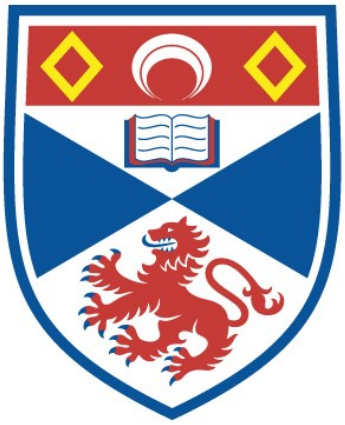
SOME EFFECTS OF ANTICONVULSANT DRUGS ON SUGAR UPTAKE BY CEREBRAL CORTEX SLICES

Peter Gray

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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SOME EFFECTS OF ANTICONVULSANT DRUGS

ON SUGAR UPTAKE

BY CEREBRAL CORTEX SLICES

A thesis presented by Peter Gray

to the University of Saint Andrews

in application for the degree of Doctor of Philosophy.

ABSTRACT

'SOME EFFECTS OF ANTICONVULSANT DRUGS ON SUGAR UPTAKE BY CEREBRAL CORTEX SLICES'

Xylose, a sugar not metabolised by brain, and glucose may be transported into brain by a common mechanism (Gilbert, 1965). In the present studies the effects of some anticonvulsant drugs on the uptake of xylose by the non-raffinose compartment of cerebral cortex slices have been examined.

The slices were pre-incubated for 30 minutes in oxygenated bicarbonate media, containing pyruvate as nutrient, and raffinose, before transfer to similar media containing xylose.

Employing an incubation time of 9 minutes, and a xylose concentration in the medium of 50 mM, it was found that the anticonvulsant drugs acetazolamide, 20 μM, and ethosuximide, 500 μM, caused an increase in xylose uptake, while diphenylhydantoin, 100μM, was without effect in this respect. -SH group blocking agents (5,5- dithiobis (2-nitobenzoic acid), 100μM, or iodoacetamide, 100μM), did not appear to affect 'basal' xylose uptake by the slices, but prevented stimulation of xylose uptake by acetazolamide or ethosuximide.

Studies of the initial velocity of xylose uptake by the slices, over a range of medium xylose concentrations, indicated that the xylose uptake process appeared to conform to Michaelis-Menten kinetics; the apparent Km (under control conditions) for xylose being 87 mM, and the Vmax. being 30 millimole. liter of intracellular water^{-1}.minute^{-1}. Acetazolamide increased both the Km and Vmax. of the xylose transport process. Both these parameters were decreased by ethosuximide. The effect of phenobarbitone on the kinetics of xylose uptake by cerebral cortex slices has been found (Gilbert, Ortiz, and Millichap, 1966) to resemble that recorded here for ethosuximide.

The anticonvulsants which have been shown to be capable of increasing xylose uptake by brain slices also increased brain glucose content 'in vivo'

(Gilbert, Gray, and Heaton, 1971), - probably as a consequence of a stimulation of glucose transport into brain rather than a depression of cerebral metabolism. The uptake of glucose may, under certain conditions, be the rate - limiting factor in its metabolism by brain (Joanny, Corriol, and Hillman, 1969; Buschiazzo, Terrell, and Regen, 1970). Glucose may also have a direct stabilizing effect on brain cells (Coldman and Good, 1969). The stimulation of sugar uptake brought about by the drugs might thus contribute to their anticonvulsant effect However, the interaction of these drugs with cerebral membranes may, in addition to affecting permeability to sugars, result in other effects more directly resposible for their anticonvulsant efficacy.

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Declaration

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been previously presented for a higher degree.

The research which provides the basis of the present thesis was conducted, under the direction of Dr. J.C. Gilbert, at the Department of Biochemistry of the University of Saint Andrews.

Certificate

I hereby certify that Peter Gray has spent nine terms in research work under my direction, that he has fulfilled the conditions of Ordinance General No. 12 and Resolution of the University Court (1967) No 1, and that he is qualified to submit this thesis for the degree of Doctor of Philosophy.

Academic Record

I was awarded the degree of Batchelor of Science, with Second Class Honours (Division Two) in Biochemistry, by the University of Saint Andrews in June, 1967. I first matriculated as a research student at the University of Saint Andrews in November, 1967.

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The author wishes to thank all those who gave assistance and advice during the planning and execution of the work described in this thesis, and, in this context, wishes particularly to express his gratitude to Dr. J.C. Gilbert.

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SOME EFFECTS OF ANTICONVULSANT DRUGS ON SUGAR UPTAKE BY CEREBRAL CORTEX SLICES

CHAPTER I

PART 1 - THE PRESENT INVESTIGATION IN THE CONTEXT OF PREVIOUS STUDIES

PART 2 - THE DESIGN OF THE PRESENT INVESTIGATION

Part 1 - The Present Investigation in the Context of Previous Studies

In the course of the present studies, some effects upon the 'in vitro' metabolism of cerebral cortex, of three anticonvulsant drugs (diphenylhydantoin, ethosuximide, and acetazolamide) have been examined.

The aim of these studies was to contribute towards a fuller understanding of the mechanism of action of anticonvulsant drugs. Before proceeding to a discussion of the present work, therefore, a brief summary will be given of the events occuring in the brain, leading to the development of seizure activity, and of the ways in which anticonvulsant drugs may achieve control of such activity.

The origin of 'spontaneously' occuring seizure activity lies, in most instances, in the abnormal behaviour of neurons in a small area of the brain. These aberrant neurons of the 'epileptogenic focus' may, from time to time, produce spontaneous, prolonged, high frequency bursts of 'spike' discharges.

If the spontaneous discharges of the epileptogenic focus are of sufficient intensity and duration, the activity may spread, by the normal processes of transmission to both adjacent and distant normal neurons, thus leading to the development of an epileptic seizure.

A number of drugs are used clinically in the control of epilepsy. The chemical formulae of some of those commonly employed are shown in Fig. I, page 2 . There is a marked similarity of structure between many of the anticonvulsant drugs. This structural affinity suggests that the drugs may have a common mechanism of action. The anticonvulsant drugs do, however, differ from one another in the effectiveness with which they control the various types of epilepsy.

Consideration of the sequence of events in the brain leading to the development of seizure activity suggests that there are three principal sites at which the anticonvulsant drugs might act. The possibilities are:-

(1) Action upon abnormally functioning neurons to prevent hyperactivity.

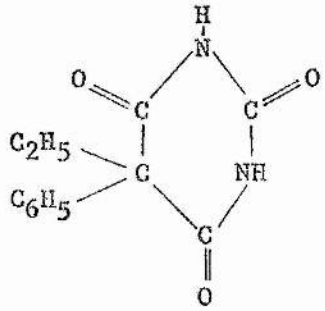
(2) Action upon the surrounding normal tissue to prevent the spread of seizure activity.

(3) Action upon non-neural systems to produce an environment in the central nervous system unfavourable to seizure activity.

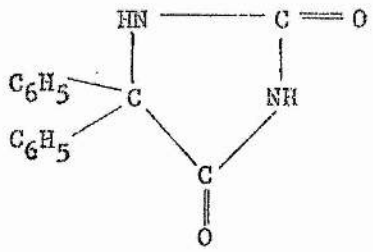
With regard to some of the anticonvulsants employed in the control of epilepsy, it has been suggested that acetazolamide may exert its/

FIGURE 1

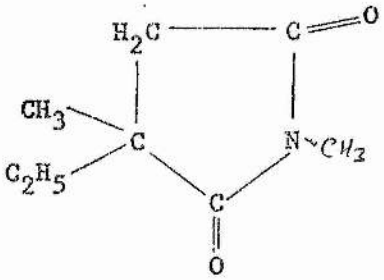
SOME ANTICONVULSANT DRUGS



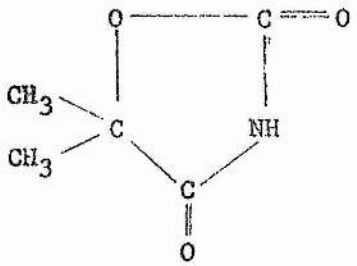
phenobarbitone.



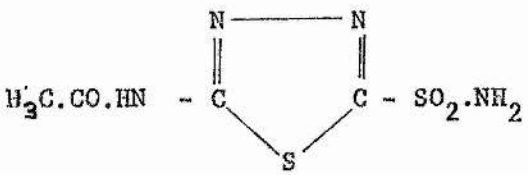
diphenylhydantoin.



ethosuximide.



dimethadione.



acetazolamide.

its anticonvulsant action by depressing the activity of the neurons of the epileptogenic focus. (Bowman, Read, and West. (1968) (3)). Phenobarbitone may well have a stabilising effect on abnormal, as well as on normal, neurons (Tower. (1960)). However the anticonvulsant drug diphenylhydantoin does not appear to act directly upon the abnormal, epileptogenic area, but prevents the stimuli from this area from exciting normal surrounding neurons. Thus with this drug, clinical seizure control is often not associated with the suppression of epileptiform activity in the electroencephalogram (Tower. (1960) (54)).

The site of the anticonvulsant action of trimethadione has been the subject of conflicting reports (Tower. (1960) Morell, Bradley and Ptashne (1959) (44)). On balance the bulk of the evidence would seem to suggest that the major anticonvulsant action of this drug is via an effect on normal neurons rather than via an effect on epileptogenic foci.

The observed effects of anticonvulsant drugs on the metabolism of normal cerebral tissue, and on its response to electrical stimulation in vitro, suggests that the stabilisation of normal neurons is a major factor in the actions of most anticonvulsant drugs. Extra-cerebral effects do not appear to be of major importance. Hydantoins, such as diphenylhydantoin, are the only common anticonvulsants to have an effect of this type. But although the administration of diphenylhydantoin causes a rise in circulating aldosterone and deoxycorticosterone, (Woodbury. (1958) (63)), and although these hormones appear to stimulate sodium transport out of the cerebral cortex cells, thus causing them to be hyperpolarised and decreasing cerebral excitability (Woodbury and others. (1958) (65)), an adrenal hormone mediated action can only be a contributory factor in the anticonvulsant action of diphenylhydantoin, since this drug will control seizures in adrenalectomised animals (Woodbury, Koch, and Verndakis. (1958) (67)).

It appears, therefore, that the principal effect of many anticonvulsants may be to cause neurons to be less responsive to the repeated, often intense, electrical impulses originating from an epileptogenic focus. The reduction in excitability could be brought about in a number of ways. Impulses could be less readily transmitted at synapses if transmitter substances were less readily released, or once released, broken down at a greater rate. A reduction in excitability would also be caused by hyperpolarisation of the neuronal membrane, as this would increase the magnitude of the depolarising stimulus required to evoke an active response. A reduction in sensitivity to high frequency stimulation would occur if there were a lengthening of the refractory period after the passage of a nerve/

nerve impulse. An increase in the glucose level of brain cells might cause a stabilisation of the cell membrane by either of two mechanisms not involving glucose metabolism. A brief discussion will therefore be given of each of these several ways by which anticonvulsants may stabilise normal neurons.

Anticonvulsants and Transmitter Substances

Studies of the effect of anticonvulsants on the release of transmitter substances have suggested that phenobarbitone and diphenylhydantoin may reduce the production of free acetyl choline by incubated guinea pig cerebral cortex slices (Tower. (1960) (54)). This effect, however, was only observed at a concentration of either drug which was five times that estimated to be attained in the brain. The significance of these observations is therefore uncertain (Tower. (1970) (55)).

The possibility that anticonvulsant drugs may affect brain acetyl choline metabolism is however of interest, as human cerebral tissues removed during surgical treatment of epilepsy have in some cases shown abnormalities of acetyl choline metabolism, and a high cerebral content of acetyl choline has been noted in a strain of mice unusually susceptible to seizures (McIlwain. (1966) (40)).

While the administration of acetyl choline intravenously or directly to the brain (intracisternally) may produce convulsions, intracisternal administration of noradrenaline may result in anaesthesia or sleep (McIlwain. (1966) (40)), and at certain levels of the central nervous system the cholinergic and adrenergic systems appear to maintain a balance by exerting mutually antagonistic effects (Koelle. (1970) (34)).

It has been suggested (Rudzik and Mennear. (1966) (49)) that the anticonvulsant action of acetazolamide may be via a catecholamine-mediated mechanism. Evidence in support of this view includes the following observations, (a) Amine-depleting agents antagonise the anticonvulsant effects of acetazolamide, (b) This antagonism by 'amine-depleters' can be reversed by dopa (3-hydroxy tyrosine) or prevented by the prior administration of monoamine oxidase inhibitors, (c) The anticonvulsant action of acetazolamide is antagonised by adrenergic blocking agents of both the α and β type. The anticonvulsant action of diphenylhydantoin is also antagonised by the amine-depleting agent reserpine. However, other amine-depleting agents do not antagonise the anticonvulsant action of this drug, and both α and β adrenergic blocking agents have no antagonistic effect. It has therefore been concluded that catecholamines do not mediate the anticonvulsant action on diphenylhydantoin.

Effects of Anticonvulsants on Cellular Ion Levels

The ionic gradients between cells of cerebral cortex and their surroundings are increased by the anticonvulsant drugs acetazolamide and diphenylhydantoin (Woodbury, Koch, and Veraadakis. (1958) (67), Woodbury and Kemp (1970) (66), Gilbert (1971) (20)). Acetazolamide appears to achieve this effect by decreasing the rate of influx of sodium ions into the cells. This drug is a potent inhibitor of carbonic anhydrase, and it has been suggested (Tower (1960) (54)) that in consequence of the inhibition of carbonic anhydrase, there is an accumulation of bicarbonate ions at or within the cell membrane, and that these bicarbonate ions hinder the passage of sodium ions through the membrane. However, the effect of acetazolamide on cellular ion levels need not necessarily operate via inhibition of carbonic anhydrase. Acetazolamide inhibits the active transport of bicarbonate by turtle bladder, a tissue apparently devoid of carbonic anhydrase activity (Gonzalez (1969) (26)).

Diphenylhydantoin is believed to increase the ionic gradients of sodium and potassium between cerebral cells and their surroundings by stimulating the (Na^+, K^+,) activated ATPase of the cell membrane (Woodbury and Kemp (1970) (66)).

The clinical usefulness of anticonvulsants depends on their ability to depress the particular types of cerebral activities associated with convulsions, while at the same time having minimal action as general depressants. The observed effect of some anticonvulsants on the ion levels of brain cells suggests a mechanism whereby these drugs might render neurons less excitable, by hyperpolarising the neuronal membrane. However the selective action of anticonvulsants in preventing the spread of seizure activities in the brain, while having minimal effect on normal activity, remains unexplained. One approach which has yielded some evidence as to how this effect might be achieved has been the study of the effect of anticonvulsants on the response of brain slices to high frequency electrical stimulation.

Anticonvulsants and the Response to High Frequency Stimulation

Anticonvulsant drugs, when present at therapeutic concentrations, do not have any effect on the oxygen consumption of unstimulated incubated cerebral cortex slices (McIlwain (1953) (39), Greengard and McIlwain (1955) (28)). Observations during clinical use of anticonvulsants lead to a similar conclusion - no change in cerebral oxygen consumption being observed (Kennedy and others (1958) (33)).

The anticonvulsant drugs, however, did have a marked stabilising effect on the oxygen consumption of electrically stimulated cerebral cortex slices (McIlwain (1953) (39), Greengard and McIlwain (1955) (22)). Whereas the oxygen consumption of untreated slices rose by about 50% when they were stimulated by electrical pulses (3.5v., 2000 cycles per sec.), slices incubated in the presence of anticonvulsants (phenobarbitone, diphenylhydantoin, or trimethadione) showed little or no increase in oxygen consumption when similarly stimulated. The anticonvulsant drugs did not protect the slices against electrical pulses of a lower frequency (50 cycles per sec.). By contrast, the drug butobarbitone which has a general depressant action on the central nervous system, antagonised the respiratory response to both high and low frequency stimulation.

The respiratory increases observed in these experiments may be indicative of an increase in electrical activity, the energy expended in the propagation of nerve impulses being restored by an increase in oxidative metabolism. It has been suggested that the effect of anticonvulsants of antagonising the respiratory response specifically to pulses at high frequency may be due to a lengthening of the refractory period between specific impulses (McIlwain (1966) (40)). This could provide an explanation for the property that distinguishes anticonvulsant drugs from other central nervous system depressants, that, while they depress excessively frequent cerebral discharge, they allow normal activity since, during normal activity, impulse rates of more than 200 per second are rarely encountered (Hodgkin (1964) (31)). Since the length of the refractory period depends upon the time required for the membrane permeability to ions (particularly potassium) to return to the 'resting' level, after the passage of a nerve impulse, (Hodgkin (1964) (31)) it seems possible that the effect of anticonvulsant drugs in protecting cerebral tissue against high frequency stimulation may be mediated by an effect on membrane permeability.

The Effect of Anticonvulsants on Sugar Uptake

It has been reported (Gilbert, Ortiz, and Millichap (1966) (24)) that the anticonvulsant drugs phenobarbitone and dimethadione, but not diphenylhydantoin, increase the uptake of the non-metabolised sugar, xylose, by guinea pig cerebral cortex slices. Xylose will compete with glucose for uptake by cerebral cortex slices and is presumably transported by the same mechanism (Gilbert (1965) (17)). Anticonvulsant drugs also increase the brain glucose level of mice, 'in vivo'. This appears to be due to an enhancement of glucose uptake, rather than a depression of metabolism (Gilbert, Gray and Heaton (1971) (23)). The converse effect, a decrease/

decrease in sugar uptake, appears to be brought about by convulsants. Thus cerebral cortex slices from animals decapitated during convulsions induced by electroshock, pentylene tetrazole, or penicillin, when incubated, showed a reduced uptake of xylose compared with controls (Ortiz, Gilbert and Millichap (1966) (47)).

The effect of an anticonvulsant drug on sugar uptake could be a direct consequence of an interaction of the drug with the cell membrane, causing an alteration in its permeability properties. An alternative possibility is that the effect of the anticonvulsants on sugar uptake may be a consequence of an effect on the sodium gradient across the cell membrane, as sugars appear to enter some types of cell by a sodium dependant mechanism (Crane (1965) (10), Schultz and Curran (1970) (50)). Sugar uptake by brain cells might take place by such a mechanism. Gilbert (15) observed a reduction in the uptake of xylose by cerebral cortex slices when the sodium ions of the incubation medium were wholly or partially replaced by trishydroxymethylaminomethane. However, in a similar experiment, Cooke (9) found that the partial replacement of the sodium ions of the incubation medium by choline had no effect on the uptake of 3-O methyl glucose by cerebral cortex slices.

Studies of the effects of anticonvulsants on sugar uptake in brain cells are therefore of interest for the following reasons:-

(i) Such studies may provide information about the nature of the interactions of the drugs with the cell membrane.

(ii) Any effect of anticonvulsants on sugar transport might result from, or cause, effects on sodium ion gradients between brain cells and their surroundings, and these effects could be of significance in relation to the anticonvulsant activity of the drugs.

(iii) Glucose molecules may stabilise cell membranes by direct physio-chemical mechanisms, not involving metabolism. (Coldman and Good (1969) (8)).

In view of the possibility that an effect on sugar transport into brain cells may be involved in the mechanism of action of anticonvulsant drugs, it was decided to investigate further the effect of anticonvulsants on sugar uptake. From the results of these studies, and of those of previous studies by other workers, it was hoped to establish whether or not effects on sugar transport are a general feature of anticonvulsant drugs.

In addition, it was decided that where a drug was found to have an effect on sugar transport, the mechanism whereby such an effect was produced should be investigated.

Part 2 - The Design of the Present Investigation

Choice of Tissue Preparation

It was decided, in the present work, to use cerebral cortex slices as the test system for investigations of sugar uptake by brain.

Possible alternatives to the use of brain slices in such studies are the use of a preparation of separated brain cells, the measurement of sugar uptake by perfused brain, or the measurement of sugar uptake by brain 'in vivo'.

