DETERMINATION OF COLLOIDAL CHARGE DENSITIES OF NERVOUS TISSUES BY AN ELECTROMETRIC METHOD

by

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INTRODUCTION

Controlling Purpose

This study seeks to demonstrate the presence of fixed ionic charges in mammalian nervous tissues, to quantitate them, and to consider their possible functional importance. The primary method chosen for use in this study is applied to the nervous system for the first time. The method and the results will be evaluated in this context, principally by inference from information derived by other means.

Developments in electrophysiology have focused attention on the importance of the ion distributions across the cell membrane in the functions of irritable cells (100). The correlation of function with depolarization is strong in muscle and nerve. Conduction, depolarization and ion migration across cell membranes are thought to be intimately related if not in some cases synonymous (84).

The argument for a functional importance of fixed ionic charges in nervous tissue, then, relates to the search for a mechanism to account for ion distributions in excitable tissues. It is not presumed here to supply the specifications of this mechanism. Evidence will be presented, however, to support the contention that the unique chemical composition and the unique physiology of the mammalian central nervous system are related.

The neurochemical literature contains a wide variety of information about the unique chemicals and chemical groups found in the nervous system. Each development in the study of chemical

architecture has brought more interest and speculation about the relationship of ultrastructure to function (29, 82, 94, 95). Much of modern physiological thinking is concerned with the close association among impulse propagation, nerve cell membrane potential, and partitioning of Na^+ and K^+ across the nerve cell surface.

Much speculation deals with possible mechanisms which may govern distribution of ions in the intimate environment of the nerve cell (16, 50, 63). Of particular interest in this regard have been the findings of metal ions in association with extracts of nervous tissue. Similar interest arises when chemical components of nervous tissue are shown to have selective affinities for certain elemental (107) and organic (38, 93) ions.

Water and Fixed Ionic Charges

Water is considered first because it is the most abundant and perhaps the most important component of nervous tissue. White matter is about 70% water, while gray matter is about 83% water (71), according to gross analytical figures. When the scope of analysis is narrowed, however, the proportions change. Finean (29) has considered the myelin sheath alone using the x-ray diffraction methods. He has given 40 - 50% as the likely range for water content in this prominent structure. The study of physical chemistry of electrolytes has developed largely in terms of an aqueous milieu. As a result considerations of ions and ionic groups at the submicroscopic level of tissue macromolecules may give the presence of water a much more critical importance. It may be necessary to consider advisedly tissue electrolytes as dilute aqueous solutions (10, 75, 76).

Lipids and Fixed Ionic Charges

The second prominent class of compounds in nervous tissue is lipid. Lipids are reported to constitute 17% of fresh white matter and 5% of fresh gray matter (71).

Koch and Pike noted in 1911, as Thudichum (73) had before them, that an abundance of unique lipid was obtainable from mammalian brain. Interest in establishing the physiological importance of cerebral lipids was high at the time in the wake of the Meyer-Overton theory of narcosis. Narcotic and anesthetic actions were theoretically correlated with the lipid solubility of the pharmacologic agent.

Koch's group (62) attempted to demonstrate physical changes in phosphatides when these polar lipids were exposed to organic solvents, particularly to anesthetics, in vitro. They did not demonstrate such changes in water emulsions of lecithin or "kephalin" when these fractions were shaken with anesthetics. They did, however, establish an experimental precedent in attempting to correlate changes in cerebral structural components with physiological events.

The belief that a physiologically important, fixed, colloidal, anionic charge exists in nervous tissue also had an early origin in neurochemistry. It arose with the finding dating back to Thudichum in 1884 that K^+ and Na^+ occur in association with lipid extracts of nervous tissue obtained with neutral solvents. (77). Koch contended in 1911 that the cation affinities of acidic "structural" lipids of brain were responsible for the high potassium content of neurons (62).

Subsequently other workers elaborated upon associations of lipid and alkali metal ions. Christensen and Hastings (14) demonstrated the calcium-binding potency of cephalin. At about this time

Folch reported that cephalin was in fact a mixture of phosphatidyl serine, phosphatidyl ethanolamine, and diphosphoinositide (32). Drinker and Zinsser determined the dissociation constant of "calcium cephalinate" experimentally (19).

Scott (83,77) reported that most of the Ca++ and Mg++ of frog sciatic nerve is localized to the myelin sheath. He based his conclusions on the results of a microincineration technique.

Folch, Lees, and Sloane-Stanley have described a method for determining the relative affinities of various alkali and alkaline earth cations for acidic lipid fractions of brain (34). When lipids were allowed to partition between chloroform-rich and water-rich phases, only sulfatides and phosphatides were found in appreciable amounts in both phases. Addition of NaCl, KCl, $CaCl_2$ to this system resulted in net shifts of appreciable quantities of lipid into the chloroform-rich phase. $MgCl_2$ and $CaCl_2$ demonstrated the greatest ability to cause this redistribution. A concentration as low as 10^{-4} M. $CaCl_2$ decreased lipid content of the water-rich phase by 20%. Presumably an association of cation with lipid in the form of an acidic lipid-salt favors solubility in the chloroform-rich phase.

Generally the sulfatide, cerebroside sulfuric acid ester, is extracted as the potassium salt (6). Phosphatidyl serine and diphosphoinositide are said to occur as neutral salts of Na, K, Mg, and Ca when extracted with organic solvents from fresh brain (34, 107).

The work of Dervichian (17) supports this concept of lipid-cation complex formation. He "titrated" aqueous phosphatidyl serine and phosphatidyl choline preparations separately against salts of Na+, Ca++, and Cu++. pH decreased in a manner resembling an acid-base titration. The most pronounced effects in each case occurred upon

addition of $CuSO_4$ to the lipid preparation. These findings were interpreted as being consistent with the formation of lipid-metal ion complexes. In the course of the "titration" H+ was presumably displaced from the acidic lipids into solution.

Drinker and Zinsser (19) also noted a fall in pH when they added neutral $CaCl_2$ to their cephalin emulsion, but did not attempt to interpret this finding. As key evidence of complex-formation, they noted that after addition of Ca++ to cephalin preparations, Ca++ determination by frog heart bioassay indicated virtual absence of free Ca++.

Christensen and Hastings reported Na+ and K+ binding by a similar cephalin fraction. They calculated that 0.5 eq. cation is taken up for each gram-atom of lipid P in the preparation (14). There is question as to which subfractions of cephalin participate in this phenomenon, (17, 34, 86).

It is probable that Na+ and K+ as well as other ions are to some extent physically compartmentalized in brain. Extraction procedures may encourage associations between cations and organic anions which are normally prevented by the anatomic and ultrastructural integrity of nervous tissue.

The compartmentalization of cation among cells of various kinds, or, indeed, within parts of cells should be considered. There is evidence for example, that certain glial cells contain unusually high concentrations of Na+. If certain glia are high sodium cells (55), while neurons are high potassium cells, and if Ca++ and Mg++ are significantly localized in layers in the myelin sheath (83), the disorganization brought about by extraction procedures may be

an important cause of chemical artefacts.

As an approach to this problem Katzman and Wilson (56) were able to extract 50% of cerebral lipid content from quick-frozen brain slices. Their solvents were chloroform and hexane. In their dry lipid extracts the ratio of cation to P was 2 : 1. For cortex alone Na : K was 2.9 : 1 while for white matter the ratio of Na : K was 1.3 : 1. Cation content correlated well with serine content, in these extracts. This points to phosphatidyl serine as an important complex-former.

The preponderance of Na in these ratios has not been explained. The solubilities of the K and Na salts of phosphatidyl serine were found to be approximately equal in the cold solvent used in this study. In any event, the association of lipid and cation in both gray matter and white matter is again demonstrated.

Study of lipid monolayers has also indicated that there is a general reaction between certain acidic lipids and metal ions. In this method a solution of a certain lipid (2) or of purified lipid extracts (87) is layered on the surface of a liquid in which it will not dissolve. The resulting film is then compressed in a horizontal plane. The lateral compressive force or other parameters are appropriately measured. Force-area, voltage-area, etc. curves can be related under controlled conditions to molecular cross-sectional area and to molecular orientation at the interface.

Ion-lipid interactions have been demonstrated with synthetic lecithin and alkyl phosphates, as well as other compounds (2, 74). Ionizing air electrode study of lecithin monolayers suggested to Anderson and Pethica that the zwitterion structure of this lipid is

destroyed by K+ and Na+, the mechanism presumably being the "binding" of these ions to the phosphate groups of the lipid in a ratio of 1 to 1. Strong effects were found with Mg++, Ca++, Ba++, and Cu++, but in both the lecithin and alkyl phosphate studies the uranyl (UO_2++) and Al+++ ions showed the most powerful complexing tendencies.

Proteins and Fixed Ionic Charges

The third component of brain which must be included in any consideration of fixed ionic charges is protein.

Protein constitutes about 8% of fresh gray or white matter (71, 77). When ionic properties of proteins are considered in relation to metal ions the chelating abilities of certain amino acids assume fundamental importance. As an example, the affinity of glycine for copper is well known (104). Steric and hydrational influences greatly alter such properties when peptide moieties become protein constituents (59). Necessity to control states of denaturation and secondary and tertiary structure make meaningful study of central nervous system protein reactions with ions difficult (63, 100). Solubility characteristics have discouraged investigation by extraction, partition, and monolayer techniques in the manners described above for nervous system lipids.

The possibility that structural rearrangements of cellular proteins is instrumental in processes of impulse conduction and ion redistribution has been considered for many years (101).

Ungar, et al. have measured changes in ionization of brain protein side groups in association with 20 minutes electric stimulation (102). They have employed two techniques. One, ultraviolet absorption, has been supported by the second, amperometric titration.

Results with the two methods indicate a significant increase in ionized sulfhydryl groups of cysteine and perhaps also -OH groups of tyrosine. Because of resemblances to denaturation with urea, these phenomena have been interpreted as indications of structural rearrangement.

These investigators report that these changes are reversible but that proof of rapid reversibility is necessary to strengthen the contention that these findings are importantly related to nerve conduction.

The finding of protein alterations with nervous activity is supported histochemically. Staining changes after prolonged excitation are reportedly "consistent with an increased number of reducing groups." (31).

Metal complexes of proteins are well known and studied (61). In general order of decreasing ability to form tight protein complexes are Mg^{++}, Ca^{++}, Na^{+} and K^{+} (60). The occurrence of protein-lipid combinations in fractions of brain extract has drawn attention to protein in a structural capacity. (77). Proteins of albuminoid, globular, and fibrous types have been isolated from brain (71). When trypsin is allowed to act upon medullated nervous tissue, myelin is found to disaggregate as the protein is hydrolyzed (98). This supports the opinions of the ultramicroscopists whose studies indicate that a protein layer (29) is one of several keystones in the jellyroll structure of the myelin sheath.

Polysaccharides and Fixed Ionic Charges

The fourth chemical component to consider as matrix for fixed colloidal charges is polysaccharides. A portion of the total

accompanies blood vessels and is not truly parenchymal (77). Glycolipids and mucopolysaccharides are found in the brain. Glycoprotein is a prominent component of peripheral nerve (84).

Though brain mucopolysaccharides are reported to exist at concentrations of only .08 to .10% of fresh brain, speculation about function in nerve conduction and participation in the blood-brain barrier has been entertained (9). Since an ion exchange property of ground substance carbohydrate was first proposed (69), considerable supporting evidence has accumulated. A biological ion exchange polymer would be most appropriate in conjunction with cells in which selective ion distribution and cell function appear intimately related (1).

Abood and Abul-Haj (1) isolated a hyaluronic acid-like substance from bovine brain. It was a hyaluronidase digestible, sulfur-free, polysaccharide which proved to have a moderate potassium ion selectivity in vitro. Their evidence that it originated from the neuron is histochemical and requires further documentation. It is of interest that the mucopolysaccharide-like material was found histologically not only in the axon, but also extending into the myelin sheaths.

Metachromasia and Fixed Ionic Charges

Histochemical evidence of fixed anionic charge is furnished by the presence of metachromatic material in nervous tissue. That the myelin sheath is intensely metachromatic, even at pH 2, is of considerable interest (4, 108). The term metachromasia describes a color change occurring in certain dyes when these dyes act upon appropriate substrates, e.g. toluidine blue turning reddish-purple

upon contacting chondroitin sulphate of connective tissue ground substance. The most prominent metachromasia is demonstrated by polysulfated compounds such as the sulfated polyhexoses of seaweeds and of ground substance. The important common factor in metachromatic dye substrates appears to be the presence of strongly anionic, regularly spaced, hydrated groups (5). Toluidine blue has been used as an indicator of high molecular weight compounds with many dissociated, negatively-charged groups (4).

Polyphosphates of bakers yeast (81) are metachromatic. Nucleic acids (8) and proteins require increasingly delicately controlled conditions to demonstrate metachromasia (5).

White matter extracts which give a metachromatic reaction comparable to the histochemical reaction are sulfatides and the cephalin subfraction, diphosphoinositide. Study of sulfatide has been hampered by its insolubility. Diphosphoinositide is readily soluble in both aqueous and organic solvents as noted above. It is strictly confined by a cellophane dialysis bag, though its tentative minimal molecular weight is only 640. This behavior, were it not for the question of charge, might suggest that it is a polymer. Indeed, its tentative formula can readily be construed as such (43). The metachromasia of both sulfatide and diphosphoinositide suggests that both are strong polyanions. The metachromasia of a sulfatide spot on filter paper persists at pH 2. The anionic group apparently dissociates strongly in order to persist in this acid environment. Dervichian (17) suggests that cerebral lipids aggregate in physical association as micelles in aqueous medium rather than as polymers.

Cartilage is the other prominently metachromatic mammalian tissue. In this tissue sulfated mucopolysaccharides are well-established as the dye-substrates which give the metachromatic reaction. Ground substance in other locations behaves similarly, but less conspicuously in its histochemical reactions. Ion exchange properties of cartilage have been independently demonstrated by a number of methods, thereby establishing an association between cation-exchange potentiality and metachromasia. Both phenomena are known to require the presence of a relatively high concentration of essentially fixed anionic groups (5, 11).

A brief outline of some of the evidence for ion exchange properties of cartilage will be given because of its relevance to the thesis. The connective tissue studies presented here have had two primary interests, both related to clarification of biological ion-exchange. They are: (1) the mechanism of calcification and (2) the mechanism of control of extracellular electrolyte.

Boyd and Neuman (7) used a dried, ground, decalcified cartilage preparation. After treatment with solutions of $CaCl_2$, $BaCl_2$, or NaCl the gram-equivalent ratio of cation to sulfate was consistently 1 : 1. Extraction of chondroitin sulfate reduced both cation uptake and sulfate content but the 1 : 1 ratio of cation to sulfate persisted. Ba^{++} was shown to exchange quantitatively with "bound" Ca^{++}.

Dunstone used minced, dried, decalcified cartilage. Exposure of the cartilage to various salt solutions gave cation: sulfate ratios of 1 : 1 in the cases of Na^{+}, K^{+}, Mg^{++}, Ca^{++}, Sr^{++}, and Ba^{++}; 1.4 : 1 with Cu^{++}; 2 : 1 with Be^{++}; and 0.8 : 1 with NH_4^{+}. Ca^{++} uptake and

sulfate content were again closely parallel.

Dunstone observed phenomena which he attributed to "irreversible binding" of a certain minor fraction of total cation. In order of decreasing net affinity for cartilage he reported Cu^{++}, Be^{++}, Ba^{++}, Sr^{++}, Ca^{++}, Mg^{++}, and K^{+} (22).

Farber (27) dialyzed solutions of Na, K, and Ca chondroitin sulfates against NaCl, KCl and $CaCl_2$. Analyses showed that more cation entered the chondroitin sulfate side (inside) of the semi-permeable membrane than could be ascribed to a Donnan distribution. This was interpreted as a lowering of activity coefficient of the inside cation by chemical union between cation and fixed anion of the chondroitin sulfate. Nonpolar methyl chondroitin did not have these effects.

Joseph, Engel, et al. used an electrometric method in studying cartilage and other connective tissues (51), epidermis (52) and muscle (25) in the living animal. Using Donnan considerations and the Henderson equation for liquid junction potentials they determined the density of fixed ionic charges. They found highest values of negative charge density in cartilage. Their figure is .16 eq./kg.

Using the electrometric method, Joseph and Engel confronted living connective tissues with .02 N. concentrations of metal ions. There were modifications in electromotive force values which they interpreted as decreases in colloidal anionic charge densities in the tissue surface. Free energies of formation of ion-colloid (tissue) complexes were calculated showing connective tissue affinities for cations in the following descending order (13): Pb^{++}, Sr^{++}, Mg^{++}, K^{+}, Ca^{++}, and Na^{+}.

As noted above, certain long-chain inorganic polyphosphates are metachromatic. Several investigators have submitted evidence that metal salts of these polyanions do not completely dissociate. Dissociation constants have been calculated on this basis. These constants indicate the extent to which cation binding occurs with polyphosphate and that the affinity for Na^+ exceeds that for K^+. Both Ca^{++} and Mg^{++} are reported to bind more tightly than K^+ and Na^+ with polyphosphate, as would be expected from experience with other polymers (91).

In summary to this point:

I. Biological associations of metachromasia and ion-exchange properties.

   A. Properties of white matter.

      1. Metachromatic material is found prominently in the myelin sheath.

      2. A number of acidic lipids form cation complexes which dissociate to a limited extent.

      3. Two metachromatic myelin lipids have been found — cerebroside sulfuric acid ester and diphosphoinositide.

   B. Properties of cartilage.

      1. Cartilage is also strongly metachromatic.

      2. Cartilage forms metal ion complexes.

      3. The metachromatic polyanion, chondroitin sulfate, plays a prominent role in both the metachromasia and in the ion-binding characteristics.

   C. Inorganic polyphosphate displays metachromasia and ion-binding characteristics.

II. Ion activity gradients are related to function of nerve cells.

III. The proximity of metachromatic material to the cation gradients and fluxes at the axon membrane suggests that fixed anionic groups participate in the ionic events which occur there.

Myelin and Function

In support of this possibility is the association of development of function with myelination in the mammalian nervous system. For example Ulett, Dow, and Larsell (99) found in infant rabbits that observable myelination regularly followed the onset of conduction in certain brain pathways. It is likely that functional myelination preceded the observed myelination. Once observable myelination was present, the incidence of response to stimulus increased and latency of response decreased appreciably. In spite of strongly suggestive morphological and physiological findings, attempts to relate chemical content of brain to maturation and finally to function have not been particularly fruitful.

In certain disease states such as multiple sclerosis, the initial involvement appears to affect only myelin. The axon cylinder appears to be completely spared while the surrounding sheath is destroyed by disease. With the damage to the myelin sheath, function is compromised (39).

Species and tissue comparisons show, however, that many irritable cells are not medullated, e.g., muscle fibers, squid axons, and eel electroplaques (40). The presence of a lipoprotein encasement about the irritable cell has been considered by many to be a relative phenomenon—that all cell surfaces are composed minimally of a bimolecular leaflet. This leaflet is said to be composed in large part of lipid and to have special properties in the irritable cell (24,28). The jellyroll structure of myelin (35) presents an opportunity to study an extensive natural collection of membrane material (82) in an organ characterized by physiological excitability.

Cations in Nervous Tissue

Determinations of the physicochemical states of ions in nervous tissues have been made — most in axons of the cephalopods. Hodgkin (49) found that K+ mobility approximates that of the ion in aqueous solution. However, the phenomena Hodgkin observed have also been interpreted as a manifestation of electroendosmosis (94). The latter interpretation would imply the presence of a "fixed" negative charge in axoplasm. Ca++ has been reported to cause both liquefaction and gelation of axoplasm (94). Solomon and Tobais studied migration velocities of K+, Na+, and Ca++ with direct current in dog sciatic nerve (90). The finding that some of the Ca++ moved anodally suggested that this element existed in a complex of net negative charge.

The diffusion constants of sucrose, Na+, and K+ were determined in brain slices by McLennon (65). The only remarkable deviation from the corresponding aqueous values was retardation of K+ diffusion — a pattern that was shared by muscle, but not by liver. The relatively slow appearance of radioactive K+ in brain after intravenous administration has also been noted (85).

The overall physical state of brain solute-solvent relationships has been studied by determination of freezing and melting points (55). Results coincided directly with those of plasma, indicating that brain tissue — which is most, if not entirely cellular — is essentially isosmotic with extracellular fluid.

Analyses of whole brain have given the following values collected by McIlwain (71). Figures are in mEq./kg.

TABLE 2.

cations		anions	
Na	57	Cl	37
K	96	HCO_3	12
Ca	2	phosphate	16
other	12	other	22
	166		87

difference: 80 mEq./kg. tissue

The figures imply a lack of electrical neutrality which has been called the "anion deficit" (32). This "deficit" is usually attributed to a neutralization of mobile cations by fixed anionic groups of organic molecules such as:

1. carboxyls of proteins and hyaluronic acid
2. phosphoric acid esters of phospholipids
3. sulfuric acid esters of sulfolipids
4. sulfhydryls of cysteine residues of proteins
5. phenolic hydroxyls of tyrosine residues of proteins

Using data from elemental analyses, Katzman calculates that brain cation osmolarity exceeds plasma cation osmolarity by 20-30 milliosmols in the face of the freezing- and melting-point strides which indicate isosmolarity. This, too, is thought to be a manifestation of "anion deficit."

Among the alternative, but not necessarily mutually exclusive hypotheses put forth in explanation of the "anion deficit" and related phenomena are: (1) fixed anionic sites form covalent or semi-ionic bonds with cations causing a decrease in cationic activity

in solution (55); (2) macromolecules with many anionic sites contribute substantially to electrical neutrality but little to osmolarity (55); (3) the spatial and electrostatic organization of solvent and solutes within the tissue alters the hydration atmosphere so that concepts of activity in dilute aqueous solution no longer pertain (27, 76).

In summary there is a considerable weight of direct and indirect evidence indicating the presence of fixed, nondiffusible organic anionic groups in nervous tissue. Data of circumstantial nature implies a functional significance to these groups. A direct evaluation of the fixed ionic charge density of intact living nervous tissues is in order. Such an evaluation would attempt to establish the presence of such ionic groups and then to quantitate them. The ultimate goal is to relate submolecular structure to biological function.

MATERIALS AND METHODS

General Methods

the electrometric method

The electrometric method used in the present study was taken directly from the one developed and described by Joseph, Engel, et al. in their studies of connective tissues, skin, and muscle (25,31,32). With this method tissue ionic charge densities *in vivo* can be calculated. Knowledge of charge densities has permitted comparisons between different tissues. Of even greater interest is that the method permits measurements under a number of experimental conditions affecting a given tissue. Such comparisons have led to important inferences about the ultrastructural state of connective tissues and the physiological import of tissue colloids (12,23,36).

