiac action potential in the intact heart, under normal and pathologic conditions. An important first step in this direction is illustrated in Figure 12, from *Salama* and *Morad* (1976). In this experiment, the excitation beam was focused on different regions of the same heart; ventricular, atrial, and sinus venosus, and the characteristic differences in duration and shape of the action potential are reflected in the optical records. When the light

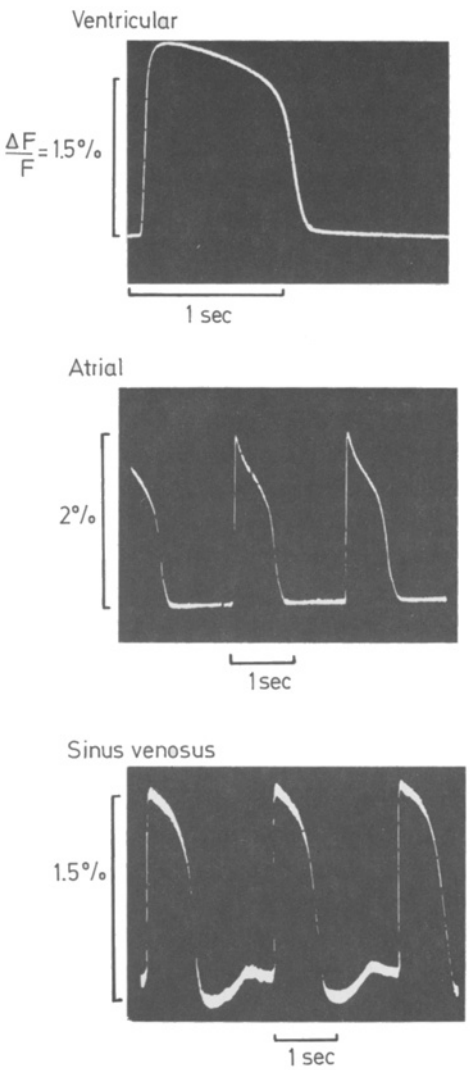

Fig. 12. Fluorescence changes from three regions of a frog heart stained with a merocyanine dye (dye I). The incident beam was positioned so that the recording was from ventricular, atrial, and sinus venosus regions. In all three cases the optical recording has a shape characteristic of electrically recorded action potentials from these regions. The light measuring system had a response-time constant of about 170 μs. (*Salama* and *Morad*, 1976)

beam was displaced 0.25 mm from a region displaying pacemaker action potentials, the "pacemaking" activity was lost, indicating adequate spatial resolution.

C. Suspensions of Cells and Subcellular Organelles

In this section, we review the application of optical methods of recording membrane potential to suspensions of cells and subcellular organelles. These applications involve time resolutions on the order of seconds; such signals are classified as redistribution signals (Sect. II, A, 2) and, as such, are

closely related to methods employing labeled lipophilic cations (e.g., *Bakeeva* et al., 1970; *Grinius* et al., 1970; and see below). As we have discussed earlier (Sect. III), interpretation of this category of optical signal is beset by a variety of pitfalls, and, therefore, conclusions regarding alterations in membrane potential in suspensions of membrane-bound systems must be accepted with caution. We have divided the literature into eight categories, which will be discussed in turn. These categories are (1) red blood cells, (2) synaptosomes, (3) extrinsic probes of photosynthetic or respiratory systems, (4) bacterial cells, (5) *neurospora,* (6) Ehrlich ascites tumor cells, (7) lymphocytes and platelets, and (8) *Paramecia.*

1. Red Blood Cells

Hoffman and *Laris* (1974) and the subsequent studies on mechanism established that in suspensions of human and *Amphiuma* red blood cells, extrinsic fluorescence changes on the order of 1%/mV could be measured when the state of electric polarization of the cell membranes was altered by the addition of a potassium ionophore (valinomycin). Many of these results have been summarized in Sections II, A, 2, and II, C.

An oxacarbocyanine (dye 64) was used (*Hartman* et al., 1976) in an effort to correlate reported circadian and semicircadian rhythms in enzyme activity in human red blood cells (*Ashkenazi* et al., 1975) with variations in membrane potential. Since these cells have neither nuclei nor machinery for protein synthesis, the observed oscillations in enzyme activation could not be dependent on DNA regulation, gene transcription, or protein synthesis. Membrane potential seemed a reasonable alternative. The fluorescence of dye 64 changed in synchrony with an apparent circadian rhythm in the activity of acetylcholinesterase and a semicircadian rhythm in the activity of glucose 6-phosphate dehydrogenase. However, the binding of the dye to osmotically shocked red blood cells exhibited very similar changes. This finding, together with the absence of any rhythmic variation in extracellular K^+ concentration in the presence of intact "rhythmic" red blood cells, suggested that the cyclic fluctuation in fluorescence intensity reflected changes in internal binding sites on the membrane, rather than membrane potential. This kind of result emphasizes the importance of comprehensive control experiments.

2. Synaptosomes

Following the introduction of cyanine dyes as membrane-potential probes in squid axons and their exploitation in red blood cells, *Goldring* and *Blaustein* used dipentyloxacarbocyanine (dye 64) to detect changes in membrane potential in synaptosomes prepared from rat brain homogenates

(*Goldring* and *Blaustein,* 1973; *Blaustein* and *Goldring,* 1975). They found that the fluorescence emission of a suspension incubated with dye 64 was proportional to log ($[K^+]_o$ + 0.05 $[Na^+]_o$) and that rubidium could substitute for potassium on an equimolar basis, whereas cesium was about 1/4 as effective as extrasynaptosomal potassium (i.e., $P_k \simeq P_{Rb} \simeq 4\,P_{Cs}$), indicating that synaptosomes have resting potentials that are determined primarily by the potassium diffusion potential. The depolarizing effect of increasing extrasynaptosomal $[K^+]$ was found to be reversible, and evidence was obtained suggesting that $[K^+]_i$ may be in excess of 100 mM. Two agents known to depolarize excitable cells by increasing their ionic permeability, gramicidin D and veratridine, both led to increases in fluorescence. The gramicidin D effect was that expected of an agent the principal action of which is to increase the permeability of the membrane to sodium (*Podleski* and *Changeux,* 1969). Veratridine is thought to act by maintaining the open state of the Na^+ channels that participate in action potentials (*Ohta* et al., 1973). Addition of 75 μM veratridine to the suspension medium produced a significant increment in suspension fluorescence, which was reduced by 3 x 10^{-7} M TTX, paralleling the results of *Ohta* et al. (1973) on excitable cells. Thus, optical methods indicated that synaptosomes maintain resting potentials sensitive to a variety of extracellular cations but are primarily potassium diffusion potentials, and that synaptosomes have functional sodium channels.

However, the use of permeant dyes on multimembrane systems like synaptosomes (or muscle, or *neurospora,* or Ehrlich ascites tumor cells, or lymphocytes, or platelets, or *Paramecia*) means that some way must be found to determine which of the membrane systems is involved in any signal, and it is not obvious how this can be accomplished. One way to avoid the dilemma is the use of impermeant dyes. *Kamino* et al. (1978), using dye I, reported many results on synaptosome ghosts that are similar to the findings of *Goldring* and *Blaustein,* suggesting that the results with dye 64 mainly represent changes in potential across the outer membrane of synaptosomes.

3. Extrinsic Probes of Photosynthetic or Respiratory Systems

In this section, we consider the application of optical methods to the measurement of transmembrane potential in a variety of respiratory and photosynthetic systems. These include mitochondria and submitochondrial particles, chromatophores and reaction center complexes, and vesicles containing purple membrane. This area of research is complex, and nowhere in this review is the caveat concerning our lack of expertise more applicable.

To a considerable extent, interest in membrane potentials in respiratory and photosynthetic systems can be traced to a hypothesis having far-reaching implications for respiration and photosynthesis put forward by *Peter Mitchell.* His chemiosmotic hypothesis (*Mitchell,* 1961) represented a new view of coupling between electron and hydrogen ion translocation and oxidative and photosynthetic phosphorylation. This hypothesis was intended to circumvent a number of difficulties in the orthodox chemical conception of coupling between ATPases and the electron and hydrogen ion transfer chains involved in catalysis. The hypothesis remains controversial, and its current status has been reviewed (*Greville,* 1969; *Skulachev,* 1971; *Slayman,* 1974). Essentially, the chemiosmotic hypothesis states that phosphorylation is driven by a proton-motive force established across a membrane, having electric and chemical components – a membrane potential and a pH difference. In mitochondria it is postulated that under most conditions the membrane potential is the principal component of the electrochemical gradient, and therefore the measurement of this potential is important for understanding the mechanism of oxidative phosphorylation and mitochondrial ion transport. Thus extrinsic potential-sensitive probes might provide a useful experimental technique for checking the predictions of this hypothesis.

Mitochondria and submitochondrial particles were, in fact, among the first systems in which extrinsic probes were used to detect presumed changes in membrane potential. A technique to monitor changes in membrane potential was developed by groups in the Soviet Union which,while not an optical method, uses the same phenomena (reequilibration of permeant anions or cations) that are the basis of the redistribution signals (Sect. II, A, 2 and C, 2). In this method (*Grinius* et al., 1970; *Bakeeva* et al., 1970), the accumulation of ions that penetrate phospholipid membranes was used to monitor membrane potential. *Grinius* et al. (1970) showed that the penetrating anions phenyl dicarbaundecaborane, tetraphenylboron, and picrate were accumulated in an energy-dependent fashion by sonicated submitochondrial particles. This accumulation was inhibited by rotenone, antimycin, and cyanide if the particles were respiring and was inhibited by oligomycin if ATP was used as the energy source. In intact mitochondria, *Bakeeva* et al. (1970) studied the energy-dependent accumulation of penetrating cations (dibenzyl dimethyl ammonium, tetrabutyl ammonium, and triphenly methyl phosphonium) and the accompanying acidification of the incubation medium. Since submitochondrial particles are thought to have "inside-out" membranes with respect to intact mitochondria (*C.-P. Lee* and *L. Ernster,* 1966) it was not surprising that the accumulation of anions into submitochondrial particles was very similar to the accumulation of cations into intact mitochondria in the following respects: (1) energy could be provided either by respiration or ATP hydrolysis, (2) the utilization of ATP energy (but not that of respiration) was

inhibited by oligomycin, (3) ion accumulation was rather nonspecific with regard to the structure of the penetrating specie, except for the sign of the charge, and (4) the ion transport in both instances was coupled to the movement of H^+. The first observation, that cations are accumulated in mitochondria and anions in "inside-out" particles, is taken as evidence that the mitochondria are negative inside and submitochondrial particles, positive inside, thus agreeing with Mitchell's hypothesis (1961, 1966). [The concluding sentence in *Bakeeva* et al. (1970) has its polarities inverted, in error].

About a year later, the fluorescence of the dye, 1-anilino-8-naphthalenesulfonate (ANS), was used to monitor nonenzymatic generation of a membrane potential in mitochondria and submitochondrial particles in what appeared to be an optical variation on the technique employing synthetic penetrating ions. *Jasaitis* and his collegues (1971) induced membrane potentials by the addition of an ionophore (valinomycin for K^+, tetrachlorotrifluormethylbenzimidazole for H^+) under conditions where there were K^+ or H^+ gradients across the mitochondrial membrane. Other authors had demonstrated that energization of mitochondria and submitochondrial particles is accompanied by changes in the fluorescence of ANS with a decrease in mitochondria and an increase in SMP (*Chance* and *Lee,* 1969; *Chance* et al., 1969; *Montal* et al., 1969; *Azzi* et al., 1969; *Packer* et al., 1969; *Azzi* and *Santato,* 1970; *Brocklehurst* et al., 1970), and *Azzi* (1969) had observed that submitochondrial particles take up and mitochondria extrude ANS in an energy-dependent manner (with the fluorescent cation, auramine O giving opposite responses). However, *Jasaitis* et al. (1971) seem to have been the first to attribute these changes to a membrane potential induced redistribution between the mitochondria and the water phase. Thus, the studies of *Jasaitis* et al. also tended to support the chemiosmotic hypothesis. At about the same time, *Azzi* et al. (1971) studied the interactions of ANS and 6-toluidino-2-naphthalene sulfonate (TNS) with mitochondrial membranes under different conditions of energization. These authors also examined the effects of valinomycin-induced diffusion potentials (anticipating *Hoffman* and *Laris*, 1974), and showed that ANS could be used to monitor membrane potential [fluorescence changes varying linearly with log $(K^+)_o$]. They concluded that there were differences in binding of ANS and TNS to mitochondrial membrane, depending upon its state of energization and that these differences could be attributed either to a conformational change or a membrane-potential change. In addition *Sone* et al. (1976) have used ANS fluorescence as an indicator of membrane potential in vesicles reconstituted from lipid and a purified ATPase. These results, obtained on mitochondrial membranes using these probes, must be interpreted with caution, however, in light of the observation of *Ferguson* et al. (1976) that the rate at which ANS

responds to ATP energization is significantly slower than its response to a diffusion potential, even when allowance is made for the time for the ATP-supported, energized state to reach equilibrium.

However, since ANS is relatively impermeant in squid axons (*Davila* et al., 1974) and artificial lipid bilayers, ANS reequilibration may be between membrane and solution rather than reequilibration between internal and external solutions so that ANS may not be behaving as a permeant ion in these systems (*Rottenberg,* 1975). Since the signals measured in suspensions have been several orders of magnitude larger than the fast ANS signals found in axons and bilayers, it is possible that they do not have the same origin. Furthermore, *Haynes* and *Simkowitz* (1977) have shown that ANS transport is sensitive to many factors other than membrane potential. Thus, it is uncertain at present whether ANS behaves as a redistribution dye, or if it senses membrane conformation, or if it signals potential by changes in its binding to the membrane. The use of this dye to measure membrane potential seems questionable.

Subsequently, cyanine dyes were used to estimate the membrane potential in mitochondria with conflicting results. *Tedeschi* (1974), and *Kinally* and *Tedeschi* (1976a, 1977) employed dyes I, V, XXI, 72, and 280 to argue against a significant role for mitochondrial membrane potential in phosphorylation (*Harris* and *Pressman,* 1969; *Tupper* and *Tedeschi,* 1969; *Harris,* 1973; *Tedeschi,* 1975). It should be noted, however, that although the evidence presented by *Kinally* and *Tedeschi* (1977) tended to argue against large membrane potentials, their results were inconsistent because one situation was found where the optical measurement did indicate a large potential. This kind of inconsistency is difficult to explain and it certainly suggests that it might be generally useful to check results with several different dyes and with more than one calibration procedure. However, there is no clue about what to do if different dyes indicate different potentials.

On the other hand, *Laris* et al. (1975) also used dye V to estimate the membrane potential of energized (state 4, *Chance* and *Williams,* 1955) mitochondria and, by extrapolation, obtained large values for the membrane hyperpolarization, consistent with the requirements of the Mitchell hypothesis. In addition, *Åkerman* and *Wikström* (1976) demonstrated that the spectral shifts in safranin O absorption in response to K^+ or H^+ diffusion potentials across mitochondrial membranes were identical to those observed on energization (by respiration or ATP hydrolysis) of isolated mitochondria and were linear up to at least 170 mV; they concluded that the energized state is characterized by a membrane potential of ~ -170 mV. These (metachromatic) shifts are apparently due to stacking (aggregation, including dimerization) of the dye molecules (*Åkerman* and *Saris,* 1976). Thus some researchers have obtained results supporting the Mitchell

hypothesis in mitochondria, whereas others have obtained results inconsistent with this hypothesis.

Another approach to making sense of dye experiments would be to try one dye on several preparations. *Smith* et al. (1976) have studied the response of an oxonol in intact mitochondria, submitochondrial particles, and reconstituted ATPase vesicles. They were able to interpret the absorption signals more easily than the fluorescence changes. Although they concluded that addition of substrate did change membrane potential, they did not estimate the magnitude of the change.

Two groups have used dipentyloxacarbocyanine (dye 64) to monitor light-induced changes in potential in membranes from photosynthetic bacteria. *Renthal* and *Lanyi* (1976) measured the development of the transmembrane potential in vesicles containing patches of purple membrane (*Oesterhelt* and *Stoeckenius,* 1971; *Blaurock* and *Stoeckenius,* 1971) from the cell envelope of the halophilic bacterium, *Halobacterium halobium,* and concluded that the total proton-motive force ranged between 150–200 mV, including a membrane potential contribution of 34–120 mV (inside negative), depending on the salt composition used. Thus, the concept of the purple membrane as a light-activated electrogenic proton pump (*Stoeckenius* and *Lozier,* 1974; *Bogomolni* and *Stoeckenius,* 1974) that can replace the respiratory system as an energy source for ATP synthesis was supported. The same dye was used by *Pick* and *Avron* (1976) to measure changes in membrane potential in chromatophores (fragments of the cell membrane containing the photochemical apparatus) from the photosynthetic bacterium *Rhodospirillum rubrum.* They demonstrated reversible changes in dye fluorescence and absorption when the chromatophores were illuminated (with actinic light filtered through a Schott RG715 filter). The signals were sensitive to metabolic inhibitors, permeable ions, and uncouplers. The light-induced polarizations of the chromatophores were estimated to be approximately 90–110 mV in the steady state (inside positive). An absolute calibration of the signal was difficult on three counts: (1) nonlinearity of the optical response at low external K^+ concentrations (reflecting either an intrinsic nonlinearity or the involvement of other ionic permeabilities), (2) dependence on factors other than membrane potential (including the redox state of membrane components), (3) probe modification of the membrane permeability and hence potential. Nevertheless, *Pick* and *Avron* concluded that, with appropriate precautions, the use of dyes compared favorably with any techniques employed previously to measure transmembrane potential in these chromatophores.

4. Neurospora

Because potential-dependent fluorescence changes measured with redistribution signals often exhibit severe nonlinearities at high membrane potentials, *Naparstek* and *Slayman* (1976) used the merocyanine dye (dye I) in an investigation of metabolism-dependent changes in membrane potential in the mold, *Neurospora crassa.* They calibrated their extrinsic absorption measurements of membrane potential against microelectrode measurements of membrane potential made in separate experiments. *Naparstek* and *Slayman* found that cells could be depolarized from their resting potential of about – 200 mV by successive addition of glucose, cyanide, and azide, with a differential absorption change (570–525 nm) of 5 x 10^{-4} OD corresponding to a 200 mV depolarization. The absorption change was found to vary linearly with membrane voltage over this range. These studies were the first to test the feasibility of using fast signals from merocyanine dyes on cell suspensions.

5. Bacterial Cells

Active transport in nonphotosynthetic bacterial cells has been studied by optical means in a number of laboratories (*Kashket* and *Wilson,* 1974; *Laris* and *Pershadsingh,* 1974; *Schuldiner* and *Kaback,* 1975; *Brewer,* 1976). *Kashket* and *Wilson* (1974) and *Laris* and *Pershadsingh* (1974) used the oxacarbocyanine dye (dye V) to monitor metabolically induced changes in membrane potential in *Streptococcus lactis,* and *Streptococcus faecalis,* respectively, Both investigations found that glucose (*Laris* and *Pershadsingh,* 1974) and arginine and glucose (*Kashket* and *Wilson,* 1974) metabolism is accompanied by a decrease in probe fluorescence and concluded that the membrane became hyperpolarized. These results are consistent with the chemiosmotic view of coupling of metabolic energy to the active transport of nutrients by means of a proton-motive force, having a transmembrane electric potential as an important component.

Schuldiner and *Kaback* (1975) used the method of permeant lipophilic cation accumulation (*Bakeeva* et al., 1970) to detect changes in membrane potential accompanying the oxidation of D-lactate and other electron donors in membrane vesicles from *Escherichia coli* and to evaluate the role of the membrane potential in driving active transport in these cells. One of the lipophilic cations employed was the dye safranin O, and in this instance, the technique became an optical method, since dye accumulation could be monitored by following the fluorescence of the suspension. The observed changes were discussed in the context of a chemiosmotic mechanism for active transport.

Brewer (1976) also used dye V to monitor membrane-potential changes in *E. coli*, in this instance probing the effect of the bacterial antibiotic protein colicin K on intact *E. coli.* Colicin K was found to induce a partial depolarization of the cell membrane (indicated by an increase in fluorescence), which may implicate an uncoupling of active transport in the mechanism of colicin-K-mediated cell death.

6. Ehrlich Ascites Tumor Cells

Fluorescence (dye 72) was used to estimate membrane potential under a variety of ionic and metabolic conditions in Ehrlich ascites tumor cells (*Laris* et al., 1976) in an effort to study amino acid transport in these cells. While calibration procedures using potassium and sodium ionophores were similar to those employed in red blood cells and synaptosomes, there were unexplained shifts in apparent resting potential depending on the conditions of incubation. Nevertheless, resting potentials measured fluorometrically (– 50 to – 60 mV "late dilution", compared to – 18 to – 42 mV, "early dilution", *Laris* et al., 1976) were consistently larger than those estimated by microelectrode measurements (*Lassen* et al., 1971; *Johnstone,* 1959). Indications were obtained of an electrogenic entry of amino acids coupled to sodium entry. Also, hyperpolarization followed the addition of glucose to cells having electron transport inhibited by rotenone, providing that the cellular Na^+/K^+ ratio was increased (and ouabain withheld), suggesting the involvement of a pump having variable stoichiometry.

7. Lymphocytes and Platelets

The application of optical methods using dye 64 to the measurement of membrane potentials in thymic and splenic lymphocytes and in platelets was established by *Bramhall* et al. (1976). The effects of extracellular calcium concentrations, as well as steroids, on the membrane potential of lymphocytes was then studied (*Morgan* et al., 1976). It had been observed earlier (*Smith* et al., 1975) that elevation of plasma calcium concentrations stimulates thymic lymphopoiesis in the male, but not in the female rat. However, in vitro, increasing extracellular calcium stimulated proliferation without regard to sex. The suppression of the response to elevated Ca^{2+} of female lymphocytes has been attributed to the presence, in vivo, of estradiol-17β, a steroid that also blocks calcium induced mitogenesis in vitro (*Morgan* and *Perris,* 1975). *Morgan* et al. (1976) showed that the membrane potential hyperpolarized upon the addition of extracellular Ca^{2+}, with the maximal effect occurring at about 1.8 mM. (Magnesium strontium, zinc, manganese, and tin had no effect.) The Ca^{2+}-induced hyperpolarization could not be reversed by adding EGTA, nor could it be

induced by the calcium ionophore A23187 (*Morgan* et al., 1976). However, estradiol-17β (10 μM) reduced the Ca^{2+} hyperpolarization by some 50% compared to controls. A series of structure-function experiments were carried out with the general result that the steroid structures that blocked calcium-induced mitogenesis tended also to block the Ca-induced hyperpolarization. The authors suggest that estrogens may block changes in membrane potential in lymphocytes that activate, directly, or through intermediate processes, DNA synthesis.

The mechanism of hemostasis (platelet aggregation in response to thrombin stimulation) has been investigated using two fluorescent probes, dyes 72 and 78 (*Horne* et al., 1977). Dye 72 was shown to bind to platelets and to indicate membrane-potential alterations, whereas dye 78, a doubly sulfonated analogue, did not bind and was used as a control for the effects of changes in light scattering of the platelets. (In the corresponding squid axon experiments, no change in fluorescence was found with dye 78, whereas a signal was found with dye 72; *Cohen* et al., 1974). This kind of control would seem to be generally useful in experiments of this nature. In this system, EGTA (1 mM) blocks platelet aggregation, and the addition of thrombin (0.2 μM) in the presence of 1 mM EGTA produced a 40% increase in the fluorescence of stained, freshly washed, human platelets, corresponding to a depolarization of approximately 7 mV (*Horne* et al., 1977), and the magnitude of the effect depended on the thrombin concentration. In addition, the effects of ADP and collagen, two other aggregating agents were studied. ADP produced a rapid decrease in fluorescence ($\sim$ 2 min) followed by a slow increase to a final level higher than the control. This change is also concentration dependent. Collagen, on the other hand, caused no change in fluorescence and the authors are unable to account for the differences among the three aggregating agents.

8. Paramecia

Several studies of the membrane basis of chemotactic responses in ciliate protozoa have been conducted using standard microelectrode techniques (*Naitoh* and *Eckert,* 1968, 1974), and these have indicated that negative chemotaxis in *Paramecium,* resulting from exposure to certain chemical agents, is accompanied by a depolarization. These investigations have been susceptible to the suggestion that, due to either mechanical restraint, or chemical suppression of ciliary beating, the animal might not be free to give normal expression to its behavior. Recently, a thiadicarbocyanine dye (dye 72) was used to monitor potential in response to a variety of chemical stimuli in free-swimming populations of the ciliates *Paramecium caudatum* and *P. aurelius* (*Doughty* and *Dodd,* 1976). At concentrations less than $\sim$ 1 μM, the probe affected neither the viability nor the motility, nor did

it induce observable chemotaxis. Cations known to induce a negative chemotaxis in *Paramecia* (*Naitoh* and *Eckert,* 1974), K^+, Na^+, Ba^{2+} as well as Ca^{2+}, TEA^+, and $choline^+$, produced a rapid (measurable optically in less than 10 s) depolarization of the membrane, which then remained constant for several minutes. The changes in membrane potential measured using optical means were found to correlate well with measurements employing microelectrode impalement, although a calibration of the magnitude of the fluorescence and absorbance changes in terms of membrane potential was not attempted. *Doughty* and *Dodd* (1976) suggested that the membrane potential shifts rapidly during the period of chemotactic response and then is maintained in the face of continued exposure to the stimulus.