Since brain cells are closely packed together, and have many long processes, any attempt to separate the cells from one another usually results in considerable damage to the cells. In an investigation of the permeability properties of separated brain cells prepared by a variety of methods (Gilbert, Adcock and Crieve (1970) (22)) it has been found that only a very small proportion of the total cell volume was inaccessible to extracellular space marker substances. Such preparations are therefore unsuitable for use in permeability studies. Perfused brain, which is not easy to prepare, and is metabolically unstable (Geiger (1958) (15)) was also considered to be an unpromising preparation for use in the present studies. Of the remaining possibilities, the use, in investigations of sugar transport, of incubated cerebral cortex slices was preferred, since the use of this preparation makes possible the control of the environment outside the cells to a degree which would not be possible in studies of brain sugar uptake by the administration of sugars to animals 'in vivo'. Thus extracellular sugar concentration can be adjusted to a desired value, for kinetic studies; or chemicals such as inhibitor substances, to which the blood-brain barrier may be impermeable, may be added. When assessing the effect of the drugs, statistical variability arising from differences between individual animals may more readily be allowed for in 'in vitro' studies, as each 'test' sample may be compared with a control sample of tissue obtained from the same animal. A simpler permeability barrier (the cell membranes of the various types of cell) is being studied, when working with tissue slices, than is the case in 'in vivo' studies, when the more complex blood-brain barrier system is studied.

Slices of cerebral cortex display many of the metabolic characteristics of the intact tissue (McIlwain (1966) (40)). If placed in a suitable medium, with glucose as nutrient, they will maintain a stable respiratory rate (60 μ moles $O_2 . g^{-1} . hr^{-1}$) for several hours. This rate is lower than that of intact brain. However, the application of electrical/

electrical pulses increase their respiratory rate to a value (120 μ moles $O_2 . g^{-1} . hr^{-1}$) comparable to that of the grey matter of intact brain. Intracellular potentials of around -65mV with respect to the environment may be observed in incubated cerebral cortex slices. 'Spike' discharges, caused by the entry of the recording microelectrode, have been observed.

The tissue potentials are susceptible to electrical stimulation, and reversible depolarisation may be produced by addition of 200mM potassium chloride to the bathing medium. Examination of cerebral cortex slices by electron microscopy (Wanco and Tower (1964) (61)) has revealed that when the slices are incubated under favourable conditions, comparable with those employed in the present study, they contain intact cells of all types encountered in cerebral cortex 'in vivo'. The morphology of these cells appears comparable with that of corresponding cells in intact brain.

Incubation Medium

It has been found that the morphological and physiological features of cerebral cortex slices are best maintained by incubation at 37°C in an oxygenated, phosphate or bicarbonate buffered medium, containing a suitable nutrient (usually glucose). (Wanco and Tower (1964) (61), McIlwain (1966) (40)). Bicarbonate medium was used for incubation of the slices in all experiments involving the measurement of sugar uptake, and was preferred to phosphate medium on two counts, these being that - (i) it appears that cerebral cortex slices can accumulate xylose against a concentration gradient in bicarbonate medium, but do not appear to do so in phosphate medium (Gilbert (1971) (20)), and (ii) that the ionic composition of the bicarbonate medium is similar to that of blood plasma, and of cerebrospinal fluid.

Measurement of 'Extracellular' Space

It was intended that the rate of uptake of sugar into intact cells of cerebral cortex slices should be measured. For this it was necessary to obtain an estimate of the volumes of the apparently intracellular, and apparently extracellular, compartments of the tissue. The trisaccharide raffinose was therefore included in the incubation medium as a marker of the apparent extracellular space. Previous studies (Gilbert, (1966) (18)) have shown that when cerebral cortex slices are incubated in a raffinose-containing medium, the volume of the non-raffinose compartment of the slices remains constant (after an initial equilibration period in the first ten minutes of the incubation) for a period of at least fifty minutes. Moreover, if the osmolarity of the external medium be altered, changes in/

in the volume of the non-raffinose compartment occur, which are in agreement with the volume changes predicted on the assumption that the components of this compartment behave as perfect osmometers.

Pre-Incubation of the Slices

In order to allow the ion levels of the slices to stabilise, slices were pre-incubated for thirty minutes at 37°C in bicarbonate medium containing a nutrient, and an extracellular space marker (raffinose), but no xylose, before transfer to a xylose-containing medium. The pre-incubation period also provided time for raffinose to equilibrate with the compartment accessible to it, and in experiments where a drug was present, allowed equilibration of the drug with the preparation.

Use of a 'Glucose' Analogue in Permeability Studies

Direct measurement of glucose uptake by tissues is made difficult by the complicating factor of loss of glucose by metabolism. In the present investigation therefore, the uptake of xylose, a sugar not metabolised by brain (Haddock (1939) (37)) was studied. Xylose appears to be transported into the 'intracellular' compartment of cerebral cortex slices by the same mechanism as glucose, since these two sugars compete for uptake by this preparation (Gilbert (1965) (17)). The method employed for determination of the xylose content of incubated cerebral cortex slices is described in Chapter II.

Selection of a Nutrient for the Incubated Slices

Glucose cannot be employed as a nutrient for the incubated slices, since it would compete with xylose for entry into the cells. Sodium pyruvate was therefore included in the incubation media as nutrient, since pyruvate is reported to have an effectiveness approaching that of glucose in maintaining the functioning of incubated cerebral cortex slices (Kratzing (1953) (35)).

The Use of Kinetic Studies to Investigate the Nature of the Effect of Anticonvulsants on Xylose Uptake.

The kinetics of the process of xylose uptake by cerebral cortex slices are compatible with the operation of a carrier-mediated process (Gilbert, Ortiz, and Millichap (1966) (24), Joanny, Corriol1, and Hillman (1969) (32)). If anticonvulsant drugs affect the parameters of this process, the nature of the effect observed, (stimulation or inhibition of xylose uptake) may depend on the concentration of xylose present in the medium, (as well as on the concentration of the drug). It was proposed/

proposed, therefore, to study the initial velocity of xylose uptake into the apparently intracellular compartment of cerebral cortex slices, at a number of xylose concentrations, and for a variety of anticonvulsant drugs, thereby obtaining an estimate of their effect on the apparent Km and Vmax of the xylose transport process. Similar studies have already been carried out for the drugs phenobarbitone and dimethadione (Gilbert, Ortiz, and Millichap (1966) (24)).

The Investigation of Possible Drug-Binding Sites

Since a change in the permeability of cerebral tissues to sugar, as well as many of the other observed effects of anticonvulsant drugs, might be brought about by interaction of the drugs with cell membranes causing an alteration of membrane structure, it was decided that, in cases where a drug was found to have an effect on xylose uptake, the effect, on the response to the drug, of agents which could be expected to block specific receptor sites on the membrane, should be investigated.

SOME EFFECTS OF ANTICONVULSANT DRUGS ON SUGAR UPTAKE BY CEREBRAL CORTEX SLICES

CHAPTER II - EXPERIMENTAL METHODS FOR THE MEASUREMENT OF XYLOSE UPTAKE.

Experimental Methods for the Measurement of Xylose Uptake

Introduction

Studies of the uptake of xylose by cerebral cortex slices entail the preparation and incubation of the tissue, and the measurement of the water, raffinose, and xylose content of the incubated tissue.

The experimental procedures employed are described in this chapter.

The Preparation and Incubation of Cerebral Cortex Slices

Media for use in the preparation and incubation of the slices was prepared in the following manner:-

0.44 grams of sodium pyruvate were dissolved in about 100 ml. of water in a 1 litre volumetric flask. The following volumes of salt solutions were then added -

NaCl	131g/500ml.	25ml.
$NaHCO_3$	21.8g/500ml.	50ml.
$KH_2\ PO_4$	3.20g/500ml.	25ml.
KCl	9.25g/1250ml.	10ml.
$MgSO_4\ 7H_2O$	7.50g/1250ml.	10ml.

The volume of the solution in the flask was made up to about 600ml. by the addition of distilled water. 10ml. of a solution of $CaCl_2\ 6H_2O$, 6.50g/250ml. were then added slowly, with mixing. The volume was then made up to 1 litre.

Where medium containing raffinose was required, 5.95g. of raffinose pentahydrate was first dissolved in about 200ml. of warm distilled water in a 1 litre volumetric flask. The contents of the flask were then cooled before the addition of the pyruvate. From this point the procedure described above was followed.

In some preliminary experiments, the pyruvate concentration was 12mM. Since the presence of this concentration of pyruvate in the medium was found to cause interference in the colorimetric determination of xylose, however, 4mM was the concentration of pyruvate included in all incubation media employed in studies of xylose uptake.

The composition of the bicarbonate medium, with pyruvate as nutrient, used in all the xylose uptake experiments was thus -

NaCl 112mM : $NaHCO_3$ 26mM : KCl 5mM : $KH_2\ PO_4$ 1.2mM

$MgSO_4$ 1.2mM : $CaCl_2$ 1.0mM : and sodium pyruvate 4mM.

This medium was termed 'medium M'. Media similar in composition to the above, but containing, in addition raffinose (10mM); or raffinose (10mM) and xylose

xylose, were also prepared. These media were termed 'medium M1' and 'medium M2' respectively.

Preparation of the Cerebral Cortex Slices

A male guinea pig, aged between six and eight weeks, and of the Dunkin-Hartley strain, was decapitated, using a guillotine. The skull was opened, using strong scissors, to expose the brain. Using the rounded end of a spatula the membrane surrounding the brain was removed, and the cerebral cortex separated from the underlying tissue, and placed in a dish containing bicarbonate medium at room temperature. The cerebral cortex was washed briefly in this medium. It was then placed in the barrel of a sawn-off syringe, which was held with the lower (open) end of the barrel upward. The barrel of the syringe had previously been moistened with a little of the medium. A stainless steel slicer with wires spaced 0.3 m.m. apart, was placed over the end of the barrel, and the syringe returned to the upright position, keeping the slicer in place. The slicer and syringe were then placed over a beaker, containing about 5ml. of the medium, and the tissue sliced by pressing down the plunger of the syringe, while keeping the barrel of the syringe firmly against the wires of the slicer, as illustrated in Fig. 2. The slices so produced fell into the medium contained in the beaker. Any unsliced tissue remaining was removed by drawing the blade of a spatula across the upper surface of the slicer, along the axis of the wires. The blunt end of the spatula was then moved backwards and forwards between the wires to loosen any slices still adhering to the lower surface of the slicer.

The medium containing the slices was then stirred briefly to break up any clumps of slices and to give, as far as possible, a uniform mixture of slices from the various regions of the cerebral cortex. The slices were then separated from the medium by the use of a sieve, fashioned from wire gauze of one sixteenth of an inch mesh. The sieve was blotted on the under side to remove excess medium. Portions of the slices were then quickly transferred to suitable media, for incubation.

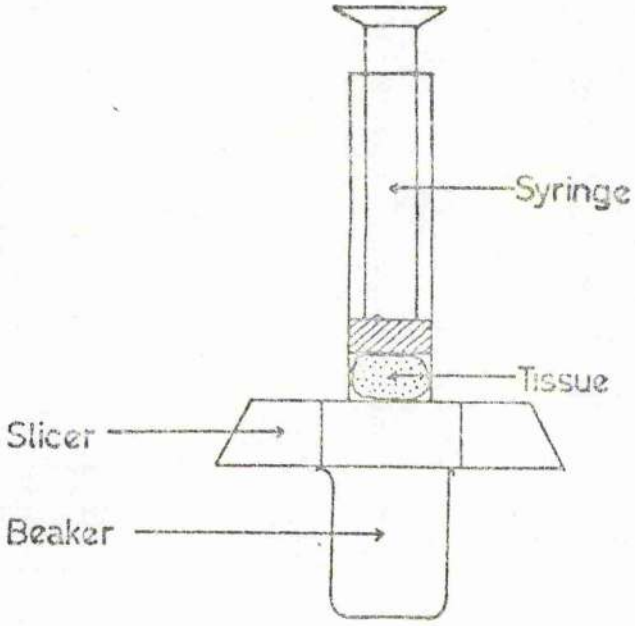
Incubation of the Cerebral Cortex Slices

Portions, each about 0.3g, of freshly prepared guinea pig cerebral cortex slices were transferred to incubation vessels of the type illustrated in Fig. 3. The vessels contained medium 'M1' (bicarbonate medium with pyruvate, 4mM, and raffinose, 10mM). The incubation medium was maintained at 37°C and was stirred by bubbling a gas mixture, containing 95% oxygen and 5% carbon dioxide, through the medium from a/

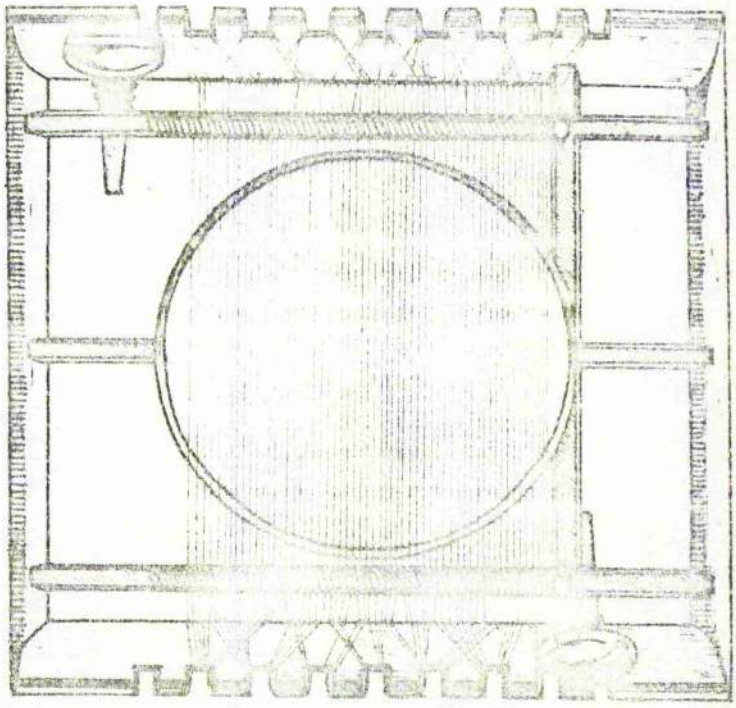
FIGURE 2

THE PREPARATION OF CEREBRAL CORTEX SLICES

(By the method of Gilbert, 1969, (19))



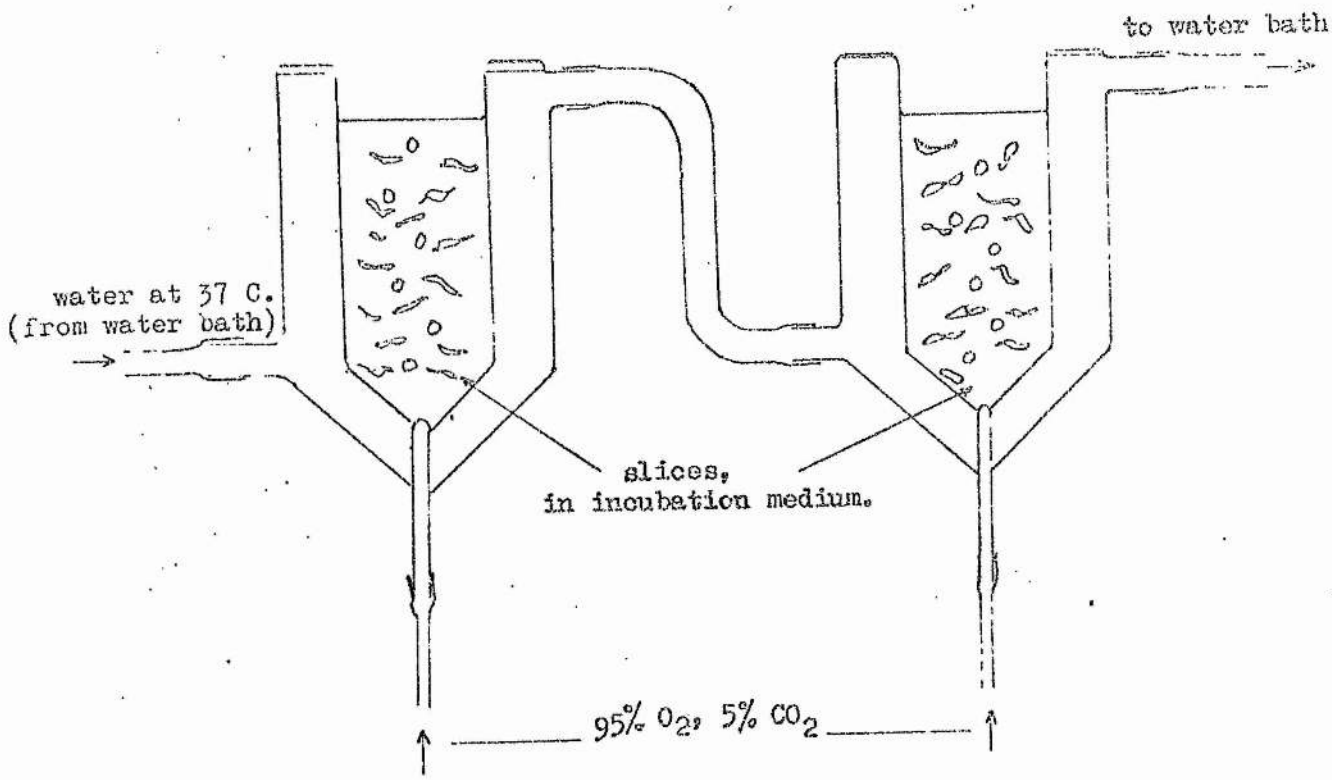
A - An illustration of the method employed for rapid slicing of the tissue.



B - The tissue slicer, viewed from above.

Fig. 3.

THE INCUBATION OF CEREBRAL CORTEX SLICES



a vent at the bottom of the incubation vessel.

This 'pre-incubation' lasted for thirty minutes, at the end of which time the gassing lead was disconnected from the bottom of the incubation chamber. The medium and slices then flowed out through the gassing vent and passed through a sieve, held just below the incubation chamber, so that the slices were retained by the sieve. The medium passed through the sieve and was discarded. Any excess medium adhering to the slices was removed by gently blotting the underside of the sieve.

The slices were then transferred to a second incubation chamber, similar to the first, but containing medium 'M2' (bicarbonate medium with pyruvate, raffinose and xylose). The slices were incubated in the xylose containing medium for a time appropriate to the purpose of each experiment. At the end of this the slices were rapidly collected in the same manner as in the previous incubation. The sieve containing the slices was blotted four times on the underside, to remove any surplus medium adhering to the slices. The slices were then transferred to a weighed conical centrifuge tube, graduated to 10ml. The tube and contents were re-weighed and placed in a deep freeze cabinet at 30°C. The water, raffinose and xylose content of the slices were subsequently determined by the methods described in the following pages.

The Determination of the Water Content of Incubated Slices of Guinea Pig Cerebral Cortex

The comparative merits of oven-drying, and of freeze-drying, for the determination of slice water content were investigated. The use of freeze-drying permits subsequent measurement of the sugar content of the freeze-dried material. If, however, slices are oven-dried at 105°C, slight charring occurs. Material dried in this way is thus unsuitable for subsequent sugar determinations, and so, if oven-drying is used to determine water content all samples have to be divided into two portions, one for drying, the other for sugar assays.

A freeze-drying procedure would therefore appear to be most suitable for use in the present studies. Since, however, oven-drying had been employed in previous studies it was thought prudent to make a brief comparison between this method of determining water content, and freeze-drying. The following procedure was adopted.

A Comparison of Oven - and Freeze - Drying Methods for the Determination of Tissue Water Content

Cerebral cortex slices were prepared and incubated as already described, except in that the slices received a single thirty-nine/

nine minute incubation in medium 'M' instead of thirty minutes in 'M1' followed by a shorter incubation in 'M2'.

The incubated slices were then divided into six equal portions. Each portion was placed in a weighed 10ml. conical centrifuge tube, and the tube and contents weighed. The dry weight of the slices in three of the tubes was then determined by oven-drying and that of the remaining three by freeze-drying.

Procedure for Oven-Drying

The slices in their tubes were heated in an oven at 105°C to constant weight. (It was found that they attained constant weight on being dried for about fifteen hours). The tubes and their contents were then transferred to a desiccator, allowed to cool, and weighed.

Procedure for Freeze-Drying

The weighed slices, in their tubes, were transferred to a deep freeze cabinet at 30°C. When the slices were completely frozen (after about ninety minutes) the tubes, with the contents, were placed in the drying chamber of the freeze drier where they were allowed to stand under vacuum for at least eight hours. (It had been found that samples had usually dried to a constant weight after three to four hours). The tubes containing the slices were then transferred to a desiccator, for transport to the balance room, and weighed.