No single previous approach to determination of fixed ionic charge in the nervous system has been fully satisfactory (95). None has dealt with intact tissue.

The electrometric method described here is simple in practice. It was employed to give another perspective to study of ionic macromolecules of living nervous tissue.

The electrometric method depends upon the concentrations of Na^+ and Cl^- in the tissue as they are influenced by fixed ionic charges. This influence is described by the Donnan equilibrium

distribution. The potentials measured by the method depend upon these concentrations and upon the fact that Cl- diffuses 50% more rapidly than Na+ through a dilute aqueous concentration gradient.

All that is required is measurement of two electrochemical potentials across the tissue surface. The first potential (E_1) is obtained with 0.15 N. NaCl at the tissue surface. This establishes a reference point.

The second potential (E_2) arises when the 0.15 N. NaCl is replaced with a one-to-ten dilution (0.015 N. NaCl). This provides a concentration gradient.

The difference, E_d, between the two potentials may then be used in a basic working equation for the calculation of tissue charge density, g. Because fixed negative charge density is a calculated value, it will be refferred to for convenience in this report as g.

The nucleus of the experimental arrangement is an electrolytic concentration cell constructed at the tissue surface. Electromotive force measurements are made potentiometrically in a circuit which includes alternately two modifications of the concentration cell. The difference, E_d, between potentials obtained with the two modifications is called the dilution potential and is given in millivolts.

The dilution potential, E_d, is related mathematically to the density of fixed colloidal ionic charge in equivalents

per kilogram tissue water (g). The relationship depends upon theoretical considerations developed by Henderson (44,45,79), Donnan (72), and Nernst (54,47), and was derived by Joseph (54).

electrochemical cells and circuit

The experimental preparation (Figure 1) requires an electric potential measuring device, calomel electrodes, and an animal preparation with a suitable exposed tissue surface. The central concentration cell consists of two half cells.

(i.) Recording half cell: dilute NaCl solution in contact with the external tissue surface and with the recording calomel electrode.

(ii.) Reference half cell: tissue electrolytes of the animal preparation in liquid continuity with the reference calomel electrode.

Thus the tissue surface forms the liquid boundary between these two concentration half cells. The complete cell is a composite. It may be written (Eq. 1, next page):

(Eq. 1.)

<u>Recording half</u> ; <u>Reference half</u>

Hg;	Hg_2Cl_2;	KCl;	NaCl	; tissue;	NaCl;	KCl;	Hg_2Cl_2;	Hg
	(s)	(sat'd)	(.15 N. or .015 N.)		(.15 N.)	(sat'd)	(s)	

Figures 1 and 2 show a representative preparation for determination of colloidal charge density of cerebral white matter of the cat. Saturated KCl solution of electrode arms extends through tubing to the tissue surface. At the tissue surface a cotton pellet or a cylindrical reservoir holds the saline solution. The dilute NaCl solution of the recording half cell is interposed between the KCl and the tissue surface by one of these two methods.

More specifically, the measurements of colloidal charge densities, $\bar{x}$, are derived from potential differences, E_d's. E_d's are obtained by constructing two modifications of the external (recording) half cell in these ways:

(i.) Apply 0.15 N. NaCl; determine EMF (E_1).

(ii.) Remove the first solution.

(iii.) Apply 0.015 N. NaCl to the tissue surface; again determine EMF (E_2).

The difference between the two EMF determinations provides the dilution potential, i.e., $E_1 - E_2 = E_d$. The dilution potential, E_d, is substituted into a basic working equation (Eq. 9) derived by Joseph (54) to obtain the colloidal charge density of the tissue in equiv./kg. tissue water, $\bar{x}$.

Four liquid junctions occur in the complete cell as written above (Eq. 1). Two are found between a dilute solution of NaCl and saturated KCl. The potentials at these junctions have been considered to be inherently small, opposite in sign, and approximately equal in magnitude.

A third liquid junction occurs between the tissue and 0.15 N. NaCl on the reference side of the cell. The potential developed

here is unknown, but since this portion of the circuit is not altered at any time, it is assumed to be constant.

The fourth liquid junction constitutes the recording site at the tissue surface. At this point the two concentrations of NaCl (.15 N. and .015 N.) are applied in succession. The difference, E_d, between electromotive forces of these two modifications of the concentration cell is then considered in the following manner (51):

i. with 0.15 N. NaCl at the tissue surface,

$$E_1 = \Sigma E + E_{j(.15)}$$

ii. with 0.015 N. NaCl at the tissue surface,

$$E_2 = \Sigma E + E_{j(.015)}$$

iii. the calculation of E_d is

(Eq. 2) $$E_d = E_2 - E_1 = E_{j(.015)} - E_{j(.15)}$$

where E_d is the dilution potential as described above.

E_1 is the total potential difference between calomel electrodes when .15 N. NaCl is applied to the tissue surface.

E_2 is the total potential difference between calomel electrodes when .015 N. NaCl is applied to the tissue surface.

ΣE is the sum of all potential differences in the circuit excluding the liquid junction potential at the tissue surface. This sum is considered constant for reasons given above.

$E_{j(.15)}$ and $E_{j(.015)}$ are the liquid junction potentials at the

tissue surface when each of the two concentrations of dilute NaCl solution is applied.

The tissue surface is first exposed to .15 N. NaCl and is considered to be in thermodynamic equilibrium with this solution. The corollary assumptions are that

$$E_{j(.15)} = 0 \quad \text{and} \quad E_{j(.015)} = E_d.$$

The equation of a NaCl concentration with transference is

(Eq. 3) $$E = 2\, t_+ \, RT/F \ln m_1g_1/m_2g_2. \quad (67)$$

where the electrodes are reversible to chloride. In this equation m_1g_1 and m_2g_2 are the molalities and activity coefficients of the electrolyte on the two sides of the liquid junction. t_+ is the cation transport number, i.e., the fraction of current carried by Na^+. E is the EMF in volts. R, T, and F are the gas constant, absolute temperature, and the Faraday electrochemical equivalent, respectively.

Since $t_+ + t_- = 1$, where t_- is the anion transport number, substitution into Eq. 3 gives

$$E = (t_+ - t_- + 1)\, RT/F \ln m_1g_1/m_2g_2 \text{ or}$$

(Eq. 4) $$E = \underset{\text{(i.)}}{RT/F \ln m_1g_1/m_2g_2} + \underset{\text{(ii.)}}{(t_+ - t_-)\, RT/F \ln m_1g_1/m_2g_2}.$$

Nernst's original interpretation of Eq. 4 (47) considers the two right hand terms as representing (i.) difference between electrode potentials, and (ii.) liquid junction potential, respectively. Inserting numerical values into the latter term (ii.) and equating

it to the dilution potential, E_d in millivolts gives

(Eq. 5) $E_d = (t_+ - t_-)\ 61.7 \log m_1g_1/m_2g_2$ at 37° C.

derivation of the basic working equation for g

Joseph (52) has taken the value of m_1g_1 as 0.15 for tissue surface in equilibrium with 0.15 N. NaCl. When 0.015 N. NaCl is then applied to the tissue surface

$$E_d = (t_+ - t_-)\ 61.7 \log 0.15/0.015 \text{ and}$$

(Eq. 6) $$E_d = 61.7\ (t_+ - t_-).$$

In Eq. 6 the concentration of 0.015 N. NaCl is substituted numerically for m_2g_2.

E_d is then shown as related only to the transport numbers of Na^+ and Cl^- at the tissue surface. Transport numbers are related in turn to colloidal charge density of the tissue in the following way.

i. assume independent ionic conductances

ii. take relative aqueous mobilities as: $Na^+ = 1.0$; $Cl^- = 1.52$. (42, 51).

iii. define transport numbers of the two ions as

(Eq's. 7) $$t_+ = 1.0\ (Na^+)/[1.0\ (Na^+) + 1.52\ (Cl^-)] \text{ and}$$

$$t_- = 1.52\ (Cl^-)/[1.0\ (Na^+) + 1.52\ (Cl^-)],$$

where (Na^+) and (Cl^-) are tissue surface concentrations of the two ions.

According to Donnan the presence of fixed ionic charges in a two-phase system affects the equilibrium distribution of ions be-

tween phases. A fixed anionic charge density in a colloidal (inside) phase increases the local concentration of cation while decreasing anion concentration. Changes in concentrations are considered relative to their equilibrium concentrations in the noncolloidal (outside) phase. When x represents a small concentration of non-diffusible anion, the following approximations of diffusible, colloid-phase ion concentrations may be made (72).

(Eq's. 8)
$$(Na^+) = .15 + x/2$$
$$(Cl^-) = .15 - x/2$$

These approximations are given in their specific application to a phase in equilibrium with .15 N. NaCl.

Eq's. 8 may be combined with Eq's. 7 to obtain expressions for transport numbers (t_+ and T_-) in terms of x. Transport number is related to E_d in turn by Eq. 6. In summary Eq's. 8, Eq's. 7, and Eq. 6 are combined to express a direct relationship between liquid boundary potential (E_d) and colloidal charge density (x).

Joseph (51) has derived a relationship between E_d and x beginning with the ideal term from Henderson's theory of mixture boundary potentials. All of the conditions and expressions cited here are employed. He arrives at a <u>basic working equation</u> (Eq. 9) for the electrometric equation

(Eq. 9)
$$E_d = E_d^o + 206\ x.$$

<u>Implications of the working equation</u>

In this expression E_d^o is the value of E_d at zero colloidal charge density. This is considered as constant under controlled

conditions, most importantly, pH*. It is calculable with Eq. 6 and Eq's. 7. The proportionality factor, 206, depends on standard mobilities of Na+ and Cl⁻, and assumes that they are independent of X. According to Joseph, Engel, and Catchpole (54), the derived relationship between dilution potential and colloidal charge density (Eq. 9) "should be regarded as a first approximation, leading to tentative values of X." (54).

In summary a relationship has been derived by Joseph which relates dilution potentials obtained at tissue surfaces to the corresponding colloidal charge densities. Determinations are made potentiometrically giving values in millivolts. Charge density is calculated by means of the derived basic working equation (Eq. 9) giving results in equivalents of anionic charge/kg. tissue water.

Conditions and assumptions of the method include:

conditions i. Electrodes should be reversible to Cl⁻.

ii. Conductance pathway should be established through the tissue surface.

iii. Tissue surface should be exposed initially to 0.15 N. NaCl.

iv. 0.15 N. NaCl should be replaced by 0.015 N. NaCl for determination of E_d.

assumptions i. Irreversible processes occurring at the three peripheral liquid junctions should not interfere signi-

---

* The value -18.9 mV. is not valid below pH 3.7 because of the high conductance of H+. Joseph obtained other figures for lower pH's by including H+ mobility in concentrations in the calculation of t_+ (Eq. 7). His values are -10.7 mV at pH 2.9 and -4.4 mV at pH 2.2 (51). These values were used in the present study when X was calculated at these pH levels.

ficantly with determination of liquid junction potentials at the tissue surface (47, 72, 79).

ii. Tissue surface should be in thermodynamic equilibrium with 0.15 N. NaCl (53).

iii. Tissue surface should provide a continuous mixture boundary, satisfying conditions for application of the Henderson equation (44, 79).

iv. The approximations describing ion distributions under Donnan membran conditions should be valid at the tissue surface.

v. Ionic mobilities in the tissue surface should be adequately approximated by their values at infinite aqueous dilution (54).

vi. Tissue colloid should be immobile.

vii. Other considerations of free energy change, e.g., water, should not interfere (79).

Materials

Subjects:

The cat provided the principal experimental subject in the present investigation. Both sexes and all ages past the weaning stage were used. Most were adult females. Electromotive force determinations required surgical exposure of cerebral cortex, cerebral white matter, spinal cord, dermis and costal cartilage. Colloidal gel preparations containing agar, carrageenin, and/or gelatin were used for studies in vitro.

Equipment:

Electromotive force measuring apparatus was either

Figure 1.

Diagrams of material used in electrometric determinations of charge densities of CNS of the cat.

a. Frontal section of brain showing exposed cortex of hemisphere on the left and exposed white matter on the right. Saline-moistened cotton pellets are shown attached to the eyedropper-tips of the electrode arm. The cotton is lightly touching the tissue surface to form the recording liquid junction.

b. The electrochemical circuit used in the determinations. A cylindrical brass reservoir is shown implanted in the centrum semiovale. The eyedropper-tips of the recording electrode (on the right) contact the tissue surface by dipping into saline held in the reservoir. The second method of forming this recording junction is shown in (a.). The cat's tail contacts the reference calomel electrode through a beaker of saline.

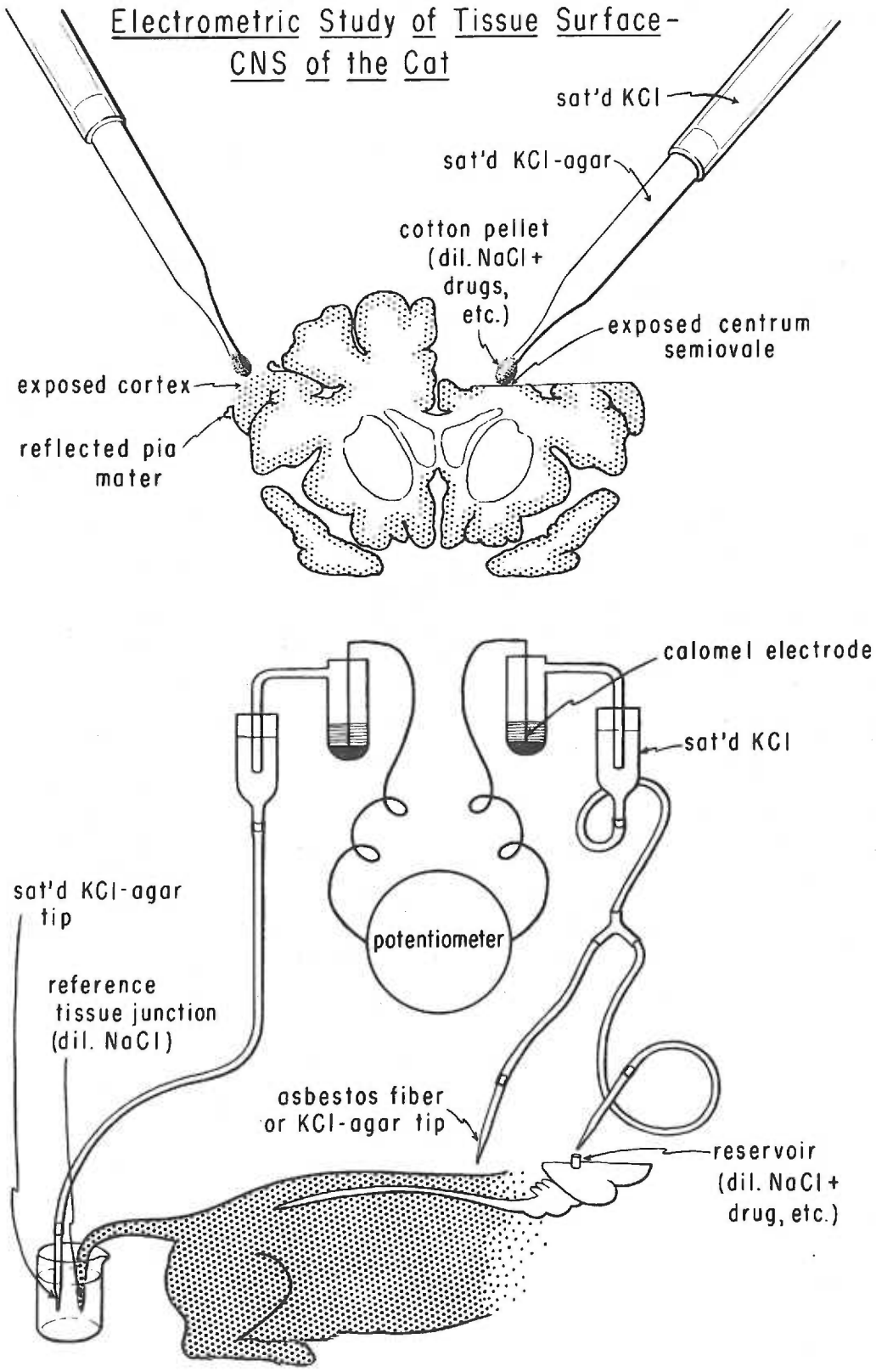
Electrometric Study of Tissue Surface-
CNS of the Cat
sat'd KCl
sat'd KCl-agar
cotton pellet
(dil. NaCl +
drugs,
etc.)
exposed centrum
semiovale
exposed cortex
reflected pia
mater
calomel electrode
sat'd KCl
sat'd KCl-agar
tip
potentiometer
reference
tissue junction
(dil. NaCl)
asbestos fiber
or KCl-agar tip
reservoir
(dil. NaCl +
drug, etc.)

Figure 2.

A photograph of electrometric determinations in progress. The decerebrate cat shows the exposed spinal cord. The potentiometer and galvanometer are seen on the left. The calomel electrodes are in the upper center.


RAYOVAC
RAYOVAC

i. Beckman Zeromatic pH meter with millivolt metering circuit modified to increase sensitivity by a factor of 10, or

ii. Leeds-Northrup K-1 potentiometer with Rubicon galvanometer. The potentiometer was balanced and read by an assistant - a process requiring 5-10 seconds for each reading.

Electrochemical potentials derived in part from the tissue surface were transmitted in the following sequence from tissue to measuring device. See Figures 1 and 2.

i. dilute NaCl solution at the tissue surface. This is held in a beaker at the reference surface (cat's tail), and in (a) brass reservoir or (b) cotton pellet at the recording surface (brain or cord).

ii. saturated KCl contained in

a. fine glass pipet-probes tipped either with a saturated KCl-5% agar plug or a fine asbestos fiber.

b. flexible tubing continuous with the sidearms of the calomel electrodes.

iii. saturated KCl (20° C.) calomel cells.

iv. insulated wire to the potential-measuring device.

Solutions:

Solutions used at tissue and gel surfaces were prepared with distilled water and had the following specifications:

i. titration buffers at graded pH intervals from pH 2 to pH 9. Each is prepared to consist of .1425 to .150 equiv. Na^+/L. and .135 to .150 equiv. Cl^-/L. One or two of these compounds were added in amounts appropriate to produce the desired pH in a solution of ionic strength .15: HCl, acetic acid,

sodium acetate, NaH_3PO_4, Na_2HPO_4, $NaHCO_3$, and Na_2CO_3. Methods of preparation of each buffer are tabulated in appendix 3 (51, 53).

ii. .015 N. NaCl and .15 N. NaCl.

iii. drug solutions in relevant concentrations.

a. saturated solutions of diphenylhydantoin sodium* in .15 N. NaCl and in all of the pH graded saline buffers (appendix 3). Solubility of this drug is reported to be only 14 µg./ml. (.00005 M.) at pH 7 and below (18). In all solutions used concentrations of less than .0004 M. were assured in preparation. When diphenylhydantoin (Dilantin) is used in the treatment of epilepsy its expected brain concentrations are reported to be .00004 M. to .0001 M. (96).

b. cocaine HCl .00015 M. in each of 7 pH graded saline buffers. Lethal dose in man is 1.2 gm. (89), which would give .00007 M. if distributed in body water.

c. ouabain .0001 M. in each of 7 pH graded saline buffers. This is the maximal concentration used by Yoshida et al. (110) to effect biochemical changes in brain slices, including K^+ depletion and Na^+ retention. Solutions were protected from light.

d. ethylene (dinitrilo) tetracetic acid, disodium salt (EDTA·2Na) .02 M. solution in a saline buffer, pH 4.6 (appendix 3).

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* Dilantin sodium.

Specific Methods

preparation of the colloidal surface

Preparation of cats, tissues, and gels is most succinctly presented in outline form.

I. Preparation of the cat for the experimental procedure requires 1 - 2 hours.

A. A 2 to 4 kg. cat is anesthetized by one of two general procedures.

1. Pentobarbital sodium* 30 mg./kg. is given intrahepatically-intraperitoneally or intravenously as needed throughout the procedure.

2. The cat is etherized. Gallamine triethiodide**, 1 mg./kg. is then given intravenously as needed to immobilize the animal while anesthesia is continued by one of two other means.

a. Gasserian ganglion is blocked bilaterally with 0.5% tetracaine HCl*** - 1:100,00 epinephrine for study of the cerebrum (111).

b. Decerebration is effected in the tentorial plane for study of the spinal cord.

B. The cat is secured in a Czermak head holder.

C. The following surgical approaches are made as indicated by the anesthetic and tissue procedures. Incisions are sewn closed following cannulation, decerebration, etc. Tetracaine-soaked sponges are left in closed wounds when local anesthetic procedure is used.

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* veterinary Nembutal

** Flaxedil

*** Pontocaine

1. A tracheal cannula is always inserted and secured. Carotid arteries are identified with loops of thread, or ligated if the animal is to be decerebrated.
2. A femoral cannula is inserted and secured for injection of drugs.
3. An area of dermis is exposed at tip of cat's tail to provide a low-resistance path to the reference calomel electrode.
4. The cerebrum is exposed by a midline dorsal skin incision and division of muscles at their origins. Bone of the skull is removed with a rongeur and the dura mater is reflected. Bone wax stops diploic bleeding.
5. Spinal cord is exposed through a midline dorsal skin incision. Muscles are divided at their origins on the dorsal spines and dissected laterally from the laminae and lateral processes with a scalpel handel. Complete lumbar laminectomy is accomplished with a rongeur. Spinal dura is reflected.

D. Preparation of surfaces for determination of E_d depends upon the tissue concerned.

1. Cerebral cortex is exposed by stripping the pia mater from a broad gyrus using a fine, toothless forcep. A minute incision of the pia mater provides a grip. Vessels must be avoided. Folded pia mater bleeds little. Gross bleeding usually stops with gentle cotton packing. Oozing usually persists and is commonly

troublesome.

2. Cerebral white matter is exposed by excising all or part of the upper 8 - 12 mm. of cerebral substance along a plane intersecting the frontal and occipital poles. Bleeding is often considerable but is stopped with gelatin sponge and gauze packs.

3. Spinal cord white matter of the dorsal funiculus of the spinal cord is readily exposed by stripping back the pia mater. This is facilitated by the relative toughness of spinal pia mater. Surface drying rather than bleeding or oozing is more of a problem with this tissue.