D. Other Tissues

Optical methods for monitoring membrane potential have been applied to tissue slices from the bovine adrenal medulla (*Baker* and *Rink,* 1975). Using a thiacarbocyanine dye (dye 72), they investigated the release of catecholamines from chromaffin cells in response to maintained depolarization. After establishing that thin slices of adrenal medulla show responses to elevated extracellular potassium similar to those obtained with venous perfused glands, i.e., a transient secretory response, the authors wanted to demonstrate that the transient nature of the response was not due to a failure of the high K^+ (72 mM) to maintain depolarization of the chromaffin cells. They showed that a maintained exposure to high extracellular K^+ produced a stable increase in fluorescence of the stained medullary slices, indicative of a maintained membrane depolarization. In addition, isosmotic replacement of sodium with sucrose or choline resulted in a small decrease in fluorescence, a result that was in agreement with the hyperpolarization measured with electrodes on isolated chromaffin cells (*Douglas* et al., 1967). In control experiments on dyed adrenal medullary slices that had been osmotically lysed in distilled water, there was no alteration in fluorescence on exposure to high K^+. This seems to us to be another kind of control that would be generally useful.

V. Summary

Optical measurement of membrane potential is a new tool for physiologists and has already found many applications. However, the number of possible pitfalls is alarming, particularly in situations where comparison with electrode measurements is impossible. Exhaustive and elaborate controls

are clearly necessary; and yet they never provide complete assurance that an optical signal represents a change in membrane potential. In our opinion, the use of redistribution signals, which are slower, and thus more likely to represent to secondary effects of changes in membrane potential, and require permeant dyes with access to the internal millieu, may be more hazardous than the use of either fast or intrinsic signals. However, the larger size of the redistribution signals has endowed them with obvious appeal. If more sensitive fast signals can be found, the use of this kind of signal would be facilitated.

Even though optical methods for measuring membrane potential were introduced relatively recently, their uses have multiplied rapidly and will doubtless continue to proliferate. It seems likely that, in several instances, optical techniques will prove to be quite powerful and, used with caution, should provide information unobtainable by other methods.

Our use of the first person plural in this review is not intended to be exclusive, Our own experiments were carried out in collaboration with *Vicencio Davila, Amiram Grinvald,* and *Bill Ross* and we have benefited from discussions with them and with *Alan Waggoner* and *Jeff Wang.* We thank *S.M. Baylor, J. Freedman, J. Kaplan,* and *R.K. Orkand* for helpful discussions concerning the manuscript.

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Cell-Cell Adhesion Studies with Embryonal and Cultured Cells*

LUIS GLASER **

Contents

I. Introduction

It has been demonstrated in a variety of systems that when an embryonic organ is dissociated into single cells these cells can reaggregate and, in certain favorable conditions, these aggregates will differentiate so that histologically and biochemically they resemble the original tissue from which the cells were derived (*Moscona,* 1965). Very often the same cells, when not present in an aggregate, fail to show the same pattern of biochemical differentiation (see, e.g., *Seeds,* 1973; *Vogel* et al., 1976; *Ramirez,* 1977a,b; *Ramirez* and *Seeds,* 1977).

The process can be divided into several steps which occur on a different time scale. The first step is cell-to-cell adhesion, which we will define as taking place when a cell is attached to another cell. This process is complete in minutes and probably consists of two steps, one reversible, one

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irreversible (*Umbreit* and *Roseman,* 1975). The second step has less clear boundaries in time and represents the increase in aggregate size to ultimately form a stable, relatively large aggregate, consisting of hundreds to thousands of cells. This process probably occurs on scale time of the order of 12–24 hours (*Lilien,* 1968; *Garber* and *Moscona,* 1972a; *Hausman* and *Moscona,* 1975; *Moscona* and *Hausman,* 1977). Finally, there is the differentiation step, where the cells within the aggregate migrate to different regions of the aggregate and undergo biochemical and morphological differentiation. This process may overlap with the second stage and may take place over a period of several weeks (*Garber* and *Moscona,* 1972a; *Seeds,* 1973; *Ramirez,* 1977a,b; *Ramirez* and *Seeds,* 1977) (Fig. 1).

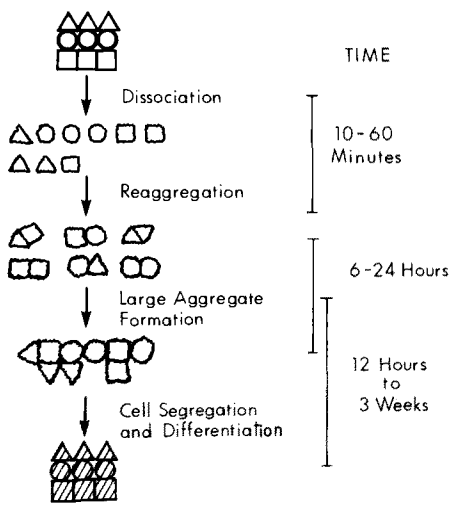


Fig. 1. Schematic chronology of the events in cell adhesion. An organized tissue of several cell types is dissociated into single cells with a possibly altered cell surface (indicated by changes in cell outline). These cells associate rapidly to form small aggregates, then large aggregates, and ultimately undergo cell sorting and histotypic differentiation indicated by the cross hatching of the cells

Specific cell adhesion[1] may be of importance in each of these three events. The initial adhesion between cells may be specific, as may be the formation of large aggregates. Cell segregation, sorting out of cells, and differentiation within an aggregate may arise as a result of differential cell affinities (*Steinberg,* 1963; *Steinberg* and *Garrod,* 1975; *Phillips* et al., 1977).

[1] For purpose of this review, specific cell adhesion will refer to the preferential adhesion of cell A to cell B as compared to cell C. Where possible, we will try to distinguish the possibilities that: (1) the preferential adhesion arises from the fact that cell A has a higher affinity for sites on cell B than on cell C, (2) the preferential adhesion arises from the presence on cell B of determinants absent from cell C, or (3) cell B contains more sites than cell C, although the sites are chemically identical. Preferential adhesion to homologous cells is frequently observed, in which case cell A and B would be identical.

It is generally assumed, but by no means proven, that the cell surface components responsible for each of these events under laboratory conditions are also functional and important during embryonal development. It is clear that variables other then cell surface adhesive specificity must be important in determining the pattern of cell adhesion and differentiation under culture conditions, since one might expect that nutritional and hormonal effects will be superimposed on the adhesive events. Thus, for example, cells may adhere to each other under certain conditions and yet fail to form large aggregates because their metabolism is in some way altered. Cells which have been dissociated under different conditions may have different alterations in surface components and their adhesive properties may differ. Thus it is not surprising that apparently small differences in experimental protocol may result in large differences in experimental results.

It is reasonable to think of cell-to-cell adhesion in the same terms as one considers the interaction of an enzyme with a specific ligand, or a hormone with a receptor (*Roseman,* 1970; *McMahon,* 1973; *Whittenberger* and *Glaser,* 1977). Thus the components which are responsible for cell adhesive specificity would always consist of a protein which can specifically bind to a ligand on an adjacent cell. This ligand could be another protein, but could also be the carbohydrate moiety of a glycolipid, a glycoprotein, or some other as yet unidentified membrane component. In cases where cell-to-cell binding induces morphological (*Overton,* 1977) and biochemical differentiation (*Seeds,* 1973; *Ramirez,* (1977a,b,c; *Vogel* et al., 1976) the induction is a result of either the binding of the ligand to the binding protein, or subsequent cell surface interactions brought about by the close apposition of two cell surfaces. A highly specific model of cell adhesion assumes that the two ligands are a glycosyl transferase and its substrate (*Roseman,* 1970; *Shur* and *Roth,* 1975).

There are a number of systems in which cell-to-cell adhesion has been studied; each has unique advantages and disadvantages. These systems include the study of cell adhesion in sponges, aggregating cellular slime molds, cells obtained by dissociation of embryonal tissues, cells obtained from teratomas, flagellar adhesion in chlamydomonas (*Snell,* 1976), sperm-egg interactions (*Schmell* et al., 1977), and cultured cells, often established permanent cell lines. The ideal system would be one in which cells rather than as a mixture of cell types, and where the adhesive events studied in the laboratory can be shown to mimick normal steps in development or differentiation. None of the systems listed is ideal from this point of view.

We will confine our discussion in this review to studies with dissociated embryonal cells and cultured cells. Several recent papers and reviews can be consulted for adhesion studies in sponges (*Burger* and *Jumblatt,* 1977)

and slime molds (*Barondes* and *Rosen,* 1976; *Frazier,* 1976, 1977) and this work will be mentioned in this chapter only for comparative purposes.

An examination of the adhesive events shown schematically in Figure 1 suggests that only the initial adhesive events will be easily available for an examination of the chemical nature of the adhesive molecules and that subsequent steps will be much more difficult to examine at the molecular level.

II. Assay Methods and Their Limitations

The following general assays have been used for the study of the initial events in cell adhesion:

a) Measure of disappearance of single cells. In this assay a suspension of single cells is placed in a suitable medium and incubated at fixed temperature and under control agitation (usually a rotary shaker bath); at intervals the number of single cells remaining are determined either in a hemacytometer or in an electronic particle counter (*Orr* and *Roseman,* 1969). Such an assay does not directly provide information regarding adhesive specificity, but can provide information on the specificity of agents which block or modify cell adhesion (*Merrell* and *Glaser,* 1973; *Brackenbury* et al., 1977).

b) A variety of assays patterned after the original work of *Roth* and *Weston* (1967) can be used to measure cell adhesion. These involve either the binding of single cells to large aggregates (*Roth,* 1968; *Roth* et al., 1971; *McGuire* and *Burdick,* 1976), or to cells attached to a solid support (*Walther* et al., 1973; *Gottlieb* and *Glaser,* 1975; *Rutishauser* et al., 1976). In all of these assays it is possible to measure the rate of adhesion of radioactivity labeled cells of one type to aggregates or immobilized cells of either the same or different type. The limitations of this kind of assay are primarily that it is difficult to assess the affect of formation of the large collecting aggregate or the attachment of the cells to a support on their adhesive properties. For example, aggregates are usually formed by incubating cells for 24 h in a rotating flask. The effect of this incubation on cell adhesion is unknown and difficult to control. One of the monolayer adhesion assays overcomes this difficulty by forming monolayers rapidly (Fig. 2), but has other limitations as discussed below. It is also possible that attachment of ligands such as concanavalin A at one place on the cell surface can affect the properties of surface molecules at a different location at the cell surface, probably by affecting the interaction of these proteins with components of the cytoskeleton (*Edelman* et al., 1973).

If the adhesive sites on the surface of cells are mobile, or restricted to a few areas on the cell surface, then it is possible that all of these assays are measuring the adhesive sites remaining exposed on cells after maximal

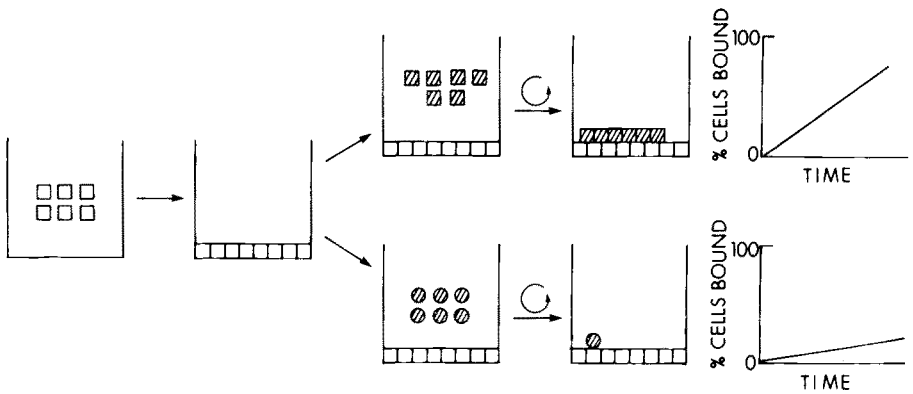


Fig. 2. Schematic outline of monolayer adhesion assay. Monolayer is prepared by centrifuguing cells on a glass surface which has been sequentially treated with γ amino propyl triethoxysilane and glutaraldehyde. Two different probe cell types are tested for their ability to adhere to the monolayer at a fixed rate of shear force generated by a rotating shaker. Preferentially cell adhesion is represented by a faster rate of adhesion of one cell type as compared to another. It is usually useful to carry out reciprocal experiments where the same cells are used in the monolayer as well as probe cells (*Gottlieb* and *Glaser*, 1975)

interaction with neighboring cells in an aggregate or in a monolayer (Fig. 3). If the sites are mobile this would be analogous to the capping phenomenon observed when multivalent ligands bind to cell surface molecules (*Taylor* et al., 1971).

Both the assays described under a) and b) are presumably critically dependent on the possible alterations to the cell surface brought about by the dissociation of the original tissue to single cells. If this dissociation has

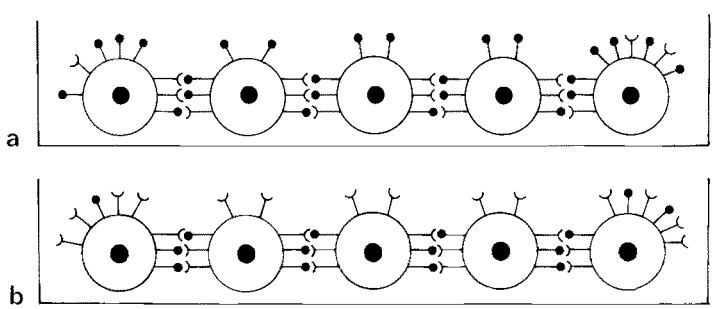


Fig. 3 a and b. Possible interaction of cells in a monolayer. A schematic illustration of how cell-cell interaction in the monolayer may change the apparent specificity of the cells if the assay only measures those ligands which are present in excess. (a) ♀ present in excess; (b) ♂ present in excess. A monolayer with excess ♀ would bind preferentially cells containing ♂ and the converse would be true for a monolayer containing ♂; yet both cells contain basically the same ligands

been carried out under very drastic conditions, extensive recovery periods may be required (see, e.g., *Barbera* et al., 1973; *McGuire* and *Burdick*, 1976; *McClay* et al., 1977).

c) One of the original cell adhesion assays measures the size of aggregates obtained after dissociation of cells and reaggregation for prolonged periods of time (*Moscona*, 1965; *Garber* and *Moscona*, 1972a,b; *Hausman* and *Moscona*, 1975, 1976). Unlike the assays listed under a) or b), this assay measures not only initial cell recognition events but also subsequent events which may include cell differentiation and the effects of trophic factors on cell metabolism and survival. Thus, as will be discussed later, factors that influence this assay may include "hormonal" as well as adhesive factors. The fact that these factors are tissue specific makes them both interesting and of obvious biologic importance.

d) Adhesion of membranes to cells. If cell-to-cell adhesion involves the adhesion of two complementary ligands on opposing cell surfaces, then it should be possible to carry out assays in which one measures the binding of isolated plasma membranes to cells. Such assays have been carried out in the embryonal nervous system (*Merrell* and *Glaser*, 1973) with cultured neuronal cells (*Santala* et al., 1977) and with hepatocytes (*Obrink* et al., 1977). The limitations of this assay are the purity of the membrane fraction and the assumption (probably incorrect, see below) that all cell surface ligands remain functional in the isolated plasma membrane fraction.

A different version of the assay has been used in which membranes are used as agents which either block (*Gottlieb* et al., 1974) or promote cell-to-cell aggregation (*Obrink* et al., 1977a,b). Membranes may inhibit cell aggregation if they contain only a single component of the two complementary ligands required for cell-to-cell adhesion and if, therefore, two cells which are completely coated with membranes can no longer adhere to each other. Membranes will promote cell aggregation if they can effectively act as cell-to-cell bridges. In theory, membranes could promote aggregation at a relatively low concentration and inhibit aggregation at a high concentration, but no system which shows this behavior has yet been described.

The membrane inhibition assay is a competition assay in which cells are either coated with membranes and remain as single cells or bind to each other; the speed of binding as well as the number of binding sites will influence the results, In this assay, a large excess of membranes will interfere with cell counting and membrane concentrations which allow the binding of only a few membrane vesicles per cell will not affect the rate of cell aggregation. Thus the assay is only useful over a very limited range of membrane concentrations. Therefore any quantitative changes in cell adhesion components which bring them outside the range of the method will be indistinguishable from qualitative changes in which certain adhesive components appear or disappear from the cell surface.

The membrane-to-cell binding assay is also a competition assay and the apparent rate of binding membranes to cells may be influenced by the rate at which cells bind to each other to form aggregates which may bind membranes less well than the corresponding cell suspensions. The promotion of cell aggregation by membranes will only be a useful assay in those systems where the rate of cell-to-cell binding is slow, so that a substantial effect on cell adhesion rate can be brought about by the additon of membranes. The binding of flagella to chlamydomonas is a special version of a membrane-to-cell binding assay, and should be mentioned here because the initial binding is rapidly followed by release of flagella which are no longer competent to bind to fresh cells (*Snell* and *Roseman,* 1977). This is so far an unique example where binding results in the irreversible destruction of the adhesive site, but it emphasizes the necessity for careful kinetic measurement to assess binding competence.

III. Specificity of Cell-Cell Adhesion

The embryonal nervous system has been a favored object for the investigation of cell-cell adhesion. The reasons for this are as follows: (1) Technically, it is relatively easy to obtain populations of highly adhesive cells from early (7–12-day) chick embryos. (2) There is an often unspoken assumption (or hope) that cell adhesion measured by one of the many variations of the methods discussed in Section II may be relevant to some aspect of synaptogenesis, which is the most precise form of cell-cell recognition known. However, it should be clear that the chemical landmarks by which an axon ultimately finds a target cell may be very different from the molecules by which cells adhere to each other.

The nervous system, even of very young embryos, consists of many functionally and morphologically different cells, and current methods of cell separation are generally not very effective in separating these different cell types, especially in the relatively large quantities required for cell adhesion assays. It is possible to demonstrate experimentally the presence of a number of regional adhesive specificities in the embryonal nervous system. However, it is not clear whether each of these experimentally defined components is not in fact composed of a number of different pairs of adhesive molecules unevenly distributed among the cells isolated from a particular region of the nervous system.

Although cell recognition was first studied in the embryonal nervous system before it was studied in cultured cell lines, it seems most reasonable to first discuss model systems which use cultured neural cell lines. These model systems have the advantage that the cell adhesion studies are carried out using homogeneous cell populations and the results are there-

fore more readily interpretable than those obtained in the nervous system. This review will focus primarily on the study of initial adhesive events. At the end of this section we will discuss measurements which clarify the later stages of cell adhesion (Fig. 1).

A. Adhesion of Cultured Neural Cell Lines

In this section we will discuss the adhesive properties of a series of neuronal cell lines mostly isolated in the laboratory of Dr. *D. Schubert.* These cell lines were all obtained by treating pregnant rats with a mutagen (nitrosoethylurea), the offspring that showed neurological symptoms was sacrifices and cells were cloned from tumors present in the central nervous system. The cells have been characterized as neural by a variety of criteria including morphology, antigenicity, their ability to synthesize neurotransmitters, the presence of sodium channels, and their content of nervous system specific proteins such as S-100 and 14-3.2. In this basis, these cells can be classified into those that appear to be primarily neuronal and those that appear to be primarily glial (*Schubert* et al., 1974; *Schubert,* 1974; *Stallcup* and *Cohn,* 1976).

A number of these cells have been tested in a monolayer adhesion assay as well as in a membrane-to-cell adhesion assay for their ability to adhere to cells derived from the embryonal nervous system. All the cells tested adhere preferentially to monolayers prepared from cells obtained from the embryonal nervous system (whether from rat or chicken) and a similar preferential adhesion can be demonstrated when one measures the ability of plasma membranes prepared from these cells to adhere to suspension of various cell types. Representative data are shown in Figures 4 and 5. These cells will also adhere to each other, as shown in Figure 4 (*Santala* et al., 1977).

An examination of the ability of plasma membranes prepared from these cells to adhere to either homologous or heterologous cells shows interesting nonreciprocal adhesive behavior. We will discuss these data in detail with regard to two cell lines, B103 and B65, and then present a hypothetical model which will account for cell and membrane adhesion properties of all of these cell lines.

B103 and B65 cells will adhere to each other in a monolayer adhesion assay. Plasma membranes prepared from B103 cells will adhere to either B103 cells or B65 cells, but plasma membranes prepared from B65 cells adhere to B65 cells, but not to B103 cells. Clearly, therefore, B103 and B65 membranes are different.

We rationalize these observations by assuming that the cell surface contains a series of adhesive molecules designated as A, a, B, b etc. These

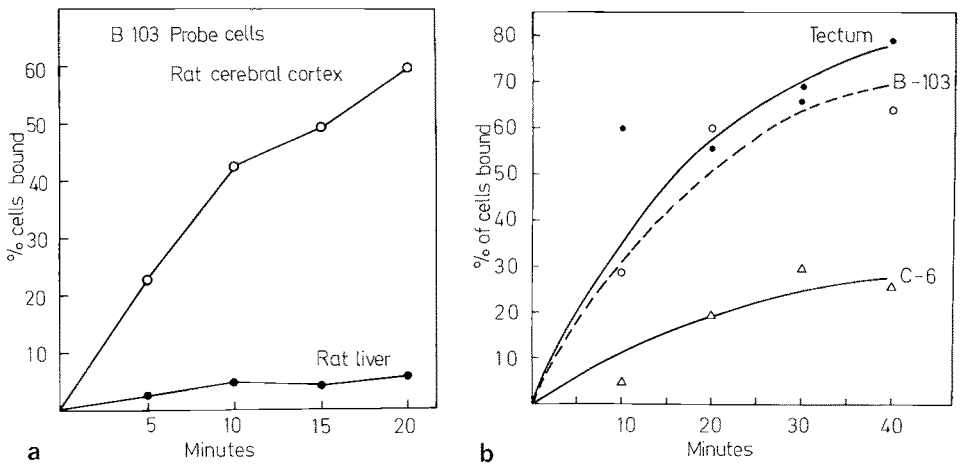


Fig. 4 a and b. Adhesion of neural cells to different monolayers. The assay measures the rate of adhesion of radioactive probe cells from cell line B103 (*Schubert* et al., 1974) to the indicated monolayers prepared from either cultured or from chick embryos. (Reproduced with permission from *Santala* et al., 1977)

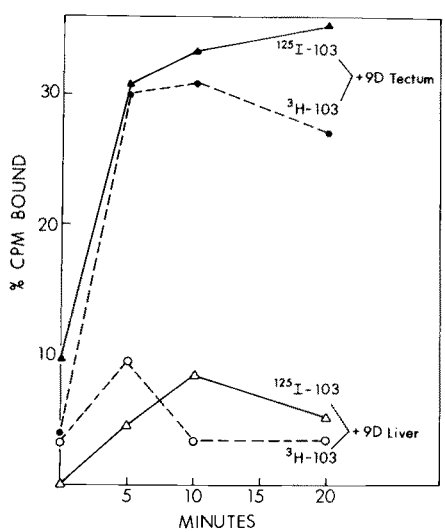


Fig. 5. Adhesion of membranes from B103 cells to cells. The adhesion of purified plasma membranes to suspensions of the cells indicated was measured. The membranes were labeled with [^{3}H] metabolically or prepared from cells isolated with glucose oxidase and lactoperoxidase. (Reproduced with permission from *Santala* et al., 1977)

molecules are such that A and a are molecules that interact and bind to each other in the same way as an enzyme binds to a substrate, or an antibody to an antigen. We assume that only the components designated as a, b, etc., remain functional in the plasma membrane. In this scheme B103 cells would have the components A, a, and b, while B65 cells would have the components B and b. B103 membranes would retain functional "a" and "b" components and could therefore adhere to both B65 and B103

cells. On the other hand, B65 membranes would only retain "b" and therefore could only bind to B65 cells, not to B103 cells. This speculation is illustrated diagrammatically in Figure 6. Since all of the cells and membranes show a low level of adhesion to non-neuronal cells such as liver, we hypothesize that a third adhesive pair, C, c, of low tissue specificity may also be present in these cells. By a totally different method, *Stallcup* (1977) has reached very similar conclusions regarding the adhesive properties of these cells. His results will be presented in detail below.