Results of the Comparison

Closely similar values were obtained when the water contents of cerebral cortex slices were determined by oven-drying and by freeze-drying (Table 1). Freeze-drying was therefore used in subsequent experiments as the means of determining slice water content.

The Determination of the Raffinose and Xylose Content of the Incubated Slices

Tissue samples were deproteinised, using zinc sulphate and barium hydroxide, as recommended by McIlwain and Rodnight (41).

To the freeze dried slices, in conical centrifuge tubes graduated to 10ml., 1ml. of 5% zinc sulphate was added. The slices were then homogenised using a 'perspex' pestle which had been machined to fit the tube. The pestle was then washed with distilled water, the washings being directed into the tube. 1 ml. of 0.3N barium hydroxide was then added. (The concentrations of the $ZnSO_4$ and $Ba(OH)_2$ reagents had been adjusted/

TABLE 1

A Comparison of Freeze Drying and Oven Drying Methods in Determining the Water Content of Cerebral Cortex Slices

	Water Content of the Slices ul per g. wet tissue	
	by oven-drying	by freeze-drying
Experiment 1	865	861
	858	863
	863	865
Mean ± Standard Error	862 ± 2	863 ± 1
Experiment 2	856	859
	857	856
	861	859
Mean ± Standard Error	858 ± 2	858 ± 2

In each of the experiments, the six portions of slices whose water content was determined were drawn from the same pool of incubated cerebral cortex slices.

adjusted so that they neutralised each other, volume for volume, when titrated with phenolptalein as indicator).

The volume in the tubes was then made up to 10ml. by the addition of water, and the tubes capped with 'parafilm' and inverted twice to mix the contents. After being allowed to stand at room temperature for thirty minutes, the contents of the tubes were again mixed.

The tubes were then centrifuged for two minutes on an M.S.E. clinical bench centrifuge. The supernatants were then withdrawn from the tubes by pipette and portions of the supernatant used for raffinose and xylose estimations. (Duplicate samples of approximately 1 ml. were required in each case). If necessary, these solutions were diluted to bring their content of raffinose or xylose into the desired concentration range (25-75 ug/ml. for raffinose, 7.5-22.5 ug/ml. for xylose - i.e. 50-150 µM for either sugar).

Estimation of Raffinose

Principal of the Method Employed

Raffinose is a trisaccharide containing galactose, glucose and fructose. In the colorimetric procedure used for the determination of raffinose, the fructose produced by acid hydrolysis of raffinose is determined by the Seliwanoff (51) method for ketohexoses.

This involved mixing the samples with a reagent containing hydrochloric acid, resorcinol and ferric chloride and heating the mixture. The acid hydrolysed the raffinose to its constituent monosaccharides. At the concentration of acid employed, the ketohexose, fructose, was rapidly dehydrated to hydroxymethyl furfural, whereas aldohexoses such as glucose and galactose were only very slowly dehydrated. The hydroxymethyl furfural formed then condensed with resorcinol, to give an orange-red coloured compound. The intensity of the colour obtained was enhanced by the presence of ferric ions in the medium.

Method

The following procedure was followed for raffinose estimations using a 'Technicon' autoanalyser. The selection of this set of conditions for the raffinose estimation is discussed in a subsequent paragraph.

'Standard' solutions containing known concentrations of raffinose, and the sample solutions whose unknown raffinose content was to be determined, were placed in 1 ml. polystyrene cups on the sampler plate of the autoanalyser. Between each standard or unknown solution was placed a cup containing distilled water. It was arranged that the order in which/

which the raffinose solutions should be sampled should be S1, S2, S3, S4, S5, U1, U2, U3, ... Un, Un, ... U3, U2, U1, S5, S4, S3, S2, S1, - 'S' denoting a standard solution and 'U' a solution of unknown raffinose content. The nominal sampling rate employed was 50 samples per hour. As alternate cups contained water, the effective sampling rate was thus 25/hr.

The reagent employed in the raffinose determinations had the following composition.

Concentrated hydrochloric acid 40% v/v
Resorcinol 0.1% w/v
$FeCl_3$ $6H_2O$ 0.0023% w/V

Fresh reagent was made up once weekly, or as required. 0.5mls. of a 30% (V/V) solution of the wetting agent, Brij-35, were added to every litre of the reagent.

Reagent was pumped at the rate of 2.00ml./min, and air segmentation (0.60ml./min.) introduced into the reagent stream before addition of the sample at the rate of 0.42 ml./min. The combined sample and reagent stream was then passed through a mixing coil before being led through a heating bath at 98°C. The mixture passed through two glass coils, each of 28ml. capacity, immersed in the bath. The time of heating was thus about eighteen minutes. After heating, the air bubbles segmenting the stream were removed, by a debubbling advice. The optical density of the mixture at 480nm was then monitored as it passed through the flow cell of the colorimeter, and the results recorded graphically by the recording device. The salient features of this method for the automated determination of raffinose are illustrated in Fig. 4, page 21.

Derivation of the Method

The method used was first adapted for automated determination of raffinose by Gilbert (16). The procedure used by Gilbert has been followed in most essentials. However, in consequence of recent alterations in the design of the autoanalyser sampler and colorimeter modules, different sampling procedures and flow rates have been employed.

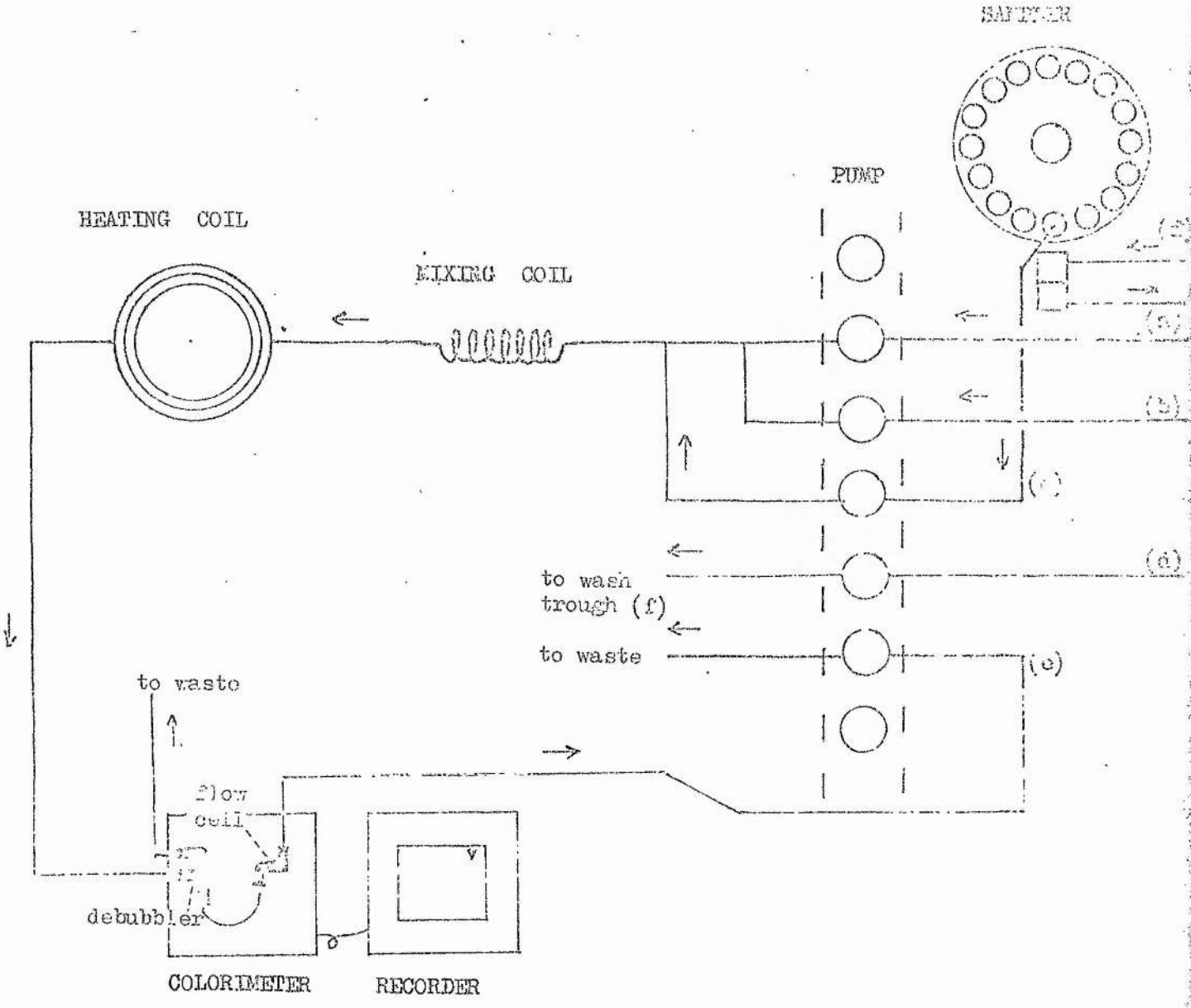
Sampling

The sampler unit of the autoanalyser possesses a wash trough , into which the sampler probe moves between the taking of samples, aspirating water for a period equal to one half the 'sampling time'.

It was found, however, that this wash period was not sufficient to entirely eliminate cross contamination between successive samples, even when the sampling rate was reduced to the lowest rate (20 samples/hr). Cont/

FIGURE 4.

THE AUTOMATED ESTIMATION OF RAFFINOSE:- FLOW DIAGRAM.



(a) reageant 2.00 ml./min. (b) air 0.60 ml./min.

(c) sample 0.42 ml./min. (d) water 2.00 ml./min.

(e) from colorimeter 1.60 ml./min. (f) water to wash trough - from (d)

Cups containing distilled water were therefore alternated with the sample cups on the sampler turntable. It was now found that the nominal sampling rate could be increased up to, but not beyond 50/hr., without any carryover between successive samples being detected.

Reagent and Colour Development

In the method developed by Gilbert, 1 volume of sample was mixed with 4.5 volumes of a reagent of the following composition - concentrated hydrochloric acid 60% v/v and ferric chloride .0050%w/v. The mixture was then heated at 98°C for six minutes and the optical density at 480nm then recorded.

Using this method, Gilbert found that the optical density recorded for a solution of pentose (arabinose) was 0.4% of that recorded for a raffinose solution of equal molarity.

The possibility of interference by pentose in the raffinose estimation could be reduced still further by reducing the reagent HCl concentration by one third, although, under these conditions, the colour obtained with a given solution of raffinose was less intense than formerly. This lower reagent acid concentration was selected for the present studies.

At the same time the heating time of the sample reagent mixture was increased thereby obtaining colour intensities comparable with those obtained by use of the '60% conc. HCl' reagent.

The flow rates of samples and reagent throughout the autoanalyser system were determined by the following considerations - (i) the desirability of introducing air segmentation to the system prior to the addition of sample, the volume of air being approximately one quarter of the combined liquid volume, (ii) provision for a volume of liquid at least equal to that of the air to be allowed to escape from the debubbler, (iii) the maintenance of a sample reagent ratio of about 4.5 : 1. The first two considerations apply equally to most other autoanalytical methods, and are procedures recommended in the 'Technicon' handbook.

Using the present method for raffinose estimation, Beers Law was found to be obeyed at least up to raffinose concentrations of 75 µg/ml. (the highest concentration tested). The presence, in some samples, of xylose did not appear to cause any interference in the raffinose estimation, for molar ratios of xylose/raffinose of up to 10/1 (the highest ratio tested).

Correction for Endogenous Chromogens

When calculating the raffinose content of incubated slices it is necessary to make allowance for the presence of substances in the slices/

slices which gave a raffinose-like reaction with the reagent. To determine the value of this factor, guinea pig cerebral cortex slices were prepared and incubated for thirty-nine minutes in a bicarbonate medium containing 4mM pyruvate, but no raffinose. The slices were then collected, freeze-dried, homogenised and deproteinised, as previously described. Before making the volume of the deproteinised homogenate up to 10ml., however, 1 ml. of a solution containing 505 µg/ml. of raffinose was added. The volume in the tube was then made up to 10ml. The contents of the tube were then mixed. After thirty minutes, the tubes were centrifuged as previously described and raffinose estimations performed on portions of the supernatants.

Results

Eight samples of incubated cerebral cortex slices, each taken from a different animal, were re-examined. The mean content of substances which reacted as raffinose was found to be 37.1 $\pm$ 5.3 µg. of raffinose per g. of incubated slices.

The results obtained in the individual experiments, expressed in the same units, were 62.3, 46.2, 46.8, 44.4, 26.1, 19.9, 28.6, 22.5.

Estimation of Xylose

Principal of the Method Employed

Xylose was estimated by a colorimetric method. The samples were mixed with a reagent containing hydrochloric acid, orcinol, and ferric chloride. The mixture was then heated. The pentose was dehydrated to furfural, which then condensed with the orcinol.

The colour of the condensation compound produced was intensified by the presence of ferric ions.

Method

The following procedure was adopted for xylose estimations using an autoanalyser. The derivation of this method from previous manual methods is described in a subsequent paragraph.

Reagent

A single reagent was employed in this method. To make 1 litre of the reagent, 2 grams of orcinol were dissolved in about 100ml. of water in a litre volumetric flask. 8.5ml. of a 1% solution of $FeCl_3 . 6H_2O$ were added, then 770ml. of concentrated hydrochloric acid (density 1.18g per ml. and containing 36.5% by weight of hydrogen chloride) were added, with mixing. The volume was made up to one litre with distilled water. To each litre of reagent 0.5ml. of a 30% solution of the wetting agent, brij-35, were added.

Fresh reagent was made up once weekly.

Sampling

The xylose-containing standards and unknowns in 1 ml. cups were placed in alternate positions on the sampler plate. The intervening cups contained water. As in the method for raffinose determination, a sampling rate of 50/hr. was employed.

Layout of the Autoanalyser System

Air segmentation at 0.6 ml/min. was introduced into the stream of reagent, flowing at 2.0 ml/min, before addition of the sample stream, flowing at 0.6 ml/min. The combined stream was then passed round a mixing coil, and then through a single 28ml. glass coil lying in a heating bath at 98°C.

The time taken for the mixture to pass through this heating coil was about eight minutes. The air bubbles segmenting the stream were then removed by a debubbling device and the optical density of the bubble free mixture monitored at 660nm as it passed through the flow cell of the colorimeter. The results were recorded graphically by the recording unit. A flow diagram illustrating the procedure above is given in Fig. 5, page 25

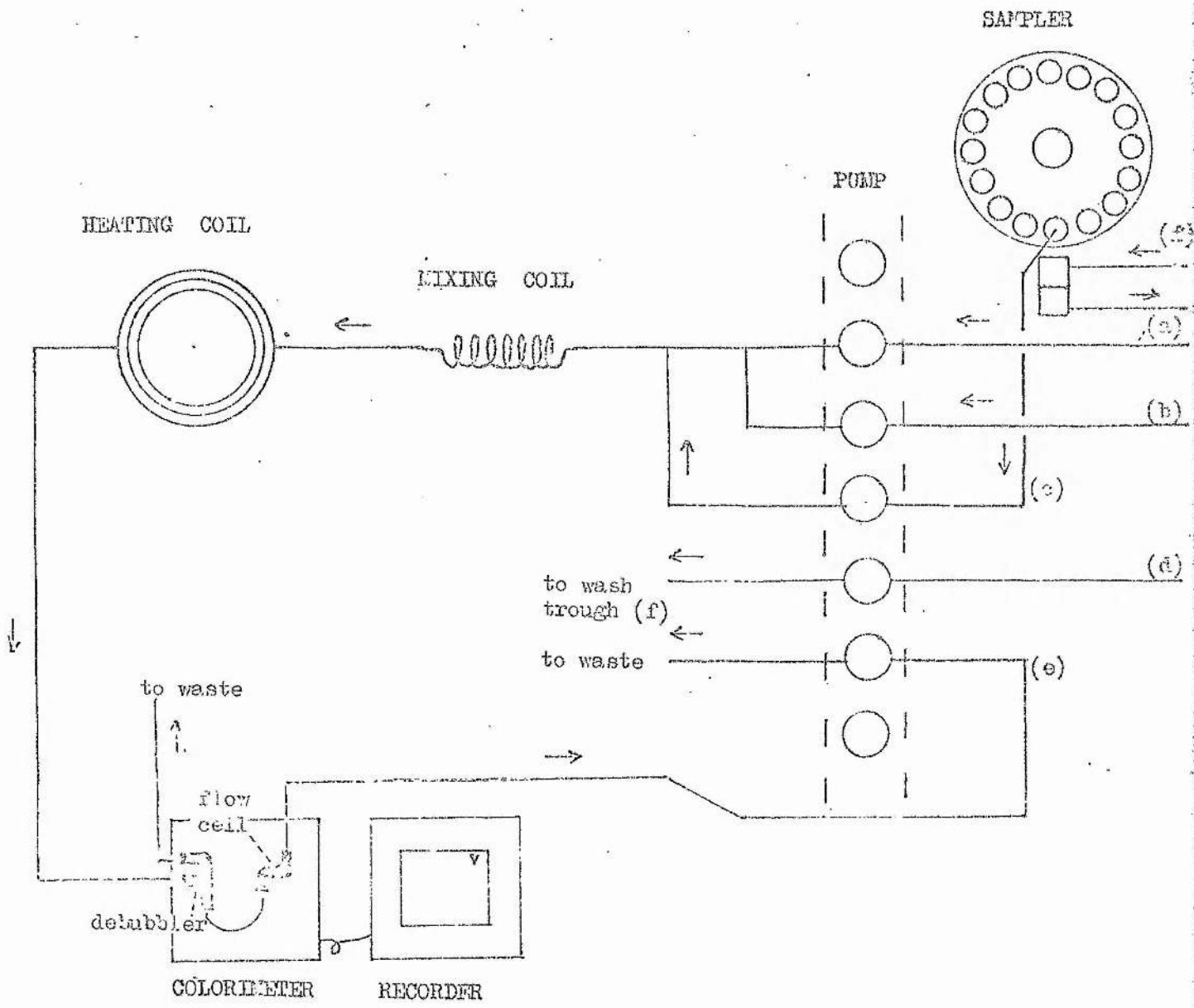
Derivation of the Method

A method has been described (Fisher and Gilbert (1970) (14)) for the automated determination of pentose using a p-bromoaniline reagent. However, on account of the toxicity of the p-bromoaniline reagent it was decided that the use of an alternative method should be investigated in the present work. The most promising alternative available appeared to be the use of an orcinol-hydrochloric acid-ferric iron reagent. This reagent, with pentoses, produces colour quite rapidly, a feature which facilitates the adaption of the method for use with the autoanalyser.

A manual method of this type has been described (McRary and Slattery (1945) (42)) employing a single reagent, made by dissolving 2 grams of orcinol in 50ml. of a solution containing 1.5% w/v of ferric chloride hexahydrate. 30% hydrochloric acid ('concentrated' hydrochloric acid diluted with one fifth of its volume of water) was then added to make the volume up to 1 litre. Portions of the reagent were then mixed with the pentose standards and unknowns in the proportion of 3 volumes of reagent to 1 volume of pentose solution. After mixing, the samples were heated at 100°C for twenty minutes, then cooled, and the optical densities read at 660nm. It was subsequently reported (Drury (11)) that interference by hexoses in this assay could be much reduced by shortening the/

FIGURE 5

THE AUTOMATED ESTIMATION OF XYLOSE:- FLOW DIAGRAM



(a) reageant 2.00 ml./min. (b) air 0.60 ml./min.

(c) sample 0.60 ml./min. (d) water 2.00 ml./min.

(e) from colorimeter 1.60 ml./min. (f) water to wash trough - from (d)

the heating time to about seven minutes. A ferric iron concentration of about one third of the value quoted above was also recommended.

In the light of these recommendations, the use, for autoanalysis, of a reagent containing 2g. of orcinol, 0.25g. of $FeCl_3 \cdot 6H_2O$, and 770ml. of concentrated hydrochloric acid per litre, was investigated. Apart from the composition of the reagent, the details of this first method of autoanalysis tested are the same as for the method finally adopted, which has already been described.