II. Gels were prepared for determinations of E_d and calculations of χ from colloidal surfaces in vitro. Gelatin,* a soluble derivative of collagen, agar**, and carrageenin*** - soluble derivatives of certain seaweeds, were used as powders. The seaweed derivatives are noted for sulfuric acid ester groups, especially carrageenin. The powders were weighed, added to a salt solution, and heated near boiling until clear. The gels are cast in polyethylene tubes of 3 mm. inside diameter and 10 cm. length. This is accomplished by immersing the tubes in molten gel and hardening by cooling. The filled tubes are then removed from the gelatinized mass. Gel surfaces are prepared for electrometric study by cutting through a tube obliquely with a clean blade.

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* Baker, U.S.P.

** Difco Bacto-Agar

*** Seakem Moss 14

titration curves

Titration curves are obtained by applying the series of pH graded saline buffers. (Appendix 3). Corresponding values of $\bar{x}$ are calculated against pH. The points are joined to give a titration curve of a colloidal surface.

junction methods; determination of $\bar{E}_d$

Two slightly differing procedures were used in obtaining values of E_d from tissue surfaces. (a) reservoir method and (b) cotton pellet method. Both give a mean value for dilution potential, $\bar{E}_d$.

(a) In the first series' of determinations of cerebral white matter a cylindrical reservoir was plunged part way into the exposed surface of centrum semiovale. The lumen of the cylinder formed a water-tight cup with the cut white matter surface as its floor. A 4 x 8 mm. brass cylinder was used. It had a 0.5 mm. wall and a sharply bevelled cutting edge. Its surface was made satisfactorily hydrophobic by vaseline.

Solutions are introduced into the reservoirs with needle and syringe, and removed by aspiration through a blunt #27 needle.

One of the solutions approximating 0.15 N. NaCl is placed in the reservoir. A reading of E_1 to the nearest 0.1 mV is quickly (about 30 seconds) taken. The solution is removed. The 0.015 N. NaCl solution is introduced and E_2 quickly determined and recorded. The dilute solution is aspirated and replaced by 0.15 N. NaCl. E_1 is then redetermined. If the two values of E_1 differ by less than 1.0 mV they are averaged. Otherwise the trio of readings is disregarded and the process repeated until three successive values of E_d lie within a range of 1.0 mV.

These rejection limits are arbitrary, but are considered justified because they indicate a change in baseline potential. Baseline potential, E_1, depends upon a number of unknown factors ($\mathcal{E}$) in the electrochemical circuit. Rejects in this procedure amount to 10 to 20% of all readings.

The three successive accepted values of E_d are averaged, giving the mean dilution potential, E_d, for use in further calculations.

(b) In some of the determinations on cerebral white matter and all of the work with other tissues saline solutions were applied to tissue surfaces in moistened cotton pellets. A number 1, 2, or 3 pellet of dry dental cotton is affixed to the fine glass pipet-tip of the calomel electrode arm. The pellet is immersed momentarily in the desired solution, blotted with a clean gauze sponge and applied gently to the tissue surface. The EMF is then determined.

One of the solutions approximating 0.15 N. NaCl is applied until E_1 is regularly reproducible. This usually takes 1 to 5 minutes. Generally cerebral cortex required the longer interval to "equilibrate". Myelinated tissues were more rapid. Cotton pellets containing the approximately 0.15 N. NaCl solutions and pellets containing 0.015 N. NaCl are then applied alternately. Smaller pellets are used with the 0.015 N. NaCl to assure that all of the recording tissue site has been exposed to the 0.15 N. NaCl.

Alternate applications of the two solutions give a pair of readings of E_1 and E_2 in less than 2 minutes. Successive values of E_1 are averaged if they agree within 1 mV. The average to two E_1 values is subtracted from the intervening E_2 to give the dilution

potential, E_d. Alternate readings continue until three or more successive values of E_d agree within a range of 1 to 2 mV. The mean of successive E_d's provides $\bar{E}_d$ for use in further calculations. Such a procedure is recognizably vulnerable to the entrance of subjective factors regarding the quality and validity of successive determinations of E_d. Therefore, means are used to control this factor, i.e., the coding of solutions and study of their comparative effects on E_d. In this manner specific identities of the contents (drugs, etc.) of the solutions are concealed from the experimenter until after the determinations have been made. This device will be discussed further in connection with individual results.

Determinations with gels *in vitro* is accomplished using moistened cotton pellets to apply recording solutions. A reference surface is established by immersing the gel-filled tube and the arm of the reference electrode in a common vessel of .15 N. NaCl. The gel-filled tube is secured and solutions applied. Only one reading of E_1 is necessary. Experience showed that a second reading contributes no additional information. E_1 rarely changes. Dispersion of results is low because surfaces cannot ooze blood and do not tend to dry rapidly. Standard deviation estimates (s) are regularly 0.3 to 0.6 mV. Compression of the surface is a hazard. Compression is observed to alter E_d and lead to higher calculated values of χ.

Use of cotton pellets at the tissue surface was adopted after the methods of Joseph, Engel, et al. Reasons were fourfold: (i.) to save time, (ii.) to increase accuracy and precision, (iii)

to reduce trauma to the tissue surface, (iv.) to eliminate any possibility of electrode effects with metal surfaces (reservoirs). The trauma associated with the use of cylindrical reservoirs was believed to be due in large part to the use and misdirection of needles at the tissue surface.

precision of E_d determinations

E_d values were noted to have changed with the adoption of use of cotton pellets. See Results section. Precision was not appreciably affected. Standard deviation estimates (s) remained in the range .6 to 1.6 for tissues. Tissue trauma was lessened. Working time for determination of a single $\bar{E}_d$ decreased from 5 - 15 minutes to 2 - 10 minutes.

statistical procedures

Four statistical procedures were used in evaluation of results. Examples of parts of the calculations appear in the tables and appendices.

In the use of these tests the null hypothesis was rejected (the difference was considered significant) in any instance in which the test being used indicated that the experimental results were unlikely to occur by chance alone. A probability of occurrence of not more than 5 times in 100 such experiments was considered unlikely or "significant." The result of a test will be summarized in the Results section of this paper by the word, "significant," followed by a symbol with a subscript. The symbols will be either t, F, R, or P, and the subscript will usually be .05. The symbols will indicate the statistical test employed (see below). The subscript will indicate the cutoff probability level used in

deciding whether or not a result is unlikely by chance.

The four tests are described briefly here, and their symbols are given.

I. t — The t distribution is used in the t test (Table 14). The t test (20) compares two means and is designed for samples of 25 or less.

II. F — The F distribution (20, 80) is used in the analysis of variance (Table 12). The analysis of variance compares means of more than two samples. It may be used to test several aspects of categorized data. It becomes most powerful when data is highly categorized and all possible variables are considered in the test.

III. R — The distribution of R is an essential part of a test devised by Duncan (21) to obtain more information from analyses of variance. This test will be referred to for convenience as the "Duncan analysis." It compares individual means in an analysis of variance one with another.

IV. P — The distribution for the sign test (20) is designated here by P. The sign test is best described by an example. Suppose that an experiment yields results that can be paired, A_1 with B_1, A_2 with B_2, etc. If the A member exceeds the B member in a large proportion of the experimental pairs, it is likely that A exceeds B. The sign test simply considers the probability of occurrence of this proportion.

In summary a result may be described as "significant (t .05)." This will indicate that the t test shows a probability of occurrence by chance alone of less than 5 times in 100.

RESULTS

Evaluation of solutions and instruments

Simple concentration cells constructed with filter paper provide tentative evaluations of solutions, instruments and general method. Saline-moistened Whatman #1 filter paper mounted on clean microscope slides was used for this purpose. The liquid junction was formed by placing two slides with moistened filter papers side by side, then bridging the space between them with a third moistened paper. Table 2 shows EMFs measured with reference solutions, 0.15 N. NaCl. For reference purposes zero is obtained experimentally using the following cell:

electrode; .15 N. NaCl; .15 N. NaCl; electrode

This fundamental value may deviate as much as 1 mV from the potentiometer null in the measuring system used.

Figures in Table 2 are means of single measurements carried out on 3 - 5 fresh preparations of the given cells. Ranges of experimental values were 1 - 2 mV with .015 N. NaCl as the recording half cell, and 0.2 - 0.3 mV with .15 N. NaCl as the recording half cell.

TABLE 2. EMFs OF FILTER PAPER CONCENTRATION CELLS.

recording half cell	reference half cells containing solutions approximating 0.15 N. NaCl							
NaCl	.15 N. NaCl	dilantin* in NaCl	divalent metal ions .02 N. in .13 N. NaCl					
			Ca++	Ba++	Mg++	Cu++	Mg++	Pb++	EDTA
.15 N.		0.0	-0.7	-0.7	-0.8	-0.8	+0.4	0.0	+0.8
0.015 N.	-8.5**		-10.5	-10.7	-11.3	-11.2	-8.3	-9.9	-9.4

* diphenylhydantoin Na

** -8.2 mV with .13 N. NaCl

Figure 2.

Electrometric titration curves of connective tissues. The token determinations made during the present study are shown to be similar to the published results of Engel et al.

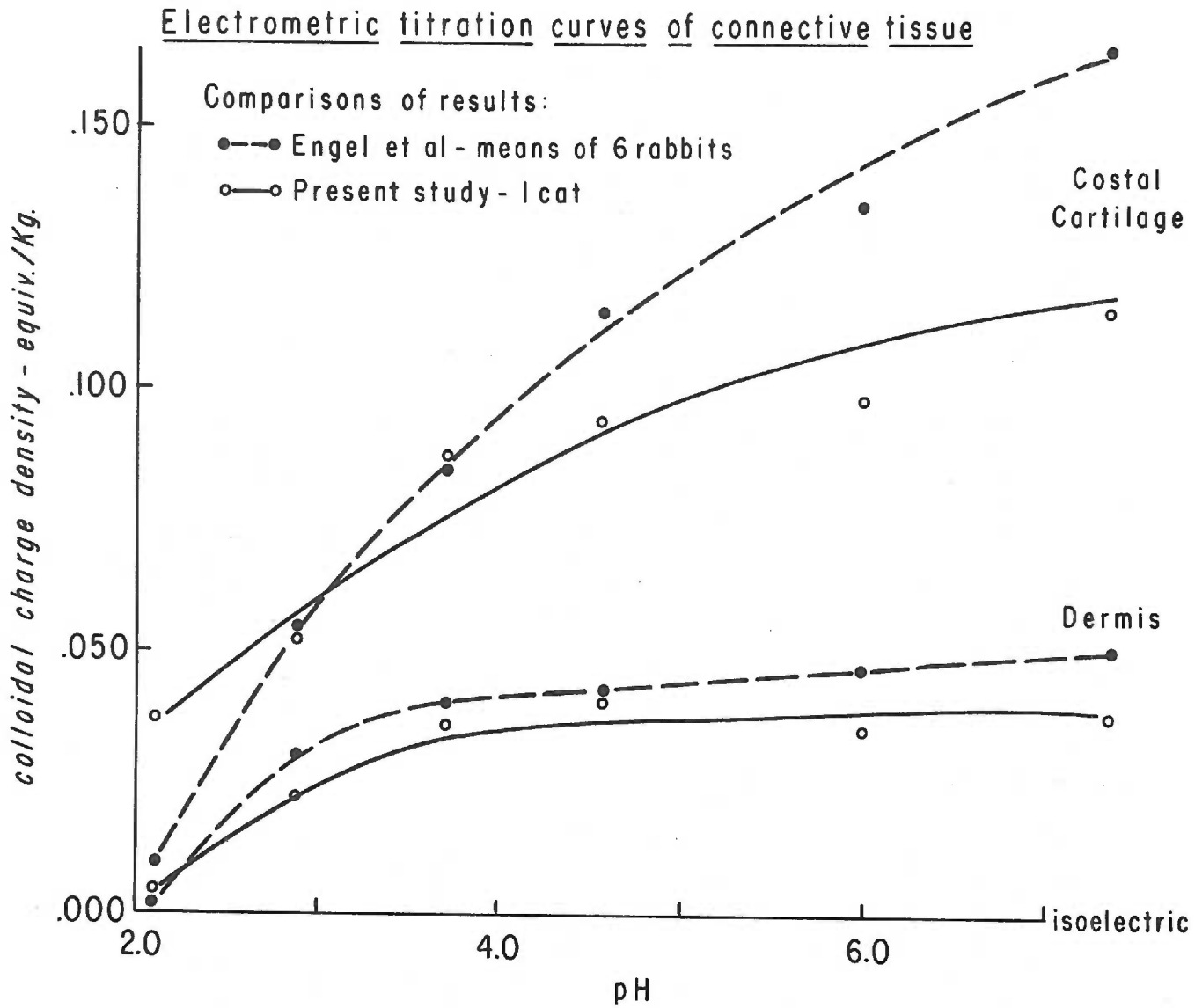

Electrometric titration curves of connective tissue
Comparisons of results:
Engel et al - means of 6 rabbits
Present study - 1 cat
Costal Cartilage
Dermis
colloidal charge density - equiv./Kg.
.150
.100
.050
.000
2.0
4.0
6.0
isoelectric
pH

Table 2 shows a range of $\bar{E}_d$ values obtained with solutions used to approximate 0.15 N. NaCl in the following studies of biological colloids. Calculated value for E_d in the cell

electrode; 0.015 N. NaCl; 0.015 N. NaCl; electrode

is -12.3 mV. Most of the results in this study fall between 0 and -10. The closer the negative values of E_d approach zero, the greater is the calculated value of negative colloidal charge density, x, according to the basic working equation (Eq. 9).

Comparison with previous studies.

Previous work with the electrometric method was done by its developers. Their primary subjects have been living connective tissues, rather than tissues of the nervous system. Figure 2 shows titration curves obtained during the course of the present investigation. The data were obtained from an adult cat. Single surfaces of dermis of the abdomen and costal cartilage were exposed for this purpose. Figure 3 compares data from these two connective tissue surfaces with the published data of Engel, Joseph, et al. (51). The latter were derived from six rabbit preparations. Both the dermis and cartilage curves obtained in the present study of the cat imply lower values of x than those given by Engel, Joseph, et al. Similarities in form and magnitude are, nevertheless, present.

Equilibration of K+ containing colloid surface with .15 N. NaCl.

Study of gelatin and sulfated polysaccharide gels was undertaken as another means of evaluating the electrometric method in vitro.

The fact that the central nervous system has a high K^+ content must be reconciled with one of the basic assumptions of the electrometric method. This assumption requires that the tissue be in thermo-

dynamic equilibrium with .15 N. NaCl, which for these purposes approximates serum or extracellular fluid (51). Two different gel preparations were used in the tentative evaluation of the "equilibrium" assumption. Both were originally made to contain a mixture of 1.5% agar and 0.6% carrageenin. One was prepared with 0.15 N. NaCl, the other with 0.095 N. KCl, 0.055 N. NaCl. The latter, K^+-containing, gel preparation approximates the concentrations of the major cations in whole brain. Carrageenin was chosen because it is a strongly metachromatic polyanion containing galactose sulfuric acid esters similar to those in white matter. Agar was chosen because of its strength and its similarity to carrageenin.

Each gel was hardened in a 1 cm. layer before being covered with 5 volumes of its respective salt solution at 4° C. After five days of equilibration at 4° C., the clear salt solutions were carefully decanted. The decanted liquors from both preparations were equally metachromatic (toluidine blue) by inspection, indicating that the salt solutions had extracted a small amount of polyanionic material from the gels.

The K^+-containing gel was firmer and stronger than the gel containing only Na^+. Measurable swelling occurred in both preparations. There were increases of weight of 120% in the K^+ gel and 140% in the gel containing only Na^+.

The two equilibrated gel preparations were then covered and promptly melted without boiling. Twenty minutes after decantation in the cold, the molten gels were cast in polyethylene tubes. All procedures, including subsequent electrometric study, were performed on the two preparations in parallel fashion.

Glass electrode measurements of each gel preparation showed pH 7.4. The gel-filled polyethylene tubes were refrigerated while immersed in their corresponding salt(s) solutions until their use in the electrometric procedure. The gels did not appear to swell during 7 days refrigeration. They were not weighed.

Application of a series of 7 graded pH saline buffers to 6 surfaces of each of the 2 gels gave the series of means of E_d shown in Table 3. Corresponding values of calculated charge density appear graphically in Figure 4. In this procedure each gel surface was placed for 90 minutes in 0.15 N. NaCl for "equilibration", prior to determinations of E_d.

TABLE 3. COMPARISONS OF E_d's FROM GEL WITH AND WITHOUT APPRECIABLE K'

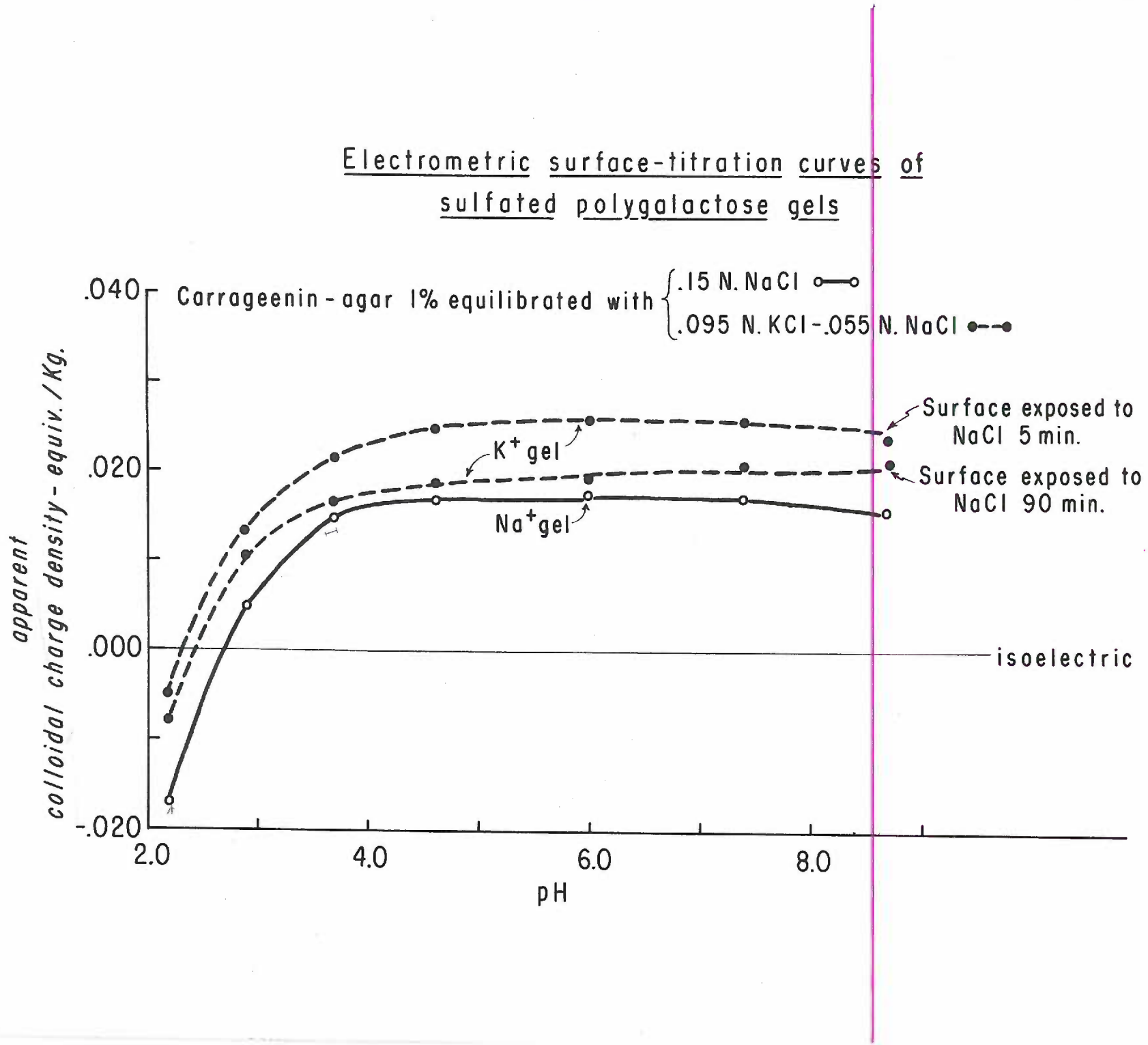
	Original gel cations		
	(A)	(B)	(C)
	.15 N. Na^+	.095 K^+, .055 N. Na^+	(B) corrected for volume
pH 2.2	-7.6	-6.0	-5.9
pH 2.9	-9.6	-8.1	-8.3
pH 3.7	-9.2	-8.8	-9.1
pH 4.6	-9.2	-8.4	-8.7
pH 6.0	-9.2	-8.2	-8.6
pH 7.4	-8.7	-7.5	-7.9
pH 8.7	-8.8	-7.6	-8.0

E_d values somewhat nearer to zero are noted consistently with the K^+ gel, even with correction for the 20%/140% less swelling sustained during preparation. Such a consistent disparity is significant ($P_{.01}$) according to the sign test (20).

The disparity between the $K^\pm$ containing and $Na^\pm$ only gels is

Figure 4.

Electrometric surface-titration curves of sulfated polygalactose gels. The upper two curves were obtained from gels prepared with a high K^+ salt solution. The lowest curve was obtained from a gel prepared with a solution containing no K^+.


Electrometric surface-titration curves of sulfated polygalactose gels
Carrageenin - agar 1% equilibrated with
.15 N. NaCl
.095 N. KCl - .055 N. NaCl
Surface exposed to NaCl 5 min.
Surface exposed to NaCl 90 min.
K+ gel
Na+ gel
isoelectric
apparent colloidal charge density - equiv. / Kg.
.040
.020
.000
-.020
2.0
4.0
6.0
8.0
pH

widened when the same K+ gel is exposed to .15 N. NaCl for only 5 minutes. Figure 4 shows the high values of apparent anionic charge density, $\bar{x}$, obtained when the exposure of the K+ gel to .15 N. NaCl was only 5 minutes. A 90-minute equilibration gave substantially lower values of calculated $\bar{x}$. Lowest values of apparent $\bar{x}$ were obtained with "infinite" equilibration — a gel already prepared with .15 N. NaCl and containing no K+.

The mean of 12 electrometric determinations of E_d at pH 7.4 gave a calculated value of $\bar{x} = .017 \pm .004$ equiv./kg. for .15 N. NaCl gel. Sulfate content was not determined analytically. Calculations from analyses in the literature (97 and 105) indicate a sulfate content of about .009 M./kg. gel H_2O.

Figure 4 shows an apparent .016 equiv./kg. positive charge density in the .15 N. NaCl gel at pH 2.2. This is unexplained. No fixed cationic groups were to be expected in this gel preparation.

Chemical integrity of tissue surfaces.