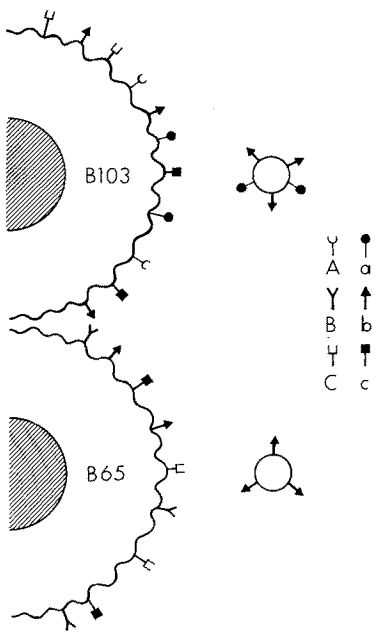


Fig. 6. Schematic model of the surface of B65 and B103 cells and the plasma membrane vesicles prepared from them. For details, see text

As can be seen in Table 1, the components designated as A, a, B, and b are adequate to explain the adhesive behavior of all the cells examined so far. It is interesting that these components appear independently on the cell surface, although we have not yet seen a cell line which expressed "b", but not "a". If these components can be expressed independently, then 16 different cell surfaces can be generated from these four components alone.

Precise synaptogenesis requires that an axon be able to distinguish a large number of different cell surfaces. It is generally assumed that such surfaces differ in their chemical composition and contain different surface molecules (*Sperry*, 1963). The presence of different cell adhesive molecules which are expressed independently on the cell surface leads us to speculate that the large number of cell surface specificities required for

Table 1. Adhesive characteristics of neural cell lines. The data labeled *Santala* et al. are based on assays performed by measuring the adhesion of plasma membranes to cells as described by *Santala* et al. (1977) and summarize observations by *Santala* et al. (1977), *Santala* and *Glaser* (1977). and unpublished observations of *Santala* and *Glaser*. As described in the text, the data can be explained by the presence on each cell line of a series of hypothetical ligands A, a, B, and b. The data described by *Stallcup* (1977) are defined in a monolayer assay with no shear force and are based on sensitivity to trypsin, antisera, and temperature. Complementary pairs 1–2 and 5–6 are trypsin-sensitive, 1–2 are sensitive to antiserum and 3–4 and 5–6 are temperature-sensitive, as is a fraction of ligands listed under 1–2 pair.

Cell Line	Cells	Ligands (*Santala* et al., 1977) Plasma Membranes	Ligands (*Stallcup*, 1977) Cells
B103	Aa	a	1, 3, 4, 5
	b	b	
B65	Bb	b	1, 2
B50	Aa	a	1, 3, 4, 5
	Bb	b	
B82	Bb	b	–
B108	Ab	b	2
B9 [1]	Aa	a	1, 3, 6 [3]
	Bb	b	
B9 [2]	Aa	a	1, 3, 6 [3]
	b	b	
C6 [1]	Bb	b	1, 2, 3 [3]
C6 [2]	b	b	1, 2, 3 [3]
B111	b	b	–

[1] At cell densities $< 10^5$ cells/cm^2.
[2] At densities $> 3 \times 10^5$ cells/cm^2.
[3] Density of cultures unknown.

synaptogenesis may arise by the expression of a limited number of cell surface molecules. Thus if the cells had six pairs of adhesive molecules, and each cell could express any combination from zero to the full complement of molecules, this would be $2^{12} = 4096$ different cell surfaces. Such a model obviates the necessity of generating thousands of different adhesive molecules, and generates the cell surface specificity by regulating the expression of a limited number of molecules. Whether this speculation has any real merit remains to be determined.

In a number of systems the specificity of synaptogenesis appears to be defined by competition, i.e., an axon will connect with those cells for which it has the highest affinity of all the axons that have access to that cell (*Jacobson*, 1970, 1976; *Hunt*, 1975). Such a situation would be most readily explained by gradients of adhesive components. It would therefore

not be surprising that in an artificial situation synaptic connections could be formed between cells that normally do not synapse, e.g., muscle and neural retina (*Puro* and *Nirenberg,* 1976). If synapse formation is determined by the use of a limited number of molecules in various combinations as suggested above, then such abnormal synapsis in artificial situations would in fact be predicted.

In examining these cell lines as a model for development, it is interesting to consider whether the adhesive components on the cell surface are fixed or whether it is possible under laboratory conditions to alter the adhesive specificity of these cell lines.

In two cell lines, C-6 and B9, it has been possible to show that the cells undergo density-dependent changes in adhesive behavior. These are illustrated in Figures 7 and 8. In the case of C-6 cells, at low density they contain the components designated as B and b, while cells at high density appear to contain only b. In the case of B9, the cells at low density express A, a, B, and b, but at high density will cease to express B. The expression of "B" is not density-dependent in all cell lines, since it appears to be expressed in B65 at all cell densities examined.

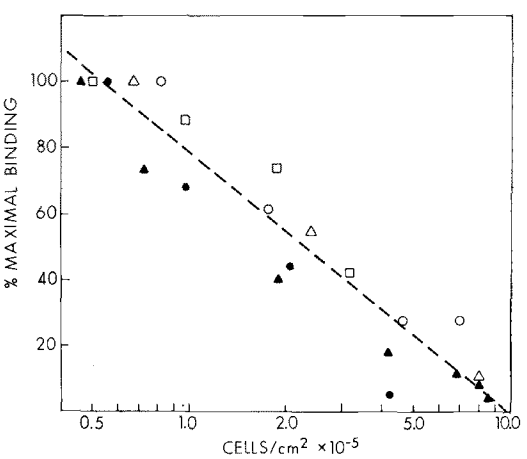


Fig. 7. Density-dependent changes of cell surface specificity in C6 cells. The data illustrate the ability of C-6 membranes prepared from either low or high density cells to adhere to cells grown to the indicated densities. A relative binding of 100% indicates the binding observed at the lowest density tested. Reproduced with permission from *Santala* and *Glaser* (1977). Different symbols indicate different experiments

The change in cell surface adhesive component in C-6 cell line is of particular interest since the cell line shows several other changes in cell metabolism only at high density. More notably, these cells only synthesize the S-100 protein at high cell density (*Pfeiffer* et al., 1970; *Labourdette* and *Marks,* 1975), and the expression of a catecholamine-dependent adenylate cyclase is also dependent on cell density (*Schwartz* et al., 1973; *Morris* and *Makman,* 1976).

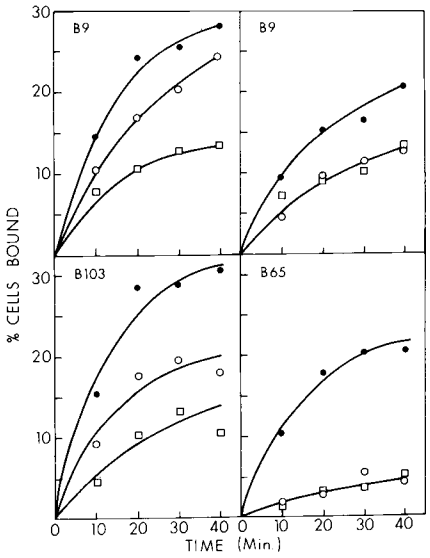


Fig. 8. Effect of cell density on adhesive specificity of B9 cells. The right and left panels are two separate experiments with membrane preparations from the indicated cell lines. At high density there is a partial decrease of the ability of B-9 cells to bind either B9 or B103 palsma membranes, but a total loss of the ability to bind B65 membranes. ● B9 cells grown to 8×10^{-4} cells/cm^2; ○ 2×10^{-5} cells/cm^2; □ 5×10^5 cells/cm^2

While these examples are relatively simple, they suggest that the expression of cell surface components in these cells is variable and therefore that these cells can serve as simple models for the more complex cell surface changes observed in the developing embryo. The loss of an adhesive component at high cell density should perhaps be considered analogous to the loss of hormone receptors from cells at high occupancy (*Lesniak* and *Roth,* 1976), an analogy which is more striking in the case of C-6 cells, where high density has hormone-like effects acting ans an inducer for the synthesis of several proteins.

In all of these systems, there occurs a low level of adhesion of the cells to heterologous monolayers. For example, although B103 cells clearly adhere preferentially to optic tectum as compared to liver, the cells nevertheless appear to show an ability to adhere to liver cells. This ability is much less pronounced or absent in plasma membrane fractions. It therefore appears necessary to postulate the presence of one additional pair of components on the surface of these cells (Cc) to account for this behavior. Under these assay conditions, this pair of components is expressed on the cells, but is essentially absent from the membrane. Thus the binding of membranes to cells represents a more selective tool for the study of all adhesion components than the study of cell-to-cell binding. Under different assay conditions, it is possible that the components designated as Cc would be the major adhesive component expressed during the assay, and no adhesion specificity would be observed under these conditions.

In an in vivo developmental situation, where the adhesive components may reach equilibrium (*Steinberg,* 1963; *Wiseman* et al., 1972; *Phillips*

et al., 1977), all the components would clearly contribute to the adhesive process and to the selection of neighbors by each cell.

Very similar conclusions regarding the presence of multiple adhesive components have been reached by *Stallcup* (1977) who has studied cell-cell adhesion in a monolayer binding assay and has been able to show, on the basis of sensitivity to trypsin, temperature, and antibody, the presence of at least three pairs of complementary adhesive molecules on the surface of several neural cell lines. Some of his results are summarized in Table 1. While the overall conclusion, i.e., multiple binding specificities are the same from both observations (*Stallcup,* 1977; *Santala* et al., 1977), it has not been possible to correlate the two sets of data. This suggests either that the two assay systems measure totally different components or that one of the assay systems can distinguish between different components which are grouped together in the otherassay system. The latter explanation seems plausible in light of the facts that all of the membrane binding assays are temperature-sensitive and the cellular component measured in the membrane-to-cell binding assay appears relatively resistant to trypsin. Because the adhesive properties of both C-6 cells and B9 cells, and possibly other cell lines, change with culture conditions, it is possible that some of the differences between the two sets of data are due, at least in part, to subtle differences in culture conditions which induce differences in cell-to-cell adhesive components. The latter possibility has not been examined systematically.

The effects of hormones on cell adhesion has not been examined in detail with cultured cell lines. A dramatic increase in the ability of a cloned pheochromocytoma PC-12 cell to adhere to plastic or to other cells has been demonstrated after addition of nerve growth factor (*Schubert* and *Whitlock,* 1977). The effect is rapid (20–30 min), occurs at physiological concentrations of nerve growth factor, and can be mimicked, at least in part, by dibutyryl cyclic AMP. While the implications of this interesting effect on cell differentiation induced by NGF are beyond the scope of this review, it is clear, from this and other observations (*Merrell* et al., 1975b), that a variety of trophic factors will influence cell adhesion. Since it is sometimes very difficult to control the concentration of some of these factors in complex growth media, this is yet another source of variability in cell adhesion assays.

The fact that this change in cell adhesion characteristics occurs rapidly and does not require the synthesis of new protein is in agreement either with, (1) the possibility that the cells have a large internal reservoir of cell surface proteins involved in cell adhesion which are externalized as a result of exposure to the trophic factor or (2) that changes in cell adhesion can be brought about simply by conformational changes of membrane proteins already exposed on the cell surface.

Several characteristics of cell-to-cell adhesion are common to many systems but can readily be discussed in the context of cell-to-cell adhesion observed with the neuronal cell lines. Most cell-to-cell adhesion systems show some sensitivity to proteolysis, although in many cases very gentle proteolysis may produce cells which retain the ability to specifically adhere to other cells (see, e.g., *Gottlieb* et al., 1976; *Grady* and *McGuire,* 1976a). In contrast to this, the adhesive properties of membrane are generally highly sensitive to proteolysis (*Santala* et al., 1977), suggesting that the relevant adhesive molecules are proteins and are more exposed in the isolated membranes than in the intact cells.

In general, cell-to-cell adhesion and membrane-to-cell adhesion are highly temperature-sensitive, and require the presence of a living cell as compared with a fixed cell (glutaraldehyde or formaldehyde) in order to occur (*Santala* et al., 1977). This suggests that adhesions as measured in these assays is more complex than the simple binding of an enzyme to a substrate, and is in agreement with a multistage binding process (*Umbreit* and *Roseman,* 1975) where the initial binding event is followed by a second step which is energy-dependent, and makes the binding much tighter or irreversible.

It will be crucial for a detailed understanding of cell adhesion to be able to translate these phenomenological observations into molecular events. For example, does cell-to-cell adhesion require a specific conformation of certain membrane proteins and does the maintenance of this conformation require energy? Are many of the cell adhesive components normally not exposed on the cell surface, and is exposure brought about by cell-to-cell contact (*Santala* et al., 1977) or by trophic factors? If so, what is the location of these molecules when they are not exposed on the surface?

The necessity for live cells in this system is in marked contrast to the cross-linking of cells by lectins, which will take place with fixed cells in a number of systems, including the lectins involved in cell adhesion in the cellular slime mold (*Barondes* and *Rosen,* 1976; *Frazier,* 1976).

The temperature dependence of cell-to-cell binding in a monolayer assay shows a steep transition. The temperature at which this transition takes place is independent of the viscosity of the lipid in the surface membrane. The midpoint of the temperature transition is dependent on the shear force used in the assay at high shear forces, but as the shear force is lowered, a limiting temperature is reached which becomes independent of the shear force. These data have been interpreted to indicate that at high shear force adhesion is limited by the rate at which multiple adhesion sites can interact after cell-to-cell collision. At low shear forces the temperature dependence may reflect the temperature dependence of an intracellular process such as energy production. In contrast, no sharp tempera-

ture transitions occur in the binding of membranes to cells since, under these assay conditions, the shear force between cells and membranes is minimal (*F. Moya* and *L. Glaser,* unpublished observations, 1977).

B. Cell-Cell Adhesion in the Embryonal Systems

Most of the work in this system in recent years has been concerned with chick embryo and has been concentrated on the retina and the optic tectum. This choice is dictated both by the ready availability of material and by the possible relationship of the adhesion of retinal and tectal cells to the problems of synaptogenesis. The ganglion cells from the retina send axons to the optic tectum. The arrangement of the retinal map on the tectum is such that there is an inversion of both the dorso- and ventrolateral axis of the retina on the contralateral tectum. Since the original observations of *Sperry* (1963), it has been assumed that this arrangement is due to the presence on the surface of the tectal cells and the retinal axons of complementary molecules in two gradients, one dorsoventral and one mediolateral. Several specific versions of the *Sperry* proposal using carbohydrate as ligands have been proposed (*Barondes,* 1970; *Marchase* et al., 1975). A diagrammatic example of such a gradient is illustrated in Figure 9.

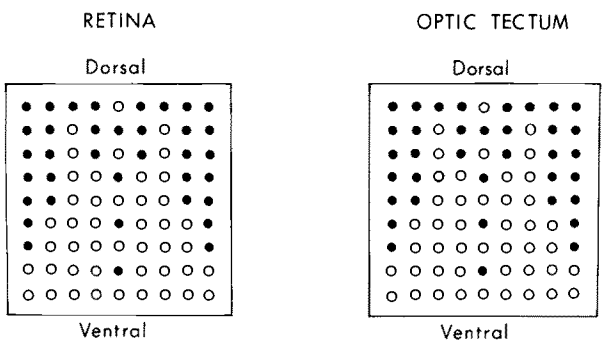


Fig. 9. Schematic demonstration of gradients of complementary surface ligands postulated to be involved in retina tectal connectivity. ● and ○ are complementary ligands. Only the dorsoventral gradient is shown, but a similar gradient is assumed to be present with different ligands along the mediolateral axis. Note that as the simplest assumption the gradients or the retina and tectum are presumed to be identical. This is not a neccessary assumption, and in fact is unlikely to be entirely correct. Ganglion cells from dorsal area of the retina have an excess of ● and would preferentially connect to tectal cells containing an excess of ○. The opposite would be true for ganglion cells from the ventral area of the retina. Note that this model is essentially a competition model and that, if a portion of the tectum were removed, the most ventral fibers from the region would still go to the most dorsal region available in the optic tectum

Such a model leads to the speculation that if cell adhesive components occur not only at the axon tips but also on the surface of the cell, then cell adhesion experiments could provide information about the adhesive specificity of cells in these systems. Ideally, such information should be obtained using only the cells in the retina involved in synapse formation with the optic tectum (i.e., ganglion cells). In fact, since pure preparations of ganglion cells are not available, all experiments to date have been carried out with mixed cell populations from the whole retina of which less than 10% are ganglion cells. Therefore, the relevance of some of these experiments to synaptogenesis should be considered with some caution.

While the retinotectal system is the one that has been examined most carefully from the point of view of cell-to-cell adhesion, other regions of the nervous system have also been examined, although in less detail, and will also be considered in this section. Four approaches have been used to study cell adhesion in these systems. The first is the study of long-term effect on aggregate size and differentiation, the second is the study of early cell-to-cell adhesion, the third is an immumological approach to cell-cell adhesion, and the fourth is the study of the effects of plasma membrane on cell-cell adhesion.

Isolation of Aggregation Promoting Factors. The pioneering work of *Townes* and *Holtfreter* (1953) and *Moscona* (1965) resulted in the demonstration that organs such as the retina could be dissociated into single cells and that aggregates from such cells would undergo histotypic differentiation. Factors which increased the size of aggregates formed after 24 h incubation of dissociated single cells were described a number of years ago (*Lilien,* 1968; *Garber* and *Moscona,* 1972a,b). These factors appear to be specific for different regions of the nervous system; one of these factors has now been isolated in pure form from neural retina and another from liver. The retinal factor has been isolated by two different methods: In the first, the factor was isolated from the supernatant fluid of retinal cells incubated in monolayer culture in serum-free medium (*Hausman* and *Moscona,* 1975); in the second, the factor was isolated from plasma membrane enriched fraction from neural retina by butanol extraction followed by conventional purification techniques (*Hausman* and *Moscona,* 1976). The two proteins appear to be either similar or identical. Incubation of retinal cells (from 10-day-old embryos) without this protein in a rotating flask for 24 h in Eagles medium results in aggregates of average volume 2×10^5 μm^2; addition of the factor results in the formation of mean volume 45×10^8 μm^2.

The precise mode of action of this factor is unknown. While the pure protein contains carbohydrate, removal of N-acetylneuraminic acid residues, treatment with glycosidases, or treatment with periodate do not

abolish biologic activity. It seems unlikely, therefore, that the carbohydrate is essential for biologic activity. The biologic activity is abolished if the factor is treated by trypsin. The protein has a molecular weight of 50,000 and appears to be a monomer. The factor has no known enzymatic activity, and has specifically been shown not to have galactosyl transferase activity (*Garfield* et al., 1974); is is specific for neural retina and has no effect on the aggregation of cells obtained from the optic tectum or cerebrum.

Similar but less well-defined factors have been isolated in other systems (*Lilien,* 1968; *Lilien* and *Rutz,* 1977; *Garber* and *Moscona,* 1972b; *Hausman* et al., 1976; *Moscona* and *Hausman,* 1977). Of particular interest is a multicomponent system which enhances the aggregation of teratoma cells (*Oppenheimer,* 1975; *Meyer* and *Oppenheimer,* 1976) where, in contrast to the factors obtained from the neural retina, carbohydrate appears to be an essential component of the aggregation factor(s). Since these factors are not pure, the precise role of the carbohydrate cannot be determined.

The mode of action of all of these aggregation factors is unclear. It is possible that the factors are ligands which specifically bind retinal cells to each other; alternatively, the factors may be considered as "hormones" or trophic factors which show extreme tissue specificity and promote cell adhesion and differentiation. The fact that these molecules are located on the cell surface does not directly prove that they function as cross-linking reagents between cells. As will be discussed in Section IV, cell-to-cell contact may have metabolic or developmental consequences which may or may not be mediated by the major adhesive components on the cell surface. Thus an isolated cell surface molecule may have trophic effects by binding to receptors on the cell surface.

It is of particular interest in this regard to discuss the very interesting observations of *Sankaran* et al. (1977). These investigators have isolated an aggregation promoting factor from liver cells using an assay similar to that described by *Hausman* and *Moscona* for retinal cell aggregation. The factor which they have purified to homogeneity greatly increases the size of liver cell aggregates formed after 24 h incubation of dissociated liver cells in rotating culture. The factor was shown to be identical to taurine. It seems highly unlikely that taurine (molecular weight 125) functions as a cell-to-cell ligand; it is much more likely that it affects cellular metabolism in such a way that larger aggregates are formed. Taurine in this system is active at a concentration of 0.05 mM. An inhibitor of the action of taurine was also detected in crude liver extracts. This material also appears to be of low molecular weight, but its chemical nature is unknown. By analogy it is possible that the aggregation-promoting factors which are proteins also function primarily by altering the metabolism of their target cells and thus represent organ-specific hormones.

1. Studies of Cell-Cell Adhesion

Cell-to-cell adhesion studies, while they do not directly provide information on the chemistry of the adhesive components, provide a great deal of information regarding the specificity of cell-to-cell adhesion. In general, adhesion to homologous cells is preferred to adhesion to heterologous cells; chick embryonal cells tend to adhere preferentially to cells derived from the same organ (see, e.g., *Roth* and *Weston,* 1967; *Roth,* 1968; *Roth* et al., 1971; *Walker* et al., 1973; *McGuire* and *Burdick,* 1976; *Buutjens* and *Edwards,* 1977), although some exceptions have been noted (*Gottlieb* and *Glaser,* 1975).

In an extensive series of studies, *Roth* and coworkers, *Barbera* et al. (1973), *Barbera* (1975), and *Marchase* (1977) have examined the adhesion of retinal cells to halves of the optic tectum. They found that cells from the dorsal half of the neural retina (or the pigmented retina) adhere preferentially to ventral halves of the tectum, and that the converse is true for cells obtained from the ventral half of the neural retina. These data would be in agreement with the known pattern of retinotectal connectivity (*Marchase* et al., 1975).

Similarly, *Gottlieb* et al. (1976) have examined the ability of neuroretinal cells obtained from the extreme dorsal of extreme ventral regions of the neural retina to bind to cells from different regions of the neural retina (Fig. 10). The data would be consistent with the presence on the retinal cell surface of two gradients of ligands very much as predicted by the model shown in Figure 9. However, no mediolateral gradient could be found in this work and none has been reported in the retinotectal system. Whether these gradients are therefore directly related to retinotectal specificity remains conjectural. Nevertheless, this represents the first demonstration of a cell surface adhesive gradient in embryonal cells which may serve to orient the nervous system along one of the major axes of the embryo.

At much simpler level, the presence of these gradients serves to illustrate the fact that even at this level of resolution the cells obtained from the retina are clearly heterogeneous with respect to their adhesive components, and it is highly likely that if more refined methods could be developed to study more discrete cell populations, even more striking differences would be found.

2. An Immunological Approach to Cell-Cell Adhesion

Studies with cultured cell lines have defined a number of neuronal or glial cell-specific antigens. None of these antibodies inhibited cell adhesion of the cultured cells (*Stallcup* and *Cohn,* 1976; *Stallcup,* 1977). Antibodies

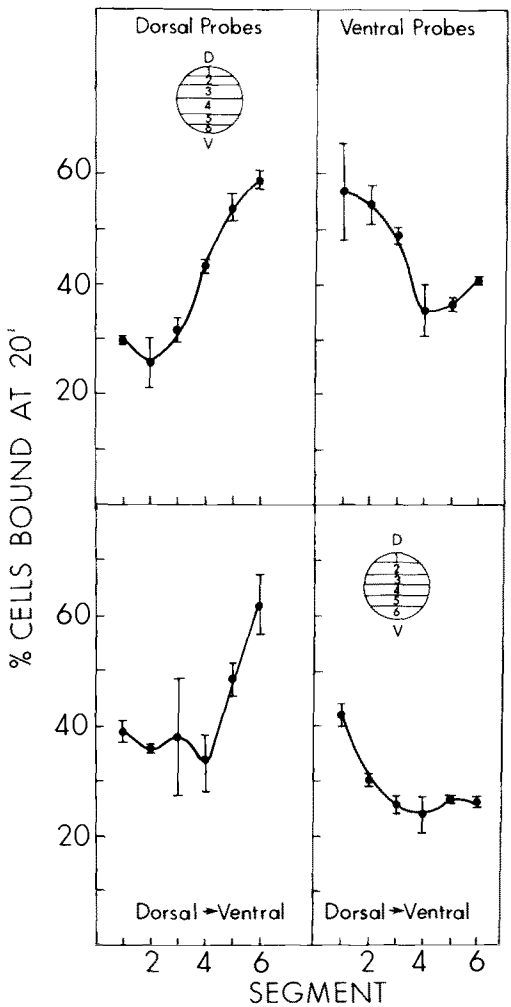


Fig. 10. Adhesion gradient in the neural retina. The data illustrate the rate of adhesion of neural retina cells obtained from the extreme dorsal area of the neural retina (segment 1) of 12-day-old chicks or extreme ventral area (segment 6) to adhere to monolayers prepared from retinal areas shown. The data are taken from the observations of *Gottlieb* et al. (1976), and have been reproducible in a number of separate experiments

specific to some of the regions of the nervous system are known, but their effect on specific cell aggregation is unknown (*Schachner,* 1974; *Schachner*et al., 1975; *Fields* et al., 1975; *Brockes* et al., 1977; *Akenson* and *Herschman,* 1974).