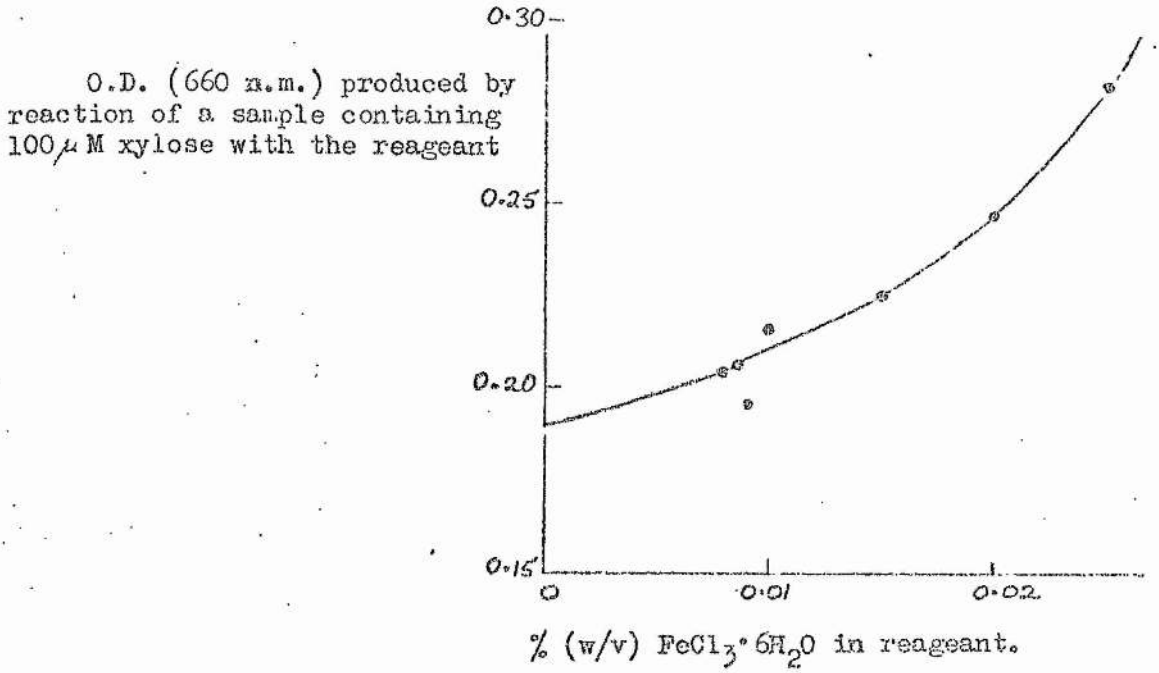
Using this reagent for xylose determination, it was found that Beers Law was obeyed within the range of xylose concentrations tested, (3.75 to 22.50 µg/ml) but, that if an equimolar concentration of raffinose was present in the xylose solutions, the apparent xylose recorded by this method was 106% of the true value. In view of a suggestion (Cgux (46)) that the concentration of ferric iron in the orcinol reagent could profitably be reduced, the effect of lowering the concentration of ($FeCl_3 \cdot 6H_2O$) in the reagent from 0.25 to 0.05 grams per litre was investigated. It was found that when the concentration of $FeCl_3.6H_2O$ was 0.085 grams per litre, or less, the presence of equimolar raffinose did not interfere with the xylose assay (see Fig. 7A) page 28. At $FeCl_3.6H_2O$ concentrations of 0.080 grams per litre or less, however, Beers Law was no longer obeyed over the required range of xylose concentrations (7.50 to 22.50 µg/ml). A reagent $FeCl_3.6H_2O$ concentration of 0.085 grams per litre was therefore chosen despite the fall of about 25% in the optical density of the same xylose standard when assayed with reagent containing 0.085, as opposed to 0.250 grams $FeCl_3.6H_2O$ per litre (See Fig. 6A). The effect of reducing the hydrochloric acid concentration of the reagent was also investigated as the life of ordinary 'tygon' pump tubing is considerably shortened, if it is employed to pump a strongly acid reagent. Reduction of the reagent acid concentration from 77% concentrated hydrochloric acid (about 330 grams per litre of hydrogen chloride) to 60% (about 260 grams per litre of hydrogen chloride) resulted in a decrease in intensity of the colour produced with xylose solutions (see Fig. 6B) and an increase in the interference produced by raffinose (see Fig. 7B). The higher concentration of acid was therefore favoured.

With the method now adopted for xylose estimation, there was no interference from raffinose. A check was then carried out to see if the presence of the bicarbonate medium in which the slices were to be incubated had any effect on the xylose estimation method. This was carried out as follows.

Fig. 6.

The Effect of Varying Reageant Fe^{+++} and HCl Concentrations on the Sensitivity of the Method for Xylose Estimation.

(A)- Effect of varying Fe^{+++} (HCl maintained at 77% v/v.)



(B)- Effect of varying HCl, ($FeCl_3 \cdot 6H_2O$ maintained at 0.0085% w/v.)

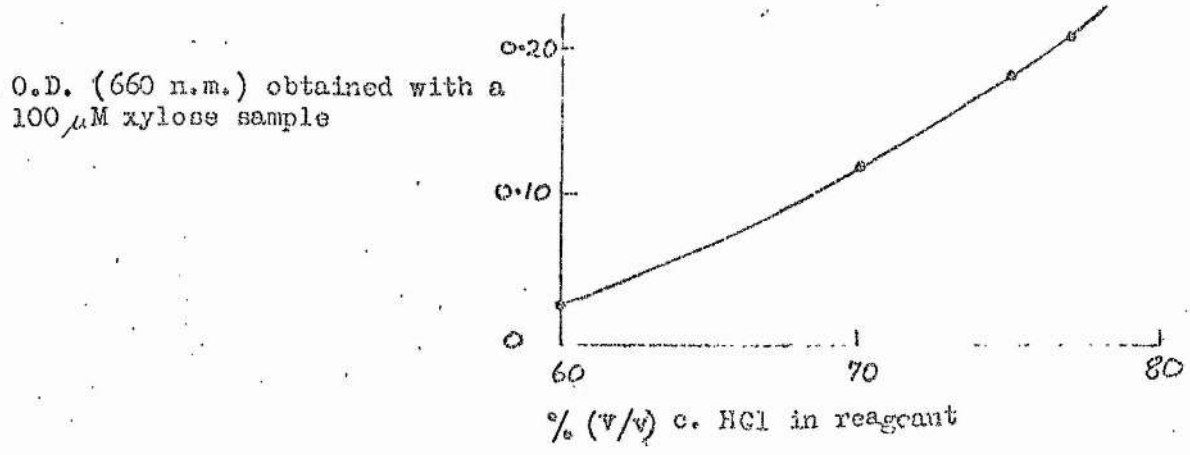
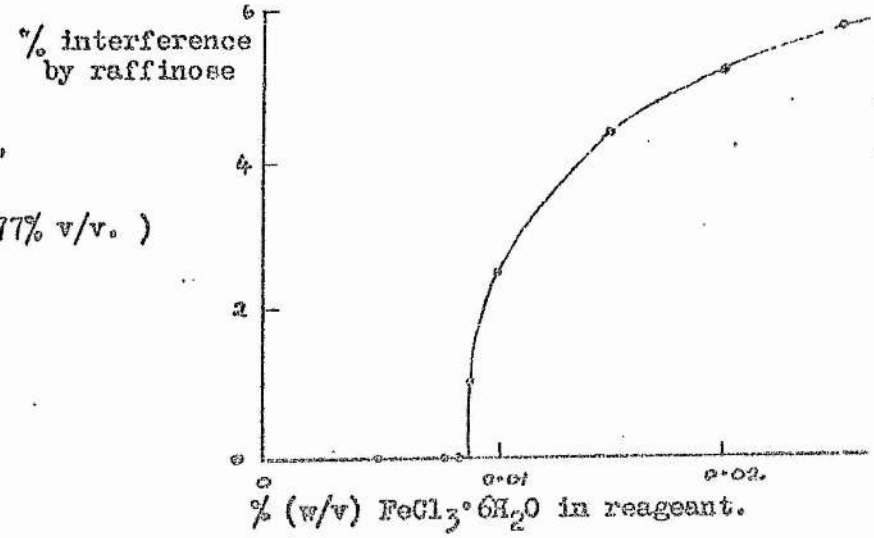


Fig. 7.

The Effect of Varying Reagent Fe^{+++} and HCl Concentrations on Interference by Raffinose in Xylose Estimations.

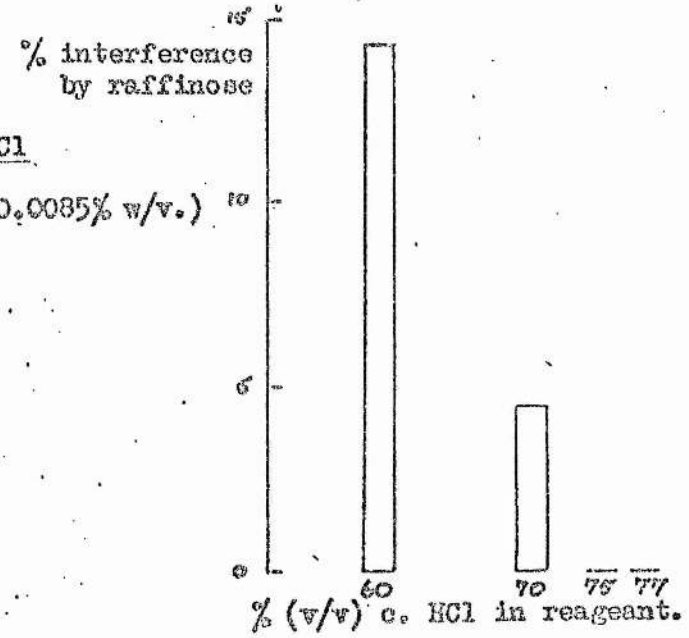
(A)- Effect of Varying Fe^{+++}

(HCl maintained at 77% v/v.)



(B)- Effect of Varying HCl

($FeCl_3 \cdot 6H_2O$ maintained at 0.0085% w/v.)



Effect of Bicarbonate Medium on Xylose Estimations

Method: Two xylose solutions, each 10mM, were made up, one in distilled water, the other in bicarbonate medium containing 12mM pyruvate. The solutions were diluted an hundredfold by addition of distilled water. Xylose contents of the dilute solutions were then measured by the method already described.

Result: The apparent xylose content of the solutions in distilled water was 15.01 $\pm$ 0.06 (4) µg/ml. The apparent xylose content of the solutions prepared by dilution from bicarbonate medium was 13.94 $\pm$ 0.09 (4) µg/ml., a decrease in 'apparent xylose' of 7%. Each of the componants of the bicarbonate medium was then tested seperately for effect on the pentose estimation method. Only sodium pyruvate was found to have any effect. 12mM sodium pyruvate depressed the apparent xylose content of the solutions by the same amount (7%) as had bicarbonate medium containing 12 mM pyruvate.

To minimise the effect of the pyruvate upon the estimation of the xylose the concentration of pyruvate in the incubation medium was decreased from 12mM to 4mM.

Correction for Endogenous Chromogens

When calculating xylose uptake by cerebral cortex slices, it was necessary to make a correction for substances already present in the tissue which reacted as pentose with the colour reagent. In order to determine this factor, guinea pig cerebral cortex slices were incubated, freeze dried and deproteinised under the same conditions as those employed for determination of endogenous raffinose-like substances. Portions of the supernatants after deproteinisation were then employed for xylose determinations.

Results: Ten samples of incubated cerebral cortex slices, each taken from a different animal, were examined. The slices were found to have a mean content of materials reacting as pentose equivalent to 58.9 $\pm$ 4.4 µg/ xylose per g. slices. The results of individual experiments, expressed in the same units, were:-

33.2, 42.7, 70.1, 64.3, 65.9, 54.4, 80.8, 63.9, 49.8, 64.1

This value for endogenuous pentoses in incubated guinea pig cerebral cortex slices is in agreement with the findings of Joanny and colleagues (32), who, using a different method of pentose estimation, that of Tracy (56) employing an aniline reagent, found endogenous pentose levels of up to 66 µg/g. It is, however, higher than the value (10 µg/g) found by Gilbert (17) using a p-bromoaniline method for pentose estimation.

The Presentation of Xylose Uptake Data

It has been mentioned, in Chapter I (page 9) that, in order to ascertain the concentration of xylose attained, at the end of an experiment, in the intracellular compartment of the cerebral cortex slices, measurement of the water, raffinose and xylose content of the slices is necessary, and methods have been described (pages 16-27) whereby these measurements may be effected. The method of calculation, from the data thus obtained, of the extent to which xylose has penetrated the intracellular compartment of the slices, will now be described.

The volumes of distribution of raffinose, and of xylose, in the slices are expressed in terms of 'solute space' where:-

$$\text{solute space (in ul. per g. incubated tissue)} = \frac{\text{tissue solute content (ug/g)}}{\text{medium solute concentration (ug/ul)}}$$

Since the distribution of raffinose is assumed to be restricted to an extracellular compartment of the slices, the volume of the apparently intracellular compartment of the slices (ICW) may be calculated thus -

'intracellular water' (ICW) = 'total tissue water' (TW) - 'raffinose space' (RS)

each of the above terms being expressed as microlitres per gram of incubated slices.

Unless the period of incubation of the slices in xylose-containing medium is very short (2.15 minutes, see page 46), the xylose space of the slices will exceed the raffinose space. Since xylose is assumed to diffuse freely through the compartment accessible to raffinose, attaining a concentration therein equal to the medium xylose concentration, it is assumed that, where the xylose space exceeds the raffinose space, it does so as a consequence of some xylose having entered the 'intracellular' compartment. An 'intracellular xylose space' may be calculated thus -

intracellular xylose space (ICXYS) = xylose space (XYS) - raffinose space (RS)

The uptake of xylose may now be expressed in terms of the fractional penetration (f) of the intracellular compartment by this sugar.

$$f = \frac{\text{intracellular xylose space}}{\text{intracellular water}}$$

Since 'f' is a ratio, measuring the extent to which xylose has penetrated the 'intracellular' compartment, rather than the concentration /

concentration of xylose attained therein, it provides a useful notation for the comparison of xylose uptakes over a range of medium xylose concentrations. Intracellular xylose concentrations may readily be calculated from values of 'f' since -

intracellular xylose concentration =
f times the medium xylose medium concentration

Osmatic Correction

The experimentally observed 'intracellular' space (ICW) is probably not the best basis on which to calculate the rate of xylose uptake, as osmotic shrinkage of this compartment occurs when the tissue is transferred to a medium containing xylose and raffinose. It has been shown (Gilbert (1966) (*18*)) that, when xylose uptake is calculated on the basis of the experimentally observed ICW, the 'intracellular' (non-raffinose) compartment of tissue in which osmotic shrinkage has been induced by the inclusion in the incubation medium of mannitol, appears to take up xylose at a greater rate than does the 'intracellular' compartment of control tissue incubated in a similar medium, but without mannitol, and therefore subject to a lesser degree of shrinkage. If, however, the xylose uptake results be calculated on the basis of a standard 'intracellular' space (ICWo) (which is taken as that which would exist if the osmolarity of the cell contents were equal to that of the medium), the discrepancy between the xylose uptake results from control media, and from media whose tonicity is increased by the addition of mannitol, disappears.

It is therefore desirable, when comparing rates of sugar uptake at different medium sugar concentrations, to relate uptake results to the osmotically corrected value (ICWo) of the 'intracellular' space. This may be calculated as follows - *(18)*

Let the osmolarity of the medium, before addition of raffinose (osmolarity r), or xylose (osmolarity x) be m. Then, assuming (i) that the xylose has, at the end of the incubation period, attained a uniform concentration, fx, throughout the 'intracellular' compartment, (ii) that the uptake of xylose by the 'intracellular' compartment does not significantly alter the total osmolarity, m+ x + r, of the incubation medium, the volume of the medium being large, and (iii) assuming that the cells behave as perfect osmometers.

$$ICWo = ICW \left(\frac{m + x + r}{m + fx}\right)$$

fo, the value for the fractional penetration of xylose into the 'intracellular' compartment based on ICWo rather than the experimentally/

experimentally determined ICW, will then be related to f as follows -

$$fo = f\left(\frac{m + fx}{w + x + r}\right)$$

SOME EFFECTS OF ANTICONVULSANT DRUGS ON SUGAR UPTAKE BY CEREBRAL CORTEX SLICES

CHAPTER III - THE EFFECT OF ANTICONVULSANT DRUGS ON XYLOSE UPTAKE

PART 1 - 'QUALITATIVE' INVESTIGATIONS OF THE EFFECT OF ANTICONVULSANTS ON SUGAR UPTAKE

PART 2 - EFFECT OF ANTICONVULSANT DRUGS ON THE KINETICS OF XYLOSE UPTAKE BY CEREBRAL CORTEX SLICES

The Effect of Anticonvulsant Drugs on Xylose Uptake

Introduction

As has been stated in Chapter I, it was proposed to survey the effect of a number of anticonvulsant drugs on xylose uptake by cerebral cortex slices, to ascertain whether such an effect was specific to the anticonvulsant drugs, phenobarbitone and dimethadione, which had been previously reported (24) to have an effect on xylose uptake by cerebral cortex slices, or whether an effect on sugar uptake was a more general feature of anticonvulsant drugs. It was also intended that where a drug was found to have an effect on xylose uptake, the mechanism of this effect should be investigated.

In the present work, therefore, the effect upon xylose uptake of two anticonvulsant drugs not previously studied in this context (ethosuximide and acetazolamide) was examined. In addition, the effect of diphenylhydantoin on xylose uptake was examined. This drug had been previously reported by Gilbert, Ortiz and Millichap (24) to have no effect on xylose uptake by cerebral cortex slices. However, the conditions of experiment employed by these investigations differed from those of the present study in that the drug was administered to the animals (guinea pigs) by intraperitoneal injection, (control animals receiving an injection of 0.9% NaCl) prior to the decapitation of the animal and measurement of xylose uptake by incubated slices of cerebral cortex.

In the present study, the investigation of the effect of an anticonvulsant drug on the uptake of xylose into the 'intracellular' compartment of cerebral cortex slices proceeded by three stages -

(i) Preliminary studies to establish whether the drug under test had any effect on xylose uptake were carried out. In these studies the conditions of incubation of the slices were such that either a stimulation of, or a depression of, xylose uptake should be readily detected.

(ii) & (iii) If the drug under test appeared to have a significant effect on xylose uptake, two further series of experiments, designed to investigate the mechanism by which this effect was achieved, were then undertaken. The effect of the drug on kinetics of the xylose uptake process was studied, and, in another series of experiments, the effect of sulphydryl group blocking agents on the response of the xylose uptake process to the anticonvulsant drug was examined.

The experimental conditions employed in studies using sulphydryl blocking agents were similar to those obtainingin the preliminary studies of the effect of anticonvulsants on sugar uptake. Both these studies, therefore, are described in part 1 of this Chapter, while the studies of the effect of anticonvulsants on the kinetics of xylose uptake are described in part 2.

Part 1 - 'Qualitative' Investigations of the Effect of Anticonvulsants on Sugar Uptake

Screening of Drugs for Effect on Xylose Uptake

A study by the author, prior to the present work, had shown that the incubation of cerebral cortex slices (which had been prepared and pre-incubated in the manner described on pages 12-16) for a nine-minute period in medium 'M2' containing 50mM xylose produced a fractional penetration by xylose of the apparent intracellular compartment of the slices, of about 0.6.

These conditions of incubation should therefore permit either inhibition or stimulation of xylose uptake by a drug to be detected, and were therefore employed in experiments to 'screen' drugs for an effect on xylose uptake. The same conditions of incubation were also employed in studies of the effect of sulphydryl group blocking agents on the response of the xylose uptake process to the drugs.