The initial electrometric determinations of colloidal charge density of central nervous system tissues employed cylindrical reservoirs to hold the recording solutions at the tissue surface. In later experiments cotton pellets were used for this purpose. Table 4 shows means of $\bar{E}_d$ values, corresponding $\bar{x}$ values, and other relevant data pertaining to 10 cats. Five of these animals were studied by the reservoir method of establishing the recording junction; five were studied by the cotton pellet method. Anesthetic method was pentobarbital Na. Surgical procedure was the same in both groups.

TABLE 4. COMPARISONS BETWEEN CEREBRAL WHITE MATTER RESULTS: RESERVOIR JUNCTIONS vs. COTTON PELLET JUNCTIONS.

	reservoir	pellet
number of cats	5	5.
hours since anesthetic induction	12-18	6-10
E_d in mV	-4.7	-0.3
$\bar{x}$ in equiv./kg.	.037	.058

The difference between E_d's is significant, $t_{.05}$.

Histologic sections of both reservoir junction and pellet junction white matter surfaces were examined after staining with toluidine blue at both pH 2 and pH 5. The surfaces within the reservoir junction showed appreciably greater distortion of tissue architecture and greater cellular infiltration.

Reference values: comparisons of nervous tissues

Three categories of nervous tissue were compared.

(i) cerebral white matter vs. cortex

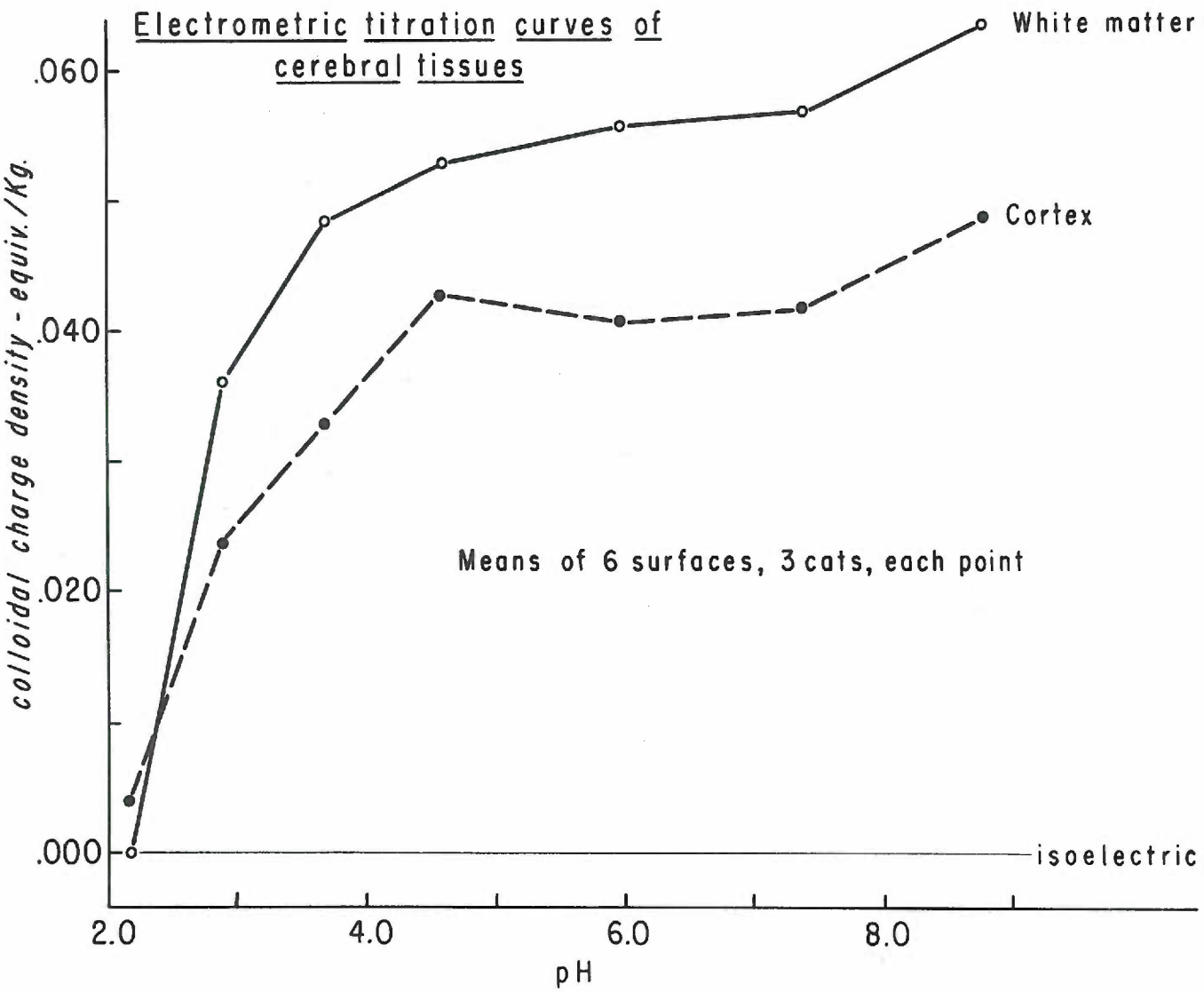
The first comparison used paired $\bar{E}_d$ values obtained from 8 cerebral hemispheres of 5 cat preparations. Procedures included pentobarbital Na anesthesia and the use of cotton junctions. Determinations of $\bar{E}_d$ began with cortex, then proceeded to the white matter of the given cerebral hemisphere. The means in Table 5 show that the calculated negative charge density of cerebral white matter exceeds that of cortex.

TABLE 5. COMPARISON OF CEREBRAL CORTEX WITH CEREBRAL WHITE MATTER.

	cortex	white matter
No. of hemispheres = 8		
E_d in mV	-3.1	-0.3
$\bar{x}$ in equiv./kg.	.045	.058

Figure 5.

Electrometric titration curves of cerebral tissues. Upper curve obtained from cerebral white matter. Lower curve obtained from cerebral cortex. The same surfaces were used for all points of each curve. The same cats provided results from both tissues.


Electrometric titration curves of cerebral tissues
White matter
Cortex
Means of 6 surfaces, 3 cats, each point
isoelectric
colloidal charge density - equiv./Kg.
.060
.040
.020
.000
2.0
4.0
6.0
8.0
pH

The difference is significant ($t_{.05}$).

Titration curves of cortex and white matter are compared graphically in Figure 5.

(ii) occipital white matter vs. frontal white matter

The two cerebral white matter poles were compared electrometrically using 4 reservoir junctions in 6 cats. Twenty-four observations of $\bar{F}_d$ on frontal white matter surfaces were compared with 24 $\bar{F}_d$'s obtained concurrently from the corresponding occipital surfaces. The mean in both groups of 24 was -5.3 mV. An identical number of observations from contralateral hemispheres was obtained using a saturated diphenylhydantoin solution at the recording junctions. Right and left hemispheres received drug alternately in the 6 successive experiments. Again the means obtained from the 24 occipital and 24 frontal observations were identical, in this instance -4.4 mV with the diphenylhydantoin Na solution. The data appear in appendix 1a. The difference between the control and diphenylhydantoin Na F_d's is considered in greater detail below.

(iii) cerebral white matter vs. dorsal funiculus

Colloidal charge densities of cerebral white matter and spinal cord white matter were compared statistically using results from 10 cats, 5 in each group. The comparison between centrum semiovale and dorsal funiculus of the cord is of interest because the two tissues present myelinated surfaces to the electrodes in two different manners. Myelinated fibers of centrum semiovale are cut obliquely and transversely. On the other hand, surfaces of the dorsal columns of the cord are exposed by dissection and are left intact. Consequently the spinal fibers run uninterruptedly through the plane of

the liquid junction. The photographs (Figure 5) of tissues from experimental animals help in visualizing this difference. Table 6 shows almost identical means for the two tissues, indicating very similar calculated values of anionic density. Anesthetic was pentobarbital Na with cotton pellet junctions.

TABLE 6. COMPARISON OF CEREBRAL WHITE MATTER WITH DORSAL FUNICULUS.

	cerebral white	dorsal funiculus
No. of cats	5	5
$\bar{Z}_d$	-0.3	-0.1
x (eq./kg.)	.058	.060

Table 7 summarizes the most probable normal values obtained thus far. No appreciable difference is seen between the two myelinated tissues, while x is lower in cortex.

TABLE 7. CALCULATED NEGATIVE CHARGE DENSITIES, 3 NERVOUS TISSUES.

Tissue	x in equiv./kg. tissue H_2O
cerebral cortex	.045
cerebral white	.058 (occipital = frontal)
spinal white	.060

Changes in experimental preparation with time or attrition.

Because experimental procedures often required 8 to 15 hours of narcotization and severe surgical trauma for the cat, the question of "deterioration" of tissues has been considered. One approach to the evaluation of "deterioration" was the making of serial determinations of $\bar{E}_d$ with time. It was thought that this procedure might detect changes in the cat preparation as time elapsed after anesthetic induction and surgical exposure. With this approach, sequence of E_d determinations is considered as an approximate measure

of time elapsed and of exposure of the preparation to the experimental procedure.

Control values such as those presented in Table 7 were always obtained during the courses of experiments designed to compare drug effects, etc. Control E_d values were arranged as T_1, T_2, ... T_n, according to the chronological order of their determination. Means of $\bar{E}_d$ given in Table 8 are taken from experimental data tabulated in appendix 1a and Table 11. Analyses of variance accompany the data (appendix 1b and Table 12) and provide significance tests for differences among these means. Significant ($F_{.05}$) differences within rows in Table 8 are indicated by an asterisk (*).

TABLE 8. COMPARISONS OF E_d's(mV.) BY TIME INTERVAL

tissue	anesthetic	T_n equals (ca.)	T_1	T_2	T_3	T_4	T_5	T_6
cortex	tetracaine-gallamine	20-40 min.	-4.0	-3.4	-3.0	-2.2	-3.5	-5.2
cortex	pentobarb.	20-40 min.	-3.0	-2.4	-3.3	-1.0	-3.4	-1.3
* cord	tetracaine-gallamine	10-20 min.	-2.3	-1.4	-0.6	-1.4	-1.3	-0.8
* cord	pentobarb.	10-20 min.	-1.8	-0.3	-0.2	-0.1	-0.0	-1.3
*centrum	pentobarb.	60 min.	-4.7	-5.7	-5.6	-5.6		
*centrum	pentobarb.#	60 min.	-3.9	-4.7	-4.5	-4.6		

values obtained with diphenylhydantoin Na in the recording solution.

Cord and cerebral white matter both show changes in mean E_d with time interval.

An extreme of "deterioration" of experimental tissue is created by sacrificing the animal by exsanguination. Breathing stops less than 5 minutes after cutting both carotid arteries. Data from one such preparation is tabulated in appendix 2. Multiple determinations

of $\bar{E}_d$ were performed with special reference to time intervals before and after death of the cat. Four reservoirs were implanted, one in the white matter surface of each frontal and occipital area. Anesthesia was pentobarbital Na. The difference, -0.8 mV., between grand means before and after exsanguination is significant ($t_{.05}$). Equally significant changes occurred in another cat preparation subjected to the same procedure. In the second instance the mean difference was -1.6 mV. Within 30 minutes after the death of each animal values of E_d were found that were more than 2 standard deviation negative to the pre-exsanguination mean.

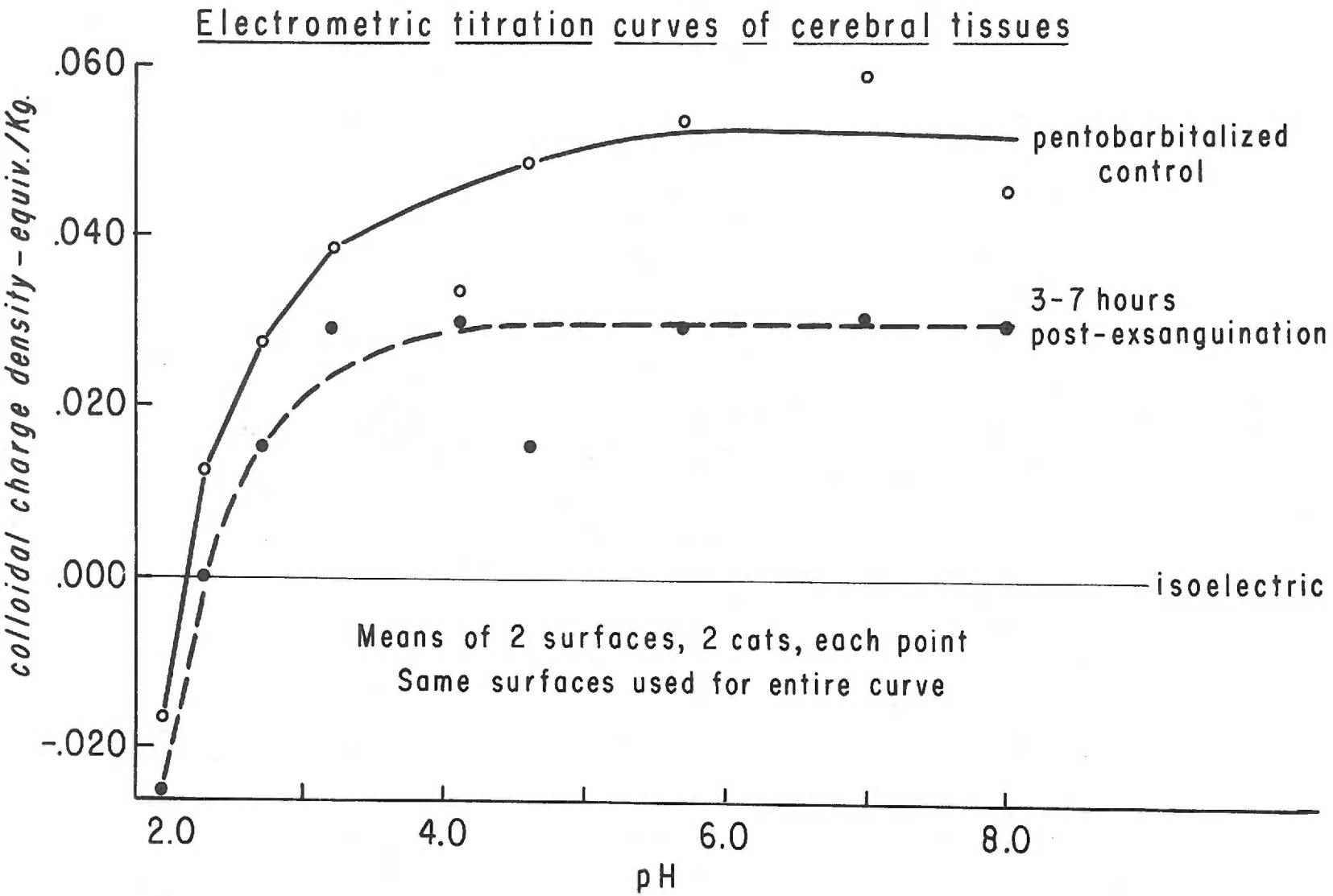
Results from the two preparations have been pooled to give values, in terms of calculated negative charge density, of .036 eq./kg. living and .028 eq./kg. post-exsanguination. This same lowering of calculated negative charge is demonstrated in the graph, Figure 6, which describes a similar experiment.

Titration curves in Figure 6 show simultaneous comparisons of littermate kittens of the same sex. One was narcotized with pentobarbital Na, then exsanguinated. The other was constantly maintained with pentobarbital narcosis. The graph in Figure 6 shows that the non-living member of the pair produces consistently lower calculated values of colloidal charge density than does the living member. Sign test of the 8 pairs of results indicates that this consistent relationship is significant ($P_{.01}$).

As an incidental finding, decreases in absolute values of EMFs (E_1 and E_2) were often noted as an animal passed through terminal stages. This was observed not only with deaths from known causes, such as exsanguination, gallamine triethiodide overdosage, pento-

Figure 6.

Electrometric titration curves of cerebral white matter. Sibling cats evaluated in parallel. Upper curve obtained from living control. Lower curve obtained from sibling which had been exsanguinated.


Electrometric titration curves of cerebral tissues
.060
.040
.020
.000
-.020
colloidal charge density - equiv./Kg.
pentobarbitalized
control
3-7 hours
post-exsanguination
isoelectric
Means of 2 surfaces, 2 cats, each point
Same surfaces used for entire curve
2.0
4.0
6.0
8.0
pH

barbital Na overdosage, but also with deaths from unknown causes.

Ouabain

Cardiac glycosides have been reported to cause a net K+ efflux from excitable tissues such as brain slices and myocardium (37, 110, 84). Ouabain .0001 M. in saline buffers was applied to cerebral cortical surfaces to see if this efflux, or any related phenomena could be detected by the present method. Anesthetic method was tetracaine - gallamine.

Paired drug-control data was obtained from 7 cerebral cortex surfaces in 3 cats. No net difference in E_d was found in association with application of ouabain. Mean of the differences between members of the 7 pairs showed no statistical ($t_{.05}$) difference from zero. Similarly, analysis of variance of $\bar{E}_d$'s obtained at 7 pH levels at single cortical surfaces, 4 cats, showed no significant differences ($F_{.05}$).

To test ouabain *in vitro* 7 pH graded saline buffers containing .0001 M. ouabain were applied to 6 fresh surfaces of sulfated polygalactose gel and E_d values compared with controls. As with the ouabain studies *in vivo*, no differences were found.

The gel preparation contained approximately 1% sulfated polygalactose. It had been equilibrated with .095 N. KCl, .055 N. NaCl as described above. Table 9 shows the means of six E_d values at each of the 7 pH levels. The K+ containing gel surfaces were each exposed to .15 N. NaCl for exactly 5 minutes before beginning electrometric study. None of the differences between members of pairs is significant ($t_{.05}$).

TABLE 9. E_d VALUES USING OUABAIN ON K+ GEL IN VITRO

	control (N=6)	ouabain (N=6)
pH 2.2	-5.2	-5.4
pH 2.9	-7.7	-7.8
pH 3.7	-7.6	-7.8
pH 4.6	-7.4	-7.2
pH 6.0	-7.4	-7.0
pH 7.4	-7.2	-7.0
pH 8.7	-7.6	-7.4

Anesthetic procedure

Inasmuch as anesthetic agents by definition affect the nervous system, it was of interest to compare the two anesthetic procedures used in this study. Details of (i.) pentobarbital Na and (ii.) tetracaine HCl- gallamine triethiodide procedures are given in the "Methods" section above. Relative results with the two procedures were evaluated by means of analyses of variance.

Table 11 shows control $\bar{E}_d$'s obtained during the course of study of effects of divalent metal chlorides at the recording surface. In both the cortex and cord data grand means of $\bar{E}_d$ are nearer zero when pentobarbital Na is the anesthetic. Table 10 summarizes the results as negative charge densities. In both tissues the pentobarbital anesthetic procedure is associated with a high value.

TABLE 10. COMPARISON OF RESULTS FROM TWO CNS TISSUES WITH TWO ANESTHETIC PROCEDURES. $\bar{x}$ in equiv./kg.

	gallamine-tetracaine	pentobarbital	difference
cerebral cortex	.042	.048	+.006
spinal cord	.054	.059	+.005

TABLE 11.

DILUTION POTENTIALS FROM CEREBRAL CORTEX AND DORSAL FUNICULUS OF THE CAT IN mV.

8 cat preparations; 2 anesthetic procedures: (1.) tetracaine HCl - gallamine triethiodide, (11.) pentobarbital Na. Data arranged according to time interval (T=20-40 min., cortex; T=10-20 min., cord) after induction with ether or pentobarbital Na. Cotton pellet method.

CORTEX

		tetra-gal					pentobarb.				
	cat	A	B	C	D	means	E	F	G	H	means
	T_1	-2.7	-3.9	-3.0	-6.6	-4.0	-6.6	-3.0	-0.3	-2.0	-3.0
	T_2	-3.8	-4.0	-2.2	-3.7	-3.4	-1.6	-5.6	-0.3	-2.0	-2.4
time	T_3	-1.4	-3.4	-4.2	-2.9	-3.0	-3.4	-5.0	-2.7	-2.0	-3.3
interval	T_4	-2.8	-1.2	-2.2	-2.7	-2.2	-2.6	+2.6	-1.5	-2.4	-1.0
	T_5	-4.6	-4.2	-2.6	-2.6	-3.5	-2.7	-1.5	-4.5	-5.1	-3.4
	T_6	-5.0	-6.8	-3.0	-6.2	-5.2	-2.0	-1.3	+0.9	-2.8	-5.2
	means	-3.4	-4.9	-2.9	-4.1	-3.6	-3.2	-2.3	-1.4	-2.7	-2.4

CORD

	cat	A	B	C	D	means	E	F	G	H	means
	T1	-4.2	-2.0	-0.2	-2.8	-2.3	0.0	-2.8	-2.5	-2.0	-1.8
	T2	-0.5	-2.6	+0.1	-2.6	-1.4	+0.8	-1.0	+0.8	-1.7	-0.3
	T3	-0.4	-1.9	+0.5	-0.4	-0.6	+2.1	-1.2	-1.5	0.0	-0.2
time	T4	-2.0	-2.0	+1.3	-2.7	-1.4	+2.3	-1.2	+1.0	-2.4	-0.1
interval	T5	-0.6	-1.8	-1.5	-1.2	-1.3	-0.6	+1.4	+0.1	-0.9	0.0
	T6	-0.5	-1.9	+0.4	-1.3	-0.8	+0.6	+2.1	+0.2	+2.2	+1.3
	means	-1.4	-2.0	+0.1	-1.8	-1.3	+0.9	-0.4	-0.3	-0.8	-0.2

TABLE 12.

Analysis of Variance of cerebral cortex E_d's: anesthetic methods/time

Source	Sum of Squares	df	Mean Square	F ratio
between Ts:	20.58	5	4.08	1.63
between anes.:	16.68	1	16.68	6.64*
interaction: (T x anes.):	21.35	5	4.27	1.70
overall between:	58.41	11	5.31	2.12*
between cats:	32.41	7	4.63	1.84
within:	72.75	29	2.51	
total:	163.57	47		

Analysis of Variance of dorsal funiculus E_d's: anesthetic methods/time

Source	Sum of Squares	df	Mean Square	F ratio
between Ts:	22.82	5	4.56	4.85**
between anes.:	14.74	1	14.74	15.68**
interaction: (T x anes.):	3.88	5	0.78	0.83
overall between:	41.44	11	3.77	4.01**
between cats:	40.88	7	5.84	6.21**
within:	27.26	29	0.94	
total:	109.58	47		

* significant, $F_{.05}$
** significant, $F_{.01}$

Therefore conclude that the data show:

1. differences between E_d's obtained with the two anesthetic methods. Occurs with both cortex and cord.