Work with cellular slime molds by *Gerisch* and coworkers first succeeded in indentifying antigens involved in cell-cell recognition, since antibodies would be prepared that specifically blocked cell adhesion (*Beug* et al., 1973; *Gerisch* et al., 1974). Recent work by *Rutishauser* et al. (1976), *Brackenbury* et al., 1977), and *Thiery* et al. (1977) has been directed at the isolation of antibodies which specifically block cell adhesion, as measured in a rotation-mediated aggregation assay, using 10-day neuroretinal cells which have been allowed to recover from trypsinization

for 24 h in vitro. Fab fragments from antibodies prepared against neuroretinal cells inhibited cell aggregation under these conditions. Proteins released from neuroretinal cells maintained in serum-free monolayer cultures block this effect of the Fab fragments, but do not themselves block cell adhesion, suggesting that these proteins are not acting directly as adhesive components.

These proteins were purified and antibodies prepared against them. Fab fragments prepared from these antibodies also inhibited aggregation. Such antibodies precipitated a single protein of apparent molecular weight 140,000 from NP-40 extracts of a crude retinal membrane fraction. Based on size difference, this protein molecule is clearly different from the adhesion-promoting factor isolated by *Hausman* and *Moscona* (1975, 1976).

The antibodies isolated in this work block adhesion in a number of neuronal systems (*Rutishauser* et al., 1976). These results can therefore be used to suggest that cell-to-cell adhesion within the nervous system is nonspecific or that the antibodies react with a common element in the cell recognition machinery. For example, if different adhesive specificities were generated by altering the carbohydrate structure of a glycoprotein, antibodies against the protein would inhibit a variety of cell-to-cell binding systems. Alternatively, if functional cytoskeleton elements are required for cell adhesion, interference with the cytoskeleton would interfere with cell adhesion in a variety of cells. The authors prefer the explanation that cell-to-cell adhesion is nonspecific, but this reviewer feels that the evidence for different adhesive specificities in the embryonal nervous system is substantial and favors the notion that these interesting antibodies recognize a common component in the cell surface adhesive mechanism. In agreement with this is the observation that the isolated protein which blocks the antibody does itself not block cell adhesion.

Similar antibodies, whose receptor has not been characterized, have been prepared by *McClay* et al. (1977). Fab fragments from these antibodies block cell adhesion in an aggregate collecting assay using 10-day neural retina cells, but the protein(s) on the cell surface to which these antibodies bind have not yet been identified.

3. Use of Membranes for the Study of Cell-Cell Adhesion

Plasma membrane enriched fractions block aggregation of homologous cells as demonstrated with embryonal chick cells primarily obtained from retina or optic tectum (*Merrel* and *Glaser,* 1973; *Gottlieb* et al., 1974; *Merrell* et al., 1976). The most likely explanation for the blocking action is that membranes functionally contain only one of the two complementary components required for cell-to-cell adhesion. This would be in agreement with the observations made with cultured neural cell lines (Sect. II).

Another, perhaps less likely explanation, is that a cell totally coated with plasma membrane vesicles may be unable to stably adhere to another cell also coated with membrane vesicles.

Using a membrane blocking assay, it has been possible to demonstrate adhesion differences between different areas of the nervous system, as well as between some areas of the nervous system at different developmental times. This, for example, membranes prepared from the neural retina of 8-day-old chick embryos inhibit the aggregation of 8-day neuroretina cells, but not that of 7- or 9-day neuroretina cells. Typical data are shown in Figure 11. The data that have been obtained with this assay are summarized in Table 2. Because of the limitations of this type of assay (discussed in Sect. I), these data cannot be simply interpreted to indicate that certain ligands have appeared or disappeared from the cell surface. They simply indicate that the cell surface has changed in such a way that the ligands can no longer be detected by this assay. It is of potential importance from the point of view of development that retinal membranes appear to inhibit the aggregation of tectal cells, but the opposite effect is not observed. Even though these specificities may reside in part in the same molecules (*Merrell* et al., 1975a), the specificity of the complementary components on the cell surface which recognize these molecules may be quite different.

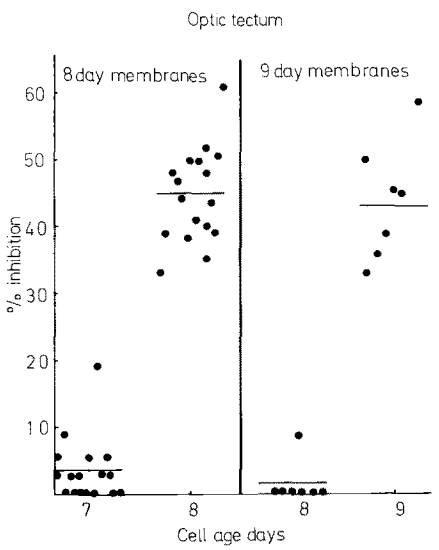


Fig. 11. Inhibition of tectal cell aggregation by membranes. Each point represents a different paired experiment to measure the inhibition of cell aggregation by membranes. The horizontal lines represent the average of all the experiments shown. All experiments were carried out at submaximal membrane concentrations to facilitate cell counting. Assay conditions as described by *Merrell* et al. (1975b)

One of the temporal changes detected by this assay has been reproduced in tissue culture (*Merrell* et al., 1975b). Incubation of seven tectal cell aggregates for 24 h in culture in the presence of 10^{-7} *M* mouse sub-

Table 2. Adhesive Specificities in the embryonal nervous system differentiated by membrane inhibition assay. The data were obtained by measuring the inhibition of cell aggregation by membranes. The limitations of this assay are discussed in Section I. Data are summarized from *Merrell* and *Glaser* (1973) and *Gottlieb* et al. (1974)

Membranes		Cells					
		Neural Retina			Opticum Tectum		
		7 day	8 day	9 day	7 day	8 day	9 day
Neural Retina	7 day	+	–	–	+	–	–
	8 day	–	+	–	–	+	–
	9 day	–	–	+	–	–	+
Opticum Tectum	7 day	–	–	–	+	–	–
	8 day	–	–	–	–	+	–
	9 day	–	–	–	–	–	+

maxillary nerve growth factor (NGF) results in a change in the adhesive characteristics of these cells so that they now resemble 8-day-old tectal cells obtained from the embryo. The effect is specific for NGF, and is not observed after the addition of a variety of other proteins or polypeptide hormones. Certain lots of fetal calf serum, which do not appear to contain NGF, will also induce this effect. The high concentrations of NGF required to induce this change suggest that it is acting as an analogue of a physiologically important trophic factor (*Merrell* et al., 1975b).

In this connection, it is important to note again that addition of NGF to a cloned pheochromocytomia induces changes in cell surface adhesive properties. These occur rapidly (30 min), unlike the changes in the tectal cells which require between 16 and 24 h. In both cases, the changes in cell surface adhesive properties are resistant to cycloheximide and appear not to require the synthesis of new surface protein (*Schubert* and *Whitlock,* 1977).

In general most of the characteristics of membrane-to-cell adhesion which have been observed in the embryonal systems have also been observed with cultured neuronal lines; this includes the sensitivity of the membrane-to-cell adhesion assay to temperature and proteases, and the apparent loss of one of the complementary adhesive components from the membrane. An analogy can also be drawn between the temporal changes in adhesion seen in the nervous system and the changes in adhesive specificity of cultured cells seen with increasing cell density (Sect. III, A).

In many, but not all cases where cell-to-cell adhesion has been examined across species, organ specificity appears to override species specificity. Recent observations have demonstrated that this is not true in the case of rat and chick hepatocytes which do not adhere to each other (*Burdick* and

Steinberg, 1969; *Burdick,* 1972; *Grady* and *McGuire,* 1976b; *Obrink* et al., 1977a). Plasma membranes prepared from each of these types of hepatocytes enhance the aggregation of hepatocytes from the homologous species, presumably by acting as cross-linking agents (*Obrink* et al., 1977a). The membranes can also be shown to bind specifically to the homologous hepatocytes (*Obrink* et al., 1977b). In order for the membranes to be active in either of these assays, they have to be pretreated with EDTA at alkaline pH. A lectin present in hepatocyte membranes binds certain glycoproteins which contain terminal galactose residues. This lectin has been investigated extensively by *Ashwell* and coworkers, *Pricer* and *Ashwell* (1971), *Hudgen* et al. (1974), and *Kawasaki* and *Ashwell* (1977). The lectin glycoprotein complex is very tight but can be dissociated under the precise conditions used by *Obrink* et al. to activate their membranes. These observations therefore encourage the speculation that the lectin present in the membrane is attached to the carbohydrate moiety of a glycoprotein, but when the membranes are activated the lectin binding site(s) become free, and this allows the membranes to attach to glycoproteins on the surface of several cells and thereby act as cross-linking agents, much in the same way as a more conventional lectin such as concanavalin A would agglutinate cells. The major lectin in rat hepatocyte requires as a necessary, but not sufficient condition for binding, a terminal galactose residue, while the main lectin in chick hepatocyte requires terminal N-acetyl-D-glucosamine residues. This difference in specificity would account for the different aggregation-promoting specificity of chicken and rat hepatocyte plasma membranes, provided that the rat hepatocyte membranes contained predominantly glycoproteins or glycolipids terminating in D-galactose residues while the chicken hepatocyte membranes contained glycoproteins or glycolipids terminating in N-acetyl-D-glucosamine residues.

IV. Functional Studies of Cell-to-Cell Adhesion

In a number of studies it has been shown that developmental changes in a variety of enzymes can be demonstrated in cell aggregates, but that such changes occur to a much lesser extent or not at all when the same cells (usually from the embryonal nervous system) are maintained in monolayer culture (*Seeds,* 1973; *Vogel* et al., 1976; *Ramirez,* 1977a,b; *Ramirez* and *Seeds,* 1977). Among the various possible interpretations of such observations is one which suggests that cell-to-cell contact is important for this developmental change. Alternative interpretations include the possibility that the microenvironment in the aggregate is very different from the environment in the monolayer culture. The changes observed in these systems usually take place over long periods of time (weeks) and are not

easily subject to biochemical investigation and to attempts to define the cell surface receptors responsible for this effect.

Several simpler systems have been investigated in which surface membranes have been added to cells in attempts to reproduce the same metabolic changes as are observed after cell-to-cell contact. The fundamental advantage of such systems is that they offer the possibility of correlating cell adhesion with a physiological response and, therefore, rule out the possibility that the observed adhesive event is a laboratory artifact, unrelated to cellular function. These experiments also provide added insight into developmental processes (see, e.g., *McMahon,* 1973).

Such experiments have been carried out using enzyme induction in the aggregating slime mole (*McMahon* et al., 1975; *Smart* and *Tuchman,* 1976; *Tuchman* et al., 1976), blocking cell division by plasma membranes in growing fibroblasts (*Whittenberger* and *Glaser,* 1977), and inducing cell division in quiescent Schwann cells by neurite plasma membranes (*Salzer* et al., 1977). The data obtained in the slime mold system are outside the scope of this review.

The inhibition of growth in spare cultures of 3T3 cells observed after addition of plasma membranes to the cultures resembles the inhibition observed at cell-to-cell contact, in that the cells are stopped early in the G1 phase of the cell cycle. The inhibition is reversible and has been shown not to be due to the removal of growth factors from the medium by the membranes (*Whittenberger* and *Glaser,* 1977; *Whittenberger, B., Raben, D.,* and *Glaser, L.*, unpublished observations, 1977). The kinetics of inhibition (Fig. 12) are complex. Maximally 50% of the cells can be blocked during

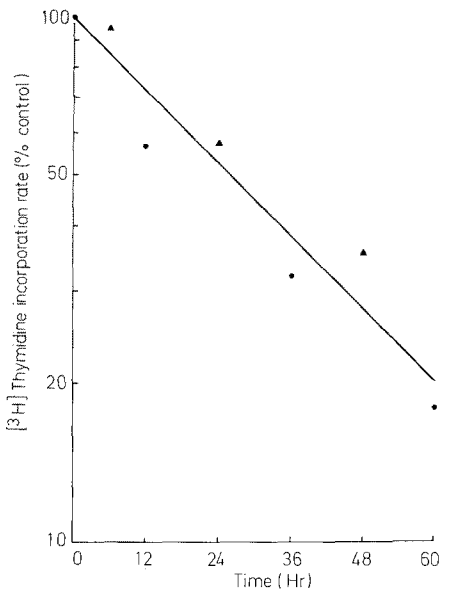


Fig. 12. Inhibition of the growth of 3T3 cells by plasma membranes. The incorporation of thymidine into DNA was measured in sparse cells exposed to saturating concentrations of membranes for the times indicated. These data summarize the observations of *Whittenberger* and *Glaser* (1977). Control experiments showed that the rate of thymidine incorporation is a true measure of the number of cells synthesizing DNA

each cell cycle, suggesting that even when the cells are receiving a maximal inhibitory signal only about 50% of the cells can be blocked during each cell cycle. Similar conclusions can be derived from an examination of the data of *Martz* and *Steinberg* (1972) who examined by cinematography the cessation of division of confluent 3T3 cells. A general model that could account for these observations would be that each cell leaves the G1 portion of the cell cycle by generating at random a different level of a signal or signals required to proceed through the cell cycle (*Smith* and *Martin,* 1973) and that only those cells with a signal level below some threshold value can be inhibited by cell contact. The membrane signal is specific, SV40 transformed 3T3 cells do not respond to the addition of membranes and membranes prepared from SV40 transformed 3T3 cells only show a very weak inhibitory effect when added to 3T3 cells.

The subject of growth control in normal and malignant cells is highly complex, and not directly germane to this review. For the purpose of this review, the datum to point out is that plasma membranes appear to retain a binding component which can interact with cells and elicit a physiological response in much the same way that a hormone interacts with a receptor to elicit a physiologically meaningful response.

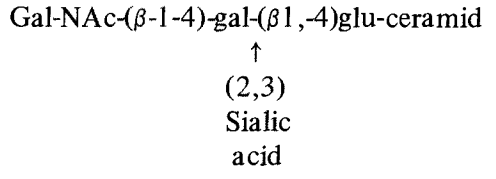
A fundamentally similar system, but one in which membranes provide a mitogenic signal, is that of Schwann cell proliferation. Schwann cells can be prepared as a quiescent (nondividing) cell population from dorsal root ganglia. Data obtained in the laboratory of Dr. *R. Bunge* have clearly shown that if such a culture comes in contact with growing neurites (*Wood* and *Bunge,* 1975; *Wood,* 1976) from dorsal root ganglia they will start to divide. The data suggested that this was a contact phenomenon. To test this further, a surface membrane fraction was prepared from neurites and dorsal root ganglia and added to quiescent Schwann cell. This membrane fraction was shown to induce proliferation of the Schwann cells. A large number of other membrane preparations and hormones were tested in the system and shown not to act as mitogenic signal. Pretreatment of the neurites (still attached to the neuronal cell) with trypsin abolished the mitogenic activity suggesting that the membrane component responsible for this effect is a protein. In agreement with this, the membrane activity was found to be destroyed by heating for 10 min at 60° (*Salzer* et al., 1977). The general conclusion from both of these data is that the cell surface contains specific receptors for ligands on adjacent cells, and that these receptors when occupied can produce either a growth inhibitory signal (3T3 cells) or a growth initiating signal (Schwann cells), presumably by generating one or more intracellular second messages as a result of the receptor ligand interaction.

V. Isolation of Cell Surface Adhesive Components

While considerable progress has been made in the isolation of adhesive components of slime molds (*Barondes* and *Rosen,* 1976; *Frazier,* 1976) and sponges (*Burger* and *Jumblatt,* 1977), the information concerning higher organisms is still very sketchy. Some success has been obtained with immunological methods (Sect. III, b), but whether the isolated component is directly involved as a cell-to-cell ligand is unclear.

Not surprinsingly, in all systems that have been investigated so far, it appears likely that at least one of the recognition components is a protein. Using an inhibition of aggregation assay, proteins (defined as trypsin-sensitive material) could be isolated from neural retina and optic tectum membranes which inhibited aggregation and showed developmental time and regional specificity (*Merrell* et al., 1975a). The quantities of material that could be obtained were too small for adequate characterization of this material.

Recently a possible identification of a cell surface adhesive component has been reported by *Marchase* (1977). *Barbera* et al. (1973), and *Barbera* (1975) had shown previously that cells obtained from the dorsal half of the neural system preferentially adhered to the ventral half of the optic tectum and conversely that cells obtained from the ventral half of the neural retina adhered preferentially to the dorsal half of the optic tectum. On the basis of studies with proteases and glycosidases, they concluded that the ligand present in excess in the dorsal half of the retina was a carbohydrate structure terminating in an N-acetyl-D-hexosamine, while the ligand present in excess in ventral half of the retina or tectum was a protein that binds preferentially to these N-acetyl-D-hexosamine residues. Based on these observations, the authors tested whether GM_2 ganglioside when incorporated into liposomes adhered preferentially to ventral halves of the tectum as compared to dorsotectal halves. A small but significant difference was observed. GM_2 has the structure



and would qualify as the hypothetical N-acetyl-D-hexosamine-containing ligand. No gradient of GM_2 could be found in the retina. However, a small difference in a galactosyl transferase which transfers Gal residues from UDP-Gal to GM_2 was found in cells from the ventral half of the retina making this transferase the putative receptor for GM_2. Since all these

differences were obtained by measuring the difference between dorsal- and ventral-retinal halves, it is possible that the differences would be greatly magnified if they could be measured in plasma membranes from extreme dorsal and extreme ventral cells. The possible relation of these observations to retinotectal connectivity, although fascinating, seems conjectural (see Sect. III, B).

VI. Conclusions and Speculations

The study of the cell-to-cell adhesion in higher eukaryotes is still to a large extent phenomenological, and its description at the molecular level is only beginning. Significant advances have been made with a number of approaches, but the problem is complicated by the fact that adhesion as observed in most assays is a multistep process, involving a number of different specificities and sequential events. Different assays or different reagents may modify these components. The use of membranes to study cell recognition is a useful approach in that it establishes that one of the adhesive components remains functional when removed from the whole cell. Increased emphasis probably will be, and should be, placed on the study of systems in which cell-to-cell contact elicits a measurable physiological response in the cell. Purification of the compounds responsible for these effects shows the greatest promise both from the point of view of assay sensitivity and the avoidance of possible in vitro artifacts. The greatest obstacle in all of these systems will no doubt be the difficulty in fractionating membrane proteins by novel techniques so that they can still interact with live cells while avoiding the toxic effects of detergents.

In considering the physiological responses of cell to cell-cell contact, it is important to consider the possibility that cell adhesion is mediated by a different set of molecules than those that elicit the physiological response. Thus, for example, cell adhesion may be brought about by the binding of two ligands of relatively high affinity, but once the two cell surfaces are in close proximity, the effective local concentration of certain molecules may be very high and some of these molecules may now be able to bind to each other and elicit a physiological response. If this speculative model actually applies it will clearly be more difficult to isolate the relevant molecues from plasma membrane.

If the cell surface (or plasma membranes isolated from the cells) contains a series of complementary ligands, that is molecules that can bind to each other with high affinity, why do these molecules not bind to each other within a single cell or within the membranes? In the case of purified plasma membranes, it is possible that most of the adhesive molecules have been bound to each other and that we measure only those molecules

present in excess. In the case of the cell surface, it is possible that the adhesive molecules do not neutralize each other because of structural considerations. Since the membrane itself is rather flexible and fluid, it may be that this restriction arises because the adhesive molecules are not present in isolated molecules in the membrane, but as a more organized adhesive structure, perhaps resembling the end plates at a neuromuscular junction. One could speculate that the morphologically defined junctions between adhering cells (*Overton*, 1977) represent a differentiated property of these adhesive structures. One might also speculate that perhaps in many cases cell populations are a mosaic and each cell contains only one-half of a complementary ligand pair. There is no immediate precedent for such a suggestion, which would have to arise as a differentiated function and not as an inherited characteristic.

None of the systems currently under investigation are ideal and it is hoped that different laboratories will continue to examine different systems using different and hopefully novel technologies. While this may appear to confuse the field, it is only through this kind of diversity that we can hope to generate significant advances in this field.

Acknowledgement. I am grateful to all my colleagues who through our daily discussion have contributed most of the ideas presented in this review, and whose data I have quoted frequently: in particular, *D. Gottlieb, R. Merrell, F. Moya, D. Raben, R. Santala, J. Salzer,* and *B. Whittenberger.* The literature on the subject of this review is large, and I have arbitrarily used selected recent papers to illustrate the main points, often neglecting for reasons of space earlier work on the same subject by the same authors. To all whose work has been accidentally overlooked, my apologies.

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Pharmacogenetics

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Contents

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1. Introduction

Differences between species in response to drugs are frequently observed. These differences are almost exclusively genetic in origin, because every constant interspecies difference has a hereditary basis. However, also within one species, every individual has a unique composition of genes ("biochemical individuality"). Man is likely to be heterozygous at about 20% of his gene loci (*Harris* and *Hopkinson*, 1972). From an evolutionary point of view, uptake of a drug may appear to be a highly artificial procedure in which a foreign agent interacts with a genetically determined biological target that may differ from person to person. Differences in the reaction to drugs, therefore, should be frequent; possibly they are the rule rather than the exception. Human genetics is the discipline that deals with inherited diversity among subjects and its mode of transmission. The field that deals with the contribution of genetics to drug response is pharmacogenetics, a term coined by *Vogel* in 1959, when the first relevant phenomena had been discovered. After entering the organism a drug interacts in a complicated way with different mechanisms, whose importance varies from case to case: absorption, protein binding, tissue storage, biotransformation, transport across membranes, excretion, drug action (interaction with receptor or other mechanisms). Each of these parameters may be subject to genetic variability.

After publication of the first book on pharmacogenetics (*Kalow*, 1962), several conferences and a number of reviews have produced detailed summaries of the state of the field (e.g., *Goedde* et al., 1965; *LaDu* and *Kalow*, 1968; *Goedde* et al., 1970; *Vesell*, 1971; WHO, 1973; *Vesell*, 1973; *Motulsky*, 1977). Here we shall review the well-known facts of pharmacogenetics in a condensed way, emphasizing more recent developments. The review will be organized according to the pharmacologic levels at which genetic differences, mono- or polygenic, may be encountered. Thereby the problem of recurrent mentioning of the same pharmacologic agent arises in some cases; nevertheless, this arrangement was preferred to the confinement of all the facts concerning a certain drug to a single space, in order to follow systematic lines.

2. A Look at Methodologic Possibilities

2.1 Pharmacologic Methods

Pharmacogenetic investigations in humans require experiments in normal subjects or in biological material of them. For such studies, drug concentrations in biological fluids can be measured. Such pharmacokinetic para-

meters as drug half-life, plasma clearance, elimination rate constant, steady-state plasma concentration, and urinary clearance can be derived from regularly timed concentration measurements. Because most drugs are appreciably metabolized, going through at least several distinct enzymatically catalyzed reactions in the process, measurement of drug metabolites will give particularly valuable information. From a genetic point of view, metabolite determination makes the discovery of new pharmacogenetic phenomena more probable: Metabolites may reflect the function of single genes more closely, because biotransformation of a drug occurs enzymatically, and the known genetic diversity in humans is expressed directly and ascertained most easily at the enzyme level. Binding of a drug or its metabolites to transport proteins may vary among subjects according to primary structure at the binding site of the protein molecule. Drug binding studies to plasma proteins can be performed in vitro along genetic lines.

Genetically determined differences in drug response as a function of the target tissue rather than drug disposition (pharmacodynamics) may come to light by measurement of pharmacologic effects in vivo after acute or chronic administration. The use of normal subjects for such investigations may impose insurmountable practical and ethical problems. Development of in vitro techniques for determination of receptors in cell cultures may circumvent some of these difficulties in the future.

2.2 Methods of Studying Human Genetics

One of the methods widely used in human genetics involves the study of twins. The advantage of the twin method is that the extent of the genetic influence on a measured trait can be easily made visible. Therefore, the twin method is useful as a first approach to the study of the genetic contribution to the phenotypic variability of a new trait. This method, which had lost much of its earlier attractiveness, gained new publicity particularly through pharmacogenetic studies. Interestingly enough, studies in twins frequently find even more interest among nongeneticists than among geneticists. As early as 1935, *F. Lenz* predicted that in most cases studies in twins of functional parameters would produce the uniform result of showing greater similarity of identical twins than of fraternal twins. Nevertheless, *Lenz* appreciated the psychological effect of studies in twins, because nongeneticists are reminded of the importance of heretary influences.

If a trait is continuously distributed in the population, it is possible to estimate the genetic contribution to the total variability of the trait from twin or family data. The genetic contribution is called "heritability" and is quantified in percent of the total variability. Calculation of heritability,

however, is problematic, because a number of assumptions (such as an additive model, lack of assortative mating, lack of interaction or covariance between genotype and environment) are made that are beyond experimental control. Conventional twin data do not allow a heritability estimate that is correct in the population sense, because between-twin variability is excluded (for a more detailed discussion see *Jinks* and *Fulker,* 1970). Nevertheless, the "heritability estimate" from twins may be useful as a first approach. It is clear that heritability is meaningful only at the population level and does not say anything about the action of single genes in a specific individual.