The Investigation of Possible Drug-Binding Sites

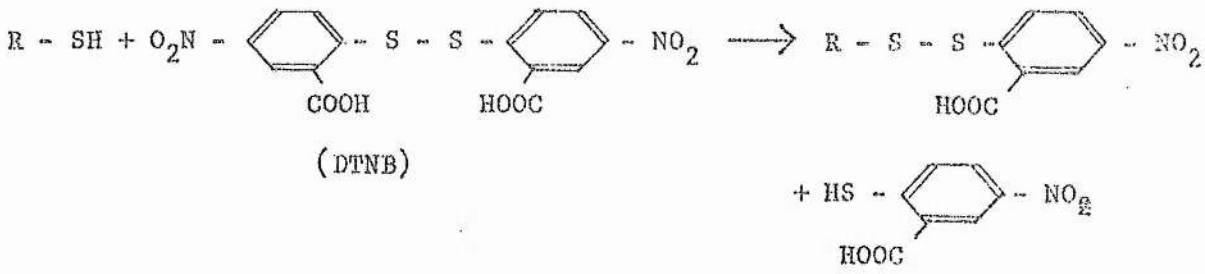
Since any effect of anticonvulsants on the movement of sugars across the cell membrane is likely to take place at or within the cell membrane it was proposed to examine some of the mechanisms by which the drugs might bind to the membrane. It was intended to incubate the slices under a number of conditions of incubation which, in a variety of different ways, might be expected to reduce the binding of the drugs to the membrane and to observe the effect of the changed conditions on the response of the xylose uptake process to the anticonvulsants. The effect of two sulphydryl group blocking agents on the response to anticonvulsants of the xylose uptake process has therefore been studied. It was not found possible, in the time available, to study the effect of other means of reducing the availability of binding sites to the drugs.

The effect of more than one sulphydryl group blocking agent on the response of the xylose uptake process to anticonvulsants was examined because no single inhibitor appears to show an absolute specificity for reaction with sulphydryl groups and because there may be wide variations in the rate at which different inhibitor species will react with the sulphydryl groups of protein. The sulphydryl group blocking agents employed in the present investigation were iodoacetamide and 5.5' dithiobis (2-nitrobenzoic acid), (DTNB). These compounds react with sulphydryl groups in the following manner.

R-/

$$R - SH + I.CH_2.CONH_2 \longrightarrow R - S - CH_2.CONH_2 + HI.$$

(iodoacetamide)



(DTNB)

The reaction of DTNB with sulphydryl groups is theoretically reversible. However, at physiological pH, the equilibrium is so far in favour of the products that the reaction may be regarded, for practical purposes, as irreversible (Ellman (12), (13)).

Iodoacetamide was chosen as a - SH group blocking agent for use in the present study because being a small uncharged molecule it might be expected to be capable of interacting with sulphydryl groups which were inaccessible to most other - SH blocking agents. Sulphydryl groups situated at the inner face of the membrane, or in pores within the membrane, for example, might be accessible to iodoacetamide. In contrast, DTNB, a relatively large and polar molecule will probably be incapable of crossing the cell membrane.

Only sulphydryl groups on the outer surface of the cell membrane will therefore be accessible to this agent. A comparison of the effects of these two - SH blocking agents may thus yield information about the distribution of any sulphydryl groups involved in the binding of anti-convulsant drugs to brain cell membranes.

Before proceeding to examine the effect of the sulphydryl group blocking agents on the response of the xylose uptake process to anti-convulsant drugs, it was necessary to verify that the inhibitors did not affect xylose uptake in the absence of the drugs. A series of experiments was therefore carried out in which the uptake of xylose by slices incubated in the presence of sulphydryl group blocking agents was compared with that of control slices.

Method

In the experiments to investigate whether certain anticonvulsants affected sugar intake, in the experiments to determine the effect of - SH group blocking agents on sugar uptake, and in the experiments to determine the sensitivity to sulphydryl blockade of the response of the sugar uptake/

uptake system to anticonvulsants, a common experimental procedure was followed -

Freshly prepared guinea pig cerebral cortex slices were divided into two portions. One portion was used for a control experiment, and was incubated in media containing no drug or inhibitor. The other portion of slices was incubated either in the presence of an anticonvulsant drug, or a - SH group blocking agent, or in the presence of both a drug and a - SH group blocking agent, according to the purposes of the experiment. The drugs and inhibitors employed were present both in the 'pre-incubation' medium (M1) and the xylose-containing incubation medium (M2). 'Test' and control experiments were carried out concurrently. After pre-incubation of the slices for thirty minutes at 37°C in medium 'M1', they were transferred to medium 'M2', which contained 50mM xylose, for a nine minute incubation period. At the end of this time, the slices were collected and their water, raffinose and xylose content measured by the methods already described. The experiment was repeated at least five times for each drug or inhibitor tested.

For each anticonvulsant, the concentration employed was that which the drug is thought to attain in brain (Millichap (1965) (43)), after administration at a therapeutic dose level, except in the case of acetazolamide, where a concentration (20μM) rather greater than the estimated brain concentration (5μM) was employed.

Diphenylhydantoin, at the concentration employed in the present work, was found to be insoluble in the medium, as has been previously reported (Gilbert, Ortiz and Millichap (1966) (24)). It was solubilised by dissolving the drug in a small volume of ethanol, prior to its addition to the medium. An equal volume of ethanol, without the drug, was therefore added to the incubation media for the control slices, in experiments to assess the effect of this drug.

To determine whether iodoacetamide or DTNB had any effect on 'basal' sugar uptake (i.e. sugar uptake in the absence of anticonvulsants) by cerebral cortex slices, the xylose uptake of slices incubated in the presence of 100μM iodoacetamide or 100μM DTNB was compared with that of simultaneously incubated controls, the conditions or incubation being as described above

In experiments to determine the sensitivity to sulphydryl blockade of the response of the xylose uptake process to anticonvulsants, the xylose uptake of slices incubated in media containing both an anticonvulsant and a - SH group blocking agent was compared with that of control slices (incubated in media containing neither anticonvulsant nor - SH group blocking agent).

Results and Discussion

The data presented in Table 2 & Fig. 8 (pages 39 & 40) indicate that under the conditions of incubation employed in the present experiments, the uptake of xylose into the 'intracellular' compartment of the cerebral cortex slices incubated in media containing acetazolamide or ethosuximide was significantly greater than that of the corresponding controls. Although some increase in xylose uptake was noted in the presence of diphenylhydantoin, this drug was not found to have any statistically significant effect in this respect.

When the uptake of xylose by the intracellular compartment of slices incubated in the presence of both an anticonvulsant and a sulphydryl group blocking agent was compared with that of control slices, (table 2 and Fig. 8) it was found that for each of the four combinations -

acetazolamide + iodoacetamide
acetazolamide + DTNB
and
ethosuximide + iodoacetamide
ethosuximide + DTNB

no significant effect on xylose uptake was observed. This contrasts with the effect of either acetazolamide or ethosuximide alone, where a significant increase in xylose uptake had occured. Since neither iodoacetamide nor DTNB appears to affect basal xylose uptake (table 2 and Fig. 8) it appears likely that these inhibitors in some way prevent the stimulation of xylose uptake by anticonvulsant drugs. The abolition, by the inhibitors, of this response to anticonvulsant drugs could be explained in a number of ways -

(i) The effect of the anticonvulsants on the movement of xylose across the cell membrane may result from an interaction of these drugs with the cell membrane. It is possible that sulphydryl groups are normally involved in the binding of the drugs to the membrane, but are rendered unavailable for this purpose by combination with the inhibitors.

(ii) It is also possible that the binding of the inhibitors to sulphydryl groups of the membrane might, by altering the structure of the protein prevent the binding of the drugs to the membrane, irrespective of whether the binding of the drugs involved sulphydryl groups.

(iii) The results of the present experiments could possibly result from the destruction of the anticonvulsant drugs by reaction in solution with the inhibitors, since members of both classes of compound were simultaneously present in the incubation media. To test this possibility, the absorption spectra (at wave lengths between 215 and 500 nm) of solutions of acetazolomide, of ethosuximide, of iodoacetamide, and of DTNB, were recorded. Solutions containing both an anticonvulsant and an inhibitor were then made and stood for/

TABLE 2

THE EFFECT OF ANTICONVULSANTS, and of -SH BLOCKING AGENTS ON THE 'INTRACELLULAR' WATER AND XYLOSE COMPARTMENT OF CEREBRAL CORTEX SLICES

Conditions of experiment and number of observations	ICW (μl/ g)	f	ICWo (μl/ g)	fo
control (7)	248±18	0.61±0.03	273±21	0.5[illegible]±0.0[illegible]
20 μM acetazolamide (7)	269±22*	0.75±0.06*	289±25	0.70±0.06*
control (7)	232±17	0.64±0.04	254±21	0.59±0.04
500 μM ethosuximide (7)	231±10	0.75±0.04*	247±11	0.70±0.04*
control with 0.5% ethanol (5)	212±13	0.78±0.03	227±15	0.74±0.03
100 μM diphenylhydantoin with 0.5% ethanol (5)	196±10	0.93±0.08	205±13	0.89±0.09
control (4)	180±9	0.72±0.07	193±12	0.67±0.07
100 μM DTNB (4)	158±5	0.72±0.08	170±6	0.67±0.07
control (5)	172±8	0.48±0.06	192±11	0.43±0.07
100 μM iodoacetamide (5)	186±9	0.49±0.03	207±12	0.44±0.0[illegible]
control	165±6	0.87±0.08	174±8	0.82±0.0[illegible]
acetazolamide + DTNB (5)	154±6*	0.85±0.07	163±8*	0.81±0.07
control (4)	184±9	0.70±0.08	199±12	0.65±0.1[illegible]
ethosuximide + DTNB (4)	178±16	0.72±0.14	192±19	0.68±0.15
control (7)	194±8	0.27±0.04	226±10	0.23±0.04
acetazolamide+iodoacetamide (7)	212±7*	0.28±0.04	245±9*	0.25±0.04
control (5)	206±16	0.62±0.09	226±2[illegible]	0.57±0.0[illegible]
ethosuximide+iodoacetamide (5)	188±15*	0.62±0.06	206±16*	0.56±0.06

ICW = the experimentally observed intracellular water content of the slices (μl. per g. wet weight),

f = the fractional penetration of the intracellular compartment by xylose, calculated on the basis of the observed ICW,

ICWo and fo are osmotically corrected values of ICW and f (see page 31),

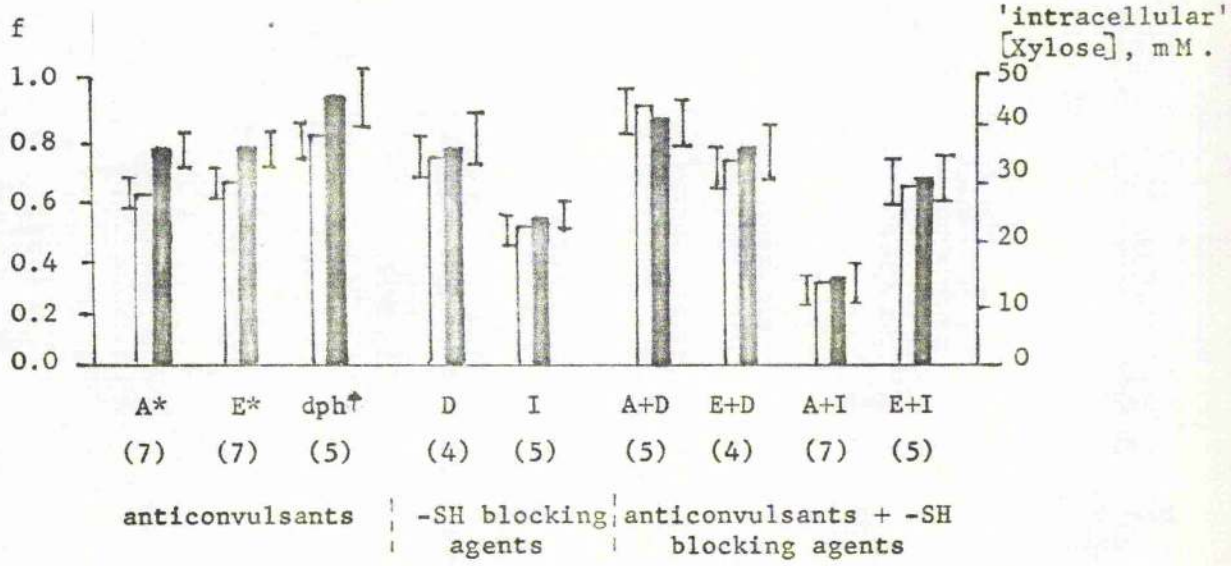
* = significantly different from the corresponding control value ('t' test, $0.05 > p$).

The slices were incubated for nine minutes in medium 'M2' containing 50 mM xylose, after pre-incubation for thirty minutes in medium 'M1' containing no xylose.

The mean f values tabulated here are also shown in Fig. 8.

FIGURE 8

THE EFFECT OF ANTICONVULSANT DRUGS ON XYLOSE UPTAKE, AND ITS ABOLITION BY -SH BLOCKING AGENTS



The dark columns show the mean xylose uptake of slices incubated in the presence of drugs and/or inhibitors, the light columns the corresponding controls. The drug tested, and number of observations, are shown at the foot of the column. The slices were incubated for nine minutes in medium 'M2' containing 50mM xylose, after pre-incubation for thirty minutes is medium 'M1' with no xylose. Standard errors of the mean values are shown to the side of each column. In experiments marked *, xylose uptake by drug treated slices was significantly different from that of controls (t test, $0.05 > p$).

Abbreviations:

A = acetazolamide: 20 μM. E = ethosuximide: 500 μM

dph = diphenylhydantoin: 100 μM D = DTNB 100 μM

I = iodoacetamide: 100 μM

† = in these experiments, ethanol (0.5%) was present in the media.

for forty minutes at 37°C. The absorption spectrum of each of these mixed solutions was then recorded, and compared with that of its component anticonvulsant and inhibitor. In each case, the absorption shown by the mixture appeared to be equal to the sum of that of its component parts (Fig 9; p.43). It therefore seems unlikely that either acetazolamide or ethosuximide react to any significant extent with iodoacetamide or DTNB under the conditions of incubation employed in the present studies.

The apparent lack of effect, on basal xylose uptake by cerebral cortex slices, shown by iodoacetamide and DTNB, together with the finding of Joanny (32) and colleagues, that iodoacetate does not affect the permeability of cerebral tissue to xylose, would seem to suggest that the sugar transport system in the brain is not sensitive to sulphydryl blocking agents. In contrast, sugar transport in a number of tissues (intestine, kidney, adipose cells and erythrocytes - Stein (1967) (52)) is inhibited by sulphydryl blocking agents, though basal glucose uptake by perfused heart is unaffected by the sulphydryl blocking agent N-ethyl maleimide (Cadenas (6)). However, the sugar transport process of some types of cell may be markedly more sensitive to some sulphydryl blocking agents than to others. Thus glucose transport in erythrocytes is inhibited by N-ethyl maleimide and by p-chloromercuribenzoate and other mercurials, but not by iodoacetate (Stein (52)).

Some of the anticonvulsant drugs and - SH blocking agents tested had a significant effect on the intracellular water content of the slices. The increase in the experimentally observed intracellular water content (ICW) of slices incubated in the presence of acetazolamide may be due to the osmotic effect of the greater intracellular xylose content of the slices, since the osmotically corrected intracellular water content (ICWo) of the acetazolamide treated slices, though higher than that of the corresponding controls, is not significantly so ($p > .05$).

Where DTNB was present in the incubation media, either alone or together with an anticonvulsant drug, there was a tendency for the ICWo of DTNB treated slices to be lower than that of controls, although this trend was only statistically significant in one series of experiments. The decrease in ICWo, where DTNB was present, could be a consequence of the disruption of the membranes of some cells by DTNB.

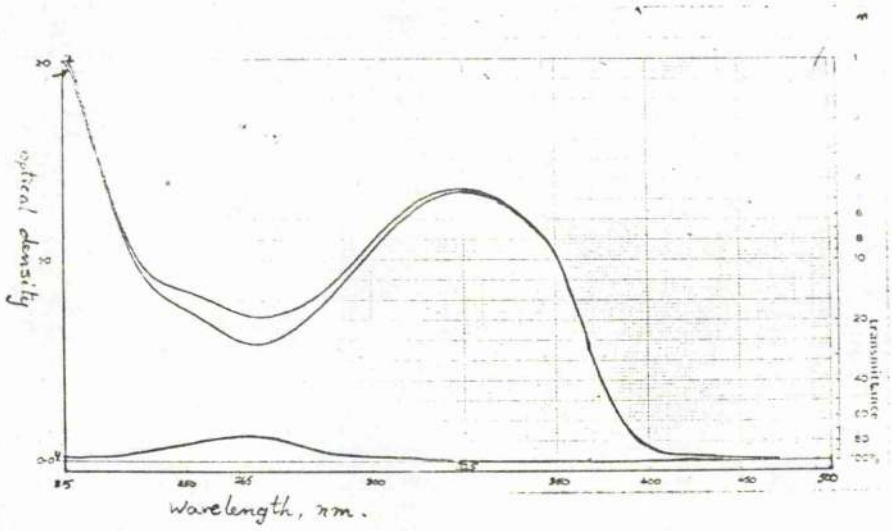
Divergent effects on intracellular water content were obtained in the various series of experiments in which slices were incubated in the presence of iodoacetamide. Thus incubation in the presence of iodoacetamide/

iodoacetamide alone did not significantly alter the ICWo of the slices, but the ICWo was significantly greater than that of corresponding controls for slices incubated in the presence of acetazolamide plus iodoacetamide, and significantly less than the control value for slices incubated in the presence of ethosuximide plus iodoacetamide. Neither acetazolamide nor ethosuximide alone significantly altered the ICWo.

The effects of iodoacetamide on the intracellular water content of cerebral cortex slices may reflect the interaction of two processes. Iodoacetamide may cause some inhibition of the respiration of the slices. This may cause swelling of the intracellular compartment of the slices, similar to that observed when brain slices are incubated in the absence of oxygen (58a). The combined effect of intracellular swelling and the binding of the inhibitor to the membrane might, however, cause a proportion of the cell membranes to rupture, in which case a decrease in the intracellular volume of the tissue might be observed.

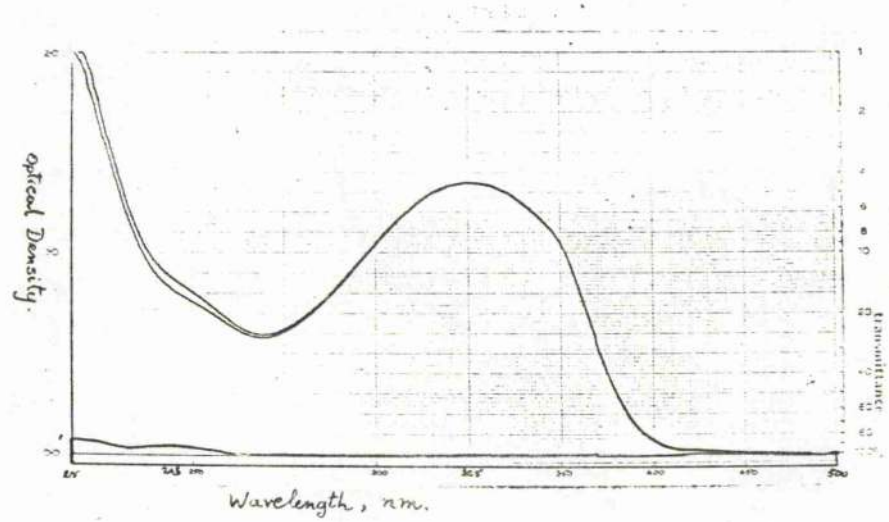
FIGURE 9.

ABSORPTION SPECTRA



A 1 = acetazolamide, 20 μM. 2 = DTNB, 100 μM.

3 = acetazolamide, 20 μM, + DTNB, 100 μM.



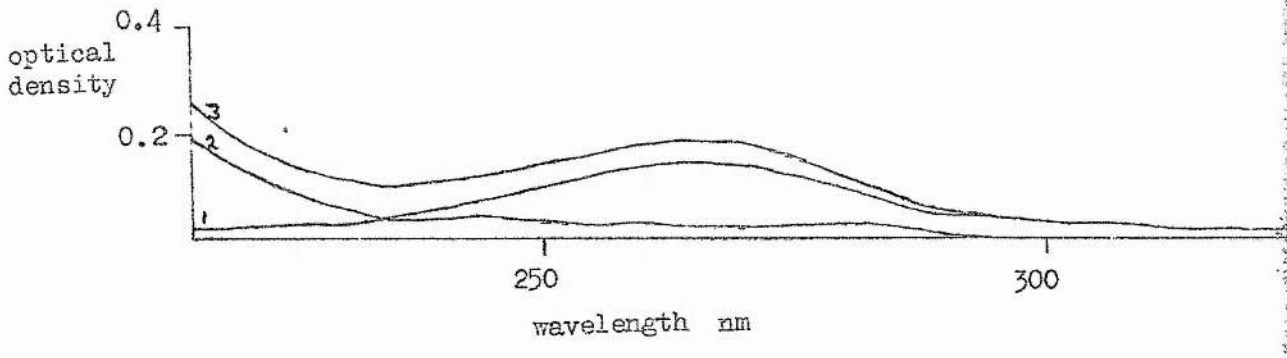
B 1 = ethosuximide, 500 μM. 2 = DTNB, 100 μM.