2. differences between E_d's obtained from different cats. Occurs with cord but not with cortex data.

3. differences between E_d's obtained at different approximate time intervals after anesthetic induction. Occurs with cord (T = 10-20 min.) but not with cortex data.

4. no anesthetic or time effects that are not additive.

Analysis of variance (Table 12) indicates that differences between E_d's obtained with the two anesthetic procedures is significant ($P_{.05}$). Each of the four cats in the two anesthetic groups contributed single observations of $\bar{E}_d$ at 6 distinct tissue sites on cerebral cortex and spinal cord. Thus there are four major groups of data (2 tissues x 2 anesthetics) each containing 24 observations. Statistical procedure is summarized in Table 10.

Effects of pentobarbital Na on electrometric determinations were also evaluated by obtaining drug-control $\bar{E}_d$ pairs from cerebral cortex. Results (Table 13) support the findings noted above (Table 10) that the presence of pentobarbital is associated with greater calculated χ. Six cortical sites were obtained in 2 cats prepared with tetracaine-gallamine. At each site a control $\bar{E}_d$ was established at pH 7.4. The $\bar{E}_d$ produced at the tissue surface with 0.0005 M. pentobarbital Na in saline, pH 7.4, was then obtained to complete the pair. The six pairs are shown in Table 13.

TABLE 13. E_d VALUES WITH PENTOBARBITAL Na APPLIED TO CEREBRAL CORTEX

	control	pentobarbital Na	difference
	-2.2	0.0	2.2
	-3.9	-3.3	0.6
	-4.2	-2.8	1.4
	-3.3	-0.5	2.8
	-4.7	-4.0	0.7
	-3.6	-3.2	0.4
mean E_d (mV)	-3.7	-2.3	1.4
χ equiv./kg.	.042	.048	+.008

The mean difference, 1.4 mV. differs significantly from zero ($t_{.05}$).

Paired data from 8 cortical surfaces, 4 cats, was used to compare E_d before and after gallamine triethiodide injection. The

drug was given intravenously in at least the average dose (1 mg./kg.) at times when the cat's movements indicated that the effects of the previous dose were waning. Results detected no changes. The mean difference between the members of the before-and-after injection pairs was less than 0.1 mV. The differences were not significant by either $t_{.05}$ or the sign test ($P_{.05}$).

Diphenylhydantoin Na

Neurophysiologic studies indicate that the antiepileptic drug, diphenylhydantoin Na, "stabilises" the axon membrane by rendering the axoplasm comparatively deficient in Na^+ (109). Effective treatment with diphenylhydantoin Na prevents convulsions. However, it may not significantly alter the abnormal cortical focus which produces the characteristic electroencephalographic pattern. Therefore, diphenylhydantoin is presumed to exert its stabilizing effect mainly on conduction pathways leading from the hyperexcitable focus. In this way a generalized seizure is said to be prevented. Consequently attention has been diverted to white matter as a locus of action of diphenylhydantoin Na. These considerations regarding the mechanism of action formed the rationale for the trial of diphenylhydantoin Na in the present study.

Diphenylhydantoin Na was applied to tissue surfaces of cerebral white matter, cerebral cortex, and spinal cord. The most detailed study pertains to cerebral white matter.

In Table 14 brains of two littermate kittens of the same sex are studied concurrently under identical conditions. Each was given pentobarbital Na. Each had four cylindrical reservoirs implanted in comparable white matter surfaces. Results show changes in E_d

TABLE 14.

Values of $\bar{E}_d$ obtained from cat preparations. Two 10 week old female siblings were used. A saturated (less than .0001 M.) solution of diphenylhydantoin sodium in .150 N. NaCl was applied to the cerebral white matter surface to obtain the experimental results. The figures represent negative millivolts.

(A) control readings from cat A
(B') diphenylhydantoin sodium readings from cat B
(A') diphenylhydantoin sodium readings from cat A

	lt. frontal	lt. occip.	rt. front.	rt. occip.	mean
(A)	3.4	4.4	5.1	3.6	4.1
	4.4	4.6	5.4	4.6	4.8
	5.7	5.2	5.3	5.0	5.3
	5.7	4.5	4.9	4.4	4.9
mean (A):	4.8	4.7	5.2	4.4	4.8
(B')	4.3	4.5	5.8	6.4	5.3
	5.7	6.7	6.2	7.3	6.5
	6.7	6.2	6.5	6.9	6.6
	5.6	5.7	5.4	7.2	6.0
mean (B'):	5.6	5.8	6.0	7.0	6.1
(A')	6.0	5.6	5.9	6.2	5.9
	5.8	5.3	5.9	5.6	5.6
mean (A'):	5.9	5.5	5.9	5.9	5.8

	(A)	(B')	(A')
Colloidal charge densities (eq./kg.):	.036	.030	.032
Standard deviations (mV):	.70	.66	.27

	(A) vs. (A')	(B') vs. (A)	(B') vs. (A')
Degrees of freedom:	23	30	21
t = :	5.26**	4.80**	1.27

* significant, $t_{.05}$
** significant, $t_{.01}$

opposite to those of pentobarbital. Diphenylhydantoin was associated with a decrease in calculated net negative charge.

The data show significant ($t_{.01}$) differences between $\bar{F}_d$ values from the control kitten (A) and corresponding values from the kitten (B') with drug applied to the white matter surface. When the diphenylhydantoin Na solution was applied to the tissue surface of the control animal (A'), $\bar{F}_d$ values became more negative, differing significantly ($t_{.01}$) from control values (A). Calculations of colloidal anionic density with the basic working equation suggest that a decrease of about .005 equiv./kg. associated with the application of diphenylhydantoin Na.

An identical change in $\bar{F}_d$ occurred in association with diphenylhydantoin in a series of experiments similar to the one described in Table 14. Using the brass reservoir junctions, 6 to 10 pairs of cerebral white matter determinations of $\bar{F}_d$ were carried out on each of 6 pentobarbitalised cat preparations. Again four tissue sites were exposed in each cat. A saturated solution (ca. .00005 M.) of diphenylhydantoin in saline was compared with a saline control at pH 7.4. Results (tabulated in appendix 1a) were paired by tissue site (occipital or frontal) and by time interval since surface exposure. The latter factors have been discussed.

Forty-eight pairs of data from the 6 cat preparations were arranged for analysis of variance (appendix 1b). The means of diphenylhydantoin and control values are given in Table 15.

TABLE 15. DIPHENYLHYDANTOIN Na VS. CONTROL, CEREBRAL WHITE MATTER, RESERVOIR JUNCTIONS

	control	diphenylhydantoin Na	difference
$\bar{P}_d$ (mV)	-4.4	-5.3	-0.9
$\bar{x}$ (eq./kg.)	.038	.034	-.004

The difference is significant ($P_{.01}$).

Three of these 6 cat procedures incorporated measures to exclude experimental bias. Labels of drug and control solutions were coded beforehand by a person other than the experimenter. At no time during the determinations or calculations were the specific contents of the two solutions known to the experimenter. The "blinding" measure did not materially affect the results. Calculated $\bar{x}$ from control $\bar{P}_d$'s still exceeded $\bar{x}$ in presence of diphenylhydantoin. In 20 of the 28 "blind" pair determinations, the diphenylhydantoin Na $\bar{P}_d$ was negative to the control $\bar{P}_d$. This relationship is significant ($P_{.05}$) according to the sign test.

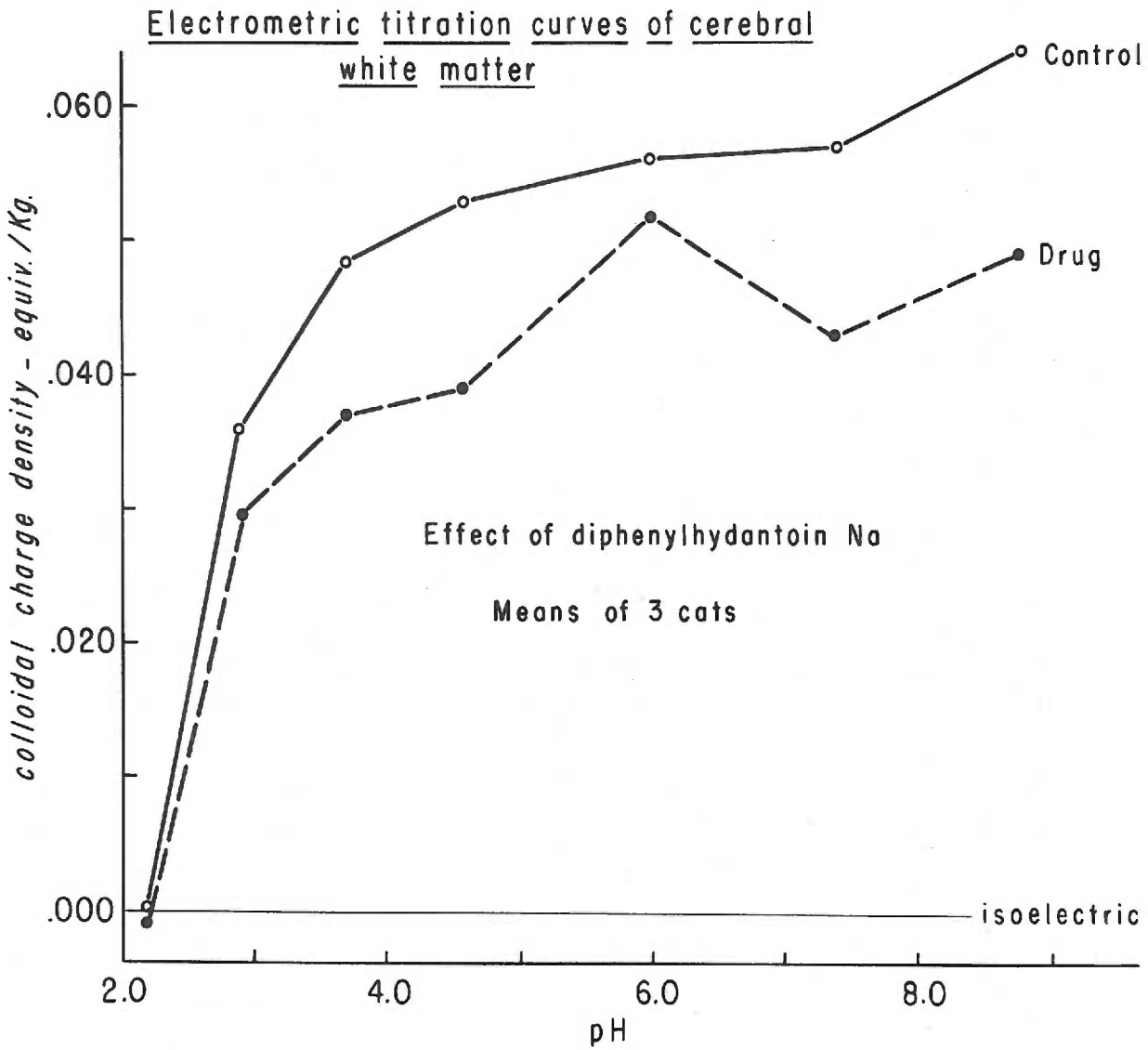
Corroborative results obtained with diphenylhydantoin Na and cotton pellet junctions used on cerebral white matter surfaces appear in Figure 7. The curves compare negative charge densities determined in the absence and in the presence of saturated diphenylhydantoin Na in the recording solutions. A consistent difference between the means of the drug and control groups is seen through the range, pH 2.2 to pH 8.7, in the 3 cats. Anesthetic method was tetracaine-gallamine.

Cocaine and diphenylhydantoin

The local anesthetic and central stimulant, cocaine, is purported to compete reversibly with Na^+ at the nerve cell membrane

Figure 7.

Electrometric titration curves of cerebral white matter. Upper curve obtained with control solutions. Lower curve obtained with diphenylhydantoin Na solutions. The same surfaces were used for all points of each curve. The same cats provided results with both diphenylhydantoin Na and control solutions.


Electrometric titration curves of cerebral white matter
Control
Drug
.060
.040
.020
.000
colloidal charge density - equiv./Kg.
Effect of diphenylhydantoin Na
Means of 3 cats
isoelectric
2.0
4.0
6.0
8.0
pH

(15, 84). In contradistinction to diphenylhydantoin, which is an anion, cocaine is cationic. In the manner of the last-mentioned diphenylhydantoin study .0001 M. cocaine HCl solutions in pH graded saline buffers were applied to cerebral white matter for electrometric evaluation of effects.

Cocaine results bear a marked resemblance to those of diphenylhydantoin. Calculated values of χ were lower in the presence of cocaine (Table 17). Means of cerebral white matter results from three cat preparations are compared in Table 16. Anesthetic method was tetracaine-gallamine.

TABLE 16. COCAINE HCl VS. CONTROL, CEREBRAL WHITE MATTER, COTTON JUNCTIONS, E_d's in mV.

	control	cocaine
pH 2.2	-4.3	-4.8
pH 2.9	-2.8	-5.7
pH 3.7	-2.9	-3.8
pH 4.6	+0.2	-1.6
pH 6.0	+0.9	0.0
pH 7.4	-0.7	-1.9
pH 8.7	+0.4	-2.5

Analysis of variance (appendix 4b) shows that the cocaine group differs significantly ($F_{.05}$) from the control group. Table 17 shows calculated values of negative charge density at pH 7.4.

TABLE 17. COCAINE HCl VS. CONTROL: CHARGE DENSITIES IN CEREBRAL WHITE MATTER AT pH 7.4

	control	cocaine	difference
E_d (mV)	-0.7	-1.9	-1.2
χ (eq./kg.)	.056	.051	.005

Similar procedures were undertaken on cerebral cortex with diphenylhydantoin Na and cocaine, HCl, but in this instance results were much less clear.

As with cocaine on cerebral white matter the samples, again, were small. Data covers the same pH range. There were 21 pairs of observations, 3 cats with cocaine HCl; 42 pairs of observations, 5 cats with diphenylhydantoin Na. In contrast to the clear differences in E_d with white matter, neither group showed significant ($P_{.05}$) difference between drug and control observations of cortex when evaluated by analysis of variance. Statistical procedure was identical to that used in analysis of cocaine - cerebral white matter data.

The sign test, however, showed a significant ($P_{.05}$) difference between members of the 42 diphenylhydantoin Na - control pairs. This difference corresponds to a lower anionic charge density in the presence of diphenylhydantoin, such as was found with cerebral white matter. Mean values of E_d from the diphenylhydantoin - cerebral cortex data appear in Table 18.

TABLE 18. DIPHENYLHYDANTOIN Na VS. CONTROL, CEREBRAL CORTEX, COTTON JUNCTIONS, E_d's in mV.

	control	diphenylhydantoin Na
pH 2.2	-3.6	-4.3
pH 2.9	-4.7	-5.0
pH 3.7	-4.3	-4.8
pH 4.6	-2.7	-3.2
pH 6.0	-4.0	-3.8
pH 7.4	-3.3	-3.5
pH 8.7	-2.2	-3.2

The control E_d at pH 7.4 corresponds to a calculated negative charge density of .044 eq./kg.

The same drug solutions (< .0001 M. diphenylhydantoin Na or .0001 M. cocaine HCl) were then tested in vitro with sulfated polygalactose gels. Results coincided readily with those obtained with cerebral white matter in vivo. Applications of the two drugs were associated with changes indicating lowered χ. Diphenylhydantoin Na and cocaine HCl could not be distinguished quantitatively from one another in this respect.

The drugs were applied in pH graded saline buffers to gel preparations of three kinds: (i.) 1.5% agar, 0.6% carrageenin in .100 N. KCl, .050 N. NaCl, (ii.) 1.5% agar, 0.6% carrageenin in .150 N. NaCl, and (iii.) 1.0% agar, 10% gelatin in .150 N. NaCl. Fixed ionic charges in preparations (i.) and (ii.) should be attributable almost entirely to the sulfuric acid ester groups of carrageenin. Gelatin in preparation (iii.) was expected to combine to some extent with the sulfuric acid ester groups of agar, and also to add fixed charges of protein side chains and terminals. Results indicate that replacement of carrageenin with protein in the gel composition caused no change in the E_d alteration with drugs.

Control determinations of E_d were made concurrently on identical gel surfaces. There were 12 gel surfaces used for the 84 observations represented. Table 19 summarizes the various drug vs. control comparisons.

TABLE 19. DIPHENYLHYDANTOIN Na AND COCAINE HCl VS. CONTROL, GEL SURFACES IN VITRO.

gel preparation	cation	result: relative apparent x(eq./kg.)
agar-carrageenin	K+, Na+	control > diphenylhydantoin Na
agar-carrageenin	Na+	control > cocaine HCl
agar-gelatin	Na+	control > diphenylhydantoin Na
agar-gelatin	Na+	control > cocaine HCl

All four drug vs. control differences are significant ($F_{.01}$). Each difference corresponds to a lower calculated net negative colloidal charge in the presence of drug.

Table 20. shows means of 6 observations of E_d obtained from the agar-gelatin gel preparation. It demonstrates drug effect through a considerable pH range in vitro.

TABLE 20. DIPHENYLHYDANTOIN Na AND COCAINE HCl VS. CONTROL IN VITRO. Figures are mean E_d's in mV.

	control	diphenylhydantoin Na	cocaine HCl
pH 2.2	-5.2	-6.7	-6.7
pH 2.9	-7.3	-8.3	-8.7
pH 3.7	-7.4	-8.2	-8.7
pH 4.6	-6.9	-7.3	-7.7
pH 6.0	-6.7	-7.4	-7.2
pH 7.4	-6.7	-7.1	-6.5
pH 8.7	-6.6	-6.3	-6.9

Analysis of variance of the constituent data from the gel preparations appears in appendix 4a. It shows significant ($F_{.05}$) differences among the three groups, and among the 7 pH levels. It shows no statistical evidence ($F_{.05}$) of interaction between pH and

drug factors, i.e., no effects that are not explicable by simple addition of pH and drug effects. Duncan analysis (21) shows significant ($R_{.05}$) differences between each of the two groups of drug E_d's and the control group. However, no significant ($R_{.05}$) difference between the diphenylhydantoin Na and cocaine HCl data was found.

Divalent metal ions

The neurotoxicity of a number of heavy metals is well known.

Studies suggesting the importance of the alkali earth metal ions in neuronal function was presented in the introduction of this report. Cations of both the toxic and the physiologic metals have been thought to have the ability to form complexes with organic colloid. These considerations prompted the testing of a series of 6 divalent metal chlorides on white matter and cortex of cat cerebrum. Complex-formation was expected to lower anionic density and cause a negative shift in E_d.

Solutions used contained .02 M. metal ions in .13 M. NaCl. The metal ions were Ca^{++}, Ba^{++}, Mg^{++}, Cu^{++}, Hg^{++}, and Pb^{++}, as the chlorides. A control solution of pH 7.4 saline buffer and a solution of EDTA·2 Na .02 M. in pH 4.6 saline buffer were added later to bring the series to 8 in number. Each was given a code label so that the experimenter was "blinded" to the specific content of each solution.

Results as E_d's have been used in calculations of changes in tissue negative charge densities (g). The calculations indicate that g is lowered in the presence of Pb^{++}, Mg^{++}, and Cu^{++} and perhaps also with Ca^{++}, and Ba^{++}, but not with Hg^{++}. The data also suggest that EDTA has the opposite effect by increasing g. It has been possible to arrange results in a series of increasing displace-

ment of E_d and to show which aspects of the series are statistically significant (Table 22).

Experiments with divalent metal ions were performed in two consecutive groups of four cats each. In the first group tetracaine-gallamine was the anesthetic procedure. Pentobarbital Na was used in the second group of four. Each of the 8 cats was represented equally with cerebral cortex and spinal cord data. Data is tabulated and subjected to analyses of variance in appendix 5. The prime object of this series was to test for a difference between E_d's determined in the presence of metal ion, as compared with a control solution. For simplicity this difference will be denoted ΔE_d.

Thus, there were four groups of ΔE_d results (2 tissues x 2 anesthetics) to be subjected to analyses of variance. The initial analysis (appendix 5a) showed that there were significant ($F_{.01}$) differences between cats and between ΔE_d's obtained with the various metal ion solutions. ΔE_d's from cortex and cord were not significantly ($F_{.05}$) different.

A rearrangement of data (appendix 5b) facilitated a second analysis of variance (appendix 5c) to allow comparison of the two anesthetic methods regarding ΔE_d. This second analysis showed significant ($F_{.01}$) differences between ΔE_d's obtained with pentobarbital as compared with tetracaine-flaxedil. Table 21 summarizes ΔE_d's obtained with the two anesthetic methods. It shows the greater negativity of most of the pentobarbital group ΔE_d's, including the control ΔE_d. As noted above, the calculated negative charge densities found with the pentobarbitalized cats had been higher than those of

the tetracaine-gallamine group.

The means of ΔE_d's from 8 tissue surfaces from each of 4 cats, appear in Table 21. These are the combined results of ΔE_d determinations on both cerebral cortex and on the dorsal funiculus of spinal cord. This pooling of results was considered justifiable as a tentative step because ΔE_d's from the two tissues had not been found to be significantly ($F_{.05}$) different. In addition there had been no statistically demonstrable interaction between any metal ion or additive and either tissue.

TABLE 21. COMPARISONS OF ΔE_d's OBTAINED WITH ANESTHETIC PROCEDURES: TETRACAINE-GALLAMINE VS. PENTOBARBITAL Na.

	tetracaine-gallamine	pentobarbital Na
control	0.0	-0.9
Ca^{++}	-1.2	-3.6
Ba^{++}	-1.2	-2.6
Mg^{++}	-2.6	-2.3
Cu^{++}	-3.7	-2.9
Hg^{++}	0.0	-2.0
Pb^{++}	-2.4	-2.8
EDTA	+2.3	-0.8

The ΔE_d's from the two anesthetic groups shown in Table 21 differ significantly ($F_{.01}$) from one another.

Analysis of variance of the constituent data of Table 21 reveals no significant ($F_{.05}$) differences among members of the pentobarbital Na group. On the other hand a similar analysis of ΔE_d's of the tetracaine-gallamine group reveals significant ($F_{.01}$) differences.

The only positive results, then, came from the 4 cats prepared

with tetracaine-gallamine. By using this data only, the various metal ion results could be arranged into a series according to magnitudes of change in potential (ΔE_d). Duncan analysis was used to designate the significant ($R_{.05}$) differences among 8 means in the series shown in Table 22.

TABLE 22. ARRANGEMENT OF ΔE_d's INTO A SERIES.
Results from 4 cats prepared with tetracaine-gallamine.