The field of pharmacogenetics gained wide-spread interest after publication of the twin studies of *Vesell* and his group; it became obvious that pharmacogenetic influences were not confined to just a few drugs. However, the main disadvantage of the twin method is that it is usually impossible to ascertain the nature of the genetic differences and the mode of inheritance. If the twin sample is not too small, it is possible that a deviation from unimodal distribution (e.g., bi- or trimodal) may suggest a Mendelian mode of inheritance. However, family studies are necessary to establish the mode of inheritance of the trait under study. Twin studies can be a useful first step in identifying those individuals in whom family studies would prove most revealing. When the trait is rare in the population, family studies have to start from affected individuals. From the genetic point of view it is desirable to measure parameters reflecting as directly as possible the functions of single genes. Otherwise family studies are unlikely to permit detection of the action of single genes. For example, determination of plasma half-life of a drug or its steady-state concentration will lead to discovery of a Mendelian pharmacogenetic trait only in those cases in which a single metabolic step has a dominating influence (e.g., acetyltransferase polymorphism; see Sect. 4.1.1). In most cases many factors will contribute to half-life or steady-state concentration, thus masking underlying monogenic traits. Admittedly, development of laboratory techniques or availability of biological materials sets limits upon the intention of the geneticist. On the other hand, plasma half-life, clearance, or steady-state concentration measurements of the parent drug, rather than of the metabolites, are useful in assessing whether a monogenic pharmacogenetic difference, once discovered, will have any clinical or therapeutic significance.

3. Pharmacogenetic Findings without a Simple Mode of Inheritance

3.1 Metabolic Differences

To the best of our knowledge, *Lüth* (1939) was the first to perform a pharmacogenetic study in man: He analyzed ethanol elimination in monozygotic (MZ) and dizygotic (DZ) twins after oral administration. This study, however, was forgotten. The experiments of *Vesell* and *Page* (1968a,b,c) therefore created new interest in the genetic aspects of drug metabolism by demonstrating the important hereditary contribution to half-lives of such different drugs as dicumarol, phenylbutazone, and antipyrine. In the following years, a number of additional twin studies with drug loading were conducted. Table 1 is a summary of the main results of these investigations. In nearly all cases it was possible to calculate intraclass correlations and heritability estimates of the published parameters, if the authors had not already done so. Most of the pharmacogenetic twin studies summarized in Table 1 are based on small twin samples; practical problems in experimental pharmacogenetic work are the most probable explanation for this fact. Despite the small number of twins examined in most investigations, large interindividual variability of the measured parameters is remarkable. For the most part the twin studies revealed that genetic factors contributed mainly to maintaining these large interindividual differences in drug disposition.

Since antipyrine is bound only to a minor extent in tissues and in blood, and since renal excretion of antipyrine is negligible, variability in half-life suggests differences in metabolic degradation, i.e., in the activity of the hepatic microsomal enzymes that hydroxylate antipyrine (*Vesell* and *Page,* 1968a). In the case of phenylbutazone, which is also hydroxylated by microsomal liver enzymes, large interindividual variations in plasma half-life may be due to metabolic differences; however, differential binding to albumin may also contribute to the observed variability. The three studies by *Vesell* and *Page* (1968a,b,c) were conducted in the same twins so that half-lives of the three drugs could be correlated within the same subjects. No correlation between antipyrine and phenylbutazone half-lives was obtained, suggesting that both drugs are metabolized by different enzymes. Protein binding of phenylbutazone, however, could mask an existing metabolic correlation. The correlation between half-lives of phenylbutazone and dicumarol can be related either to the fact that both drugs are almost completely bound to plasma proteins, or that they are degraded by similar metabolic steps. If the twin data show a bi- or trimodal distribution, this can serve as a useful hint for monogenic inheritance. In the case of antipyrine, phenylbutazone, and dicumarol the twin data were more compatible with a unimodal distribution, thus pointing to polygenic inheritance.

Table 1. Studies in twins on drug elimination rate or under steady-state conditions

Drug	Authors, No. of twin pairs	Measured Parameter	Range	r_{MZ}	r_{DZ}	H
Antipyrine 18 mg/kg p.o. (single dose)	*Vesell* and *Page* (1968a) 9 MZ, 9 DZ	Plasma half-life (h)	5.1 – 16.7	0.93	– 0.03	0.99
Phenylbutazone 6 mg/kg p.o. (single dose)	*Vesell* and *Page* (1968b) 7 MZ, 7 DZ	Plasma half-life (days)	1.2 – 7.3	0.98	0.45	0.99
Dicumarol 4 mg/kg p.o. (single dose)	*Vesell* and *Page* (1968c) 7 MZ, 7 DZ	Plasma half-life (h)	7.0 – 74.0	0.99	0.80	0.98
Halothane 3.4 mg i.v. (single dose)	*Cascorbi* et al. (1971) 5 MZ, 5 DZ	Urinary excretion of sodium trifluoroacetate in 24 h (% of injected dose)	2.7 – 11.4	0.71	0.54	0.63
Ethanol 0.5 g/kg p.o. (single dose)	*Lüth* (1939) 10 MZ, 10 DZ	β_{60} (mg/ml · h) EDR (mg/kg · h)	0.051 – 0.141 50.00 – 109.63	0.64 0.77	0.16 0.45	0.63 0.67
1 ml/kg p.o. (single dose)	*Vesell* et al. (1971) 7 MZ, 7 DZ	β_{60} (mg/ml · h)	0.11 – 0.24	0.96	– 0.38	0.98
1.2 ml/kg p.o. (single dose)	*Kopun* and *Propping* (1977) 19 MZ, 21 DZ	Absorption rate (mg/ml · 30 min) β_{60} (mg/ml · h) EDR (mg/kg · h)	0.20 – 1.12 0.073 – 0.255 57.6 – 147.6	0.56 0.71 0.76	0.27 0.33 0.28	0.57 0.46 0.41
Diphenylhydantoin 100 mg i.v. (single dose)	*Andreasen* et al. (1973) 7 MZ, 7 DZ	Serum half-life (h)	7.7 – 25.5	0.92	0.14	0.85

Lithium 300 mg/12 h p.o. (for 7 days)	*Dorus* et al. (1975) 5 MZ, 5 DZ	Plasma concentration (mEq/l)	0.16 – 0.38	0.94	0.61	0.86
		Red blood cell concentration (mEq/l)	0.050 – 0.102	0.98	0.71	0.83
		RBC/plasma concentration (each after 3 days of treatment)	0.18 – 0.56	0.84	0.62	0.92
Amobarbital 125 mg i.v. (single dose)	*Endrenyi* et al. (1976) 7 MZ, 7 DZ	Plasma clearance rate (ml/min)	16.0 – 67.2	0.87	0.55	0.83
		Weight-adjusted clearance (l/kg · h)	1.76 – 6.16	0.92	0.60	0.80
		Elimination rate constant (h^{-1})	2.09 – 8.17	0.93	0.03	0.91
Nortriptyline 0.6 mg/kg · d p.o. (for 8 days)	*Alexanderson* et al. (1969) 19 MZ, 20 DZ	Steady-state plasma level (ng/ml)	8 – 78	Published data do not allow calculation, but MZ twins are much more similar to one another than DZ.		
Sodium salicylate 40 mg/kg i.v. (single dose)	*Furst* et al. (1977) 7 MZ, 7 DZ	Slope of serum salicylate decay (mg/dl · h)	0.64 – 1.02	0.64	0.32	0.86
Aspirin 65 mg/kg · d p.o. (for 3 days)		Plateau serum salicylic acid (mg/dl)	11.9 – 36.4	0.90	0.33	0.98
		Salicylurate excretion rate (plateau) (mg/kg · h)	0.84 – 1.91	0.94	0.76	0.89

r_{MZ}, r_{DZ} = intraclass correlation coefficient;

$$H \text{ (heritability)} = \frac{V_W (DZ) - V_W (MZ)}{V_W (DZ)}$$

V_W = variance within twin pairs.

In a family study on phenylbutazone half-life, *Whittaker* and *Evans* (1970) showed that this parameter is under polygenic control. The authors determined half-lives of the drug in the same subjects twice: once after a drug-free period of six months, and then after induction of microsomal liver enzymes with phenobarbital in order to render the environment more uniform. Obviously, many factors contribute to phenylbutazone half-life, so that it is not possible to work out the influence of single genes.

A small percentage of halothane is metabolized to sodium trifluoroacetate, which is excreted in the urine. Testing urine after halothane administration, *Cascorbi* et al. (1971) reported a fourfold difference among their 20 twin subjects. Identical twins were more similar to one another than fraternals in the percentage of intravenously administered halothane excreted in the urine as this metabolite. The study gave no evidence for action of a single gene; the sample, however, was small and no family studies have as yet been carried out.

Ethanol is the drug that gained the greatest pharmacogenetic interest, insofar as three twin studies have been performed. In each study (*Lüth,* 1939; *Vesell* et al., 1971; *Kopun* and *Propping,* 1977), the twins were unmedicated and healthy; they had to drink the ethanol dose within a short, standardized time. With respect to ethanol elimination [disappearance rate of ethanol from blood = β_{60} (mg/ml · h); ethanol degradation rate = EDR (mg/kg · h)], each investigation revealed a remarkable genetic contribution to interindividual variation; the precise heritability estimate, however, differed in the three studies. This is not surprising, since every heritability estimate is valid only for those conditions under which the study was performed. The study by *Kopun* and *Propping* (1977) suggested that the usual drinking and smoking habits exhibited by twins prior to the investigation probably influenced ethanol elimination rates, since environmental factors contributed heavily to interindividual variability. The extent to which the heritability estimate is influenced by these environmental differences among the subjects remains uncertain.

The twin study on amobarbital elimination (*Endrenyi* et al., 1976), which combines more sophisticated kinetic models and a critical evaluation of different heritability estimates, has given new impetus to pharmacogenetic research. Almost all of this barbiturate can be accounted for in metabolized form in the urine. The main metabolites are 3-hydroxyamobarbital, a product of side-chain hydroxylation, and a metabolite that was interpreted as N-hydroxyamobarbital (*Tang* et al., 1975). These two metabolites account for about 92% of amobarbital metabolites in urine. Each of the measured elimination parameters, such as biological half-life, plasma clearance rate, weight-adjusted clearance, and elimination rate constant gives high heritability values. However, the authors emphasize that for characterization of drug metabolism, plasma clearance is most valuable,

since it is independent of kinetic model assumptions. Thus, genetic control of drug elimination can be studied much more appropriately by comparison of clearance values in twins. The detection of individuals that lack one step of amobarbital metabolism is an interesting and stimulating discovery (*Kalow* et al., 1977; see below).

Lithium has been used primarily in the treatment or prophylaxis of bipolar affective illness. The distribution of the lithium ion across the red blood cell (RBC) membrane as assessed by the RBC/plasma lithium ratio has received increasing interest in relation to therapeutic response and toxicity. It is well known that large interindividual differences occur in blood levels, therapeutic response, and toxicity after lithium administration (see below); within the same subject, however, lithium distribution remains fairly constant (*Greil* et al., 1977).

A twin study measuring lithium distribution across the RBC membrane both in vitro and in vivo revealed that genetic factors contribute mainly to interindividual variability (*Dorus* et al., 1974, 1975). *Greil* et al. (1977) presented evidence that variations in the efficiency of the Na^+-dependent Li^+ countertransport system are responsible for variability among subjects, thus pointing to genetic differences within cell membrane transport characteristics. The recent observation of a presumably monogenic Li^+ transport abnormality (*Pandey* et al., 1977; see below) suggests that only a few genes are implicated in maintaining variation in Li^+ transport.

Tricyclic antidepressants such as nortriptyline, amitriptyline, imipramine, or desipramine show an enormous variability in steady-state plasma levels between persons. The steady-state plasma level of a drug depends mainly on the rate of metabolism and its volume of distribution. *Alexanderson* et al. (1969) investigated the influence of genetic factors on nortriptyline metabolism in twins under steady-state conditions. Nortriptyline plasma concentrations were repeatedly determined in twins 6–8 days after beginning of treatment. The authors concluded that most of the variability in steady-state plasma concentrations between subjects is genetically determined, the most important source for this variability being rate of drug metabolism. The published paper does not provide the nortriptyline concentrations necessary to calculate heritability. Having obtained the individual data, *Vesell* (1973), however, calculated an H value of 0.98 for the nortriptyline steady-state concentrations. The cross-over study with desipramine and nortriptyline (*Alexanderson*, 1972) indicates that the metabolism of both drugs is controlled by the same genetic factors and that steady-state plasma levels can be predicted from single-dose plasma level data. The great interindividual variability of blood levels of tricyclic antidepressants is also of particular clinical importance, since on any standard dosage a certain number of patients will have plasma levels too low to be effective, and some, even on low dosage, develop high

levels that are toxic (*Asberg,* 1974). As in the case of lithium, monitoring plasma levels of tricyclics may help to increase the efficacy of treatment, although the plasma level of an antidepressant is of course not the only factor responsible for therapeutic response (cf. *Müller-Oerlinghausen,* 1978). *Asberg* et al. (1971) attempted to detect monogenic inheritance with respect to nortriptyline metabolism. Using the steady-state approach, they examined nortriptyline plasma levels in families of three propositi who developed extremely high plasma concentrations, and in random subjects. However, no tendency toward bimodality was observed. From this study, nortriptyline steady-state plasma concentrations appear to be under polygenic control. Many factors contribute to steady-state concentrations of this antidepressant so that the effects of single genes may be masked.

3.2 Pharmacodynamic Differences

3.2.1 Effect of Alcohol on the EEG

Two main requirements have to be fulfilled to establish genetically determined pharmacodynamic differences: reliable quantification of the drug effect and control of pharmacokinetic factors. The twin study on the effect of alcohol on the human electroencephalogram (EEG) (*Propping,* 1977) may be regarded as an attempt to meet these conditions, because alcohol elimination can be fairly well quantified in the individual case, and the EEG is one of the most sensitive methods for measuring central drug effects in man, especially when combined with computational analysis. The normal human resting EEG exhibits enormous interindividual variation that is nearly totally genetically determined (*Vogel,* 1970). The EEG has a high stability during adulthood within the same individual; identical twins have identical EEG patterns, even in old age (*Heuschert,* 1963) or when reared apart (*Juel-Nielsen* and *Harvald,* 1958), and certain variants have a simple mode of inheritance (*Vogel,* 1970). Figure 1 shows the mean intrapair differences in the number of β, α, and ϑ waves per 120 s and the mean frequency in the resting EEG of MZ and DZ twin pairs after automatic analysis, compared with repetitive tests in the same subjects on different occasions. With respect to these parameters, there is no difference between identical twins and repetitive experiments in the same subjects. An ingested psychotropic drug therefore interacts with a genetically determined trait.

The twin investigation (*Propping,* 1977) on genetic aspects of ethanol action on the EEG comprised 52 adult healthy male twin pairs (26 MZ, 26 DZ). The siblings underwent alcohol loading in a standardized way: Having ingested a light breakfast, they had to drink 1.2 ml/kg ethanol (100% as a 30% solution in orange juice) within 15 min; this dose produces a maximal blood alcohol concentration of about 1.1‰ after about

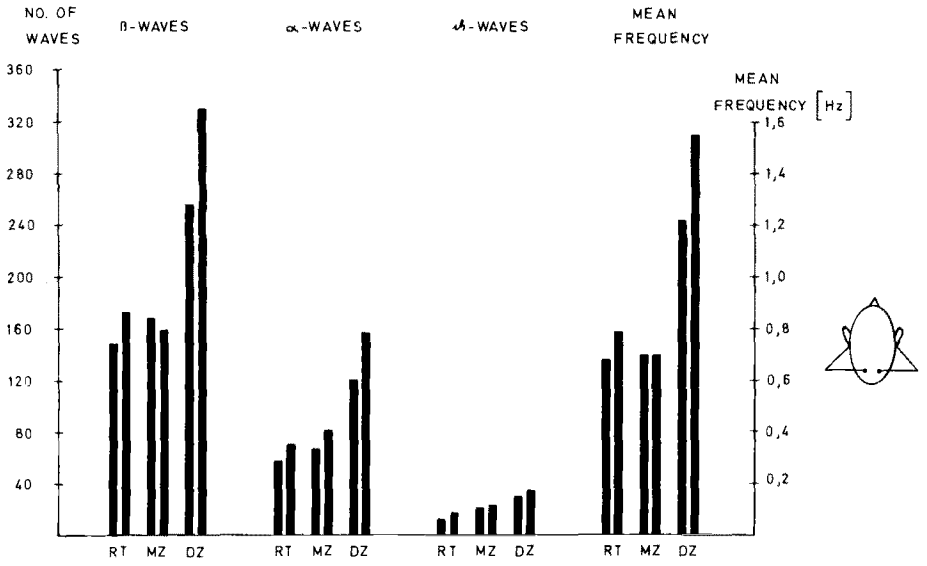

Fig. 1. Mean intrapair differences in number of β, α, and ϑ waves and in mean frequency in the resting EEG in MZ and DZ twins and in repetitive tests in the same subjects (*RT*). Left (*left column*) and right (*right column*) unipolar occipital leads

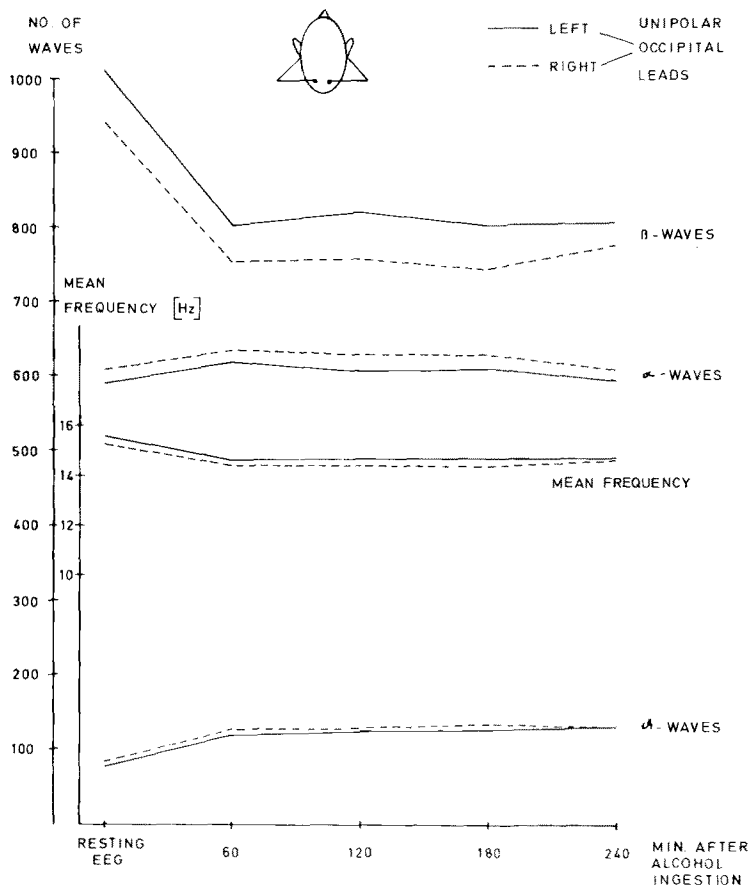

Fig. 2. Effect of 1.2 ml/kg ethanol on number of β, α, and ϑ waves and on mean frequency. For the calculation of the alcohol effect only the No. I of each twin pair was used

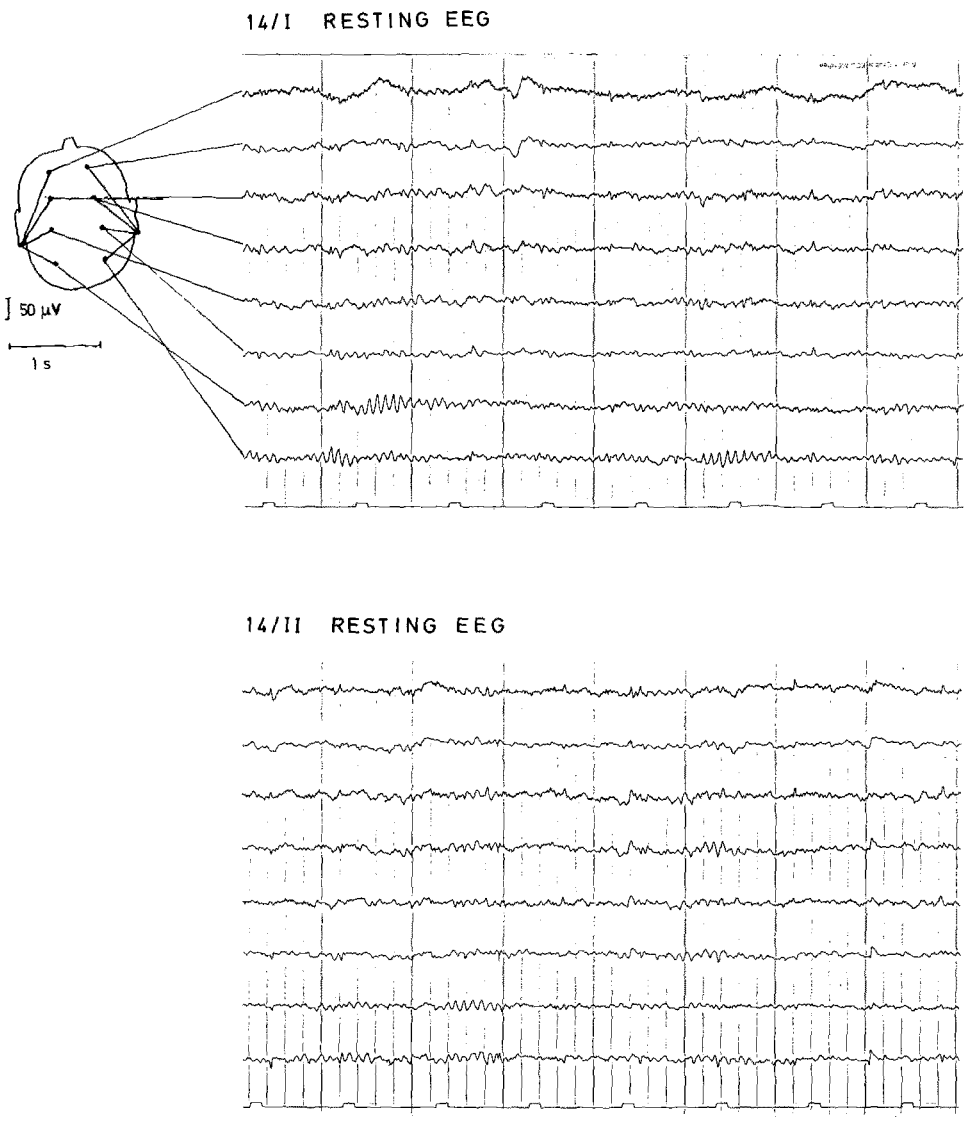

Fig. 3. Effect of alcohol on an average α EEG in a pair of identical twins

90 min. The EEG was recorded before and 60, 120, 180, and 240 min after the beginning of drinking. It has been known for more than 30 years that alcohol improves synchronization of the EEG, i.e., the number of α and ϑ waves as well as the amplitudes for all frequency classes increase, whereas the β activity is diminished. Figure 2 shows the alcohol effect in the twin sample after automatic computational EEG analysis: The mean frequency decreases by about 1 Hz (cycles/s); this effect persists during

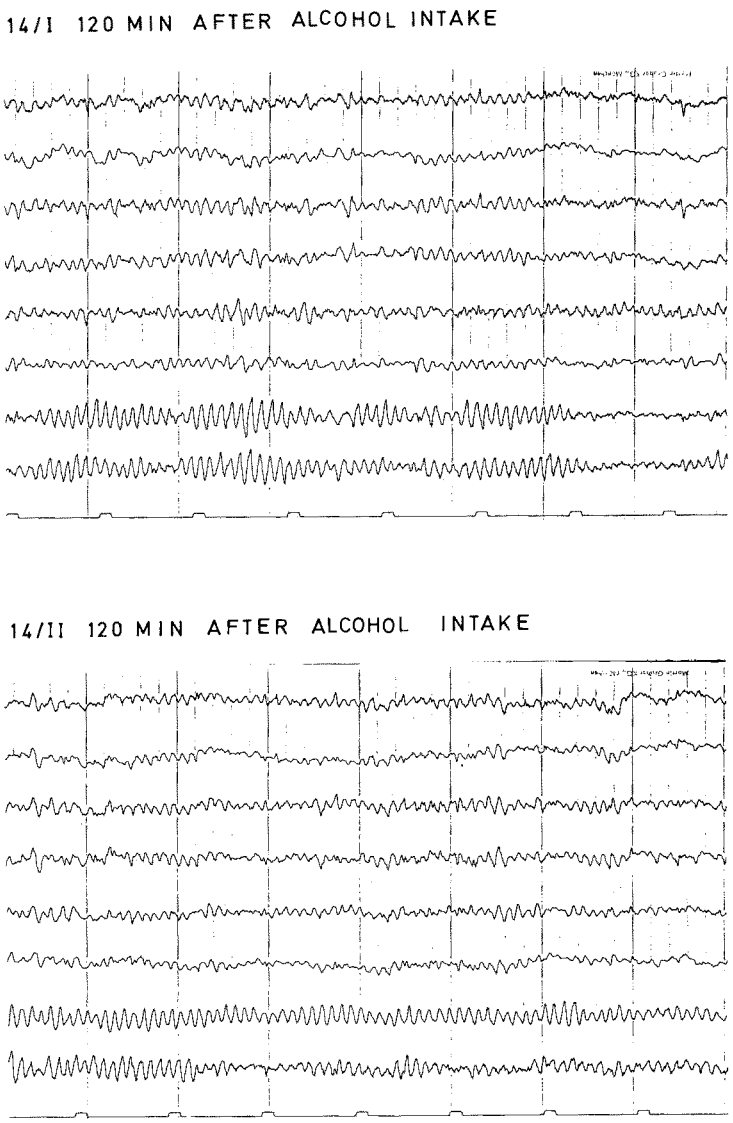

Fig. 3 cont.