3 = ethosuximide, 500 μM, + DTNB, 100 μM.

(continued)

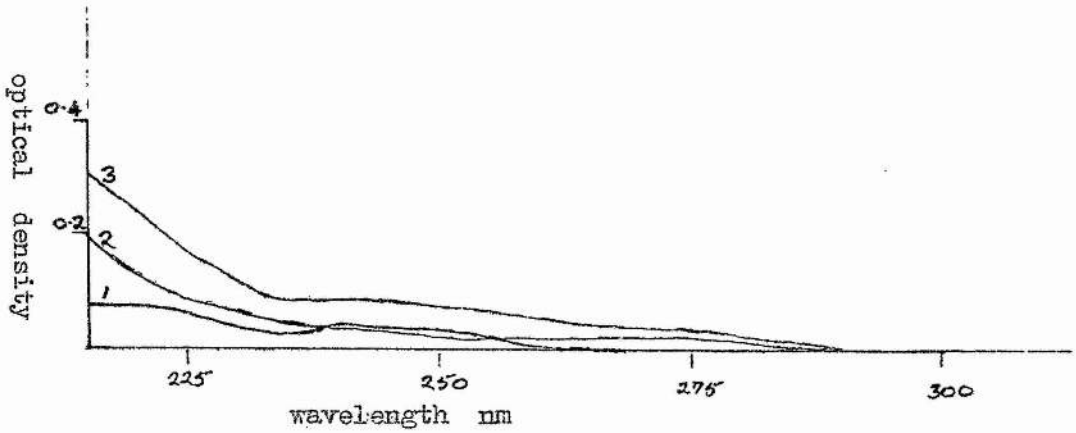
FIGURE 9 (Continued)

ABSORPTION SPECTRA



C 1 = acetazolamide, 20 μM. 2 = iodoacetamide, 100 μM.

3 acetazolamide, 20 μM, + iodoacetamide, 100 μM.



D 1 = ethosuximide, 500 μM. 2 = iodoacetamide, 100 μM.

3 = ethosuximide, 500 μM, + iodoacetamide, 100 μM.

Part 2 - Effect of Anticonvulsant Drugs on the Kinetics of Xylose Uptake by Cerebral Cortex Slices

Introduction

An estimate of the parameters, Km and Vmax, of the process of xylose uptake by the 'intracellular' compartment of the slices has been obtained both for slices incubated under control conditions and for slices incubated in the presence of anticonvulsant drugs. The method employed entailed the measurement of the initial velocity (V) of xylose uptake at a number of different xylose concentrations (X). Values of Km and Vmax were then calculated from a Lineweaver-Burk plot of $^1/_V$ against $^1/_X$. Values of 'V' were calculated by dividing the final xylose concentration attained in the 'intracellular' compartment by the time during which xylose was entering the compartment. Since, when the slices are first placed in xylose containing medium, the xylose will take a finite time to diffuse through the 'extracellular' compartment, the time during which xylose may enter the 'intracellular' compartment of the slices will be equal to the incubation time minus the time of diffusion of xylose through the 'extracellular' compartment. It is therefore necessary to determine this 'diffusion time' experimentally before embarking on initial velocity studies. It is also desirable that the incubation time selected for initial velocity studies should be such that the rate of entry of xylose into the intracellular compartment of the slices should not have fallen away significantly from the initial value. For these reasons the time course of uptake of xylose by the cerebral cortex slices was determined.

Methods

The Time Course of Uptake of Xylose

Slices of a guinea pig cerebral cortex were prepared and pre-incubated in medium 'M1', as previously described (page 13). The slices were then transferred to medium 'M2', which contained 100mM xylose, for a further period of incubation. In a series of experiments incubation times of 1, 2, 4, 6 and 8 minutes were employed for the incubation in M2. At the end of this incubation, the slices were recovered and their water, raffinose and xylose content measured by the methods previously described.

In order to reduce the possibility of the results of the experiments being affected by day to day variations in the environment of the experimental animals, one incubation at each of the incubation times selected was carried out during the course of one day, and the order in which the incubations were carried out during the day was varied between successive groups of/

of experiments.

Initial Velocity Studies

Cerebral cortex slices were prepared from the brain of one guinea pig. The slices were divided into two portions. One portion was pre-incubated in medium M1, and subsequently transferred to medium M2, both media containing no drug. This portion of slices served as a control. At the same time, the other portion of slices was incubated in similar media in which acetazolamide (20 μM) or ethosuximide (500 μM) had been dissolved.

The time of incubation of the slices in the xylose containing medium M2 was four minutes, the 'time course of xylose uptake' studies having indicated that this incubation time was appropriate for 'initial velocity' studies (see page 47). In a series of paired experiments the xylose uptake of the 'intracellular' compartment of both control and drug treated slices, incubated in media containing xylose at concentrations of 20, 30, 50 or 100mM, was estimated. At least five pairs of experiments were performed at each medium xylose concentration.

Results and Discussion

The Time Course of Xylose Uptake by the Slices

The results of experiments to determine the time course of uptakeof xylose by cerebral cortex slices are shown in Fig. 10 page 52. It is assumed that when xylose has diffused through the 'extracellular' compartment, but has not entered the 'intracellular' compartment to any appreciable extent, the proportion of tissue water accessible to xylose will be equal to the proportion accessible to raffinose. By a plot of the percentage of the tissue water accessible to raffinose and to xylose at the various times of incubation in 'M2' employed in these experiments, the time taken for xylose to diffuse through the 'extracellular' compartment of the slices may be estimated. A value, for the diffusion time of xylose, of 2.15 minutes, was obtained by this method. On referring to Fig.10.page 52 , it will be seen that the entry of xylose into the slices may be divided into two phases - (i) diffusion of xylose through the extracellular space during the first 2.15 minutes of the incubation, and (ii) subsequent entry of xylose into the intracellular compartment.

There will, however, be some overlap between the two phases, as xylose will have begun to enter some of the more accessible cells during the 'diffusion' period. Therefore, although the volume of distribution of xylose equals that of raffinose after 2.15 minutes, the distribution of xylose at this time will include a small intracellular componant, while that/

that of raffinose will be entirely extracellular. Xylose may therefore still be diffusing into the less accessible portions of the extracellular compartment after 2.15 minutes.

For initial velocity studies, an incubation of the slices in xylose-containing medium of four minutes duration was employed, as the uptake of xylose into the intracellular compartment of the slices remains fairly constant until the end of the fourth minute of incubation. The rate of increase in xylose content of the slices is greater between 2.15 and 3.50 minutes than subsequently, but this may reflect some diffusion of xylose from the medium to the extracellular compartment.

Studies of the Initial Velocity of Xylose Uptake

From the data obtained as to the water, raffinose and xylose content of slices incubated in the course of the 'initial velocity' studies, values were calculated for the fractional penetration (f) of the intracellular compartment of the slices by xylose, at each of the four medium xylose concentrations. Since the uptakes of xylose at different medium xylose concentrations were to be compared, the osmotically corrected values of the fractional penetration (fo) were then calculated by the method described on page 31.

The velocity (V) of xylose uptake at each of the xylose concentrations (X) was calculated from the mean values of fo at each concentration in the following manner -

The concentration of xylose in the 'intracellular' compartment at the end of the incubation, calculated on the basis of the 'osmotically corrected' intracellular volume, will be foX. The time (t) during which xylose has been entering the 'intracellular' compartment will be the incubation time (4 minutes) minus the time taken for diffusion of xylose through the 'extracellular' compartment (2.15 minutes), i.e. 1.85 minutes. The velocity of xylose uptake can therefore be calculated from the relationship

$$V = \frac{foX}{t} = \frac{foX}{1.85}$$

where the units of 'V' are - millimoles.litre intracellular water^{-1}. hour^{-1}.

Km and Vmax, for the xylose uptake process, both in the presence and in the absence of the drugs, could then be determined from a plot of values of $1/V$ against the corresponding values of $1/X$.

The results of the initial velocity studies fall into two groups - (i) studies of the effect of acetazolamide on the kinetics of xylose uptake and (ii) similar studies, carried out at a later date, of the effect of/

of ethosuximide.

(i) The results of the first series of experiments (comparing acetazolamide treated slices with controls) are presented in Tables 3A & 3B, and Fig. 11

It will be seen (Table 3A, pages 53-55) that the mean fractional penetration of xylose into the 'intracellular' compartment of both control and drug treated slices decreased as the medium xylose concentration was increased, as would be expected if xylose were entering this compartment by a saturable mechanism. The results presented in this table show also that the experimentally determined intracellular water content (ICW) of both control and drug treated slices appears to decrease as the concentration of xylose in the incubation media increases. This decrease in the value of the observed ICW is probably due to osmotic shrinkage, as the 'osmotically corrected' values for the 'intracellular' water content are more or less constant.

When values of $^1/_v$ were derived from the results of the present experiments (see Table 3B page 56) and plotted against values of $^1/_x$ (see Fig. 11 page 57) the following values of Km and Vmax were obtained -

- for xylose uptake by control slices, Km = 87mM,
  Vmax = 30 m-moles.litre intracellular water^{-1}. min^{-1}.
- for acetazolamide treated slices, Km = 218mM,
  Vmax = 66 m-moles.litre intracellular water^{-1}. min^{-1}.

(ii) When the results of the second series of initial velocity studies, in which ethosuximide treated slices are compared with controls, are examined, it will be seen (Table 4A pages 58-62) that in rather more than half of the experiments, little or no penetration of xylose into the 'intracellular' compartment of the slices was observed. Such results are not in agreement with previous studies of xylose uptake under similar conditions (pages 52-55 of the present work, and Gilbert, Ortiz and Millichap (1966) (34)).

From the thirteen pairs (control and drug) of experiments in which there was an appreciable uptake of xylose by the intracellular compartment of the slices, some information may be obtained about the nature of the effect of ethosuximide on the xylose uptake process. Thus, although estimated values of Km and Vmax obtained from these experiments are liable to be in error, the direction in which Km and Vmax are altered by the drug may be detected. The results obtained are given in Table 4A , Table 4B , and Fig. 12. (pages 58 - 64). For control slices, Km for xylose uptake was estimated to be 153mM, Vmax 36m-moles.litre intracellular water^{-1}. min^{-1}. The corresponding values for ethosuximide treated slices/

slices were - Km, 53mM, Vmax 19m-moles/litre intracellular water^{-1}. min^{-1}.

The effect of ethosuximide, in decreasing both the apparent Km and Vmax of the xylose uptake process is very similar to that observed for the anticonvulsant drug phenobarbitone by Gilbert, Ortiz and Millichap (24). Acetazolamide, however, increases both the Km and Vmax of the transport system for xylose. The differing effects of acetazolamide and ethosuximide on the kinetics of xylose, uptake are not inconsistent with the previous findings (page 38) that both drugs increase xylose uptake, from a medium containing 50mM xylose, by cerebral cortex slices. It may be calculated, from the plots of $1/v$ against $1/x$ (Figs. 11 and 12) that slices incubated in the presence of acetazolamide will take up xylose more rapidly than controls if the external sugar concentration is greater than 20mM, while ethosuximide treated slices will show an increased rate of sugar uptake if the external sugar concentration is less than 63mM.

The prediction that acetazolamide should decrease xylose uptake, where slices are incubated in media containing xylose at a concentration less than 20mM is in agreement with a recent report (Gilbert (1971) (20)) that acetazolamide (20μM) decreases the uptake of xylose by cerebral cortex slices, from a medium containing 1mM xylose.

It seems unlikely that the effect of acetazolamide or of ethosuximide on the kinetics of xylose uptake by cerebral cortex slices can be accounted for by any action these drugs may have on cellular sodium levels. If the uptake of sugars by cells of cerebral cortex were by a sodium dependent process of the type described by Crane (10) and Schultz and Curran (50), then the flux of sugar into the cell would be expected to show a dependance on the concentration of sodium in the external medium, while the outward flux of sugar would depend on the intracellular sodium concentration. Since the experimental conditions for the initial velocity studies have been arranged so that the influx of xylose into the cells should be large compared with the efflux from the cells, and since the sodium concentration of the medium remains constant (the volume of the medium being large compared with that of the tissue), any changes in intracellular sodium level would not be expected to alter the values obtained for the Km and Vmax of xylose uptake.

<u>An Interpretation of the Results on the Kinetic Studies in terms of a 'Carrier-Mediated' Hypothesis of Sugar Transport</u>

The effect of the anticonvulsant drugs on the kinetics of xylose uptake may be interpreted in the following manner. Assuming that -

(1)/

(i) Sugar traverse the membrane in association with a membrane component, the carrier.

(ii) The carrier molecules move freely within the membrane due to the thermal agitation, and that this movement is unaltered by combination of the carrier with the sugar.

(iii) The rate of movement of the carrier can influence the equilibrium between the carrier and the sugar at the membrane surface

The movement of xylose across the cell membrane may be represented by the model described in Fig. 13 page 65. It can be shown (Bowyer (1957) (4), Stein (1967) (52)) that under these circumstances

$$Km = \frac{k-1 + k2}{k1} \quad \text{and} \quad Vmax = \frac{k2\ k-1}{(k2 + k-1)} \cdot \frac{Ct}{2}$$

where k1, k-1 and k2 are the rate constants for the processes described in Fig. 13, and Ct is the total concentration of carrier in the membrane (Ct = Co + Ci + Go + Gi). The expressions for Km and Vmax both contain the terms k-1 and k2. The increase in Km and Vmax brought about by acetazolamide might therefore follow either from an increased rate of breakdown of the sugar-carrier complex, or from an increased rate of movement of the sugar-carrier complex across the membrane. Similarly the decreased Km and Vmax observed in the presence of ethosuximide might be due to a decrease in the rate of either (or both) of these two processes.

Interpretation of the Results of the Kinetic Studies in Terms of a "Polar Pore' Hypothesis of Sugar Transport

An alternative to the carrier hypothesis of sugar transport has been proposed (Naftalin (1970) (45)) in which the movement of sugars across cell membranes is likened to the diffusion of solutes within a lattice, the membrane lattice being an array of fixed binding sites situated within water filled channels which span the membrane. It is assumed that one dimensional diffusion takes place between the sites, and that exchange between different solutes may occur between neighbouring binding sites or between the solution on either side of the membrane and the boundary sites. The kinetics of sugar flow through erythrocyte membranes appear to be more completely predicted by a computer simulation of this model system than by the carrier hypothesis. Studies of the structure of the membranes of a number of types of mammalian cell provide some support for the 'polar pore' hypothesis of sugar transport in that they indicate that water filled channels lined with polar side chains may be present in the membrane/

membrane (Wallach and Gordon (1961) (40)).

If the results of the present 'initial velocity' studies are interpreted in terms of Naftalins 'polar pore' hypothesis of sugar transport then Km becomes a measure of the efficiency of the sugar in occupying free binding sites on the lattice.

A lowering of the affinity of a molecular species for the binding sites (which would increase the Km for this species) will increase the inter-site transition probability of the species, and hence it will move more rapidly across the membrane lattice. The increase in the Km and Vmax of xylose transport seen in the presence of ethosuximide might result from a decrease in the affinity for xylose of the binding sites lining the membrane pores. Similarly the decrease in the Km and Vmax of xylose transport brought about by acetazolamide could be a result of an increase in the affinity of the binding sites for xylose.

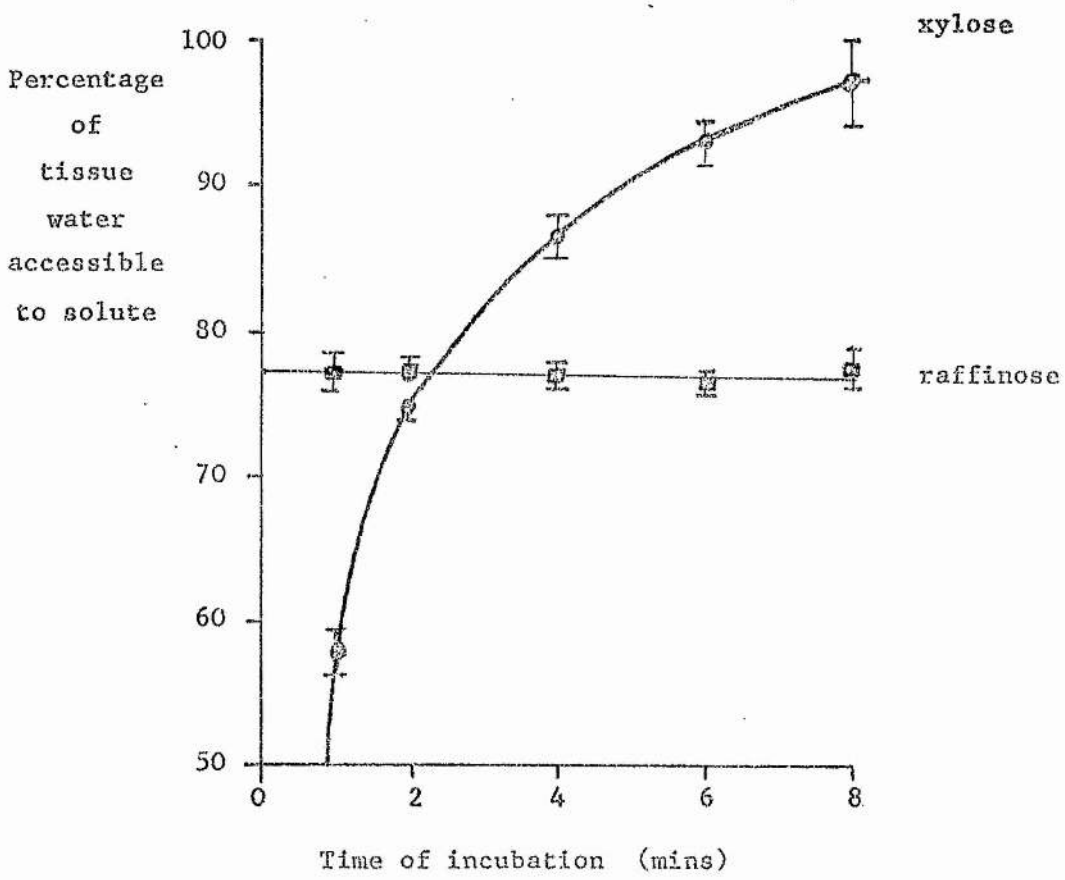
The Variability of the Xylose Uptake Results

The variability of the uptakes of xylose recorded in the course of the 'initial velocity' studies is much greater than that reported in some comparable studies (1,24,32). In some series of experiments, unexpectedly low uptakes of xylose by portions of incubated slices are seen, this being most clearly demonstrated in the results presented in Table 4A pages 58-62. (a study of the effects of ethosuximide on the initial velocity of xylose uptake). Since in each paired experiment, unusually low uptakes of xylose are either seen in both control and drug treated slices, or not at all, it appears that the factor causing the reduced uptake of xylose operates prior to the incubation of the slices, since from that point onwards the two samples of slices are processed separately.

Unusually low values for xylose uptake were also recorded in some of the several series of experiments described in part one of this chapter, as may be seen by reference to Fig. 8 page 40, in which each of the unshaded columns represent the mean xylose uptake by control slices for one series of experiments. The conditions of preparation and incubation of the control slices were the same for all the series of experiments, although each series of experiments was carried out at a different time and using guinea pigs from a different batch. It is possible that the variation between the xylose uptake values obtained in successive series of experiments could reflect variations in the environment to which successive batches of guinea pigs had been exposed.

FIGURE 10

THE TIME COURSE OF XYLOSE UPTAKE



Each point on the graph represents the mean of 4 or 5 results.
The cerebral cortex slices were pre-incubated in a medium (M1) without xylose for thirty minutes, then incubated in medium (M2) containing 100 mM xylose, for the times shown.