	ΔE_d (mV)	rank (cortex series)	rank (cord series)
NDTA	+2.3	1	1
control	0.0	2	3
Mg++	0.0	3	2
Ca++	-1.2	5	4
Ba++	-1.2	4	5
Pb++	-2.4	6	8
Mg++	-2.6	7	6
Cu++	-3.7	8	7

When the combined ΔE_d results from cortex and cord are subjected to Duncan analysis, the following observations are statistically significant ($R_{.05}$):

(i.) Cu++ differs from all others.

(ii.) control, Mg++, Ca++, Ba++ differ from Pb++, Mg++, Cu++

(iii.) NDTA differs from Ca++, Ba++, Pb++, Mg++, Cu++

Table 23 shows an expanded series of metal ion effects on calculated values of χ and E_d. Calculations are based on mean control E_d values obtained from 28 observations on each of the two tissues. The control means are -3.6 mV (cerebral cortex) and -1.0 (dorsal funiculus). E_d's in the presence of the various metal ions were calculated by adding ΔE_d to the control mean for the particular tissue.

TABLE 22. E_d VALUES AND CORRESPONDING NEGATIVE CHARGE DENSITIES IN THE PRESENCE OF DIVALENT METAL IONS.

	cortex E_d	cortex x	cord E_d	cord x
EDTA	-1.3	.054	+1.3	.066
control	-3.6	.042	-1.0	.055
Mg++	-3.6	.042	-1.0	.055
Ca++	-4.8	.036	-2.2	.049
Be++	-4.8	.036	-2.2	.049
Pb++	-6.0	.031	-3.4	.043
Mg++	-6.2	.030	-3.6	.042
Cu++	-7.3	.024	-4.7	.037

Table 22 shows a series of metal ion additives in ascending order of their abilities to decrease negative charge density in nervous tissue. The series applies to cerebral cortex and to dorsal funiculus. It is based on experimental data and Joseph's basic working equation. It shows outstandingly low calculated anionic densities in the presence of Cu++.

DISCUSSION

Evaluation of solutions

The use of filter paper concentration cells as a testing procedure gave results which pertain to several aspects of this discussion. The only drug solution tested, diphenylhydantoin Na, behaved as expected. It did not appear to modify the liquid junction potential.

In several instances, however, the filter paper concentration cells gave results (Table 2) which would not be predicted from the electrochemical principles cited in the "Methods" section. The EMF of the control cell,

(Eq. 10.) electrode; 0.015 N. NaCl; 0.15 N. NaCl; electrode,

was only -8.5 mV. This is to be compared with the -12.3 mV. calculated by Joseph et al. (51) from standard conductance data. The added presence of divalent metal ions (.02 N.) in .13 N. NaCl in the right half cell gave EMFs of about -11 mV. in several instances, while EDTA gave only -3.4 mV. This is to be compared with the -8.5 mV. control and the -12.3 mV. "ideal." When 0.13 N. NaCl is used in the right-hand half cell of Eq. 10, the EMF is -8.2 mV.

Joseph reports in a personal communication (53) that discrepancies of this magnitude have not occurred in tests he has made. These tests were conducted with concentration cells of somewhat different design. Joseph's testing design gives an EMF of -11 mV. for the cell of Eq. 10 in the system used in the present study as well as in Joseph's.

The most obvious difference between the two concentration cell designs is the presence of a paper matrix (on microscope slides) in both half cells in the present study. The more "ideal" EMFs came from the design recommended by Joseph in which two beakers of free salt solutions form the half cells. A KCl - agar tipped electrode arm contacts each salt solution. The liquid junction is formed at one end of a filter paper which dips into both half cells and forms a bridge between them (53).

A full explanation of this discrepancy between the two designs is not available at present. Among the factors which may be considered are those relating to (i.) formation of the liquid junction and to (ii.) properties of the solutions.

In reference to (i.) junction potentials are noted for unpredictable changes of up to 2 - 3 mV. occurring when the method of formation of the liquid junction is altered (64). Factors such as a fixed ionic charge density of the filter paper, or impurities in the filter paper would not be excluded.

In reference to (ii.) the presence of .02 N. metal ion at the liquid junction may be most important. A "swamping effect" (46) on junction potentials is said to occur when sufficient concentrations of a second electrolyte are present.

The effect of EDTA on junction potentials with filter paper half cells is most pronounced (Table 2). The relationship of this, and of the divalent metal ion findings, to ΔE_d's of tissue surfaces are of great interest in the final interpretation of the results of the tissue - metal ion series (Table 23). The complexities of factors (i.) and (ii.) above preclude such an interpretation in

this report.

ΔE_d's of the divalent metal ions

If ΔE_d's obtained at CNS tissue surfaces are attributed to tissue changes, the series of tissue charge densities (Table 23) may be used to make other inferences. An inferential process was developed by Joseph, Engel, et al. (13, 26) which considered an equilibrium reaction between tissue charges and divalent metal ions. The reaction may be written

$$Me^{++} + X^{=} = Me{\cdot}X \quad (51)$$

where Me^{++} is the divalent metal ion, $X^{=}$ is the ionizable tissue colloid, and Me·X is the colloid-metal complex. The concentration of Me^{++} is predetermined by the experimenter. 0.02 N. metal chlorides were used in the present study. $X^{=}$ is the colloidal charge density determined from the control E_d values using the basic working equation for χ (Eq. 9). Me·X is determined experimentally as ΔE_d. Knowledge of these values allows calculations of the equilibrium constant of the reaction ($K_{equilibrium}$), and of free energies of formation (ΔF^{o}) of ion-colloid complexes. The latter is a measure of binding capacity of dissociated colloid anion for specific cation. It does not take into account exchange reactions such as

$$Me^{++} + K_2X = Me{\cdot}X + 2\,K^{+} \quad \text{or} \quad Me^{++} + Mg{\cdot}X = Me{\cdot}X + Mg^{++}$$

where K^{+} and Mg^{++} are indigenous cations.

The free energies of formation of cation - CNS colloid com-

plexes were calculated from data of the present study (Table 23) by the relationship $\Delta F^{\circ} = -RT \ln K_{equilibrium}$ (51, 67). The symbols have the same meanings as above. The calculated values were -2500 calories in cerebral cortex and -2200 calories in dorsal funiculus of the cord. The corresponding figures for the Mg^{++} - colloid complexes were zero calories for both tissues.

Cupric ion exceeded all others measured in its calculated ability to form complexes with CNS tissue colloid. This is in keeping with the findings cited in the introduction in which Cu^{++} gave the most pronounced decreases in pH when added to aqueous preparations of cerebral lipid. Cartilage also demonstrated a strong affinity for Cu^{++} in a number of different studies. Using's report is interesting to note, in connection with the electrometric findings with Cu^{++} in nervous tissues. He finds that only .000001 M. Cu^{++} will markedly inhibit "sodium pump" action of isolated frog skin (103).

The connective tissue studies of Joseph and Engel (13, 51) have not included Cu^{++}. The studies are relevant, however. They provide comparisons of tissue affinities for the few ions which have been evaluated electrometrically with both nervous and connective tissues. Comparison of electrometric results from the two tissues indicates that $K_{equilibrium}$ values are of the same order of magnitude in both. Connective tissues, however, may react with Ca^{++}, Mg^{++}, and Pb^{++} to a somewhat greater extent than do nervous tissues. Catchpole, Joseph, and Engel found ΔF° values of -2,300, -2700 and -3500 respectively for reaction of these three ions with connective tissues (13).

Equilibration of K+ - containing surfaces

Table 3 shows that the presence of K+ in sulfated polygalactose gel is associated with E_d's nearer zero. Figure 4 shows progressive decreases in calculated χ after exposures of K+ - containing gel to .15 N. NaCl for 5 minutes, 90 minutes, and "infinity."

Two possible explanations for the more negative E_d's of the 0.15 N. NaCl - containing (no K+) gel will be cited here. (i.) The first is the residual K+ remains in the surface of the K+ - containing gel even after 90 minutes exposure to .15 N. NaCl. (ii.) The second is that procedures for preparation of the K+ - containing gel and the .15 N. NaCl gel were not comparable, i.e., that physical and chemical differences between the gels fostered a greater loss of polyanion from the Na+ gel into the overlying equilibration salt solution.

The K+ - containing gel produced E_d's which were positive to E_d's of the .15 N. NaCl - containing gel even after 90 minutes equilibration with .15 N. NaCl. The disparity was even greater after only 5 minutes equilibration. Two possible explanations may be cited here as well. (i.) E_d may be expected to depend in part on the presence of K+ in the gel surfaces because of the relation of E_d to ion mobilities (Eqs. 6 and 7). A progressive negative shift in E_d with lengthened exposure to a low K+ solution is consistent with a progressive loss of K+ from the colloidal surface.

(ii.) On the other hand the basic working equation (Eq. 9) demonstrates the relationship of E_d to colloidal charge density. In view of this a negative shift in E_d during equilibration of the K+ - containing gel with .15 N. NaCl is also consistent with loss

of fixed ionic groups.

The loss of colloidal anion from K+ - containing sulfated polygalactose matrix into the saline would most probably be accompanied by a loss of K+.

In any event the relationships of F_d's obtained from the three modifications of sulfated polygalactose gel may be seen in Figure 4 to be very consistent throughout the pH range of the studies. Colloidal portions of CNS tissue may or may not behave with K+ in the manner of these soluble gels. If they do, this finding suggests that calculations of charge density from F_d in K+ - rich CNS tissues are probably somewhat limited in accuracy. The presence of diffusible K+ in appreciable amounts may be expected to give spuriously high values of tissue charge density because of the relatively high mobility (42) of K+ across the recording liquid junction.

However, the results obtained *in vitro* also show that F_d's obtained from high K+ colloidal surfaces can correlate well with values found in the absence of diffusible K+. In addition the possible elevation in calculated χ due to the presence of diffusible K+ appears to be small (Figure 4).

An important faculty of the electrometric method is the ability to record changes in χ due to small concentrations of drugs. Very possibly, changes in χ due to drugs may be expected to be more accurate than determination of tissue charge density *per se*. Comparison of drug-influenced F_d's with control F_d's would tend to cancel by subtraction such errors as have just been speculatively attributed to high K+ conductance.

Several other aspects of the present study would indicate that

observations of E_d differences in the presence of drugs is one of the strongest aspects of the electrometric method. In a given instance E_d might be suspected of being misleadingly low or high because of tissue K+ or capriciousness of junction potentials. Nevertheless, a minute amount of drug might produce a change in the tissue which could be determined with relative accuracy because of the subtracting-out process.

A statement of accuracy could be defended only by first proving lack of (i.) influence on junction potentials, and (ii.) interference by mobility of tissue K+. The evidence recapitulated below indicates that these potentially disrupting factors may not be operative in the diphenylhydantoin studies at least.

(i.) Diphenylhydantoin Na was not found to alter the junction potential in a filter paper concentration cell (Table 2). As a consequence, deviations from an "ideal" control E_d due to peculiarities in formation of the liquid junction could be expected to alter drug E_d in a like manner.

(ii.) Effects of diphenylhydantoin on E_d's of gel surfaces are measurable in vitro in the absence of metabolism and in the relative absence of K+ (Table 20). These findings, (i.) and (ii.), show that the respective interfering factors can be excluded in specific instances.

Chemical integrity of tissue surfaces

Lower values of g were found with the use of reservoir junctions as compared with cotton pellet junctions at the cerebral white matter surface. The difference evokes consideration of several possible mechanisms. Some, or all of these mechanisms, may help explain the

difference.

First, the use of brass as reservoir material may have influenced E_d's by introducing EMFs from extraneous electrochemical processes. However, a few experiments using polyethylene cylindrical reservoirs gave similarly low values of χ. This suggests the alternative that a nonphysiological explanation may be sought in the manner of forming the liquid junction. As noted above, modes of formation of liquid junctions are known to alter junction potentials.

Secondly, the state of the tissues must be considered. The process of implanting the sharp-edged cylindrical reservoir isolates the tissue anatomically from all aspects but its base. Blood flow and, indeed, viability under these circumstances may be compromised. This may be especially true after the blood loss of surgical exposure and up to 10 hours experimentation. Histopathological changes and electrometric differences between reservoir and pellet junctions may be effects of the same cause, i.e., pathological changes in the tissue.

A third explanation relates closely to the second. The greater duration and trauma of reservoir junction experiments may facilitate the extraction of polyionic compounds from the tissue. The differences observed are consistent with the results of such a process.

Fourthly, a similar "washing out" process, possibly coupled with alterations in tissue metabolism, may have served to deplete tissue K^+ within the cylindrical reservoir. Depletion of tissue K^+ would concur with the greater physiologic compromise expected in the tissue of the reservoir junction. This is to be compared with the unbroken plane of exposed white matter which forms the cotton junction.

Similar hypotheses may help interpret the changes in E_d observed when cat preparations were exsanguinated. The effect of exsanguination on χ of cerebral white matter was studied with reservoir junctions only. As a result the pathological, physiological and biochemical changes suggested above must have become even more marked with exsanguination to account for the further drop in calculated χ.

The findings of changes in E_d with time during the course of an experiment are not readily explained. No consistent pattern could be detected. This indicates that whatever changes did occur, the net effect on electrometric results was not the expected progressive one.

Reference values: comparisons of nervous tissues

The most readily available estimates of fixed anionic charge densities in nervous tissues are based upon calculations of "anion deficit" (Table 1). McIlwain's (71) figure is (0.10 equiv./kg. tissue water when it is recalculated in these units). He estimates that half of the "deficit" is attributable to protein and half to lipid anionic groups. An interpretation by the present author of analytical data from mammalian nervous tissues gives values of 0.16 equiv./kg. H_2O for whole spinal cord, 0.09 equiv./kg. H_2O for cerebral cortex, and 0.14 equiv./kg. for cerebral white matter. These calculated values were based upon data collected from the literature by Rossiter (77). The data given here are midrange values derived from a number of species by many different investigators. As a consequence, the differences between tissues, though suggestive, are probably unreliable.

"Anion deficit" estimates of negative charge density should be expected to exceed electrometric results. This is because cations

sequestered within tissues as undissociated organometallic complexes would tend to magnify the "deficit." This situation may resemble what Dunstone (22) termed "irreversible binding" in cartilage.

Chemical extraction procedures have repeatedly obtained cation-lipid complexes from CNS tissues. Folch (32, 71) reports that lipid extracts alone can account for 30% to 50% of the "deficit." He accounts for 0.065 equiv. of cation per kg. tissue water (whole brain) in three lipid groups. They are sulfuric acid ester (12% of K), Phosphatidyl serine (16% of K and 20% of Na), and diphosphoinositide (50% of Ca++ and Mg++).

The higher "anion deficit" values calculated from Rossiter may be compared with the lower experimentally-determined tissue anionic densities — 0.060 equiv./kg. H_2O in white matter and 0.045 equiv./kg. H_2O in gray matter. This comparison suggests that about one-half of net fixed tissue anionic charge is not detected electrochemically at the recording liquid junction at pH 7.4. Thus it appears that about one-half of the nervous tissue content of the physiologic metals is not ionized in dilute aqueous solution. This supports Folch's contention that the brain contains an abundance of lipid - cation complexes. It leaves, however, little metal "left over" to be ascribed to metalloproteins.

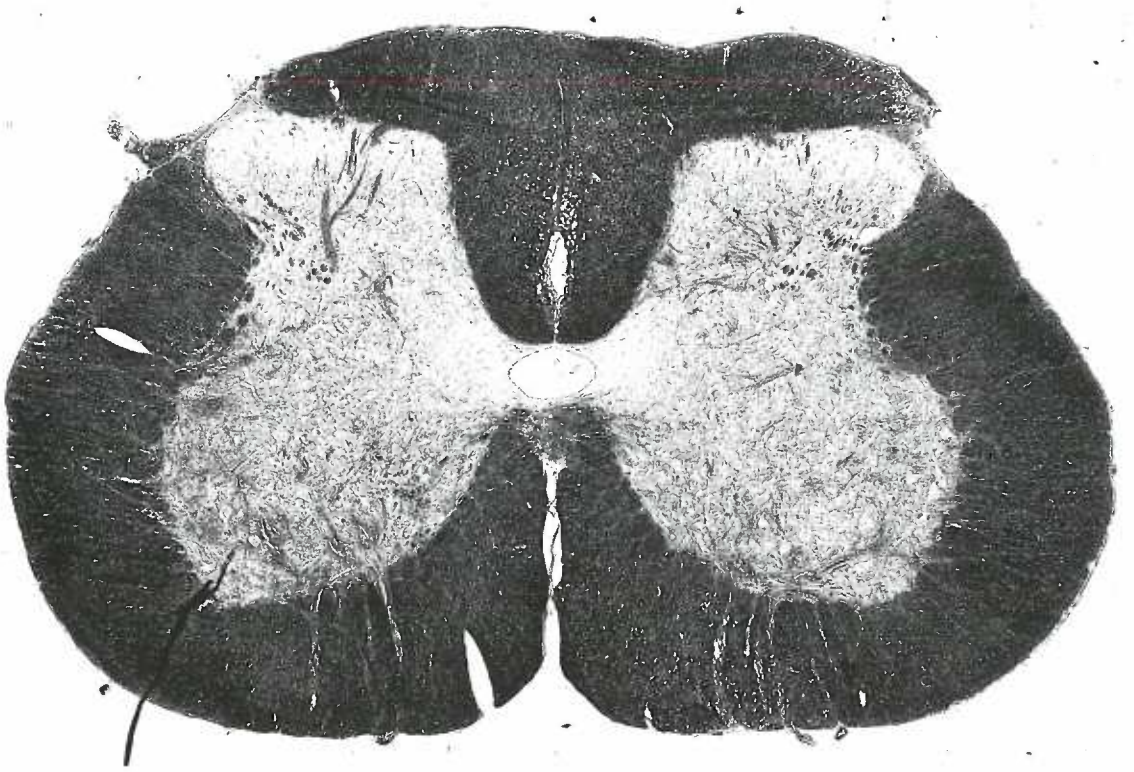
The data collected by Rossiter indicate that Ca++ and Mg++ complexes could account for much of the apparently undissociated fraction of cation, but probably not all. K_d values obtained in the present study with the chelating agent, EDTA (Table 23), are consistent with the hypothesis that Ca++ and Mg++ make up an important part of the difference between "anion deficit" and tissue anionic density, E.

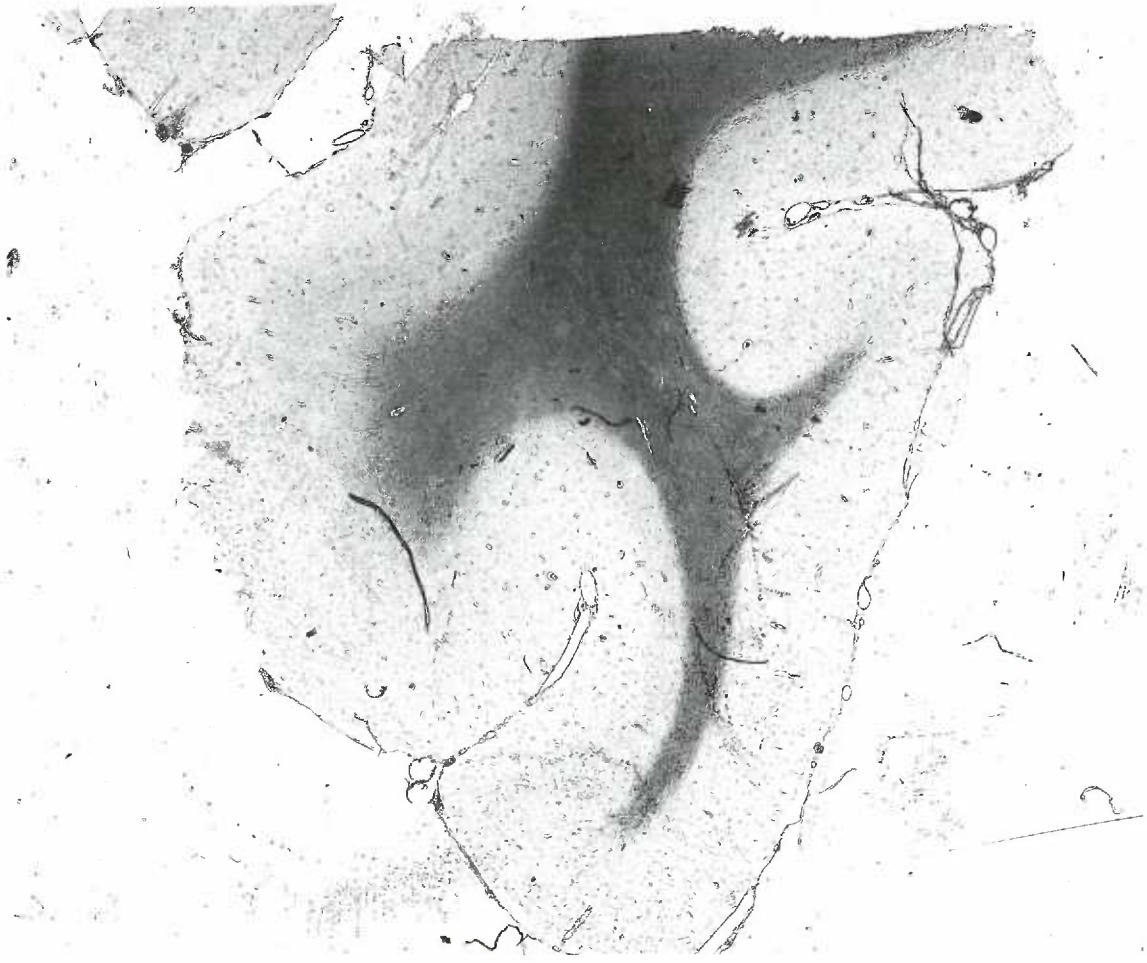
Figure 8.

Photographs of CNS tissues from experimental cats. Formalin-fixed tissues were prepared by frozen section. They were stained with toluidine blue 0.05% at pH 2. Metachromatic areas are black in the photographs. The transparencies in the manila envelope below show the same specimens in color. Holding these reproductions up to the light shows the red-purple staining from contact of the blue dye with myelinated tissue.

a. Lumbar cord with dorsal funiculus at the top.

b. Cerebrum.





The suggestion of an increase in negative charge density of the tissue with EDTA is compatible with a loss of Mg^{++} and Ca^{++} from anionic binding sites.