nearly the whole experiment. The mean amplitude increases by about 12 μV. Figure 3 contains an example of an average α EEG in a pair of identical twins in the resting state and after alcohol loading: The extent of the alcohol-induced synchronization is similar. Figure 4 shows the divergent resting EEG of a fraternal twin pair: the No. I sibling exhibits a considerable degree of synchronization after alcohol administration, whereas the α EEG of the No. II sibling is only slightly impaired by alcohol. As these examples show, the extent of the effect of the alcohol differs greatly between individuals. Identical twins, however, remain as similar

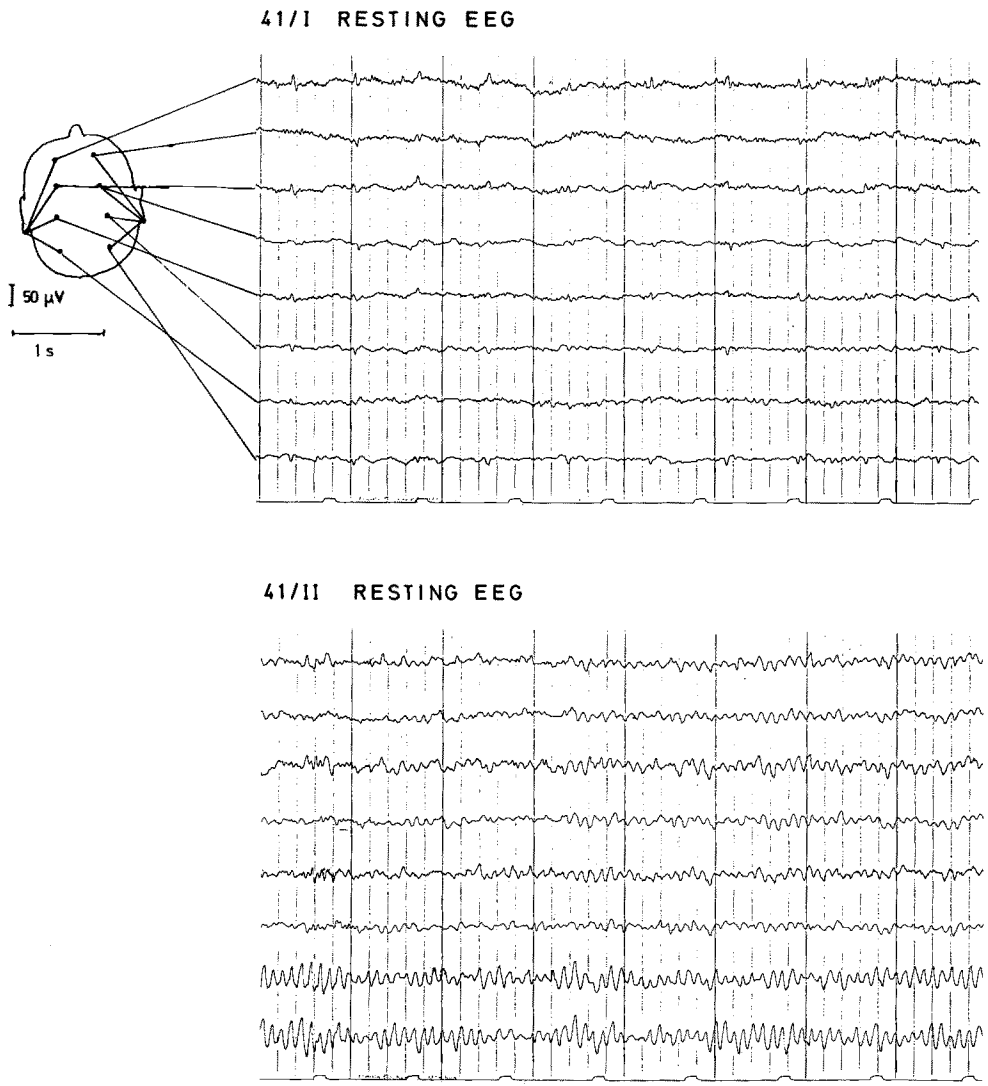

Fig. 4. Effect of alcohol on the EEG of a fraternal twin pair. The poorly synchronized EEG of the No. I sibling exhibits α waves after alcohol intake, whereas the α EEG of No. II is only slightly impaired

to one another as the same subjects in repetitive experiments. A comparison of the mean intrapair differences of mean frequency of MZ and DZ twins with intrasubject differences in repeated loading tests is shown in Figure 5. Fraternal twins become more dissimilar to one another after alcohol, thus indicating a differential reaction of the siblings.

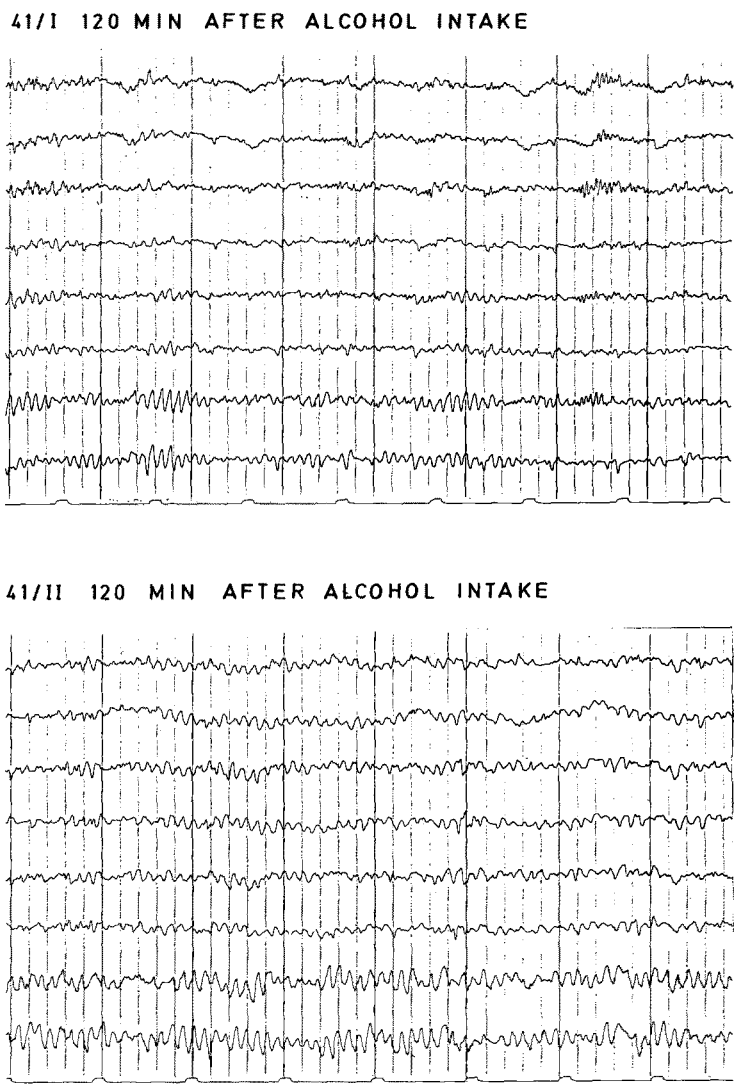

Fig. 4 cont.

An important question is whether the greater similarity of the EEG reaction to alcohol in MZ twins is due to a more similar blood alcohol concentration. During the period of the main effect, i.e., between 60 and 180 min after alcohol intake, blood alcohol curves of MZ twins are not more similar to one another than those of DZ twins. Thus, the identical reaction of the EEG in MZ twins as compared to fraternal twins is a pharmacogenetic phenomenon of the central nervous system (*Propping*, 1977).

Within the twin sample two pairs of identical twins with a low voltage EEG in the resting state were observed; this EEG type proved to be practically resistant to alcohol. The most pronounced effect of alcohol was

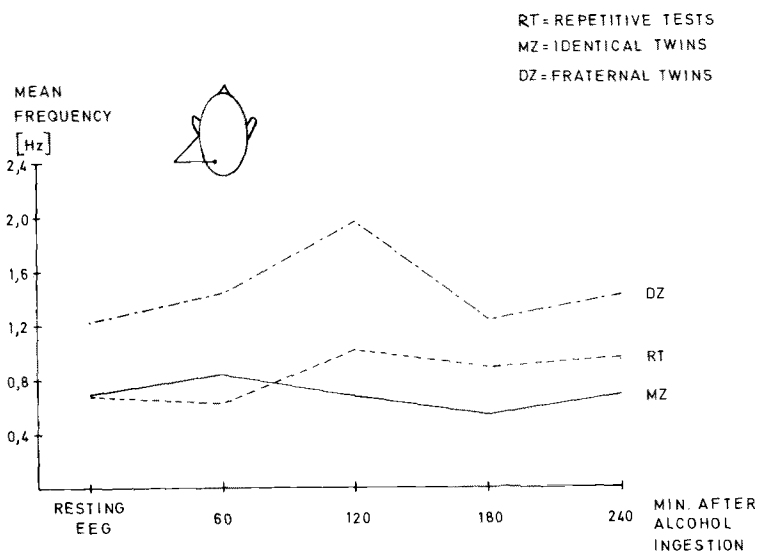

Fig. 5. Mean intrapair differences of mean frequency in MZ and DZ twins as compared to repetitive experiments in the same subjects (*RT*) during the alcohol loading test

observed in subjects with a borderline α EEG, i.e., in cases of a badly synchronized EEG, whereas the monotonous α EEG showed only a slight shift to the slower frequency bands.

The extent of the effect of alcohol on the EEG obviously depends on the nature of the resting EEG, which in turn is genetically determined. Nothing is presently known concerning the neurophysiologic basis of the variability of the normal human EEG, but it may be assumed that an important factor is the differential tonic influence of the desynchronizing ascending reticular activating system (ARAS). This notion is supported by the fact that alcohol has been shown to diminish the spontaneous activity of the ARAS, thus improving synchronization of cortical discharges (*Caspers*, 1957, 1958). Interindividual differences in the extent of the effect of alcohol on the EEG between individuals, might therefore reflect genetically determined differences in the spontaneous activity of the ARAS.

3.2.2 Differential Effects of Psychotropics on the EEG

Astonishingly little work has been done on psychotropic drug effects upon the EEG, taking into account the differences in the resting EEG. Some findings, however, suggest that differential effects of psychotropic drugs on the EEG are the rule rather than the exception. *Bente* (1973), for instance, described a differential effect of the anxiolytic agent etifoxin on the EEG, depending on the quality of the α rhythm in the resting EEG.

Itil (1974) tested the effect of a number of minor and major tranquilizers on the EEG and concluded that "individual sensitivity" is an important aspect. *Koukkou* and *Lehmann* (1976) analyzed the EEG before and repeatedly after cannabis application, and they measured subjective experiences. The applied low doses of tetrahydrocannabinol produced a shift of the EEG spectrum to the slower side during hallucinations. It seemed that there are characteristics of brain activity that indicate a predisposition to subjective drug effects. Subjects with high responsiveness to cannabinol exhibited resting spectra before and after drug application with higher modal α frequencies than subjects with a low tendency. Although these investigations were not performed with a genetic concept in mind, the observed differential effects probably reflect differences of brain function: A drug interacts with a biological system of which at least the electrical macrorhythms – the EEG – show a genetically determined variability.

3.2.3 Sedation Threshold

Another approach that succeeded in demonstrating genetic differences in the pharmacologic response of the central nervous system is the determination of the sedation threshold with amobarbital in twins (*Claridge* et al., 1973). In these experiments, the proband was required to perform continuously a concentration task, while receiving an intravenous infusion of amobarbital at a constant rate. The amount of drug per kg body weight administered until a certain percentage of errors is reached measures the sedation threshold. In this experimental procedure, the measured parameter will mainly reflect barbiturate tolerance of the central nervous system, although distributional factors are not entirely ruled out. In 11 MZ and 10 DZ twin pairs, *Claridge* et al. (1973) found an average intrapair difference in DZ twins almost three times that of MZ pairs. The authors also observed that high tolerance for the sedative drug was characteristic of neurotic, self-controlled introverts, on the one hand, and sociable, unanxious extraverts, on the other. Poor tolerance was found often in impulsive, neurotic extraverts and in withdrawn introverts who lack anxiety. Thus, pharmacogenetic methods may be used as a tool for genetic analysis of behavior.

3.2.4 Pharmacogenetic Aspects of Affective Disorders

Angst (1961, 1964), *Pare* et al. (1962), and *Pare* and *Mack* (1971) reported correlations between genetic background and the effect of antidepressive drugs: Blood relatives of patients with depression who also suffered from similar illnesses usually showed a similar response to one particular type of antidepressant: either MAO inhibitor or tricyclic antidepressant. However,

there was no such similarity of response when antidepressants of different pharmacologic groups were used. The differential response may depend on the biochemical type of depression that runs in families; one type may be responsive to MAO inhibitors, and another to tricyclic antidepressants. This notion is supported by reports that patients responsive to MAO inhibitors would suffer largely from secondary depressions, whereas tricyclics would be preferentially effective in unipolar endogenous depression (*Akiskal* and *McKinney,* 1975). Positive family history for bipolar affective illness, on the other hand, is a reliable predictor of responsiveness to lithium carbonate (*Mendlewicz* et al., 1973). However, not everybody treated with Li^+ responds in the same way to this therapy. It has been reported that the average value of the ratio of Li^+ concentrations in erythrocytes and plasma (Li^+ index) is higher in the group of patients who respond to lithium therapy than in groups of nonresponders and normal controls (*Mendels* and *Frazer,* 1973; *Mendels* et al., 1976; *Casper* et al., 1976; *Pandey* et al., 1977). This would indicate that the therapeutic effect depends on such factors as lithium transport across membranes (see below). Interestingly enough, *Rybakowski* (1977) reported a higher incidence of affective illness in first-degree relatives of patients with high RBC lithium index. This could indicate a possible link between RBC lithium index and genetic factors in manic-depressive psychosis.

The findings on a relation between lithium transport and therapeutic effect are controversial, however, since there are also reports without a difference in Li^+ ratio between responders and nonresponders to lithium treatment (*Rybakowski* and *Strzyzewski,* 1976; *v. Knorring* et al., 1976). Possible differences in diagnostic criteria may have contributed to the discrepancy.

One may expect that psychoactive agents, more than other drugs, elicit responses that are characterized by pharmacogenetic differences on a pharmacodynamic basis. However, there is the particular difficulty that in vitro experiments are impossible. Table 2 is a summary of some methodologic possibilities for measuring drug effects in the case of psychotropic drugs in man. A more detailed discussion of the genetic aspects of psychtropic drug action was presented earlier (*Propping* and *Kopun,* 1973; *Omenn* and *Motulsky,* 1976).

3.2.5 Intraocular Pressure Response to Glucocorticoids

Among randomly selected subjects topical glucocorticoid application leads to a variable response. Some persons show a temporary glaucoma-like elevation of intraocular pressure, while others show little or no response. It has been claimed that three different populations could be characterized by low, intermediate, and high levels of pressure response and that these

Table 2. Methods available for measurement of genetically determined pharmacodynamic differences of psychotropic drugs in humans

Psychological level:
Improvement of psychiatric symptoms
Behavioral responses or mood changes as measured by rating scales
Sensorimotor level:
Psychomotor and psychophysiologic performance (e.g., tapping speed, tremometer, reaction time, pursuit rotor)
Concentration performance
Sedation threshold
Autonomic level:
Cardiovascular responses (heart rate, blood pressure)
Skin conductance
Electrophysiologic level:
Resting EEG
Evoked potentials
Drug-induced biochemical responses:
Endocrine response
Enzyme inhibition
Transmitter (e.g., norepinephrine) release

phenotypes should correspond to the three possible genotypes of a diallelic system (*Armaly,* 1968). A study in twins, however, failed to support a predominant role of genetic factors: Identical twins were not much more alike than fraternal ones (*Schwartz* et al., 1972). Although genetic factors may be implicated in this penomenon, it seems evident that the hypothesis of monogenic inheritance has to be questioned.

3.2.6 Mydriatic Response

Topical application of a mydriatic in standard concentrations does not produce mydriasis in everybody to the same extent; especially in individuals with dark irides, mydriasis takes place inadequately. *Goldsmith* et al. (1977) examined dilatation of the pupil after local application of tropicamide in different ethnic groups of northern Chile. As could be expected due to the high frequency of dark irides, failure to dilate adequately occurred frequently, and familial clustering was observed. The limited family data would be compatible with autosomal-recessive inheritance, with "failure to dilate promptly" being the homozygous state. Thus far, however, polygenic inheritance is equally possible, since the degree of pigmentation of the iris is not determined by a single gene.

4. Pharmacogenetic Findings with a Simple Mode of Inheritance

4.1 Metabolic Differences

4.1.1 Polymorphic N-acetyltransferase

One of the first findings that led to the concept of pharmacogenetics was the observation that identical twins were much more similar to one another than fraternal twins with respect to excretion of the tuberculostatic drug isoniazid (INH) (*Bönicke* and *Lisboa,* 1957). The remarkable constancy within one subject combined with a high interindividual variability of isoniazid elimination had induced these authors to perform the twin study. When the plasma isoniazid concentration is measured 6 h after a single oral dose in a large sample of the normal population, a bimodal distribution results: Subjects can be classified into "rapid" and "slow" inactivators of isoniazid (*Evans* et al., 1960). The rate of isoniazid elimination is not significantly influenced by such factors as sex, tuberculosis, age, or enzyme induction. Detailed family studies have shown that the ability to eliminate isoniazid rapidly is inherited as an autosomal-dominant trait. Thus, isoniazid elimination is determined by a system of alleles causing "rapid" or "slow" inactivation (*Knight* et al., 1959; *Evans* et al., 1960). Rapid inactivators may be genetically of two types – heterozygous or homozygous. Although it is not possible to differentiate biochemically between the two genotypes in a single case, *Evans* et al. (1960) showed that the group of known heterozygotes differs significantly from the whole group of rapid inactivators, thus indicating a gene-dosage effect. In the meantime, a number of different methods have been described that allow the reliable phenotyping of a subject as a slow or rapid inactivator (e.g., *Evans,* 1969; *Eidus* et al., 1971; *Jessamine* et al., 1974; *Hoo* et al., 1977). The biochemical basis of the polymorphism of isoniazid elimination lies in the enzymic acetylation that takes place in the liver (*Evans* and *White,* 1964), but which may already occur during intestinal absorption (*Reidenberg* et al., 1973). The polymorphic enzyme transfers the acetyl group from acetylcoenzyme A to the acceptor drug molecule. Our present-day knowledge of isoniazid metabolism in man is summarized in Figure 6: The terminal metabolites are isonicotinyl glycine and diacetyl hydrazine. The most important route for isonicotinic acid formation is via acetyl isoniazid. However, isonicotinic acid can also be produced directly from isoniazid by direct hydrolysis, especially in slow acetylators. Interestingly enough, acetyl hydrazine is also acetylated by the polymorphic enzyme to diacetyl hydrazine (*Ellard* and *Gammon,* 1976). Acetylator phenotype is of no practical importance in tuberculosis therapy when daily isoniazid regimens are employed; it is important, however, when treatment is given only once weekly (*Ellard*

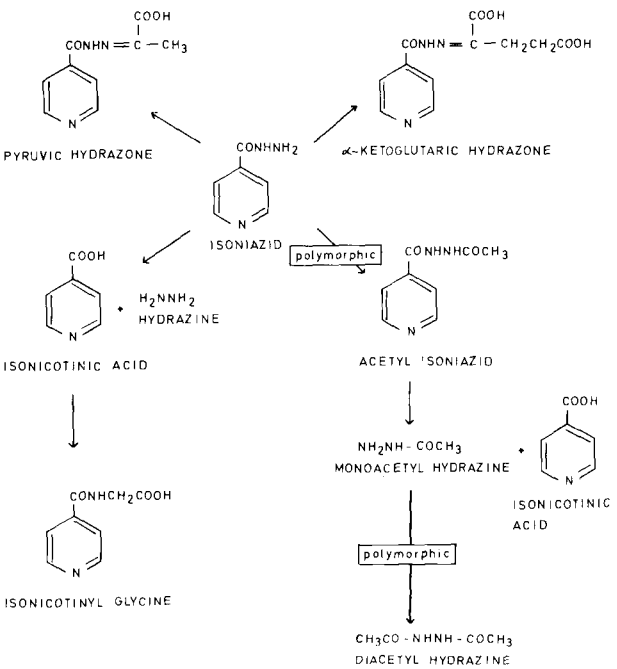

Fig. 6. Metabolism of isoniazid in man (after *Ellard* and *Gammon*, 1976)

and *Gammon*, 1976). Due to their higher plasma levels of isoniazid, slow acetylators have a higher risk of developing polyneuritis (*Hughes* et al., 1954), which, however, can be prevented by the simultaneous administration of pyridoxine (*Carlson* et al., 1956). In contrast, rapid acetylators of isoniazid have a higher risk of developing hepatitis. Approximately 10–20% of INH recipients manifest biochemical evidence of liver injury. This is in accordance with the observation of a higher susceptibility for isoniazid-induced hepatitis in Orientals than in populations with smaller proportions of rapid acetylators (*Mitchell* et al., 1975). Rapid acetylators hydrolyse about 44% of a dose of INH to isonicotinic acid, whereas slow acetylators convert only about 30% to isonicotinic acid. This hydrolysis simultaneously liberates stoichiometric amounts of the hydrazino moiety of isoniazid. *Mitchell* et al. (1975) suppose that the hydrazino moiety, especially acetyl hydrazine, is the toxic component that causes the isoniazid hepatitis. This metabolite may also be of interest because of its mutagenic potency (see below).

Recently *Timbrell* et al. (1977) presented evidence that acetylhydrazine itself should not be responsible for INH-induced liver injury. Acetylhydrazine is removed from the body by three routes: excretion, acetylation to diacetylhydrazine, and elimination by metabolism via the hepatic microsomal enzyme system. This latter pathway is thought to produce

a reactive intermediate responsible for hepatotoxicity. Since more acetylhydrazine is initially formed in the rapid acetylator, the potential for toxicity may be greater in this phenotype.

The physiologic function of the polymorphic N-acetyl-transferase is still unknown. However, there are a number of other drugs that are acetylated by this enzyme (Fig. 7). The monoamine oxidase (MAO) inhibitor phenelzine, which resembles isoniazid structurally, is also acetylated by the polymorphic enzyme (*Evans* et al., 1965). Inhibition of MAO occurs more slowly in rapid than in slow acetylators (*Johnstone*, 1976), and the antidepressant effect in neurotically depressed patients has been shown to be significantly greater in slow acetylators (*Johnstone* and *Marsh*, 1973). This group could not confirm the difference in the incidence of side effects between the two acetylator groups that *Evans* et al. (1965) had reported.

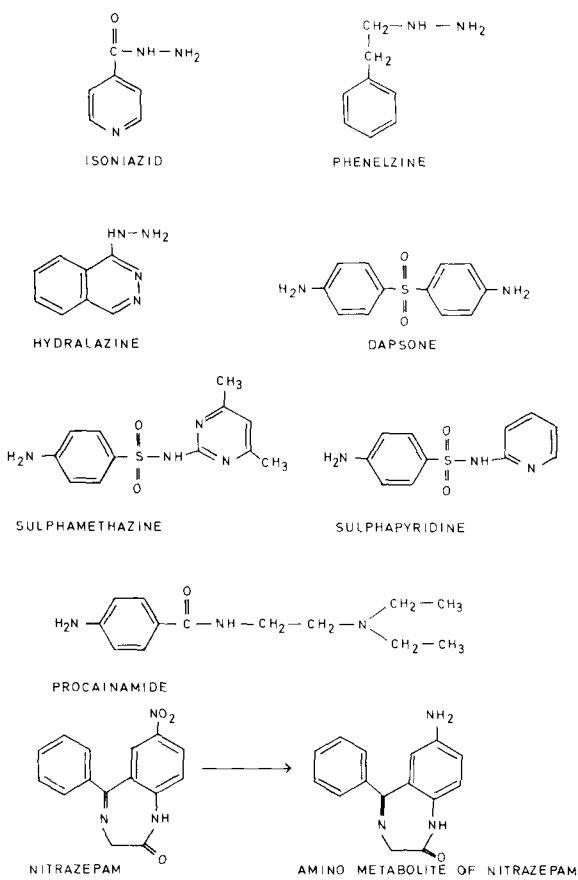

Fig. 7. Drugs that are known to be acetylated by the polymorphic N-acetyltransferase

When treated with hydralazine, an antihypertensive drug used increasingly in recent years, slow acetylators achieve higher serum concentrations, lower blood pressure, and develop more side effects, especially a syndrome that resembles disseminated lupus erythematosus (LE), than do rapid acetylators (*Perry* et al., 1970; *Koch-Weser,* 1976). However, metabolism of hydralazine is complex in man and not completely understood. *Reidenberg* et al. (1973) did not find differences between rapid and slow acetylators in plasma half-life values of hydralazine, indicating that elimination pathways other than polymorphic acetylation are the major ones for unchanged hydralazine. *Talseth* (1977) reported that N-acetylation of hydralazine probably does not govern the elimination rate of hydralazine in the postdistributive phase of the drug. The higher incidence of LE in slow acetylators might be explained by the fact that these subjects metabolize less of orally administered hydralazine than do rapid acetylators during "first pass" metabolism, thereby producing higher plasma concentrations. Slow acetylators, even when not treated with a drug, are at a higher risk of developing lupus erythematosus (*Reidenberg,* 1977). It has been hypothesized that drug-induced and spontaneous LE are the same diseases (*Drayer* and *Reidenberg,* 1977).