TABLE 3A

THE EFFECT OF ACETAZOLAMIDE ON THE INITIAL VELOCITY OF XYLOSE UPTAKE BY CEREBRAL CORTEX SLICES

Fractional Penetration of the Intracellular Space of the Slices by Xylose after a 4 Minute Incubation

Medium xylose concentration (mM)		T.W.	R.S.	XyS	ICW	ICWo	ICXyS	f	fo
20	C.	855	617	780	238	251	163	0.68	0.64
	D.	856	578	713	278	297	135	0.49	0.46
	C.	856	607	744	249	265	132	0.55	0.52
	D.	856	615	741	241	257	126	0.52	0.49
	C.	858	634	754	224	240	120	0.54	0.50
	D.	853	653	777	200	212	124	0.62	0.58
	C.	862	674	782	188	200	108	0.57	0.54
	D.	858	668	780	190	202	155	0.61	0.57
	C.	855	661	771	194	207	110	0.57	0.54
	D.	868	652	773	216	230	121	0.56	0.53
	C.	858	681	758	177	190	77	0.44	0.41
	D.	863	650	755	213	228	105	0.49	0.44
Mean ±s.e.m.	C	857±1	646±13	765±6	212±12	226±12	119±12	0.56±0.03	0.53±0.03
	D	859±2	636±14	757±11	223±13	238±14	121±4	0.55±0.02	0.52±0.02

TABLE 3A contd.

Medium xylose concentration (mM(		T.W.	R.S.	XyS	ICW	ICWo	ICXyS	£	£o
30	C.	848	599	750	249	267	151	0.61	0.57
	D.	849	614	684	235	260	70	0.30	0.27
	C.	861	647	715	214	236	68	0.32	0.29
	D.	860	624	719	236	258	95	0.40	0.37
	C.	853	624	674	229	255	50	0.22	0.20
	D.	857	622	752	235	253	130	0.55	0.51
	C.	860	661	777	199	214	116	0.58	0.54
	D.	852	704	797	148	158	93	0.63	0.59
	C.	860	632	799	178	190	117	0.66	0.62
	D.	864	707	831	157	165	124	0.79	0.75
Mean ±s.e.m.	C	856±3	642±14	743±22	214±12	232±14	101±18	0.48±0.09	0.44±0.07
	D	856±3	654±21	756±26	202±20	219±23	102±11	0.54±0.09	0.47±0.07
50	C.	846	641	700	205	237	59	0.29	0.25
	D.	847	612	733	235	262	121	0.51	0.46
	C.	851	615	694	236	271	79	0.33	0.29
	D.	857	614	705	243	277	91	0.37	0.33
	C.	864	668	789	196	214	121	0.62	0.57
	D.	856	625	698	231	264	73	0.32	0.28
	C.	852	639	715	213	243	76	0.36	0.32
	D.	865	688	798	177	193	110	0.62	0.57
	C.	859	655	813	204	218	158	0.77	0.72
	D.	861	692	812	169	182	120	0.71	0.66
	C.	863	672	745	191	217	72	0.38	0.34
	D.	862	705	816	157	169	111	0.71	0.66
Mean ±s.e.m.	C	855±3	648±9	743±20	208±6	233±9	94±15	0.46±0.03	0.42±0.06
	D	858±3	656±18	760±22	202±16	225±20	104±3	0.54±0.07	0.49±0.07

TABLE 3A contd.

Medium xylose concentration (mM)		T.W.	R.S.	XyS	ICW	ICWo	ICXyS	f	fo
100	C.	850	662	700	188	244	38	0.20	0.16
	D.	852	666	717	156	236	51	0.27	0.21
	C.	848	669	708	179	231	39	0.22	0.17
	D.	851	675	691	176	237	16	0.09	0.07
	C.	852	646	702	206	261	56	0.27	0.21
	D.	847	649	727	198	242	78	0.39	0.32
	C.	847	632	740	215	254	108	0.50	0.42
	D.	855	642	793	213	237	151	0.71	0.64
	C.	834	633	732	201	238	99	0.49	0.41
	D.	848	659	753	189	233	94	0.50	0.47
	C.	839	707	780	132	154	73	0.55	0.47
	D.	852	682	796	170	191	114	0.67	0.60
	MeanC	845±3	658±12	727±13	187±12	230±17	69±12	0.37±0.05	0.31±0.05
	±s.e.m.D	851±1	662±6	747±17	138±6	228±8	84±19	0.44±0.09	0.38±0.09

Abbreviations

T.W. = total water, R.S. = raffinose space,
XyS = xylose space, ICW = intracellular water,
ICWo = osmotically corrected value of ICW, ICXyS = intracellular xylose space,
all expressed as ul. per g. of incubated slices.
f = fractional penetration of the ICW by xylose,
fo = the osmotically corrected value of f.

TABLE 3B

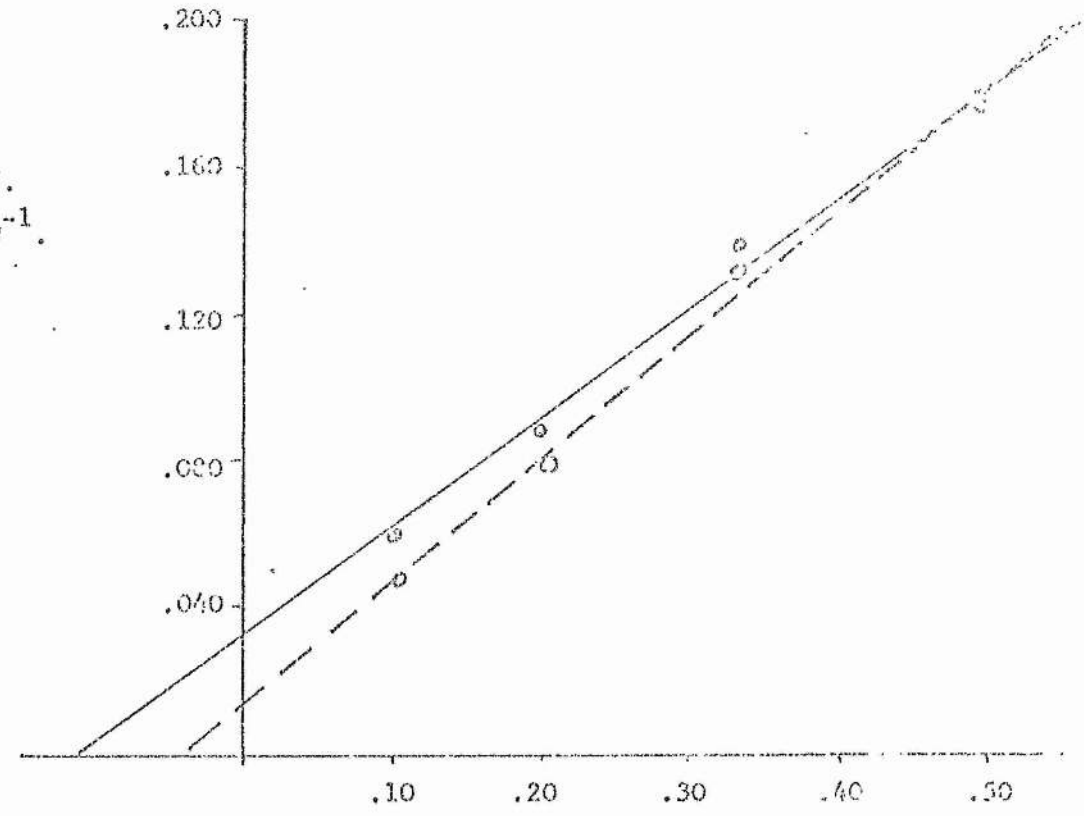
The Initial Velocity of Xylose Uptake by the Intracellular Compartment of the Slices, Calculated from the Mean fo Values of Table 3A

	X	fo	V	$^1/_X$	$^1/_V$
control	20	0.53±0.03 (6)	5.73	0.050	0.175
	30	0.44±0.07 (5)	7.13	0.033	0.140
	50	0.42±0.08 (6)	11.35	0.020	0.088
	100	0.31±0.06 (6)	16.76	0.010	0.060
with acetazolamide (20 µM)	20	0.52±0.02 (6)	5.62	0.050	0.178
	30	0.47±0.07 (5)	7.62	0.033	0.131
	50	0.49±0.07 (6)	13.24	0.020	0.076
	100	0.38±0.09 (6)	20.54	0.010	0.049

X = medium xylose concentration (mM),
fo = osmotically corrected fractional penetration of the intracellular compartment of the slices by xylose,
V = velocity of xylose uptake by the intracellular compartment, (m-moles. litre ICW^{-1}.min^{-1}.)

FIGURE 11

THE EFFECT OF ACETAZOLAMIDE ON THE INITIAL VELOCITY OF SUCROSE UPTAKE



—o— = control values
--o-- = with acetazolamide
values of 1/v at each point have been calculated from the mean of 5 or 6 results

controls - Km = 87 mM. Vmax = 30 m-moles.liter$_{ICW}^{-1}$.min^{-1}.

with 20 μM acetazolamide -
Km = 218 mM. Vmax = 66 m-moles.liter$_{ICW}^{-1}$.min^{-1}.

TABLE 4A

THE EFFECT OF ETHOSUXIMIDE ON THE INITIAL VELOCITY OF XYLOSE UPTAKE BY CEREBRAL CORTEX SLICES

Fractional Penetration of the Intracellular space of the Slices by Xylose after a 4 Minute Incubation

Medium xylose concentration (N m)		T.W.	R.S.	XyS	ICW	ICWo	ICXyS	f	fo
20	C.	(863)	(582)	(580)	(281)		-	-	-
	D.	(862)	(606)	(632)	(256)	(281)	(26)	(0.10)	(0.09)
	C.	(861)	(612)	(612)	(249)		-	-	-
	D.	(873)	(657)	(665)	(216)	(238)	(0)	(0.04)	(0.03)
	C.	872	695	770	177	190	75	0.42	0.37
	D.	867	672	787	195	207	115	0.59	0.56
	C.	862	629	727	233	251	98	0.42	0.39
	D.	872	642	786	230	243	144	0.63	0.59
	C.	858	527	648	331	357	121	0.37	0.34
	D.	861	586	692	275	296	106	0.39	0.36
	C.	(856)	(679)	(543)	(177)		-	-	-
	D.	(859)	(691)	(618)	(168)		-	-	-
	C.	(866)	(713)	(659)	(207)		-	-	-
	D.	(863)	(715)	(660)	(203)		-	-	-
	C.	(860)	(686)	(574)	(224)		-	-	-
	D.	(855)	(632)	(608)	(223)		-	-	-
Mean* ± s.e.m.	C.	864±4	617±48	715±36	247±43	266±49	98±13	0.40±0.02	0.37±0.02
	D.	866±3	633±25	756±30	233±23	249±26	122±11	0.54±0.07	0.50±0.07

Table 4A contd.

Medium xylose concentration (mM)		T.W.	R.S.	XyS	ICW	ICWo	ICXyS	f	fo
30	C.	866	654	680	212	239	26	0.12	0.11
	D.	868	641	690	227	253	49	0.22	0.20
	C.	867	650	670	217	245	20	0.09	0.08
	D.	869	652	692	217	243	40	0.18	0.16
	C.	866	727	798	139	151	71	0.51	0.47
	D.	861	699	796	162	174	97	0.60	0.56
	C.	8[illegible]	701	824	168	178	123	0.73	0.69
	D.	871	676	817	195	207	141	0.72	0.68
	C.	859	610	738	249	270	128	0.51	0.47
	D.	862	596	766	266	285	170	0.64	0.60
	C.	(868)	(719)	(635)	(149)		-	-	-
	D.	(864)	(613)	(547)	(251)		-	-	-
	C.	(857)	(623)	(644)	(234)	(265)	(11)	(0.05)	(0.04)
	D.	(861)	(633)	(613)	(228)		-	-	-
	C.	(873)	(726)	(683)	(147)		-	-	-
	D.	(862)	(722)	(692)	(140)		-	-	-
Mean ±s.e.m.	C	865±2	668±21	742±30	197±19	217±22	74±23	0.39±0.12	0.36±0.12
	D	866±2	653±17	752±26	213±17	232±19	99±25	0.47±0.11	0.44±0.11

Table 4A contd.

Medium xylose concentration (mM)		T.W.	R.S.	XyS	ICW	ICWo	ICXyS	f	fo
50	C.	(870)	(663)	(655)	(207)		-	-	-
	D.	(867)	(671)	(696)	(196)	(227)	(25)	(0.13)	(0.11)
	C.	(864)	(640)	(648)	(224)	(270)	(8)	(0.04)	(0.03)
	D.	(869)	(656)	(637)	(212)		-	-	-
	C.	852	658	671	194	232	13	0.07	0.06
	D.	864	696	727	168	197	31	0.18	0.15
	C.	861	589	764	272	296	175	0.64	0.59
	D.	850	588	637	262	298	99	0.38	0.33
	C.	858	630	746	228	253	116	0.51	0.46
	D.	861	604	694	257	293	90	0.35	0.30
	C.	(870)	(663)	(595)	(207)		-	-	-
	D.	(868)	(684)	(616)	(184)		-	-	-
	C.	(860)	(651)	(620)	(209)		-	-	-
	D.	(862)	(627)	(659)	(235)	(278)	(32)	(0.14)	(0.12)
	C.	(853)	(671)	(656)	(182)		-	-	-
	D.	(862)	(691)	(632)	(171)		-	-	-
Mean* ±s.e.m.	C	857±3	626±20	727±28	231±23	260±19	401±47	0.41±0.17	0.37±0.16
	D	858±4	629±33	685±26	229±31	262±33	73±21	0.30±0.06	0.26±0.06

Table 4A contd.

Medium xylose concentration (mM)		T.W.	R.S.	XyS	ICW	ICWo	ICXyS	f	fo
100	C.	(862)	(668)	(698)	(194)	(256)	(30)	(0.15)	(0.12)
	D.	(848)	(648)	(612)	(200)		-	-	-
	C.	(852)	(718)	(715)	(134)		-	-	-
	D.	(848)	(690)	(694)	(158)	(217)	(4)	(0.03)	(0.02)
	C.	857	659	700	198	256	41	0.21	0.16
	D.	857	639	748	218	257	109	0.50	0.42
	C.	846	664	727	182	225	63	0.35	0.28
	D.	852	650	708	202	255	58	0.29	0.23
	C.	(847)	(711)	(600)	(136)		-	-	-
	D.	(858)	(686)	(617)	(172)		-	-	-
	C.	(848)	(637)	(600)	(211)		-	-	-
	D.	(848)	(654)	(603)	(194)		-	-	-
	C.	(857)	(705)	(622)	(152)		-	-	-
	D.	(858)	(695)	(612)	(163)		-	-	-
Mean* ±s.e.m.	C	852±6	661±3	714±13	190±8	241±15	52±	0.28±0.07	0.22±0.06
	D	855±2	645±5	728±20	210±8	256±1	84±25	0.40±0.11	0.33±0.10

Table 4A contd.

* In the calculation of mean values, no account has been taken of results indicated thus - ( ).

Other Abbreviations

T.W. = total water content of the slices,
XyS = xylose space,
ICWo = osmotically corrected value of ICW,
R.S. = raffinose space,
ICW = intracellular water,
ICXyS = intracellular xylose space
- all expressed as ul. per g. of incubated slices.
f = fractional penetration of the ICW by xylose,
fo = osmotically corrected value of f.

TABLE 4B

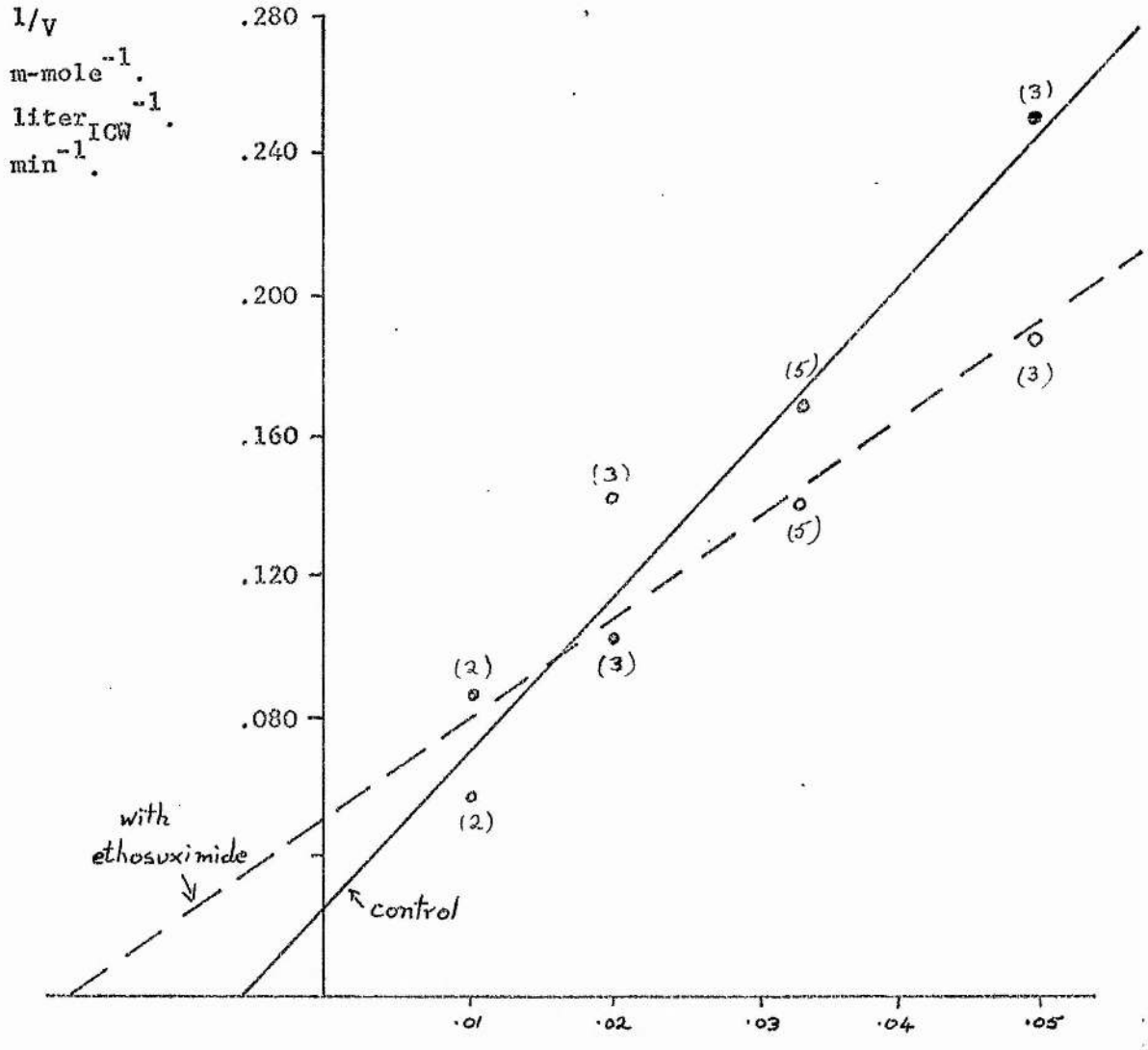
The Initial Velocity of Xylose Uptake by the Intracellular Compartment of the Slices, Calculated from the Mean fo Values of Table 4A

	X	fo	V	$^1/_X$	$^1/_V$
control	20	0.37±0.02 (3)	4.00	0.050	0.250
	30	0.37±0.11 (5)	6.00	0.033	0.167
	50	0.37±0.16 (3)	10.00	0.020	0.100
	100	0.22±0.06 (2)	11.89	0.010	0.084
with ethosuximide (500 μM)	20	0.50±0.07 (3)	5.40	0.050	0.185
	30	0.44±0.11 (5)	7.13	0.033	0.140
	50	0.26±0.06 (3)	7.03	0.020	0.142
	100	0.33±0.06 (2)	17.84	0.010	0.056

X = medium xylose concentration (mM),
fo = osmotically corrected fractional penetration of the intracellular compartment of the slices by xylose,
V = velocity of xylose uptake by the intracellular compartment, (m-moles, litre ICW^{-1}.min^{-1}.)

FIGURE 12

THE EFFECT OF ETHOSUXIMIDE ON THE INITIAL VELOCITY OF XYLOSE UPTAKE



● = control points.

○ = with ethosuximide.

( ) = number of observations from the mean of which values of 1/x and 1/v were obtained.