In the present study white matter had a greater negative charge density than did cerebral cortex. This was expected on the basis of the relative reactions of the two tissues to toluidine blue. Figure 4 shows 0.05% toluidine blue stains of histologic sections of cerebrum and sacral cord. There is marked contrast at pH 2 between the dense staining of myelinated areas and the fainter-staining gray matter. The red metachromatic reaction of white matter upon contact with the blue dye indicates a high fixed anionic density (3, 5). The contrast between the two CNS tissues in the electrometric determinations is not as pronounced (Figure 5). Perhaps this is a manifestation of greater specificity on the part of toluidine blue in the detection of certain kinds of immobile anionic groups. The dye may produce metachromasia primarily by reacting with certain chemical groups peculiar to myelin. On the other hand electrometric results may reflect fixed anionic groups less discriminantly, and consequently give less contrast.

Ouabain

Ouabain had no measured effect on electrometric results in this study. The effect of ouabain on cerebral cortex (110), then, is quantitatively and/or qualitatively different from either diphenylhydantoin or pentobarbital. It is perhaps relevant that ouabain is a nonpolar molecule. In this respect it is unlike other chemical additives employed at tissue surfaces in this study.

Cortex was chosen as the site for application of the drug be-

cause of its greater O_2 - glucose consumption (96) and presumably greater K^+ turnover. One possible effect of ouabain might be a negative shift in E_d due to K^+ efflux from the tissue surface. This could occur only if diffusible K^+ remained in the tissue surface after "equilibration." Should the expected increase in negativity of E_d have occurred, it would also have been interpretable as a lowering in fixed charge density (Eq. 9). A third interpretation would encompass the first two, saying that both describe the same process in excitable tissues (100).

The failure to observe any significant change in E_d upon application of ouabain suggests that perhaps no K^+ efflux occurred. This lack of change helps little in deciding whether or not the tissue surface can be satisfactorily equilibrated with respect to 0.15 N. NaCl under the experimental conditions.

Pentobarbital

An elevation of calculated negative charge density occurred in association with the pentobarbital anesthetic procedure (Table 10). This finding appears to be contradicted by a tentative report of Ungar et al. (102). These investigators found that electrical stimulation increased sulfhydryl ionization in brain extracts. As an incidental procedure they compared the levels of ionized sulfhydryls in two groups of rats treated with different anesthetic methods. One group of rats received pentobarbital prior to exposure and electric stimulation of the brain. The second group received the freeze-anesthesia of topical ethyl chloride. The pentobarbital group appeared to have a lower ratio of sulfhydryl ionization than did the topical ethyl chloride group.

The application of Hg^{++} to nervous tissue in the present study gave no evidence of effect on fixed charge (Table 21). This was unexpected because Hg^{++} is said to be especially attracted by sulfhydryl groups, and has been expected to "bind" within the protein lamellae of myelin sheaths and other protein-rich areas (29).

A simple explanation may reconcile the findings of Ungar et al. with those of the present study. Perhaps two different entities have been measured. The electrometric method may not "count" sulfhydryl groups of the kind Ungar et al. have reported. As a result, addition reactions with Hg^{++} may not have been detected either.

Application of pentobarbital Na to cerebral cortex was associated with a mean increase in calculated χ (Table 13). This stands in contrast with the decreases observed in nervous tissues with cocaine and diphenylhydantoin. The results with pentobarbital are not strictly comparable with those obtained with the latter two drugs, because in general different nervous tissues were studied. For discussion purposes, however, results in Table 18 may be compared with those in Table 13. These two tables show cortical E_g's to be generally more negative than controls with diphenylhydantoin and less negative with pentobarbital. Anesthetic procedure in both instances was tetracaine-gallamine.

Certain pharmacological differences between diphenylhydantoin and pentobarbital should be noted in view of these contrasting trends in influence on χ. It may be relevant that pentobarbital is a powerful hypnotic with a characteristic effect on the electroencephalogram. By contrast neither diphenylhydantoin nor cocaine have this property (97).

The chemical structures of pentobarbital and diphenylhydantoin might also be considered. A resemblance is noted by considering that pentobarbital, the hypnotic, is a malonylurea, while diphenylhydantoin, the anticonvulsant, is a glycolylurea (Appendix 6). Another compound, 5-ethyl-5 (1, 3-dimethylbutyl)barbituric acid, which is closely related to pentobarbital, regularly produces seizures (96). These phenomena point again to the importance of considering spatial and electrostatic characteristics of molecules in the study of nervous tissues.

Diphenylhydantoin Na and cocaine HCl

Both diphenylhydantoin Na and cocaine HCl were found in this study to cause decreases in calculated negative charge density in cerebral white matter (Tables 15 and 16). The cortex data is less extensive, and no comparison of the relative effects on the two tissues can be made.

The cerebral white matter findings in the diphenylhydantoin experiments concur with the studies indicating that the drug acts upon axonal conduction pathways to prevent seizure spread.

Negative shifts in F_q associated with the application of cocaine to cerebral white matter surfaces were not distinguishable from those of diphenylhydantoin. A parallelism between these two drugs is also notable pharmacologically. Both are agents which block nerve conduction (89).

The similarity of electrometric results occurred with the two drugs despite the apparent chemical dissimilarities (Figure 9). Quantitative relationships on a molar basis have not been determined. They would depend upon (1.) determination of minimally effective

concentrations of drug, and (ii.) statistical tests of electrometric results.

The finding that both cocaine and diphenylhydantoin are associated with a lowered calculated χ in sulfated polygalactose gels indicates that the measured change is not a metabolic one, though neurometabolic effects have been reported with both drugs (97).

A number of reactions between organic ions and tissues or tissue constituents have been reported. Some of these will be cited here to support the hypothesis that such reactions occur between brain and cocaine, diphenylhydantoin, and probably other molecules as well.

Interactions of biologic amines with brain and anionic brain fractions have been demonstrated by several different techniques (3, 38). The lipid-solvent solubilities of serotonin, histamine, acetylcholine, norepinephrine, and strychnine have been shown to be substantially increased by sulfatides and/or certain phospholipids (38).

Incubation of brain slices with protamine is associated with a loss in excitability. Excitability is restored by addition of ganglioside, an anionic sphingolipid, and by suramin, an anionic trisulfonated polycyclic drug (93). Austin (3) reported that a small concentration of protamine blocks and reverses the metachromasia of a sulfatide spot on filter paper.

Joseph, Engel, et al. (51) noted similar phenomena with organic cations in connective tissues. They observed reduction in χ upon the application of small concentrations of procaine, protamine, histamine, and other amines to tissue surfaces.

Mechanisms

These findings all endorse the concept of the formation of a

salt-like linkage which unites the cationic amino group of the biologic amine to anionic sites of nervous tissues and their extracts.

A salt-linkage complex can readily account for the apparent reaction of cocaine with the anionic groups of white matter and sulfated polygalactose gel observed electrometrically. The cationic amine of cocaine HCl would unite with fixed anions of the gels. Such reactions would qualitatively explain the experimental finding of a lowered fixed anionic charge reported in this paper.

An explanation of this type will not suffice for diphenylhydantoin, which is itself anionic. A physical interaction between brain tissue and diphenylhydantoin has recently been reported, however. It is cited here because it may be a manifestation of the same phenomenon observed electrometrically with diphenylhydantoin.

Diphenylhydantoin was shown by radio-assay to concentrate in cat brain to an unusual extent (30, 78). Brain levels of the drug were stated to be 10 times the concentration of diffusible drug in plasma. "Somewhat greater" concentrations of drug were said to have occurred in white matter than in gray matter. Ultrafiltrates of brain and plasma had concentrations equal to that in cerebrospinal fluid. Relative levels of drug in plasma solids and brain solids were not described.

Joseph, Engel, et al. (25, 54) have found a number of anionic aromatic compounds which produce a paradoxical lowering of calculated negative charge in a manner resembling diphenylhydantoin. The report that the anion, pentobarbital, may lower the ionization ratio of sulfhydryls in brain may also be an example of this apparent paradox.

Picrate (Appendix 6) is reported to be one of the most effective anions in the paradoxical lowering of χ. The explanation offered by Engel, Catchpole and Joseph (25) for this action of picrate and other

anions can be summarized in two steps. Let P represent a polyionic colloid with positive as well as negative groups. Let A represent the anionic additive, e.g., picrate.

(i.) A salt linkage is formed between the anionic (phenolic) group of the additive (picrate) and a fixed positive amino.

$$P^{+} + A^{-} = AP^{-} \quad (25)$$

(ii.) Hydrogen bonds are formed. In this process electronegative side groups of the additive (nitro groups of picrate) are required. These groups are said to coordinate through hydrogen bonds with anionic side groups of the colloid. This coordination would "fix" hydrogens between the side groups of the additive and the side groups of the colloid. The result is conjectured to be a net decrease in dissociable anionic groups.

$$AP^{--} + 2\,H^{+} = APH_2 \quad (25)$$

Diphenylhydantoin possesses a single keto side group which might serve as an electronegative coordinator in hydrogen bonding (53) (Appendix 6). However, the presence of only one coordinating group should limit this action to the formation of a single hydrogen bond per molecule of drug. This would not readily account for a net decrease in negative charge density. Diphenylhydantoin was seen to produce this effect in an inanimate gel containing no known amino groups with which to form salt linkages. A possible source of fixed cation is a double salt linkage between carrageenin or agar and diphenylhydantoin. It would rely upon native (104) Ca^{++} and Mg^{++} of the seaweeds to serve as its nexus.

$$(\text{polygalactose sulfate})\text{-}Ca^{+} + (\text{diphenyl-hydantoin})^{-} = Ca\begin{matrix}(\text{polygalactose sulfate}) \\ (\text{diphenyl-hydantoin})\end{matrix}$$

Interesting relationships have been found between certain cations and the two drugs cocaine and diphenylhydantoin. The few relationships with Ca++ presented below are nonspecific and diverse. They are not meant to compare the two drugs directly. They are presented to show drug - tissue - ion interactions on three levels of investigation — (i.) physiological, (ii) electrophysiological, and (iii.) physicochemical.

(i.) Ca++ depletion in peripheral nerve produces hyperexcitability. This may be reversed with diphenylhydantoin (70).

(ii.) Ca++ depletion causes a marked increase in electrical resistance through the myelin sheath (92). Cocaine in relatively high concentrations gives similar changes.

(iii.) Cocaine substantially increases the surface pressure of a monolayer of lipoidal nerve extracts. Addition of Ca++ reverses this effect (87).

These and other examples tell little about the relative specific modes of actions of the two drugs. They may suggest common neural mechanisms are influenced on a physicochemical level. The present electrometric study offers evidence supporting the existence of such a mechanism in vivo. The evidence presented in this thesis indicates that whatever is the nature of this mechanism, it is indigenous to the tissue structure.

For directness and for simplicity the results in the present study have been interpreted tentatively in terms of colloidal charge

densities. Among the other factors which have been considered in the interpretation of E_d and of the changes in E_d are:

(i.) the mobilities of the prominent ions in the colloidal surface.

(ii.) the activities of prominent ions in the colloidal surface. This would include:

a. the possible influences on activity coefficients of mechanisms other than changes in charge density. Such mechanisms might include steric, electrostatic, and hydrational factors which could affect the colloidal matrix, the solvent atmosphere, and the co-ions of a fixed-charge system.

b. the possible influences on cation concentrations in the colloidal surface. Such mechanisms may include dissociation constants of cation-colloid complexes.

(iii.) the direct or indirect effects related to the heterogeneous biological surface which participates in the formation of the recording liquid junction; other liquid junction effects.

(iv.) mechanisms which may include all four of the above and perhaps other, more fundamental, concepts which are unknown or are beyond the scope of this paper.

SUMMARY AND CONCLUSIONS

I. Colloidal charge densities of living nervous tissues have been determined by an electrometric method. The limitations and interpretations of the method and of the results have been discussed.

II. A negative charge density in myelinated tissues was found to be .06 equiv./kg. tissue water. This figure was obtained from both cerebral white matter and dorsal funiculus of the spinal cord of the cat.

A negative charge density in cerebral cortex was found to be .045 equiv./kg. tissue water.

III. Certain procedures and certain drugs and other additives are found to alter the electrometric results. One of the most useful features of the electrometric method appears to be its ability to detect changes effected by minute concentrations of drug.

(i.) Both .0001 M. cocaine HCl and < .0001 M. (saturated) diphenylhydantoin Na lower the calculated negative charge density of cerebral white matter *in vivo* and of sulfated polygalactose gel *in vitro*. Diphenylhydantoin may also lower the negative charge density of cerebral cortex *in vivo*.

(ii.) Death due to exsanguination lowers the calculated value of negative charge density in cerebral white matter, probably within 30 minutes.

(iii.) Pentobarbital Na appears to elevate calculated negative charge densities of nervous tissues. This drug was evaluated both topically and parenterally.

(iv.) No effect was detected electrometrically at the cerebral

cortex with

(a.) application of .0001 M. ouabain, or

(b.) intravenous doses of gallamine triethiodide, >1 mg./kg. cat.

(v.) Certain metal ions such as Cu^{++}, Mg^{++}, and Pb^{++} appeared to lower the calculated negative charge densities of nervous tissues. These effects need further study in relationship to the electrometric method.

IV. A parameter of tissue ultrastructure has been studied. The results with this newly-adapted method may be variously interpreted. The results suggest that this parameter may have a close relationship to the functional characteristics of the nervous system. The concept of fixed charge density may be one simplification of more complex phenomena.

APPENDIX 1a.

$-E_d$ VALUES FROM CEREBRAL WHITE MATTER OF THE CAT, IN -mV.

PAIRED DATA: DIPHENYLHYDANTOIN Na vs. CONTROL

Reservoir method of application of solutions. Pentobarbital Na anesthesia. Data grouped according to (i.) time (T = 60 min.) interval since initial pentobarbital injection: (ii.) anatomic location of reservoir in white matter surface (frontal vs. occipital).

		CONTROL					DIPHENYLHYDANTOIN Na				
cat	surface location	T_1	T_2	T_3	T_4	means	means	T_1	T_2	T_3	T_4
A	front.	3.4	4.4	5.7	5.7	4.8	5.6	4.3	5.7	6.7	5.6
	occip.	4.4	4.6	5.2	4.5	4.7	5.8	4.5	6.7	6.2	5.7
B	front.	5.1	5.4	5.3	4.9	5.2	6.0	5.8	6.2	6.5	5.4
	occip.	3.6	4.6	5.0	4.4	4.4	7.0	6.4	7.3	6.9	7.2
C	front.	4.2	3.5	2.6	3.5	3.5	4.4	5.4	3.9	4.7	3.5
	occip.	4.1	4.5	3.1	3.1	3.7	5.5	6.0	5.1	5.4	5.4
D	front.	2.5	5.3	4.0	4.7	4.1	4.1	1.3	4.4	5.2	5.5
	occip.	5.1	5.9	4.9	5.0	5.2	4.8	3.6	5.6	4.4	5.4
E	front.	4.3	7.3	7.7	6.4	6.4	6.8	5.6	7.5	6.6	7.6
	occip.	4.0	5.0	3.9	5.0	4.5	4.9	5.0	5.3	4.0	5.2
F	front.	2.3	2.8	3.0	3.6	2.7	5.1	4.9	5.1	4.8	5.5
	occip.	3.8	3.4	3.8	4.4	3.9	5.0	3.4	5.2	5.5	5.7
means		3.9	4.7	4.5	4.6	4.4	5.3	4.7	5.7	5.6	5.6

mean, frontal: 4.4
mean, occipital: 4.4

mean, frontal: 5.3
mean, occipital: 5.3

APPENDIX 1b.

Analysis of Variance of cerebral white matter $\bar{F}_d$'s: diphenylhydantoin vs. control/time

Source	Sum of Squares	df	Mean Square	F ratio
between drug and control:	21.94	1	21.94	30.09**
between Ts:	12.64	3	4.21	5.76**
interaction: (drug x T):	0.65	3	0.22	0.30
overall between:	35.23	7	5.03	6.89**
between drug-control pairs:	56.23	11	5.11	7.00**
within:	56.27	77	0.73	
total:	147.73	95		

* significant, $F_{.05}$
** significant, $F_{.01}$

Therefore conclude that the data show:

1. $\bar{F}_d$'s obtained with diphenylhydantoin at the recording surface differ from control $\bar{F}_d$'s.
2. differences between $\bar{F}_d$'s obtained at the different approximate time intervals (T = 60 min.) after starting experiment.
3. no drug or time effects that are not additive.
4. differences between drug-control pairs not attributable to anatomic location of recording junction (frontal vs. occipital).

APPENDIX 2.

Values of $\bar{E}_d$ obtained from cat preparation. Respiration ceased at 1750 hours after the common carotid arteries were severed. Results are expressed as negative millivolts.

		lte. frontal	lte. occip.	rte. front.	rte. occip.	means
LIVING:	- 5	4.8	6.0	4.5	5.0	5.1
	- 4	5.5	5.8	5.9	4.5	5.4
	- 3	4.2	5.6	5.2	4.1	4.8
	- 2	5.5	5.5	5.9	4.9	5.4
	- 1	4.9	5.3	5.8	5.6	5.4
	0	carotids cut, respiration ceased				
DEAD:	+ 1	5.6	5.8	6.6	6.0	6.0
	+ 2	7.4	7.4	7.3	6.7	7.2
	+ 3	5.8	6.7	6.2	6.4	6.3
	+15	3.8	5.0	4.8	5.2	4.7
	+16	4.6	5.2	5.6	6.2	5.4
	+17	6.1	6.7	6.5	6.7	6.5
means:						
LIVING/DEAD:		5.0/5.6	5.6/6.1	5.5/6.2	4.8/6.2	5.2/6.0 (mV)
colloidal charge densities:						.035/.031 (eq./kg.)

Standard deviations: LIVING/DEAD = .56/.92 (mV)

Degrees of freedom: 41 $t = 3.44^{**}$

** significant, $t_{.01}$

APPENDIX 2.

Table for preparations of 1 liter of saline buffers. Solutes approximate 0.15 M. NaCl at 7 different pH levels.

pH soln.	NaCl 3.00 M.	HCl 0.61 M.	Na-Ac facial	Na-Ac 0.5 M.	NaH_2PO_4 .1 M. H_2O 10% w/v	Na_2HPO_4 5% w/v	$NaHCO_3$ 5% w/v	Na_2CO_3 1% w/v
2.2	47.50	12.36						
2.9	49.50	2.48						
3.7	49.34		0.86	3.6				
4.6	45.00		0.86	30.0				
6.0	45.00				14.80	6.06		
7.4	45.00				1.88	19.38		
8.7	45.00						22.67	8.00

APPENDIX 4a.

Analysis of Variance of agar-gelatin gel $\bar{E}_d$'s: (cocaine vs. diphenylhydantoin Na vs. control)/pH.

Source	Sum of Squares	df	Mean Square	F ratio
between 3 drug groups:	15.51	2	7.76	6.99**
between pH intervals:	57.38	6	9.56	8.61**
interaction (drug x pH):	11.10	12	0.93	0.84**
overall between:	83.99	20	4.20	3.78**
within:	116.96	105	1.11	
total:	200.95	125		

Therefore conclude that the data show:

1. $\bar{E}_d$'s obtained with one or both drugs differ from control $\bar{E}_d$'s.
2. differences among $\bar{E}_d$'s at the various pH levels.
3. no drug or pH effects that are not additive.

* significant, $F_{.05}$
** significant, $F_{.01}$

APPENDIX 4b.

Analysis of Variance of cerebral white matter $\bar{E}_d$'s: (cocaine vs. control)/pH.

Source	Sum of Squares	df	Mean Square	F ratio
between drug and control:	26.72	1	26.72	7.28*
between pH intervals:	135.14	6	22.52	6.14**
interaction (drug x pH):	9.06	6	1.51	0.41
overall between:	170.92	13	13.15	3.58**
between cats:	24.78	2	12.39	3.38*
within:	95.41	26	3.67	
total:	291.11	41		

Therefore conclude that the data show:

1. $\bar{E}_d$'s obtained with cocaine · HCl at the recording surface differ from control $\bar{E}_d$'s.
2. differences among $\bar{E}_d$'s at the various pH levels.
3. differences associated with the cat preparation or the tissue surface employed.
4. no drug or pH effects that are not additive.

* significant, $F_{.05}$
** significant, $F_{.01}$

APPENDIX 5a.

$\Delta\bar{E}_d$'s from Cerebral Cortex and Dorsal Funiculus of the Cat. Figures are coded: cell value = $\Delta\bar{E}_d$ (mV) + 10. $\Delta\bar{E}_d = \bar{E}_d$ (Me++) - $\bar{E}_d$ (control).

Analysis of Variance - $\Delta\bar{E}_d$'s from 6 cats, cortex vs. cord.

Source	Sum of Squares	df	Mean Square	F ratio
between Me++#:	107.67	7	15.38	4.85**
between cortex and cord:	10.27	1	10.27	3.24
interaction (Me++ x tissue):	16.15	7	2.31	.73
overall between:	134.09	15		2.82**
between cats:	107.53	5	21.51	6.78**
within:	237.95	75	3.17	
total:	479.57	95		

Therefore conclude that the data show:

1. differences among $\Delta\bar{E}_d$'s associated with Me++ applications.
2. differences among $\Delta\bar{E}_d$'s from the various cat preparations.
3. no difference between $\Delta\bar{E}_d$'s: cortex vs. cord.

* significant, $F_{.05}$
** significant, $F_{.01}$
\# (Me++ refers to a solution of Ba++, Cu++, Mg++, Ca++, Mg++, Pb++ as .02 N. chloride in .13 N. NaCl. Also included in this analysis are EDTA•2 Na, pH 4.6 and a pH 7.4 control. $\Delta\bar{E}_d$ is the displacement in $\bar{E}_d$ when Me++ is applied to the tissue surface).

APPENDIX 5b.

Redistribution of data from same series of cat experiments in order to relate differences between cats to change in anesthetic procedure.

		tetracaine – ethylene								pentobarbital						
		Ca++	Ba++	Mg++	Cu++	Hg++	Pb++	sums	sums	Ca++	Ba++	Mg++	Cu++	Hg++	Pb++	
cortex	cat A	7.8	9.0	6.5	7.0	8.9	9.6	48.8	55.3	11.4	7.8	9.6	6.2	10.0	10.3	cat E
	cat B	9.2	9.0	9.2	5.8	9.6	8.6	51.4	47.2	7.5	4.3	8.9	8.3	10.2	8.0	cat F
	cat C	8.9	7.7	7.3	6.9	8.6	9.6	49.0	41.7	4.2	6.1	7.4	7.0	10.3	6.7	cat G
	cat D	11.3	11.8	9.3	6.2	11.3	9.3	59.2	38.8	2.9	8.9	8.1	7.3	5.7	5.9	cat H
cord	cat A	8.4	9.6	5.5	7.3	10.4	6.6	45.1	45.9	9.0	9.9	8.1	5.2	8.0	5.6	cat K
	cat B	6.9	9.0	6.7	6.4	9.2	4.1	42.3	39.6	4.3	7.4	5.8	9.0	5.3	7.8	cat F
	cat C	8.7	6.8	6.5	3.3	6.3	5.9	37.5	40.9	7.9	5.8	6.0	5.7	6.7	8.8	cat G
	cat D	9.2	9.8	7.9	7.2	12.1	7.2	53.4	41.0	4.0	8.6	7.6	8.3	7.6	4.9	cat H
	means	8.80	8.75	7.36	6.26	9.55	7.61			6.40	7.35	7.69	7.13	7.98	7.25	means
	means, mV	-1.2	-1.2	-2.6	-3.7	0.0	-2.4			-3.6	-2.6	-2.3	-2.9	-2.0	-2.8	means, mV

APPENDIX 5a.