Polymorphic acetylation of the chemotherapeutic drug dapsone can be clearly recognized only by simultaneous measurements of dapsone and its monoacetyl derivative (*Gelber* et al., 1971). An individual's capacity to acetylate dapsone parallels that for isoniazid and sulfamethazine. It has long been known that acetylation is one of the main metabolic processes of sulfonamide elimination. Two sulfa derivatives, sulfamethazine and sulfapyridine, are known to be clearly acetylated by the polymorphic system (*Evans* and *White,* 1964; *Schröder* and *Evans,* 1972). Sulfamethazine is nowadays even frequently used for determining a person's acetylator status (*Evans*, 1969). In addition, other sulfa derivatives such as sulfamethizole (*Naito* and *Nelson,* 1963), sulfamerazine (*Mattila* et al., 1969), and sulfamethoxypyridazine (*White* and *Evans,* 1968) presumably are substrates for the polymorphic enzyme. However, in these cases additional factors within the overall metabolism are supposed to prevent unequivocal recognition of the polymorphic metabolic reaction. Obviously, the polymorphic acetylation of a drug can only be recognized when this reaction is at least the dominating event. Sulfadiazine, sulfisomidine, sulfisoxazole, sulfaphenazole, and sulfameter did not share the acetylation pattern of sulfamethazine (*Mattila* et al., 1969).

In subjects who had been treated with the antiarrhythmic agent procainamide for several days, *Karlsson* et al. (1975), *Reidenberg* et al. (1975), and *Campbell* et al. (1976) reported its polymorphic acetylation by the same enzyme. Toxic plasma levels of procainamide are common in slow acetylators receiving high doses of the drug, and ineffective levels of

the drug are common in rapid acetylators receiving low doses. Dosage schedules of procainamide in clinical practice should therefore be individualized (*Koch-Weser,* 1977). The N-acetyl derivative of procainamide, which still has antiarrhythmic properties, is presumably less likely to cause LE than the parent drug. The clinical consequences – therapeutic and toxic – of drug acetylation polymorphism have recently been reviewed by *Drayer* and *Reidenberg* (1977).

The sedative agent nitrazepam has been shown to be metabolized in man in part by the successive enzymic biotransformation steps of nitroreduction to an amine, followed by acetylation to the acetamido compound. The acetylation step is under the control of the acetylation polymorphism (*Karim* and *Evens,* 1976). This may influence therapeutic effectiveness as well as the rate of side effects.

The physiologic function of the polymorphic acetylation system is not known. Because of the simple mode of inheritance, a structural difference between the two allele products at the catalytic site of the enzyme molecule appears most probable. The apparently identical Michaelis constants (*Jenne* and *Orser,* 1965; *Weber* and *Cohen,* 1968), however, gave rise to some doubt about this hypothesis. Another explanation could be the existence of a polymorphic regulator gene. Interestingly enough, there are pronounced differences in frequencies of the two alleles between Whites and Blacks on one hand and Mongolids on the other (Table 3). No convincing explanation has as yet been proposed for this phenomenon; perhaps the ethnic differences are only the result of genetic drift.

Table 3. Frequencies of rapid and slow acetylators in different populations (from *Schloot* et al., 1967)

Population	Number of subjects	Acetylation type		Gene frequency Ac^S
		rapid %	slow %	
Europeans	900	51	49	0.70
Blacks	197	46	54	0.74
Indians	442	41	59	0.77
Japanese	1808	88.5	11.5	0.34
Thais	100	43	57	0.76

4.1.2 Alcohol Dehydrogenase Polymorphism

As summarized above, twin studies have presented evidence that alcohol elimination is under more or less pronounced genetic control. These investigations do not allow any specific conclusions as to the nature of the underlying genetic factors. The two main enzymes responsible for ethanol

oxidation in man are alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) both located in the liver. Three autosomal gene loci are concerned (ADH_1, ADH_2, ADH_3) in determining the structure of alcohol dehydrogenase in man. Locus ADH_1 and ADH_3 are primarily active during fetal life; locus ADH_2 is expressed already during embryogenesis and becomes gradually more active so that in adults this locus is responsible for most of the liver ADH activity (*Smith* et al., 1971). In adults the enzyme is expressed only in the liver and kidney. It is of special interest that an "atypical" alcohol dehydrogenase (ADH_2^2) has been described that differs from the "usual" enzyme (ADH_2^1) in total activity and pH optima (*v. Wartburg* et al., 1965). The atypical allele was found in 5%–20% of European populations (*v. Wartburg* et al., 1965; *Smith* et al., 1971). In Japanese, on the other hand, the situation is quite different: About 90% of the liver samples showed the ADH_2^2 allele either in the homo- or heterozygous form (*Fukui* and *Wakasugi*, 1972; *Stamatoyannopoulos* et al., 1975) (Table 4). Acetaldehyde dehydrogenase was not found to be polymorphic, at least in the Japanese population (*Stamatoyannopoulos* et al., 1975).

Table 4. Gene frequencies at ADH_2 locus in Europeans and Japanese

Origin	No. of liver	ADH_2^1	ADH_2^2	References
England	118	0.95	0.05	*Smith* et al. (1971)
England	50	0.98	0.02	*v. Wartburg* and *Schürch* (1968)
England	23	0.96	0.04	*Edwards* and *Evans* (1967)
Switzerland	59	0.89	0.11	*v. Wartburg* and *Schürch* (1963)
Germany	35	0.97	0.03	*Klein* et al. (1962)
Germany	46	0.96	0.04	*Harada* et al. (1978)
Japan	62	0.31	0.69	*Fukui* and *Wakasugi* (1972)
Japan	40	0.39	0.61	*Stamatoyannopoulos* et al. (1975)

The data of *Klein* et al. are based on activity determinations only, since the atypical variant was not known at that time.

Although the biological significance of the ADH_2 polymorphism remains uncertain, some possible consequences appear interesting (*Stamatoyannopoulos* et al., 1975). Since at the physiologic pH the atypical enzyme is many times more active than the usual one, it is possible that persons with the atypical allele metabolize alcohol differently from the ADH_2^1 homozygotes. It is difficult, however, to correlate alcohol elimination rates with enzyme type, because additonal metabolic factors influence

alcohol elimination. *Edwards* and *Evans* (1967) did not find clear evidence for higher alcohol elimination rates in subjects who carried the atypical allele. They had infused alcohol intravenously and calculated the ethanol degradation rate; they concluded that this methodology was not a reliable way of ascertaining the type of ADH_2 that an individual possessed. These authors auggested that alcohol dehydrogenase activity should not be the rate-limiting factor in human ethanol metabolism.

It has been questioned whether ADH_2 polymorphism has any influence on the phenotype. Most Mongolids response with rapid, intense flushing of the face and with symptoms of alcohol intoxication after administration of alcohol doses without apparent effect in most Caucasians (*Wolff*, 1972, 1973). Although *Wolff* prefers to attribute these phenomena to a direct effect of ethanol, *Stamatoyannopoulos* et al. (1975) postulated that the flushing reaction is the consequence of the initially high amount of acetaldehyde produced by the highly active atypical ADH. Further support for this notion comes from the fact that alcohol intoxication symptoms in Mongolids resemble effects of disulfiram, which acts by inhibiting acetaldehyde dehydrogenase, thus leading to higher acetaldehyde levels. Recently, *Sauter* et al. (1977) reevaluated the disulfiram-alcohol reaction in man. The intensity of this reaction was found to depend on the blood level of acetaldehyde, although differences between the symptoms of acetaldehyde intoxication and the disulfiram-alcohol reaction suggest a more complex mechanism for the latter. Alcohol intoxication phenomena including flushing have been produced by the administration of acetaldehyde to normal volunteers (*Asmussen* et al., 1948). Furthermore, *Reed* et al. (1976) found particularly high acetaldehyde levels in Mongolids as compared to Caucasians after oral alcohol loading. *Ewing* et al. (1974) had reported the same result, although their race difference was not statistically significant. In spite of consistency of data, however, the acetaldehyde mediated mechanism of facial flushing in Mongolids still awaits experimental proof.

The results on race differences in the rate of ethanol elimination are conflicting (*Fenna* et al., 1971; *Ewing* et al., 1974; *Reed* et al., 1976; *Bennion* and *Li*, 1976). A crucial variable in comparison of ethanol elimination rates between races is body habitus, which is likely to show interethnic differences. Of two individuals with identical lean body mass and identical absolute rates of ethanol metabolism, the one with less fat will have a higher calculated elimination rate in mg/kg x h (*Reed* et al., 1976). *Fenna* et al. (1971), who claimed that Canadian Indians metabolize ethanol more slowly than Caucasians do, found an unusually high rate of ethanol metabolism for their Caucasian subjects. *Bennion* and *Li* (1976) reported no differences between races; however, their Indian subjects were heavier than their Caucasian subjects (82.2 v. 70.0 kg). If the Indians were

more obese than the Caucasians, then the former should have a higher rate per kilogram of lean body mass. *Reed* et al. (1976) found an appreciably higher metabolic rate in Chinese and Canadian Indians than in Caucasians. Despite the conflicting results of the different studies, this latter finding has the highest plausibility. This conclusion is confirmed by the recent studies of *Farris* and *Jones* (1978) and *Hanna* (1978), who found a significantly faster rate of alcohol metabolism in American Indians than in Whites. Carriers of the atypical ADH allele, however, such as most Orientals, obviously develop higher acetaldehyde concentrations. Thus, it might well be possible that these subjects are protected metabolically from developing alcoholism. The general level of discomfort experienced may protect especially Orientals, who are known to have a lower incidence of alcoholism, from over-using alcoholic beverages as a psychological escape mechanism (*Ewing* et al., 1974).

4.1.3 Pseudocholinesterase Polymorphism

This polymorphism was among the first pharmacogenetic discoveries, and it has been one of the classic examples of the field. The discovery of certain variants of this enzyme has been associated with clinical observations of prolonged apnea after application of suxamethonium. Pseudocholinesterase is a plasma enzyme of unknown physiologic function. A number of review articles have appeared in the past (e.g., *Kalow,* 1972; *Goedde* et al., 1967; *Altland,* 1975). Although pseudocholinesterase (EC 3.1.1.8) catalyzes the hydrolysis of ester linkages in different drugs, the clinical importance is derived from suxamethonium (succinyldicholine) hydrolysis. Suxamethonium is a short-acting muscle relaxant commonly used during surgery. The short-term action of this relaxant is normally based on the catalytic degradation of succinyldicholine to succinylmonocholine and to succinate by pseudocholinesterase. There is a good correlation between pseudocholinesterase activity in serum and duration of apnea. At least two loci have been identified as determinants of pseudocholinesterase. One locus, called E_1, is genetically well established. At least four allelic genes exist at the E_1 locus (usual = E_1^u; atypical = dibucaine-resistant = E_1^a; fluoride-resistant = E_1^f; silent = E_1^s). The variant forms of the enzyme became detectable by kinetic studies in which benzoylcholine was used as substrate; the atypical form is characterized by decreased affinity to a number of inhibitors such as dibucaine (= cinchocaine); the E_1^f enzyme exhibits a greater resistance to inhibition by sodium fluoride. Usually, the influence of the inhibitor on enzyme activity is measured as per cent inhibition, and this percentage is called the dibucaine or fluoride number. Both alleles lead to decreased enzyme activity whose extent depends on the genotype and the kind of substrate. The silent allele is associated with

complete or nearly complete absence of enzyme activity. Obviously there is genetic heterogeneity within the E_1^s allele (*Altland* and *Goedde*, 1970; *Das*, 1973). Additional alleles, which were detected by interaction with the atypical allele, have been described in single families (*Garry* et al., 1976; *Rubinstein* et al., 1978). The four well-attested allelic genes at the E_1 locus would allow ten different combinations. Table 5 is a summary of the main characteristics of the genotypes and the estimated frequencies in European populations. However, the four alleles differ in their geographic distributions (for review see *Steegmüller*, 1975), the E_1^s, e.g., being very frequent in Alaskan Eskimos, where 1.5% of the population are estimated to be homozygous for this allele (*Gutsche* et al., 1967). Table 6 gives an impression of gene frequencies of the atypical allele in some populations.

Table 5. Pseudocholinesterase types and suxamethonium sensitivity (from: WHO techn. Rep. Ser., 1973, No. 524)

Genotype of clinical significance	Cinchocaine number	Fluoride number	Phenotype frequency in "European" populations [b]	Suxamethonium sensitivity
E_1^a E_1^a	22	27	1: 3200	+++
E_1^s E_1^s	0	0	1:170000	++++
E_1^f E_1^f	66	35	1: 28000	++
E_1^a E_1^s	22	27	1: 11000	+++
E_1^a E_1^f	49	33	1: 2500	+++
E_1^f E_1^s	67	43	1: 33000	++
E_1^u E_1^u	80	59	95%	none
E_1^u E_1^a	62	48	3%	(+)
E_1^u E_1^f	74	50	1%	(+)
E_1^u E_1^s	80	59	1: 200	+a

[a] Supplemented by data of *Baker* et al. (1977).
[b] Based on the frequency of the heterozygous state of 3.5% for the atypical allele, 1.3% for the fluoride-resistant allele and 0.5% for the silent allele. The frequencies of the homozygous and heterozygous states were calculated by an expansion of the Hardy-Weinberg theorem for multiple alleles.

In about 70% of patients with prolonged apnea after suxamethonium administration, detectable variants of pseudocholinesterase can explain this unusual side effect. It had been assumed earlier (*La Du*, 1972) that there were rare variant forms of the esterase affecting suxamethonium

Table 6. Incidence of the atypical allele E_1^a in different populations (from *H. Steegmüller*, 1975)

	Number of tested individuals	Homozygotes E_1^a E_1^a	Heterozygotes for E_1^a	Gene frequency for E_1^a
Caucasoids	29348	16	1053	0.01849
Negroids	1284	–	8	0.00312
Mongoloids	2993	–	9	0.00150
Indians	2305	–	20	0.00434
Eskimos	704	–	1	0.00071
Australoids	98	–	1	0.00510
Melanesians	2731	–	65	0.01190
Lapps	890	–	26	0.01460
Pygmies	425	–	–	0.00000

hydrolysis that were not detectable with benzoylcholine as substrate. The introduction of suxamethonium into the enzyme assay indeed has recently provided evidence for the existence of hitherto unknown variants of cholinesterase with altered properties toward succinylcholine (*Agarwal* et al., 1976). The authors examined sera from 21 individuals who had suffered from prolonged apnea and whose cholinesterase nevertheless had previously been classified as normal (E_1^u E_1^u). With succinylcholine only six sera showed normal enzyme characteristics, whereas in six cases there was no apparent activity, and in nine samples enzyme activities were normal but exhibited a reduced dibucaine number. These results together with family investigations suggest a fifth allele at the E_1 locus for which E_1^{su} has been proposed (*Goedde*, 1977).

Additional variants of cholinesterase such as the Cynthiana variant with unusually high enzyme activity and the C_5 variant have been described, but are genetically not entirely understood (for discussion, see *Altland*, 1975).

4.1.4 Dehydrogenation of Sparteine

Sparteine, a drug with antiarrhythmic properties, has been described as being usually metabolized by dehydrogenation (*Dengler* and *Eichelbaum*, 1977). About 5% of the population, however, are unable to metabolize the drug. These individuals excrete, as unchanged drug in the urine, more than 95% of the sparteine administered, whereas normally only 20%–30% is excreted in unchanged form. Among about 300 subjects examined a clearly bimodal distribution with respect to urinary excretion of unchanged sparteine was obtained (Fig. 8). Nonmetabolizers exhibit a blood level

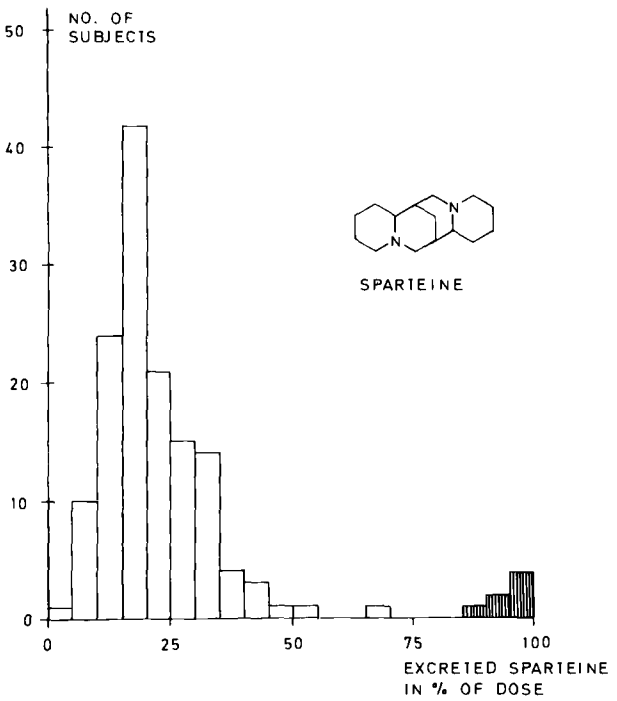

Fig. 8. Distribution of unchanged sparteine excreted in urine as percentage of the dose administered (after *Dengler* and *Eichelbaum*, 1977)

4–5 times higher than metabolizers, and the sparteine has an appreciably prolonged half-life. The metabolic peculiarity of this agent came to light when the authors observed two individuals who showed severe side effects after the usual dose of sparteine. Obviously there is no alternative step for sparteine degradation that could have masked the consequences of the defective reaction. *Dengler* and *Eichelbaum* suppose that defective sparteine dehydrogenation has a genetic basis. They have evidence for this assumption from a family study.

4.1.5 Diphenylhydantoin Hydroxylation

Diphenylhydantoin is among those drugs for which twin investigations have shown that genetic factors play an important role in its elimination rate (*Andreasen* et al., 1973). Even when a strict standard dose is employed, a wide interindividual variability in steady-state plasma concentrations can be observed (*Cunningham* et al., 1974). This means that plasma levels have to be monitored in individual patients, because different subjects need different doses to achieve the optimal therapeutic plasma level of 10–20 μg/ml. The most important cause of the observed variability is the rate of diphenylhydantoin metabolism: The primary and rate-limiting step in its metabolism is p-hydroxylation of one phenyl group (*Remmer*

et al., 1969). Whereas the majority of patients are capable of metabolizing up to 10 mg/kg diphenylhydantoin, there are some who metabolize only 1–2 mg/kg daily (*Kutt,* 1971). (The range of the clearance rates, which is a better parameter for elimination, is 0.6–3.6 l/h in the study by *Cunningham* et al., 1974.) Therefore, there is always the danger that the drug accumulates, producing toxic plasma concentrations. *Kutt* et al. (1964) described in a family three members (mother and two sons) unable to metabolize more than 3 mg/kg daily (Table 7). This author observed three families in each of which the mother and one or more siblings were afflicted. The mode of inheritance for a deficiency to hydroxylate diphenylhydantoin might be autosomal dominant, but polygenic inheritance cannot be ruled out.

Table 7. Very rare pharmacogenetic traits that are assumed to have a monogenic basis. Due to the low number of families the mode of inheritance is uncertain in each case

Genetic peculiarity	References	No. of observed subjects or families	Suggested mode of inheritance
Deficient p-hydroxylation of diphenylhydantoin	*Kutt* et al. (1964) *Kutt* (1971)	3 families	Autosomal dominant
Deficient "N-hydroxylation" of amobarbital	*Kalow* et al. (1977b)	1 MZ twin pair within 1 family	Autosomal recessive
Acetophenetidin-induced methemoglobinemia	*Shahidi* (1968)	2 sisters	Autosomal recessive
Abnormal lithium and sodium transport in erythrocytes	*Pandey* et al. (1977)	1 family: father, 3 children	Autosomal dominant
Warfarin resistance	*O'Reilly* et al. (1964) *O'Reilly* (1970)	2 large families	Autosomal dominant
Debrisoquine hydroxylation	*Mahgoub* et al. (1977)	3 families	Autosomal recessive

4.1.6 Metabolism of Amobarbital

The twin study on amobarbital elimination described above revealed that the observed interindividual variability in rate of drug decay is due mainly to genetic factors. Development of highly sensitive methods has made it possible to quantify two metabolites of amobarbital in man after a single oral dose; these metabolites appear in the urine and account for 80% of

the ingested drug. They were interpreted as C- and N-hydroxyamobarbital (*Tang* et al., 1975). In the meantime, however, doubt has arisen as to the identification of the "N-hydroxylated" metabolite[1]. Among their twin probands, *Kalow* et al. (1977) observed an identical pair that did not produce N-hydroxylated amobarbital. Examination of the whole family yielded a wide variability of N-hydroxylation capacity. The distribution of this capacity in the family is compatible with an autosomal-recessive trait: One can assume that two allelic genes exist, one of which (that for N-hydroxylation deficiency) is rare; this would allow three different genotypes. Figure 9 shows the originally described pedigree that is an interpretation of a two-allele model (*Kalow* et al., 1977). It is interesting that the authors could not find evidence of compensatory or linked activities between the two reactions of amobarbital.

Although the metabolite is not finally determined, the measured parameter presumably reflects directly the function of a single gene. Admittedly, polygenic inheritance may also be possible, as long as only one family has been observed.

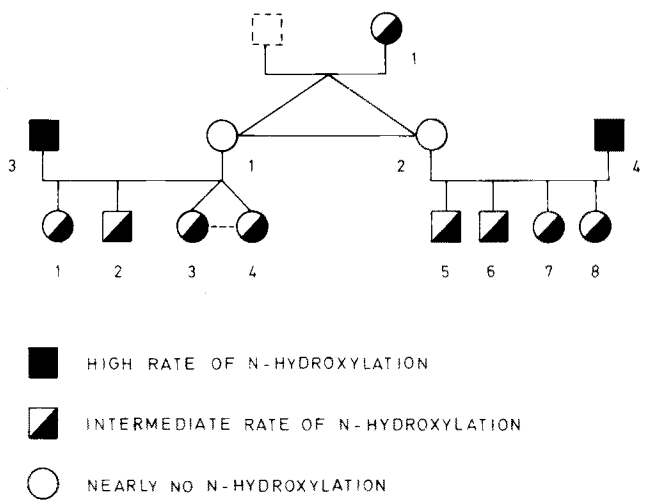

Fig. 9. Familial deficiency of N-hydroxylation of amobarbital. The interpretative marking of each subject's genotype is based on the assumption that the capacity for N-hydroxylation is determined by two autosomal allelic genes (from *Kalow* et al., 1977)

[1] Personal communication of Prof. *Kalow:* "The identification of this metabolite still rests on physical and chemical properties of the metabolite, rather than on a comparison with a synthetic product. However, even if the final proof of identity is missing, this does not affect the validity of the biological data."

4.1.7 Acetophenetidin-Induced Methemoglobinemia

Shahidi (1968) described two sisters who, after administration of acetophenetidin (phenacetin), produced different 2-hydroxy derivatives that led to increased methemoglobin formation. In contrast, the control person as well as the parents and other siblings did not produce such amounts of hydroxy derivatives. This observation is compatible with an autosomal recessive mode of inheritance, although polygenic influence is also possible. It would be reasonable to examine systematically the metabolism of phenacetin in patients with phenacetin-induced interstitial nephritis and their relatives.

4.1.8 Debrisoquine Hydroxylation

Both hypertensive patients and normal volunteers vary widely in their hypotensive response to the adrenergic-blocking drug debrisoquine. *Mahgoub* et al. (1977) showed that some people have a decreased ability to convert debrisoquine to its major metabolite 4-hydroxydebrisoquine. Among 94 normal subjects there are three who excreted only very small amounts of the 4-hydroxy derivative in the urine. Examination of the families of the three subjects suggests autosomal recessive inheritance. Since within the sample more than 3% were poor metabolizers of the drug, the gene frequency of the defective allele is rather high ($q = 0.17$). Extensive metabolizers of debrisoquine in contrast to poor metabolizers showed little or no hypotensive response to the drug (*Idle* et al., 1978). It would be interesting to determine whether poor metabolizers of debrisoquine also lack the ability to hydroxylate other drugs.

4.2 Pharmacodynamic Differences

The above-mentioned resistance of the low voltage EEG to alcohol probably is a monogenic peculiarity, since this EEG type is inherited as an autosomal-dominant trait (*Vogel,* 1970). Family studies with alcohol loading, however, have not been done so far.