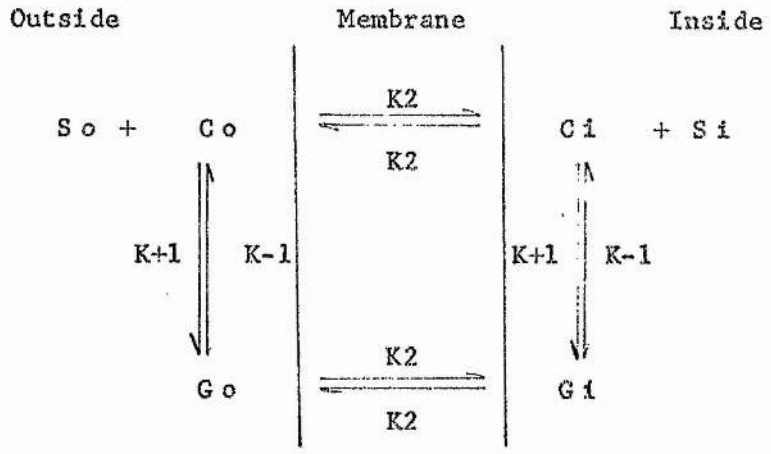
controls - Km = 153 mM. Vmax = 36 m-moles.liter$_{ICW}^{-1}$.min^{-1}.

with 500 μM ethosuximide -

Km = 53 mM. Vmax = 19 m-moles.liter$_{ICW}^{-1}$.min^{-1}.

FIGURE 13

A Model of a Carrier - Mediated Sugar Transport Mechanism



So and Si are the concentrations of sugar in the extracellular and intracellular fluids.
Co and Ci are the concentrations of the free carrier at the edges of the membrane.
Go and Gi are the concentrations of the sugar-carrier complex.
K + 1, K - 1, and K 2 are velocity constants.

(After Bowyer, 1957, (4) with minor modifications)

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CHAPTER IV - THE EFFECT OF ANTICONVULSANTS ON THE RESPIRATORY RESPONSE OF CEREBRAL CORTEX SLICES TO A HIGH EXTERNAL POTASSIUM CONCENTRATION.

The Effect of Anticonvulsants on the Respiratory Response of Cerebral Cortex Slices to a High External Potassium Concentration

Introduction

In Chapter I, mention was made of several ways in which the effect of certain anticonvulsant drugs on sugar uptake by brain might be related to the anticonvulsant activity of these drugs. In Chapter III, evidence was presented that sulphydryl group blocking agents abolish the response of the sugar uptake process (of cerebral cortex slices) to anticonvulsants. It is of interest to know whether the sulphydryl group blocking agents inhibit other effects of anticonvulsants, particularly those effects which appear to be most closely associated with their anticonvulsant activity.

One 'in vitro' effect of the drugs which appears to be closely related to their 'in vivo' anticonvulsant effect, is the ability of some anticonvulsant drugs to prevent the increase in oxygen consumption normally seen in cerebral cortex slices subjected to high frequency electrical stimulation (28, 39, 40). It would have been desirable to investigate the effect of sulphydryl group blocking agents on this property of anticonvulsants. However, suitable apparatus for producing high frequency stimulation of slices was not available within the time remaining for completion of the present project. The alternative adopted, therefore, was to examine the effects, both in the presence and in the absence of a sulphydryl group blocking agent, of the anticonvulsants on the respiratory response of slices to an increase in the external potassium concentration to about 100mM. Potassium stimulated respiration of cerebral cortex slices shows many similarities to electrically stimulated respiration (Hertz (1969) (30)), and the potassium stimulated respiratory response may be inhibited by pharmacological concentrations of phenobarbitone (McIlwain (1953) (39)), although diphenylhydantoin and trimethadione do not show this effect.

DTNB was the sulphydryl group blocking agent used in the present experiments, since it is unlikely to penetrate intact cells and is therefore less likely to inhibit respiration than inhibitors which could penetrate the cells, such as iodoacetamide or iodoacetate. The respiratory response of cerebral cortex slices to potassium is inhibited by iodoacetate (Heald (1953) (29)).

Method

In each of a series of three experiments, the basal and potassium-stimulated oxygen consumptions of portions of cerebral cortex slices obtained from one animal were measured under a variety of conditions of/

of incubation (control:with acetazolamide: with ethosuximide: with DTNB: with acetazolamide + DTNB: with ethosuximide + DTNB) The oxygen consumption of the samples was measured, at constant pressure, using a 'Gilson' differential respirometer.

Experimental procedure was as follows -

Krebs phosphate medium was prepared in the manner recommended by Umbreit, Burris and Stauffer (57). Six respirometer flasks, each having a centre well and a single side arm, were taken. 1.8ml. of an incubation medium were then pipetted into the main compartment of each flask. The compositions of the incubation media were -

Flask 1	Krebs phosphate medium
Flask 2	Krebs phosphate medium containing 20 μM acetazolamide
Flask 3	Krebs phosphate medium containing 500 μM ethosuximide
Flask 4	Krebs phosphate medium containing 100 μM D.T.N.B.
Flask 5	Krebs phosphate medium containing 20 μM acetazolamide and 100 μM D.T.N.B.
Flask 6	Krebs phosphate medium containing 500 μM ethosuximide and 100 μM D.T.N.B.

Oxygen was bubbled through the media for about ten minutes prior to the experiment. 0.2ml. of a 'molar' solution of potassium chloride was pipetted into the side arm of each of the six flasks and 0.2ml. of a 20% solution of potassium hydroxide into the centre well of each flask.

A guinea pig was then killed. Slices of the cerebral cortex were prepared and divided into six portions of about 0.2g. Each portion was quickly weighed on a watch glass then transferred, using a stainless steel spatula, to one of the respirometer flasks. After transfer of the slices, the contents of the main compartment of the flask were stirred gently to disperse the slices. The flasks were then each placed on one of the respirometer stations. An atmosphere of oxygen was maintained above the contents of the flasks which were maintained at 37°C and agitated by mechanical shaking. After a fifteen minute 'equilibration period', measurement of oxygen uptake commenced. The oxygen consumption of the slices was recorded over a twenty minute period. Additional potassium was then tipped into the incubation media, from the side arms of the flasks, and oxygen uptake determined over a further twenty minute period.

Results and Discussion

The results of the respiratory studies are presented in Table 5, page 68.

The values obtained for the basal, and potassium-stimulated, oxygen/

TABLE 5

BASAL AND POTASSIUM STIMULATED RESPIRATION OF CEREBRAL CORTEX SLICES

		O_2 consumption; μ moles. g fresh slices^{-1}.hr^{-1}. conditions of experiment					
		control	with ace	with eth	with DTNB	with ace + DTNB	with eth + DTNB
Exp. 1.	basal	60.7	56.1	56.1	50.3	57.5	65.2
	potassium stimulated	88.7	88.3	86.6	82.8	94.7	90.8
Exp. 2.	basal	64.0	58.9	71.4	66.9	66.7	70.3
	potassium stimulated	93.0	93.4	109.0	98.0	102.4	98.7
Exp. 3.	basal	56.0	50.4	56.1	54.0	50.7	54.4
	potassium stimulated	92.5	89.0	85.5	86.0	92.3	86.0
mean ±s.e.m.	basal	60.2±2.2	55.1±2.5 *	61.2±5.1	57.1±5.0	58.3±4.6	63.3±4.7
	potassium stimulated	91.4±1.4	90.2±1.6	93.7±7.1	88.9±4.6	96.6±3.0	91.8±3.7

* = significantly different from control value ('t' test, $0.05 > p$),

ace = acetazolamide, 20 μM.

eth = ethosuximide, 500 μM.

s.e.m. = standard error of the mean.

oxygen consumptions of guinea pig cerebral cortex slices are comparable to those previously reported (Gore and McIlwain (1952) (27), McIlwain(1953) (39), Greengard and McIlwain (1955),(28).

The basal oxygen consumption of the slices is unaffected by ethosuximide, but is depressed to a small but significant extent by acetazolamide. In the prescence of DTNB, acetazolamide did not significantly decrease the basal oxygen consumption of the slices. Other anticonvulsant drugs (phenobarbitone, diphenylhydantoin, and trimethadione (28,39) do not appear to affect basal oxygen cosumption. Acetazolamide appears to differ from most other anticonvulsant drugs in that it causes an increase in the glucose consumption of cerebral cortex slices incubated in bicarbonate medium. (Gilbert, Gray, and Heaton, 1971,(23). Thus acetazolamide appears to increase glycolysis (and possibly glycogen synthesis) while depressing oxidative metabolism, though neither of these effects is very great.

Neither acetazolamide nor ethosuximide appear to affect the potassium-stimulated oxygen consumption of the slices. The increased respiration of cerebral tissue which can be brought about by increasing the potassium ion concentration of the surrounding medium may result from the stimulation, by extracellular potassium ions, of the (Na^+, K^+) stimulated ATPase of cell membranes. It has been suggested that the respiratory response to increased potassium may be principally a property of glial cells (Hertz, 1969 (30), Talwar and Singh, 1971 (53)). The respiratory response to electrical stimulation could also result from an effect of potassium ions on the membrane (Na^+, K^+) ATPase, the potassium ions in this case having diffused out of the neurons during electrical activity.

The lack of effect of acetazolamide and of ethosuximide, on the respiratory response of the cerebral cortex slices to potassium thus suggests that the activity of the membrane (Na^+, K^+) ATPase, and the respiratory capacity of the tissue remain unaltered by these drugs.

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CHAPTER V - GENERAL DISCUSSION.

General Discussion

Comparison of the results of the present investigation with the results of several other studies suggests that most anticonvulsant drugs so far tested appear to be capable, under certain conditions, of stimulating sugar uptake by brain, as is illustrated by the data summarised in Table 6, page 71 .

The anticonvulsant drugs do not necessarily affect cerebral sugar uptake by a common mechanism, however. Thus the effect of acetazolamide on the kinetics of xylose uptake appears to differ from that of other anticonvulsants. It has been noted in chapter four that acetazolamide differs from the other anticonvulsants discussed in this work in its effects on the metabolism of cerebral cortex slices. These differences shown by acetazolamide may be a consequence of its molecular structure, which differs considerably from that of the other anticonvulsant drugs represented in figure I (page 2).

A point of similarity between the effects of the various anticonvulsants on cerebral sugar uptake may be their sensitivity to inhibition by -SH blocking agents. Thus in the present study it was found that the effects of acetazolamide, and of ethosuximide, on xylose uptake by cerebral cortex slices, are abolished when iodoacetamide or DTNB are present, and it has recently been demonstrated by Gilbert (21) that phenobarbitone, in the presence of iodoacetamide, is without effect on glucose transport in cerebral cortex slices.

It thus appears that the effect of anticonvulsant drugs on the transport of sugars across cerebral membranes may involve interaction of the drugs with sulphydryl groups of the membrane.

The effect of phenobarbitone on the permeability of synaptosomes to sodium and potassium ions is also sensitive to inhibition by DTNB, but is not inhibited by another sulphydryl group blocking agent, p-chloromercuribenzoate (PCMB). (Balfour, 1971, (2)).

Although some anticonvulsants increase the ionic gradients across brain membranes, several discrepancies between the effects of anticonvulsants on cellular ion levels, and their effects on sugar uptake,

TABLE 6

RESPONSE OF BRAIN SUGAR UPTAKE TO ANTICONVULSANT DRUGS

Reference	System Studied	Effect of Drug				
		Phe.	Dip.	Dim.	Eth.	Ace.
Gilbert, Ortiz and Millichap (24).	Xylose uptake by guinea pig cerebral cortex, 'in vitro'. *	+	o	+		
Gilbert, Ortiz and Millichap (24).	Xylose uptake by guinea pig cerebral cortex, 'in vitro'.	+		o		
Present Work	Xylose uptake by guinea pig cerebral cortex, 'in vitro'.		o		+	+
Gilbert, Gray and Heaton (23).	Glucose content of mouse brain 'in vivo'.	+	+		+	+

Phe. = phenobarbitone: Dip. = diphenylhydantoin: Dim. = dimethadione:
Eth. = ethosuximide: Ace. = acetazolamide.

\+ indicates that the drug was observed to have a significant effect on sugar uptake by, or sugar content of, the tissue:

o, that no significant effect was observed.

In the experiments marked *, animals had received an anticonvulsant by intra-peritoneal injection some time before decapitation. (controls received 0.9% NaCl.) Cerebral cortex slices were then prepared and incubated in medium containing xylose, but no drug. In all other 'in vitro' experiments, cerebral cortex slices from animals receiving no drug were incubated in media containing an anticonvulsant drug. (Media for control incubations had no drug).

The elevation of brain glucose level in mice, brought about by anti-convulsant drugs, did not appear to be due to decreased glucose utilisation by brain: it appears likely that it was a consequence of increased glucose uptake.

suggest that the latter effects may be independent of the former. Thus acetazolamide (20 uM) can increase the uptake of xylose by cerebral cortex slices, but, at this concentration, this drug has no effect on slice sodium levels (Gilbert, 1971 (20)). Diphenyl hydantoin increases the ratio of extracellular to intracellular sodium in brain, (Woodbury, Koch, and Vernadakis, 1953, (67)), but does not significantly affect the uptake of xylose by cerebral cortex slices: while dimethadione can increase the uptake of xylose by cerebral cortex slices (Gilbert, Ortiz, and Millichap, 1966 (24)), but has not been observed to have any effect on brain electrolytes (Woodbury, 1970 (64)).

Any attempt to relate the effect of anticonvulsant drugs on sugar uptake by cerebral cortex slices to the 'in vivo' effects of these drugs is complicated by the existence, in vivo, of a 'blood brain barrier'. This permeability barrier appears to be identical with, or adjacent to, the walls of the cerebral capillaries, (Crone, 1965 (10a), Buschiazzo Terrell and Regen, 1970 (5)), and it has been suggested that sugars may, after crossing the blood-brain barrier, permeate all the cerebral water available to them without meeting another major barrier (5).

If the capillary walls function as the blood-brain barrier, then no information as to the permeability properties of this barrier will be obtained by the study of cerebral cortex slices. If, however, the blood-brain barrier should be located in the membranes of the astrocyte processes, which surround the cerebral capillaries , the permeability properties of the slices might resemble those of the blood-brain barrier, since functionally intact astrocytes might make up an appreciable portion of the total intracellular volume of the slices.

Examination by electron microscopy of slices of cerebral cortex incubated in conditions comparable with those used in the present studies shows the presence of apparently intact neurons, astrocytes, oligodendroglial cells and microglial cells. (61). The anticonvulsant-sensitive xylose uptake system of cerebral cortex slices may be a property of non-neuronal cells of the slices, since phenobarbitone increases the uptake of xylose into slices of cerebral cortex, but has no significant effect on the entry of xylose into synaptosomes (Balfour, 1971 (2)). Synaptosomes are pre-synaptic nerve terminals, 'pinched off' from the body of their axon by a homogenisation procedure.

The damaged membrane of the synaptosome appears to reseal, and synaptosomes have been found to display permeability properties similar to those of non-myelinated neuronal membranes (62)).

The data summarised in table 6 , page 71 , suggests that the sugar uptake system of cerebral cortex slices bears a considerable resemblance to that of intact brain 'in vivo' in its response to anti-convulsants. The elevation, by these drugs, of brain glucose levels may possibly be a part of the mechanism of their anticonvulsant activity. Some ways in which an increase in brain sugar content could contribute to the anticonvulsant effect of the drugs were suggested in Chapter I, page 7 . The possibility that the effect of anticonvulsant drugs on brain sugar and sodium levels might be closely linked was raised. The evidence referred to on page 72 of this chapter, however, suggests that this is not the case, for a number of drugs.

Since the oxidation of glucose is the chief source, in nervous tissue, of the energy required for the maintainance, by means of the sodium pump, of the ionic gradient across the cells, and for other 'metabolic work' of the cell, an increased uptake of glucose by the cell might, in some circumstances, contribute towards its stability by permitting increased glucose metabolism. It has been suggested that one explanation of the relatively low glucose concentration encountered in brain might be that the transport of glucose to the cells is a limiting factor in the metabolism of this sugar by cerebral tissue. (Joanny, Corriol, and Hillman, 1969 (32)). The anticonvulsant drugs phenobarbitone, diphenylhydantoin, and ethosuximide, which increase the glucose content of mouse brain, do not however increase the utilisation of glucose by slices of guinea pig cerebral cortex. (acetazolamide does cause a small increase in glucose consumption) (Gilbert, Gray and Heaton, (1971)). A study of glucose transport across the blood-brain barrier of the rat (Buschiazzo, Terrell, and Regen, 1970 (5)), has indicated that the Vmax. for glucose phosphorylation in brain is about one third as great as the transport Vmax., and that the phosphorylation Km (0.04mM)

is thirty times less than the brain glucose concentration, while the transport Km (7mM) is approximately equal to the blood glucose concentration. Thus glucose transport may not be the limiting factor in cerebral glucose metabolism at normal blood glucose levels, though it could be so in hypoglycaemic conditions.

A way in which the increased brain glucose content brought about by anticonvulsant drugs might stabilise brain membranes by a mechanism not involving glucose metabolism is indicated by the observation that the presence of glucose can stabilise the membranes of erythrocytes against hemolysis. (Good, 1961. (25)). It is suggested that an orderly arrangement of water molecules in the membrane is stabilised by glucose, through hydrogen bonding. The effect of the convulsant, pentylene tetrazole, is the reverse of that of glucose. Pentylene tetrazole appears to decrease the stability of the orderly arrangement of water molecules in the erythrocyte membrane. Pentylene tetrazole, at a concentration similar to that which would induce convulsions, had about the same destabilising effect on the hydration structure of erythrocyte membranes as did the reduction of the glucose content of the medium from values corresponding to normal blood glucose levels, to values equivalent to those encountered in hypoglycaemic convulsions (Coldman and Good, (1969)). Cerebral membranes may, like erythrocyte membranes, be stabilised by the presence of glucose, since glucose appears to reduce the susceptability of cerebral lysosomes to lysis by mild osmotic shock. (Gilbert, Gray and Heaton, 1971). (23).

However, although the increase in brain sugar levels brought about by anticonvulsant drugs could contribute to their anticonvulsant effect by the mechanisms discussed in the preceding paragraphs, it seems likely that the major source of the anticonvulsant effect of these drugs is due to some other mechanism; and that the observed effects of the anticonvulsants on sugar uptake by cerebral tissue may be one aspect of a more wide-ranging effect of these drugs on cerebral membranes.

One possibility is that the interaction of anticonvulsant drugs with cerebral membranes might result in a slight modification of the series of changes in the permeability of neuronal membranes to ions which occur during the initiation and propagation of an 'action potential'. By such a mechanism the refractory period of cerebral neurons might be increased, rendering them less susceptible to high-frequency stimulation.

Summary.

From the results of the present studies, together with data obtained by other workers (21, 24) the following information has been obtained concerning the effect of anticonvulsant drugs on brain sugar uptake.

(i) Most anticonvulsant drugs appear to be capable of stimulating sugar uptake by brain. On the basis of their effect on the kinetics of xylose uptake by cerebral cortex slices, the drugs tested may be grouped into three categories:-

(a) Phenobarbitone and ethosuximide increase the Km and Vmax. of xylose uptake. Dimethadione may also be included in this category since, although Gilbert, Ortiz and Millichap (24) found this drug to have no effect on the kinetics of xylose uptake, these workers also recorded that, under different experimental conditions, the effect of dimethadione on xylose uptake closely resembled that of phenobarbitone.

(b) Acetazolamide decreases the Km and Vmax of xylose uptake by cerebral cortex slices.

(c) Diphenylhydantoin appears to be without effect on sugar uptake by cerebral cortex slices.

(ii) The ability of -SH group blocking agents to prevent the stimulation by anticonvulsant drugs of sugar uptake into cerebral cortex slices has been demonstrated for the anticonvulsant drugs ethosuximide and acetazolamide (in the present study) and for phenobarbitone (Gilbert, 1972 (21)). The effect of anticonvulsant drugs on the permeability properties of cerebral membranes may thus involve interaction of the drugs with -SH groups of the membranes.

(iii) Although the stimulation of brain sugar uptake brought about by anticonvulsant drugs may contribute to their anticonvulsant effect, it seems probable that the interaction of these drugs with cerebral membranes may, in addition to affecting permeability to sugars, result in other effects more directly resposible for their anticonvulsant efficacy.

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