Analysis of Variance - ΔE_d's, Ne++ additives/anesthetic procedure.

Source	Sum of Squares	df	Mean Square	F ratio
between Ne++:	37.98	5	7.60	3.16*
between anes.:	17.97	1	17.97	7.46**
interaction (Ne++ x anes.):	26.76	5	5.35	2.26
overall between:	82.71	11	7.52	3.12**
between surfaces:	102.02	15	6.81	2.83**
within:	166.23	69	2.41	
total:	350.96	95		

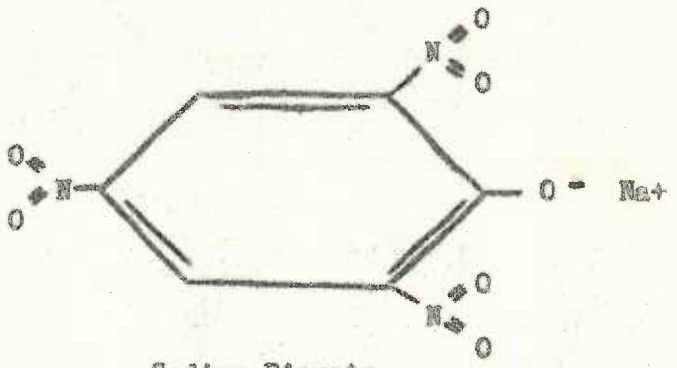
Therefore conclude that the data show:

1. difference(s) among the effects of Ne++ applications.
2. differences between ΔE_d's obtained with the two anesthetic procedures.
3. differences among tissue surfaces not attributable to cord vs. cortex, but partly to anesthetic effect.
4. no anesthetic of Ne++ effects that are not additive.

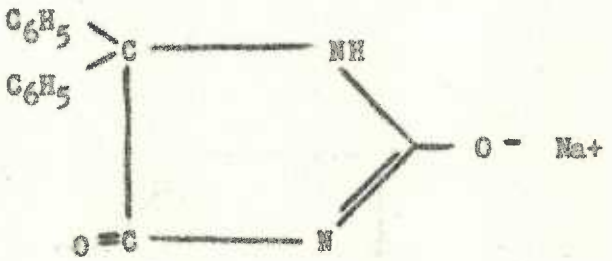
* significant, $F_{.05}$
** significant, $F_{.01}$

Appendix 6

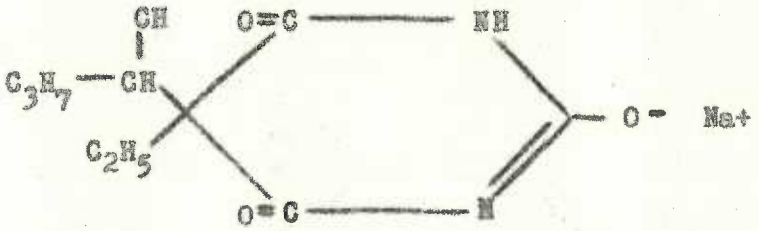
STRUCTURAL FORMULAS OF PERTINENT COMPOUNDS *



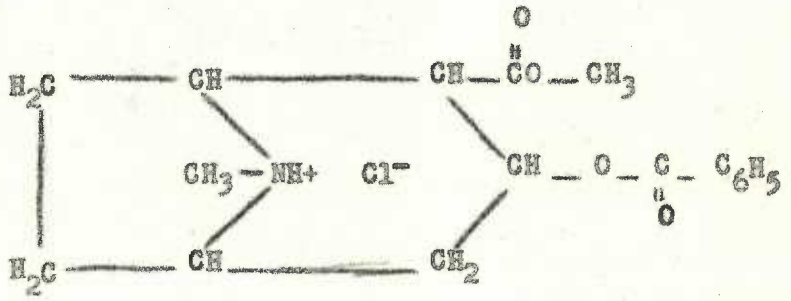
Sodium Picrate



Diphenylhydantoin Sodium



Pentobarbital Sodium



Cocaine Hydrochloride

* Merck Index., 1960.

BIBLIOGRAPHY

1. Abood, L.G., & Abul-Haj, S.K. Histochemistry and characterization of hyaluronic acid in axons of peripheral nerve. J. Neurochem., 1956, 1, 119.

2. Anderson, P.J., & Rethics, B.A. Surface chemistry of synthetic lecithin. In Popjak, G., & LeBreton, E. (Eds.) Biochemical problems of lipids. New York: Interscience Publishers, 1956.

3. Austin, J.H. Metachromatic form of diffuse cerebral sclerosis. 2. Diagnosis during life by isolation of metachromatic lipids from urine. III. Significance of sulfatide and other lipid abnormalities in white matter and kidney. Neurology, 1957, 7, 716 and 1960, 10, 470.

4. Austin, J.H. Metachromatic sulfatides in cerebral white matter and kidney. Proc. Soc. Exptl. Biol. Med., 1959, 100, 361.

5. Bergeron, J.A., & Singer, M. Metachromasy: an experimental and theoretical reevaluation. J. Biophys. Biochem. Cytol., 1958, 4, 433.

6. Blix, G. The sulfur-containing lipoids of the brain. Cerebronsulfuric acid. Z. Physiol. Chem., 1933, 219, 82; Chem. Abstracts, 1933, 27, 4549.

7. Boyd, E.S., & Neuman, W.F. The surface chemistry of bone. V. The ion-binding properties of cartilage. J. Biol. Chem., 1951, 193, 243.

8. Bradley, D.E., & Wolff, M. Aggregation of dyes bound to polyanions. Proc. Natl. Acad. Sci. U. S., 1959, 45, 944.

9. Brante, G. Mucopolysaccharides and mucoids of the nervous system. 4th Intern. Congr. Biochem., 1959, XII, 291.

10. Buswell, A.M., & Rodebush, W.H. Water. Scientific American, 1956, 4, 77.

11. Calmon, C., & Kressman, T.R.E. Ion exchange resins in organic and biochemistry. New York: Interscience Publishers, 1957.

12. Catchpole, H.R. Role of the ground substance in wound healing. In Williamson, M.B. (Ed.) The healing of wounds. New York: Blakiston, 1957.

13. Catchpole, H.R., Joseph, H.R., & Engel, M.B. Homeostasis of connective tissues. III. Magnesium-sodium equilibrium and interactions with strontium and lead. A.M.A. Arch. Pathol., 1956, 61, 503.

14. Christensen, H.N., & Hastings, A.B. Phosphatides and inorganic salts. J. Biol. Chem., 1940, 136, 387.

15. Condouris, G.A. A study on the mechanism of action of cocaine on amphibian peripheral nerve. J. Pharmacol. Exptl. Therap., 1961, 131, 243.

16. Conway, E.J. Principles underlying the exchanges of K and Na ions across cell membranes. J. Gen. Physiol., 1960, 43, 17.

17. Derviahian, D.G. Electrochimie des acides gras et des lipides ioniques. In Popjak, G., & LeBreton, E. (Eds.) Biochemical problems of lipids. New York: Interscience Publishers, 1956.

18. Dill, W.A., Kaserman, A., Wolf, L.M., & Glaser, A.J. Studies on 5,5 diphenylhydantoin (dilantin) in animals and man. J. Pharmacol. Exptl. Therap., 1956, 118, 270.

19. Drinker, H., & Zinsser, H.H. The equilibrium between calcium and cephalin in various systems. J. Biol. Chem., 1943, 148, 187.

20. Dixon, W.J., & Massey, F.J. Introduction to statistical analysis. (2d Ed.) New York: McGraw-Hill, 1957.

21. Duncan, D.B. A significance test for differences between ranked treatments in an analysis of variance. Virginia J. Sci., 1951, 2, 171.

22. Dunstone, J.R. Ion-exchange reactions between cartilage and various cations. Biochem. J., 1960, 77, 164.

23. Edwards, L.C., & Dunphy, J.E. Wound healing. I. Injury and normal repair. II. Injury and abnormal repair. New England J. Med., 1958, 259, 224 and 275.

24. Elfvin, L.-G. The ultrastructure of unmyelinated fibers in the splenic nerve of the cat. J. Ultrastructure Res., 1958, 1, 428.

25. Angel, M.S., Catchpole, H.R., & Joseph, N.R. Effects of temperature and anions on titration curves of frog muscle. Science, 1960, 132, 669.

26. Engel, M.S., Joseph, N.R., & Catchpole, H.R. Homeostasis of connective tissues. I. Sodium-calcium equilibrium. A.M.A. Arch. Pathol., 1954, 58, 26.

27. Farber, S.J. Mucopolysaccharides and sodium metabolism. Circulation, 1960, 21, 941.

28. Finean, J.B. Electron microscopy of the myelin sheath. Proc. Roy. Soc. Med., 1961, 54, 19.

29. Finean, J.B. The role of water in the structure of peripheral nerve myelin. J. Biophys. Biochem. Cytol., 1957, 3, 95.

30. Firemark, H., Barlow, C.F., & Roth, L.J. Diphenylhydantoin - 2 - C^{14} in cat brain. Fed. Proc., 1961, 20, 329.

31. Fischer, R., Zeman, W., & Irons, I. Differential dye-uptake in excited and non-excited nervous tissue after treatment with papain and neotetrazolium chloride. J. Histochem. Cytochem., 1961, 9, 103.

32. Folch, J. Biochemical problems related to psychiatry. In Psychiatric Research. Cambridge, Mass.: Harvard, 1947.

33. Folch, J. Composition of the brain in relation to maturation. In Biochemistry of the developing nervous system (1st International Neurochemical Symposium). New York: Academic Press, 1954.

34. Folch, J., Lees, M., & Sloane-Stanley, G.H. The role of acidic lipids in the electrolyte balance of the nervous system of mammals. In Richter, D. (Ed.) Metabolism of the nervous system. London: Pergamon, 1957.

35. Geren, B.B. Formation from the Schwann cell surface of myelin in the peripheral nerves of chick embryos. Exptl. Cell Research, 1954, 7, 558.

36. Marsh, I., & Catchpole, H.R. The nature of ground substance of connective tissue. Perspectives in Biol. Med., 1960, III, 282.

37. Glynn, I.M. Action of cardiac glycosides on the red cell. J. Physiol., 1955, 128, 56.

38. Green, J.P., Robinson, J.D., Jr., & Day, M. Interaction between cerebroside sulfate and amines. J. Pharmacol. Exptl. Therap., 1961, 131, 12.

39. Greenfield, J.G., et al. Neuropathology. (1st Ed.) London: Edward Arnold Ltd., 1958.

40. Grundfest, H. Discussion of paper by Schmitt, F.O. Ultrastructure of nerve myelin. In Korey, S. (Ed.) The biology of myelin. New York: Hoeber-Harper, 1959.

41. Gurd, F.R.N. Some naturally occurring lipoprotein systems. In Hanahan, D.J. Lipide Chemistry. New York: Wiley, 1960.

42. Hamer, W.J., & Wood, R.E. Electrolytic conductivity and electrode processes. In Condon, E.U., & Odishaw, H. (Eds.) Handbook of physics. New York: McGraw-Hill, 1958.

43. Hannhan, D.J. Lipide chemistry. New York: Wiley, 1960.

44. Henderson, P. The thermodynamics of liquid cells. Z. Physik. Chem., 1907, 59, 118; Chem. Abstracts, 1907, 1, 2766.

45. Henderson, P. On the thermodynamics of liquid cells. Z. Physik. Chem., 1908, 63, 325; Chem. Abstracts, 1908, 2, 2892.

46. Hitchcock, D.I. Membrane potentials in the Donnan equilibrium. In Shedlovsky, T. (Ed.) Electrochemistry in biology and medicine. New York: Wiley, 1955.

47. Hitchcock, D.I. Selected principles of physical chemistry. In Hober, R. (Ed.) Physical chemistry of cells and tissues. Philadelphia: Blakiston, 1945.

48. Hodgkin, A.L., & Keynes, R.D. Experiments on the injection of substances into squid giant axons by means of a microsyringe. J. Physiol., 1956, 131, 592.

49. Hodgkin, A.L., & Keynes, R.D. The mobility and diffusion coefficient of potassium in giant axons from Sepia. J. Physiol., 1953, 119, 513.

50. Hokin, L.E., & Hokin, M.R. Evidence for phosphatidic acid as the sodium carrier. Nature, 1959, 184, 1068.

51. Joseph, N.R., Catchpole, H.R., Lamkin, D., & Engel, M.B. Titration curves of colloidal surfaces. II. Connective tissues. Arch. Biochem. Biophys., 1959, 84, 224.

52. Joseph, N.R., & Engel, M.B. Titration curves of colloidal surfaces. I. Human epidermis. Arch. Biochem. Biophys., 1959, 85, 209.

53. Joseph, N.R., & Engel, M.B. Personal communication, 1962.

54. Joseph, N.R., Engel, M.B., & Catchpole, H.R. Interaction of ions and connective tissue. Biochem. Biophys. Acta, 1952, 8, 575.

55. Katzman, R. Electrolyte distribution in mammalian central nervous system. Neurology, 1961, 11, 27.

56. Katzman, R., & Wilson, C.E. Extraction of lipid and lipid cation from frozen brain tissue. J. Neurochem., 1961, 7, 113.

57. Kimura, Y., & Nagai, Y. Preparation of hydrophilic colloids of brain kephaline. Biochem. J., 1960, 77/1, 1.

58. Kitchener, J.A. Ion-exchange resins. London: Methuen, 1957.

59. Klotz, I.M. Protein molecules in solution. Circulation, 1960, 21, 628.

60. Klotz, I.M. Metal complexes with proteins and other large molecules. In Barron, E. (Ed.) Trends in physiology and biochemistry. New York: Academic Press, 1952.

61. Klotz, I.M., & Curme, H.G. The thermodynamics of metalloprotein combinations. Copper with bovine serum albumin. J. Amer. Chem. Soc., 1948, 70, 939.

62. Koch, W., & Mike, F.H. Pharmacological studies on the phosphatids. 2. The relation of the phosphatids to the sodium and potassium of the neuron. J. Pharmacol. Exptl. Therap., 1911, 2, 245.

63. Ling, G.N. The interpretation of selective ionic permeability and cellular potentials in terms of the fixed charge-induction hypothesis. J. Gen. Physiol., 1960, 43, 149.

64. MacInnes, D.A. The principles of electrochemistry. New York: Reinhold, 1939.

65. Melamman, R. Diffusion constants in the extracellular space of brain tissue. Neurology, 1958, 8 (Suppl. 1), 67.

66. Mumery, J.F. Inorganic metabolism of the brain. In The biology of mental health and disease. London: Cassell, 1952.

67. Moron, S.H., & Prutton, C.F. Principles of physical chemistry. (3d Ed.) New York: MacMillan, 1958.

68. Mauro, A., & Finkelstein, A. Realistic model of a fixed-charge membrane according to the theory of Teorell, Meyer, and Seivers. J. Gen. Physiol., 1958, 42, 385.

69. Meyer, K., & Rapport, M. The mucopolysaccharides of the ground substance of connective tissue. Science, 1951, 113, 596.

70. Morrell, F., Bradley, W., & Pfeiffer, M. Effect of diphenylhydantoin on peripheral nerve. Neurology, 1958, 8, 140.

71. Mollmain, H. Biochemistry and the central nervous system. (2d Ed.) Boston: Little, Brown & Co., 1959.

72. Overbeek, J. The Donnan equilibrium. Progr. Biophys. Biophys. Chem., 1956, 6, 58.

73. Page, I.H. Thudichum and the chemistry of the brain. In Elliott, K., Page, I.H., & Quastel, J.H. (Eds.) Neurochemistry. Springfield, Ill.: Charles C. Thomas, 1955.

74. Perreira, H.C., & Pethica, B.A. Surface chemistry of the alkyl phosphates. Proc. Intern. Congr. Surface Activity, 2d London, 1957, 1, 141; Chem. Abstracts, 1959, 53, 19518e.

75. Pauling, L. A molecular theory of general anesthesia. Science, 1961, 134, 15.

76. Podolsky, R.J. The structure of water and electrolyte solutions. Circulation, 1960, 21, 618.

77. Rossiter, R.J. Chemical constituents of brain and nerve. In Elliott, K., Page, I.H., & Quastel, J.H. (Eds.) Neurochemistry. Springfield, Ill.: Charles C. Thomas, 1955.

78. Roth, L.J., & Barlow, C.F. Drugs in the brain. Science, 1961, 134, 22.

79. Scatchard, G. Thermodynamics and simple electrostatic theory. In Cohn, E.J., & Edsall, J.T. (Eds.) Proteins, amino acids and peptides. New York: Reinhold, 1943.

80. Scheffé, H. The analysis of variance. New York: Wiley, 1959.

81. Schmidt, G. The biochemistry of inorganic pyrophosphates and metaphosphates. In McElroy, W.D., & Glass, B. (Eds.) Phosphorus metabolism. Vol. 1. Baltimore: Johns Hopkins, 1951.

82. Schmitt, F.O. Ultrastructure of nerve myelin and its bearing on fundamental concepts of the structure and function of nerve fibers. In Korey, S. (Ed.) The biology of myelin. New York: Hoeber-Harper, 1959.

83. Scott, G.H. Mineral distribution in some nerve cells and fibers. Proc. Soc. Exptl. Biol. Med., 1940, 44, 397.

84. Shanes, A.M. Electrochemical aspects of physiological and pharmacological action in excitable cells. I. The resting cell and its alteration by extrinsic factors. Pharmacol. Reviews, 1958, 10, 59.

85. Sheppard, C.W. New developments in potassium and cell physiology. Science, 1951, 114, 85.

86. Silber, R.H., & Schmitt, F.O. The role of free amino acids in the electrolyte balance of nerve. J. Cell. Comp. Physiol., 1940, 16, 247.

87. Skou, J.C. Local anesthetics. VI. Relation between blocking potency and penetration of a monomolecular layer of lipids from nerves. Acta Pharmacol. Toxicol., 1954, 10, 325.

88. Sloane-Stanley, G.H. Lipids of the central nervous system. Biochem. Soc. Symps., 1952, 8, 44.

89. Sollmann, T. A manual of pharmacology. (8th Ed.) Philadelphia: Saunders, 1957.

90. Solomon, S., & Tobias, J.M. Influence of direct current on the long axis distribution of K, Ca, and Na in dog nerve. Biol. Bull., 1951, 101, 198.

91. Steinbach, H.B. The sodium and potassium balance of muscle and nerve. In Barron, E. (Ed.) Trends in physiology and biochemistry. New York: Academic Press, 1952.

92. Tasaki, I. Nervous transmission. Springfield, Ill.: Charles C. Thomas, 1953.

93. Thomson, C.C., & McIlwain, H. An attachment of proteines to cerebral tissues, studied in relation to gangliosides, survain and tissue excitability. Biochem. J., 1961, 79, 342.

94. Tobias, J.M. On ultrastructure and function in nerve. In Barron, E. (Ed.) Trends in physiology and biochemistry. New York: Academic Press, 1952.

95. Tobias, J.M., & Nelson, P.G. Structure and function in nerve. In Zirkle, R.E. (Ed.) A symposium on molecular biology. Chicago: University of Chicago Press, 1959.

96. Tower, D.B. Neurochemistry of epilepsy. Springfield, Ill.: Charles C. Thomas, 1960.

97. Toman, J.E.P. Neurotropic drugs. In Elliott, K., Page, I.H., & Quastel, J.H. (Eds. Neurochemistry. Springfield, Ill.: Charles C. Thomas, 1955.

98. Tuqan, N.A., & Adams, C.W.M. Histochemistry of myelin. I. Proteins and lipid-protein complexes in the normal sheath. J. Neurochem., 1961, 6, 327.

99. Ulett, G., Dow, R.S., & Larsell, O. The inception of conductivity in the corpus callosum and the cortico-ponto-cerebellar pathway of young rabbits with reference to myelinization. J. Comp. Neurol., 1944, 80, 1.

100. Ungar, G. Les phenomenes chimiques de l'excitation cellulaire. Leur role dans le mecanisme de l'excitation. J. Physiologie, 1957, 49, 1235.

101. Ungar, G., Aschheim, E., Psychoyos, S., & Romano, D.V. Reversible changes of protein configuration in stimulated nerve structures. J. Gen. Physiol., 1957, 40, 635.

102. Ungar, G., & Romano, D.V. Sulphydryl groups in resting and stimulated rat brain; their relationship with protein structure. Proc. Soc. Exptl. Biol. Med., 1958, 97, 324.

103. Ussing, H.H. Ionic movements in cell membranes in relation to the activity of the nervous system. 4th Intern. Congr. Biochem., 1959, III, 1.

104. West, E.S., & Todd, W.R. Textbook of biochemistry. (3d Ed.) New York: Macmillan, 1961.

105. Whistler, R.L., & Corbett, W.M. Polysaccharides. In Pigman, W. (Ed.) The carbohydrates. New York: Academic Press, 1957.

106. Widdowson, E.M., & Dickerson, J.W.T. The effect of growth and function on the chemical composition of soft tissues. Biochem. J., 1960, 77, 30.

107. Wittcoff, H. The phosphatides. New York: Reinhold, 1951.

108. Winicki, G.B., & Singer, M. Basophilic and metachromatic staining of myelin sheaths and its possible association with a sulfatide. J. Comp. Neurol., 1950, 92, 71.

109. Woodbury, D.M., Koch, A., & Vernadakis, A. Relation between excitability and metabolism in brain as elucidated by anticonvulsant drugs. Neurology, 1958, 8 (Suppl. 1), 113.

110. Yoshida, H., Nukada, T., & Fujisawa, H. Effect of ouabain on ion transport and metabolic turnover of phospholipid of brain slices. Biochim. Biophys. Acta, 1961, 48, 611.

111. Zanchetti, A., & Brookhart, J. Measurement of electrical responsiveness of cortico-spinal efferents in cat and monkey. J. Neurophysiol., 1955, 18, 288.