4.2.1 G-6-PD Deficiency

Glucose-6-phosphate dehydrogenase (G-6-PD) is an X-linked enzyme that exhibits a high degree of polymorphism: About 130 different variants have been described so far. G-6-PD deficiency is the most common enzyme abnormality in man, since about 300 million people are estimated to be affected, when gene carriers of both sexes are included (*Luzzatto,* 1973). The genes for G-6-PD are expressed in all cells in which the enzyme activity has been tested, the most easily accessible tissue being red blood cells. In practically all the cases of G-6-PD variants, the abnormalities are

due to structural mutations resulting in the synthesis of abnormal enzyme molecules with altered catalytic activity, kinetic properties, stability and electrophoretic mobility. In the majority of cases, and under neutral conditions, G-6-PD-deficient red blood cells have an essentially normal metabolism and a nearly normal life span. They hemolyze only when challenged by an exogenous oxidant agent, such as drugs. G-6-PD polymorphism has become an expanding field of research not only in clinical and genetic research, but also in biochemistry, cellular physiology, and evolutionary research. A number of reviews have summarized the state of the field (e.g., *Kirkman,* 1971; *Beutler,* 1972; WHO tech. Rep. Ser., 1972; *Luzzatto,* 1973, 1975; *Flatz* and *Xirotiris,* 1975; *Kahn,* 1977). Here we will confine ourselves to pharmacogenetic aspects.

G-6-PD deficiency belongs to the classic examples of pharmacogenetics. Some types of this enzyme abnormality predispose to hemolytic anemia when certain drugs have been taken up. The agents inducing hemolysis in patients carrying an abnormal allele are drugs that either produce peroxide in vivo or act themselves as direct oxidants. There is evidence that an oxidant stress accentuates the activity of the hexose monophosphate shunt, and if G-6-PD is deficient qualitatively or quantitatively, reduced glutathione is not available and oxidant damage occurs to vital components of the cell. The result of the oxidant damage is Heinz body formation and hemolysis (*Desforges,* 1976). Two principal types of G-6-PD deficiency are the African (A^-) and the Mediterranean (B^-) type. The A^- enzyme differs from the normal (B) in electrophoretic motility and in vivo stability; its activity is almost normal in young red blood cells, but declines more rapidly than the normal enzyme when the cell ages. The activity of the Mediterranean type, on the other hand, is diminished even in the youngest cells and continues to decline more rapidly than the A^- type. Accordingly, drug-induced hemolytic anemia is more severe in persons with the Mediterranean G-6-PD deficiency than in the African type (*Desforges,* 1976). Enzyme activity of A^- is approximately 8%–20% of the normal, whereas B^- exhibits only 0–7% of the normal activity. Table 8 contains a list of those drugs that are known to provoke hemolysis in G-6-PD-deficient persons. As a rule of thumb, one should consider those variants with an enzyme activity of less than 10% to constitute a potential risk.

Since G-6-PD is an X-linked enzyme, all red blood cells of a male who has a deficient enzyme are at a hemolytic risk when an oxidant drug is taken up. Females will be heterozygous in most cases. Since in females one X chromosome in every cell is inactivated during early embryogenesis, heterozygous females exhibit two red blood cell populations: one with the normal enzyme and one with the deficient enzyme. In most cases, the proportion of deficient cells varies between 40% and 60%, but may deviate

Table 8. Drugs reported to induce hemolysis in subjects with G-6-PD deficiency (from: WHO techn. Rep. Ser., 1973, No. 524)

Drug	Hemolysis	
	Negro subjects	Caucasian subjects
A. Drugs producing clinically significant hemolysis		
Acetanilide	+ + +	
Dapsone	+ +	+ + +
Furazolidone	+ +	
Furaltadone	+ +	
Nitrofural	+ + + +	
Nitrofurantoin	+ +	+ +
Sulfanilamide	+ + +	
Sulfapyridine	+ + +	+ + +
Sulfacetamide	+ +	
Salazosulfapyridine	+ + +	
Sulfamethoxypyridazine	+ +	
Thiazosulfone	+ +	
Quinidine		+ +
Primaquine	+ + +	+ + +
Pamaquine	+ + + +	
Pentaquine	+ + +	
Quinocide	+ + +	+ +
Naphthalene	+ + +	+ + +
Neoarsphenamine	+ +	
Phenylhydrazine	+ + +	
Toluidine blue	+ + + +	
Trinitrotoluene		+ + +
B. Drugs reported as hemolytic agents in some cases, but usually not producing clinically significant hemolysis under normal conditions (e.g., in the absence of infection)		
Phenacetin	+	
Acetylsalicylic acid	±	+
Sulfadiazine		+ +
Sulfafurasole	+ +	
Sulfoxone	+	
Chloramphenicol	0, +	+ +
Nitrite	+	+ + +
Methylene blue	+	
Ascorbic acid	+	
Dimercaprol	+	
Chloroquine	±	
Mepacrine	±	

appreciably from this value in the individual case. Accordingly, heterozygous females are not simply carriers of the abnormal allele, but are actually susceptible to hemolysis. However, the degree of hemolysis will be less in a heterozygous female than in a trait-carrying (hemizygous) male.

4.2.2 Abnormal Li^+ and Na^+ Transport

The twin study on lithium distribution across the red blood cell membrane (*Dorus* et al., 1974, 1975; see above) pointed to the influence of genetic factors. The report of a family in which four members (father and three children) showed an abnormal lithium transport (*Pandey* et al., 1977) opens an interesting new pharmacogenetic dimension. In normal human erythrocytes, Li^+ is transported by at least three distinct mechanisms: one inhibited by ouabain, one by phloretin, and one inhibited by neither compound. The phloretin-sensitive transport of Li^+ occurs against its electrochemical potential gradient, if there is an electrochemical potential gradient for Na^+ in the opposite direction (i.e., Li^+/Na^+ counterflow can occur). Li^+ distribution between erythrocytes and plasma in vivo depends mainly on Li^+/Na^+ counterflow and on the transport of Li^+ that is resistant to both ouabain and phloretin; ouabain-sensitive Li^+ transport is negligible under physiologic conditions. *Pandey* et al. (1977) observed a male patient in whom the phloretin-sensitive Li^+/Na^+ counterflow system was almost absent in the erythrocytes, so that the steady-state ratio of Li^+ concentration in the erythrocytes to that in plasma was 2–3 times higher than in control persons. The father and at least three siblings of the patients showed the same abnormality. This would suggest a dominant mode of inheritance (the sex of the siblings is not stated). The authors could show that the abnormality was not due to plasma inhibitors; instead, they assume the involvement of a carrier molecule in the erythrocyte membrane that can bind and transport both Na^+ and Li^+. This carrier system is thought to be altered due to a genetic defect. The authors could not find evidence for a connection between abnormal Li^+/Na^+ transport and at least a subclass of affective disorders. This would mean that the observed peculiarity is a characteristic only of the red blood cell membrane.

4.2.3 Malignant Hyperthermia

During general anesthesia a severe symptomatology may arise that is characterized by tachycardia and hyperthermia; muscular rigidity occurs in three-fourths of the cases; regardless of the presence or absence of rigidity, about two-thirds of the patients die. The clustering of such cases in families implicated genetic factors. Earlier investigations of *Kalow* and his group rendered an autosomal dominant mode of inheritance probable (*Kalow,*

1972). Elevated serum activities of creatine phosphokinase (CPK) in relatives of affected patients may be helpful in identifying persons at risk. Although halothane as the most widely used anesthetic drug was frequently implicated, *Kalow* suggests that malignant hyperthermia is a consequence of general anesthesia with a variety of volatile anesthetics rather than a specific effect of halothane. The incidence of the trait (which may be regarded as a special type of muscle disease that can only be detected by anesthesia) is about 1 per 20,000; 180 cases have been described (*Kalow,* 1972). *Kalow* et al. (1977a) attempted to obtain a clue to the pathogenesis of malignant hyperthermia by a pharmacologic assay of isolated skeletal muscle. The test consists of measuring the contracture tensions of muscle fibers when exposed to various concentrations of caffeine, once in the presence, once in the absence of halothane. The contracture produced by caffeine could serve to distinguish between muscle from normal subjects and those susceptible to malignant hyperthermia. Examination of relatives of patients who had recovered from malignant hyperthermia resulted in three kinds of relatives: those indistinguishable from patients, those falling into the control group, and an intermediate group. The data are partly compatible with recessive, partly with dominant inheritance. This would lead to a genetic model with two different loci predisposing to malignant hyperthermia; however, a single locus with three alleles would also be possible (*Kalow* et al., 1977).

Schmitt et al. (1974) observed a family in which two children had died of malignant hyperthermia following halothane anesthesia. Mother and sister of the sibs exhibited only 10%–20% of normal muscle adenylate kinase activity. The authors suggest that in individuals with adenylate kinase (E.C. 2.7.4.3) deficiency, malignant hyperthermia develops due to the inability to regenerate ATP in sufficient amounts. Interestingly enough, halothane has a specific binding site deep in the enzyme molecule; the same site was found to bind the adenine moiety of AMP (*Sachsenheimer* et al., 1977). Inherited adenylate kinase deficiency may be aggravated by the inhibitory effect of halothane, thus leading to fatally decreased ATP levels. This could explain the mechanism of malignant hyperthermia in at least some cases.

4.2.4 Warfarin Resistance

Most of the large interindividual variations in dicumarol disposition are genetically determined (see above), presumably in a polygenic way. *O'Reilly* and his group (*O'Reilly* et al., 1964; *O'Reilly,* 1970) reported two large kindreds, in which the two propositi and a number of relatives required enormous doses of warfarin to achieve the usual hypoprothrombinemic response. In contrast to the half-life study in twins, here a receptor

mutation leads to decreased sensitivity toward the anticoagulant. The propositi were equally resistant to dicumarol and to the indanedione anticoagulant phenindione. The authors could experimentally prove that the carriers of the mutant gene also have a greater requirement for vitamin K than normal subjects do. Hereditary resistance to oral anticoagulants possibly belongs to the inherited vitamin-dependent states, which are also known for other vitamins (*O'Reilly,* 1971).

It is interesting to note that a similar mutation of the coumarin receptor has repeatedly been observed in rats. As breeding experiments between warfarin-resistant wild rats and normal Sprague-Dawley rats have shown, the trait is also transmitted as autosomal dominant (Fig. 10). Resistant wild rats were discovered independently in various countries. Due to the wide-spread use of coumarins as rodenticides, survival of resistant rats was favored, so that such strains became predominant in several areas (*Drummond,* 1966). In warfarin-resistant rats it has been possible to learn more about the nature of the hypothetical receptor both for vitamin K and warfarin: An epoxide reductase (which is responsible for reduction of a natural metabolite inhibitory to vitamin K) from liver of resistant rats is inhibited by warfarin to a lesser extent than the reductase from control animals (*Zimmermann* and *Matschiner,* 1974). In the described human pedigrees the same mechanism may, but need not be, responsible for warfarin resistance.

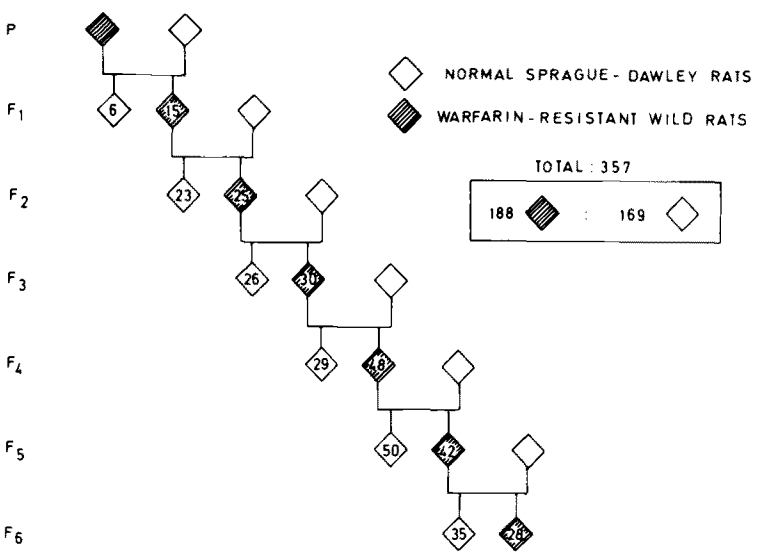

Fig. 10. Breeding experiment between warfarin-resistant wild rats (*Rattus norvegicus*) and normal Sprague-Dawley rats through 6 generations (compiled from data of *Pool* et al., 1968)

5. Pharmacogenetics and Protein Binding

As cited above, man is likely to be heterozygous in about 20% of his gene loci (*Harris* and *Hopkinson,* 1972). This should also apply to proteins that are known to bind small molecules such as drugs. Differences in the binding of a drug are expected to lead to differences in pharmacologic effect, because only the unbound fraction of a drug is pharmacologically active. Variation of protein binding should have practical consequences particularly in those drugs that are highly bound to proteins. Ordinarily, drugs bind to albumin, although other plasma proteins also bind drugs. The albumin molecule shows a remarkable genetic variability; however, all of the known variants are rare (for review see *Langenbeck,* 1975). After detection of albumin variants with a reduced binding capacity for small molecules, pharmacogenetic implications have been repeatedly supposed (*Melartin,* 1967; *Johnston* et al., 1969).

The first experimental hint for implication of genetic factors in drug binding to plasma proteins came from a twin study (*Alexanderson* and *Borga,* 1972). The binding ratio of plasma proteins for nortriptyline was measured by an equilibrium dialysis technique in MZ and DZ twins. The authors found a twofold variation of the binding ratio, MZ twins being more similar to one another than DZ pairs. However, there were also significant differences between individuals within MZ pairs, pointing to environmental influences at this level of analysis. Since the number of twin pairs was small (7 MZ, 10 DZ) and albumin variants are rare, factors other than structural differences in the albumin molecule may account for the observed genetically influenced difference. Dietary influences can alter the concentration of tightly bound endogenous ligands such as fatty acids, thus contributing to interindividual differences of drug binding (*Wilding* et al., 1977). Basic drugs, such as the β-adrenoreceptor blocker alprenolol and the tricyclic antidepressant imipramine, have recently been shown to bind avidly to α_1-acid glycoprotein, a plasma protein of low molecular weight (40,000) (*Piafsky* and *Borga,* 1977). The free fraction of both drugs correlated negatively with the plasma concentration of α_1-acid glycoprotein, while there was no correlation with albumin concentration. Although studies on the genetic contribution to interindividual variations of this plasma protein have not yet been done, it may be assumed that, in the healthy individual, plasma protein concentrations are under polygenic control.

The study of *Wilding* et al. (1977) on in vitro warfarin binding to sera of subjects with albumin variants circumvents the practical difficulties of in vivo investigations. At therapeutic serum concentrations, 99% of warfarin is bound to albumin. Equilibrium dialysis was used to measure the binding of warfarin to isolated albumin. The normal albumin (A/A)

and the Mexico variant (A/Me) were examined for their warfarin binding capacities. The mean percentages of bound warfarin for the A/A and A/Me populations were 97.8% and 97.2%, respectively, However, since only the free form of a drug is pharmacologically active, a more appropriate comparison may be between the free fractions of warfarin. The 2.8% free fraction of the A/Me population represents a 27% increase over the 2.2% free fraction of the A/A population. When whole serum was used, A/Me and A/Na (heterozygous for albumin Naskapi) sera also proved to bind warfarin to a lesser extent. No in vivo study, in which pharmacokinetic parameters of a drug with high albumin binding are examined in individuals with an albumin variant, has yet been performed. In carriers of albumin variants with decreased drug binding capacity, enhanced pharmacologic responses and toxicity might be possible (*Wilding* et al., 1977). Compensatory binding to other plasma proteins, however, is also possible. It would be particularly interesting to examine pharmacokinetic parameters of drugs that are appreciably bound to proteins in subjects with the rare analbuminemia. This trait is probably inherited in an autosomal recessive way. One might expect that elimination of such drugs is essentially faster, since, e.g., warfarin was shown to have a much shorter half-life in marked hypoalbuminemia due to nephrotic syndrome (*Lewis* et al., 1967). In hereditary analbuninemia, however, compensatory increase of other proteins (α_2- and β-globulins) (for review see *Langenbeck*, 1975) might lead to a complicated drug-binding situation.

6. Extension to Ecogenetics

While pharmacogenetics is devoted to genetic variability in the reaction to drugs, it appears probable that there is also genetic variability in the reaction to environmental agents. In a stimulating short annotation, *Brewer* (1971) coined the term "ecogenetics". This should be a discipline that examines the old problem of gene-environment interaction with respect to wide-spread or even ubiquitous industrial agents. One of the first and best known examples cited in this context is α-1-antitrypsin deficiency, which increases the risk of pulmonary emphysema. Smokers with α-1-antitrypsin deficiency have an even higher risk of developing emphysema (see, e.g., *Stanbury* et al., 1972).

6.1 Serum Paraoxonase Polymorphism

The human serum contains an enzyme that hydrolyzes the cholinesterase inhibitor paraoxon. Paraoxon is used as an insecticide per se, or it is formed within the mammalian organism by microsomal oxidation from parathion (Fig. 11). Paraoxonase shows a remarkable interindividual variability, whereas its activity remains constant within a given subject. *Geldmacher-v. Mallinckrodt* et al. (1973) described a trimodal distribution of enzyme activity with a simple mode of inheritance. The authors suggested a diallelic model with a high- and a low-activity allele. This interpretation was confirmed by *Playfer* et al. (1976) by family investigations in Britain, although these authors only found a bimodal distribution in the population. This difference may be accounted for by methodologic factors: The method used could not differentiate between heterozygotes and subjects homozygous for low activity. Nevertheless, both the German and the British groups had a gene frequency of about 0.7 for the low-activity allele. In addition, *Playfer* et al. (1976) examined other ethnic groups for paraoxonase polymorphism: Whereas Indians exhibited a similar bimodal distribution, it was impossible to define separate genotypes in the examined African, Malay, and Chinese populations. Paraoxonase has no known natural substrate, but individuals with high enzyme activity may be at an advantage when poisoned with parathion, paraxon, or perhaps structurally similar cholinesterase inhibitors.

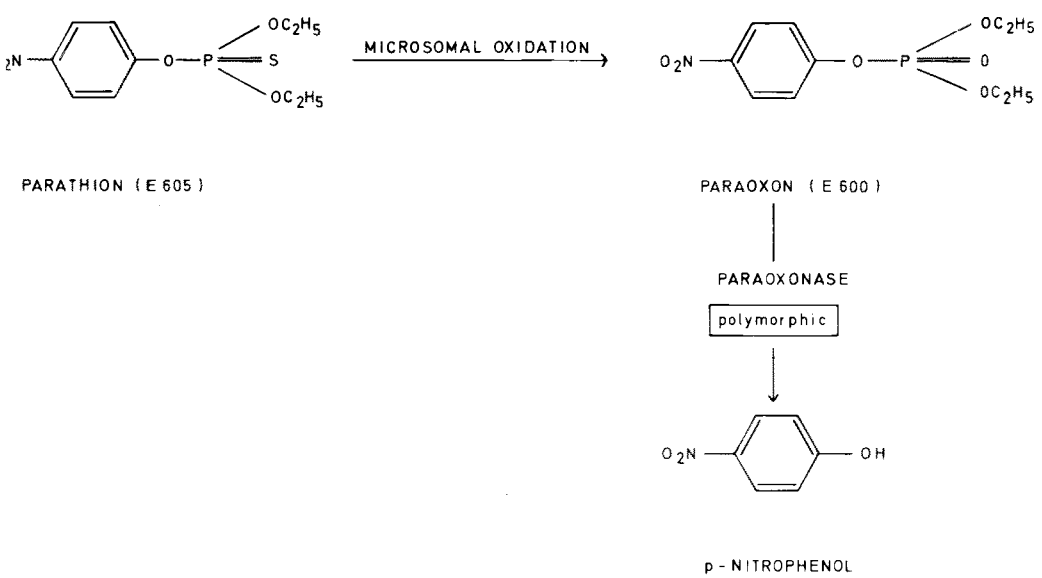

Fig. 11. Metabolism of parathion in humans

6.2 Carcinogenicity of Polycyclic Hydrocarbons

Certain polycyclic hydrocarbons, such as benzo[a]pyrene, 3-methylcholanthrene, dimethylbenzanthracene, and others, are important in the etiology of human bronchial carcinoma. These agents are not carcinogenic by themselves but need conversion to carcinogenic epoxides by aryl hydrocarbon hydroxylase (AHH). Epoxides then bind nonenzymatically to DNA. Polycyclic hydrocarbon carcinogenesis became of particular interest for the geneticist, when *Kellermann* and co-workers claimed that inducibility of the enzyme AHH could be studied in cultured human lymphocytes, and that humans could be separated into three groups of low, intermediate, and high inducibility (*Kellermann* et al., 1973a). The authors assumed a single-locus control of the enzyme's inducibility with two alleles that lead to three genotypes. In an investigation on the frequency distributions of the two alleles in patients with lung cancer, other tumors, and healthy controls, *Kellermann* et al. (1973b) found an essentially higher proportion of subjects with high and intermediate AHH inducibility among patients with bronchial carcinoma. These findings supported the concept that epoxides are the ultimate carcinogenic forms of polycyclic hydrocarbons and that persons with high inducibility of the activating enzyme AHH should bear a greater risk of developing bronchial carcinoma.

However, these results were not confirmed by *Paigen* et al. (1977). The authors were unable to measure lymphocyte AHH inducibility in half the patients with lung cancer, presumbably due to the disease. The progeny of the patients did not differ from controls with respect to AHH inducibility either. Furthermore, *Paigen* and her group did not obtain evidence of a bi- or trimodal distribution of enzyme inducibility, although they also observed a remarkable interindividual variability. Recently, however, *Emery* et al. (1978) confirmed the original finding of *Kellermann* et al. (1973b) of a higher inducibility of AHH in patients with lung cancer, presumably on a polygenic basis. Despite variability of AHH inducibility depending on culture conditions and seasonal variation, a considerable part of the interindividual variability is genetically determined (*Atlas* et al., 1976), heritability being about 0.80. These authors did not obtain evidence for single-locus control of AHH inducibility either; instead, their data were compatible with polygenic control.

The induction of sister chromatid exchanges (SCE) probably also reflects carcinogenic effects. *Rüdiger* et al. (1976) examined SCE in vitro after adding benzo[a] pyrene; the rate of SCE did not correlate with the rate of intracellular benzpyrene metabolism. This lack of correlation suggests that genetic differences in benzpyrene metabolism are not related to genetic differences in the predisposition toward bronchial carcinoma. Indeed, *Schönwald* et al. (1977) found no difference in the rates of SCE in lymphocytes of patients with lung cancer and in those of control persons. Presumably the risk for development of bronchial carcinoma in the

presence of polycyclic hydrocarbons is not only reflected by the rate of epoxide formation in cultured lymphocytes; intracellular epoxide concentration also depends on the degradation rate by epoxide hydratase and glutathione-D-transferase (*Rüdiger* et al., 1976). Much more essential might be, how much epoxide binds to DNA? It is interesting that binding of benzo[a]pyrene to DNA in cultured human bronchi varies considerably between subjects, being slightly higher for patients with lung cancer than for normals (*Harris* et al., 1976). Much more work has to be done on genetically determined differences in susceptibility toward lung cancer.

6.3 Mutagenicity of Isoniazid

Isoniazid is a weak mutagen in the host-mediated assay with *Salmonella typhimurium* as indicator organism (*Röhrborn* et al., 1972; *Grafe* et al., 1975; *Schöneich,* 1976). This test system is capable of detecting point mutations in the bacterial genome and takes the metabolism of the experimental animal into account. Since isoniazid is not mutagenic in the bacterial in vitro test, *Röhrborn* et al. (1972) hypothesized that hydrazine should be the metabolite responsible for mutagenic activity. However, hydrazine, which is a powerful mutagen, has never been identified as a metabolite of isoniazid in man. As reviewed above, acetyl hydrazine is assumed to be an intermediate metabolite in humans. Among other hydrazine derivatives, acetyl hydrazine is able to induce λ-prophage in *E. coli* (see *Kimball,* 1977), thus pointing to its mutagenic potency. Since isoniazid as well as monoacetyl hydrazine is acetylated by the polymorphic enzyme (see above), slow and rapid acetylators should have different susceptibilities to induction of mutations or even cancer. Other mutation mechanisms of hydrazine derivatives may exist in addition, such as, e.g., hydrogen peroxide formation (*Freese* et al., 1968).

7. Future Outlook

Pharmacogenetics is a fascinating interdisciplinary field that probably has uncovered thus far only a small part of the existing phenomena. Clinical pharmacologists especially should pay more attention to reproducible interindividual differences in response to drugs. It must be an important aim of pharmacogenetic research to detect the mode of inheritance of such differences in drug response and accordingly to measure parameters that reflect most directly control by single genes. Detection of specific pharmacogenetic peculiarities or defects is of theoretic as well as of clinical interest. However, pharmacogenetics is not only the field of inborn errors of drug metabolism: It can also help to tell us more about genetic diversity of functions uncovered only by the action of drugs.

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