BBA 75230

MYO-INOSITOL TRANSPORT IN SLICES OF RAT KIDNEY CORTEX

I. EFFECT OF INCUBATION CONDITIONS AND INHIBITORS

GEORGE HAUSER

Research Laboratory, McLean Hospital, Belmont, Mass. and Department of Biological Chemistry, Harvard Medical School, Boston, Mass. (U.S.A.)

(Received September 3rd, 1968)

SUMMARY

The incubation conditions affecting the energy- and sodium ion-requiring uptake and retention of *myo*-inositol by slices of rat kidney cortex have been studied. No transport could be demonstrated in other tissues. Leakage of inositol from the slices amounted to 15 % of the endogenous inositol in the first 45 min of incubation in air and at most to 8 % on reincubation for 1 h in 95 % O_2–5 % CO_2. Removal of oxygen from the gas phase increased leakage and abolished the active uptake of inositol, effects which could not be prevented by the addition of energy sources such as glucose and ATP. The accumulation of radioactivity had a Q_{10} greater than 3 and an apparent K_m of $1.57 \cdot 10^{-4}$ M. A number of inhibitors of different stages of metabolic energy production interfered in varying degree with the uptake and retention of inositol. The extent of inhibition was largely determined by the concentration of the substance used, but an anomalous response was seen with 2,4-dinitrophenol. The results are discussed with reference to current concepts of active transport.

INTRODUCTION

Concentrations of *myo*-inositol of up to 50 mM are maintained in mammalian tissues against a large concentration gradient[1,2]. Although endogenous production of inositol occurs in both the intact animal[3–8] and in tissue preparations *in vitro*[9], no information is available relating to the adequacy of this biogenic potential for providing the amounts of inositol found in each organ. Alternatively, inositol, primarily synthesized in one locus, together with the substantial quantities supplied daily by the diet and absorbed in the intestine, might subsequently be transported to and accumulated by the tissues. The kidney has the ability to reabsorb and thus conserve inositol, although the reason for this apparent need of the organism is at present unexplained.

HOWARD AND ANDERSON[10] have demonstrated the uptake of inositol against a gradient by kidney slices when final medium concentrations were between 0.75 and 9.5 mM, and we have previously shown the accumulation of radioactivity during incubations of slices of rat kidney cortex with labeled inositol[11]. This phenomenon was dependent on energy-generating processes and the presence of sodium ions in

the medium. Further details of these observations are reported in this communication. The study has been extended to include, in addition to the uptake of radioactivity from the medium, the influence of various incubation conditions and added inhibitors on the prevention of inositol leakage from the slices and the consequent preservation of relatively high intracellular concentrations. Essentially parallel effects on the two processes were found.

METHODS AND MATERIALS

Tissues. Adult male rats, obtained from the Charles River Breeding Laboratories, N. Wilmington, Mass., and weighing 200–300 g, were used in all experiments. After decapitation, their kidneys were quickly removed, decapsulated and stored on cracked ice. Slices were prepared with a Stadie–Riggs microtome. From each hemikidney the first, polar, slice was discarded, and the two following slices were blotted on hardened (S and S 576) filter paper, weighed and transferred to the incubation flasks. 200–300 mg of slices were used in each flask.

Labeled inositol. [2-^{3}H]Inositol was obtained from New England Nuclear Corporation, Boston, Mass. Most of the experiments were carried out with this material. During a period when it was not available, [2-^{14}C]inositol (Calbiochem, Los Angeles, Calif.) was used. The results obtained were identical, and no distinction is made in the text as to which substance was used in a given group of experiments.

Incubation conditions. Slices were incubated in 3.0 ml of Krebs–Ringer bicarbonate medium without added energy source in a Dubnoff shaking water bath at 37°. Standard conditions included a 45-min "preincubation" period, at the end of which the slices were withdrawn from the medium, blotted, weighed and transferred into fresh medium for incubation with labeled inositol (17 μM). Unless otherwise indicated, the gas phase during preincubation was air, and during incubation 95 % O_2–5 % CO_2. However, the flasks were gassed only briefly before the start of the incubation and at 20-min intervals thereafter. Although this gassing schedule was followed routinely, only the initial replacement of air with 95 % O_2–5 % CO_2 was essential (*cf.* Table III). Incubations were routinely carried out for 1 h.

Analyses for radioactivity and inositol. At the end of the incubation period the flasks were immersed in crushed ice and the medium removed with a Pasteur pipette. The slices were quickly rinsed with or dipped into portions of ice-cold water, Krebs–Ringer bicarbonate medium or isotonic saline in order to wash off adhering medium. The type of treatment had no effect on the results. After being blotted and weighed the slices were either deproteinized by homogenizing with 3.0 ml 0.19 M $ZnSO_4$ followed by 3.0 ml 0.3 M $Ba(OH)_2$ (ref. 12) or immersed in 2.0 ml of water and heated in a boiling water bath for 5 min (ref. 13). Aliquots for analyses were taken from the deproteinized supernatant solution or the aqueous extract of the slices and from the medium, either directly or after similar $ZnSO_4$–$Ba(OH)_2$ treatment. Radioactivity measurements were about 7 % lower for the deproteinized samples.

Radioactivity was measured with a liquid scintillation spectrometer (Tricarb, model 3214, Packard Instrument Co., Chicago, Ill.) using 0.5-ml aqueous samples and 10.0 ml of the scintillation fluid described by SILBERT[14] (43 g naphthalene; 5 g 2,5-diphenyloxazole; 0.043 g 1,4-bis-2-(5-phenyloxazolyl)-benzene in 130 ml 95 % ethanol and 870 ml dioxane). Suitable quench corrections were obtained by using an

internal standard with samples containing 2,4-dinitrophenol. Inositol was determined by bioassay with *Kloeckera apiculata* (brevis)[15].

Calculation of distribution ratios. From the radioactivity and inositol analyses, ratios of distribution between intracellular and extracellular fluid were calculated[16]. The water content of kidney slices was taken to be 82%, with 26% extracellular and 56% intracellular[17,18]. Total slice values were corrected for extracellular fluid content of inositol or radioactivity, assuming equilibration between the medium and extracellular fluid. The inositol ratios would be expected to show a much greater variability than the radioactivity ratios, since the initial intracellular inositol concentrations are high and only very small amounts are released into the medium during the incubation. These are lower than the quantities found after preincubation (see below) and are the main determinants of the magnitude of the ratio. No correction has been made for the inositol added to the medium, nor could justifiable assumptions be made to permit such an adjustment.

Although the high initial inositol content of the slices imposes limitations on the study of inositol transport, reproducible results were obtained, which can be interpreted in terms of the ability of the kidney membranes to control inositol penetration in both directions.

RESULTS

Inositol uptake by slices of different tissues. In attempting to discover the possible existence of a transport system, responsible for the high concentrations of inositol reported to exist in tissues, slices of a number of tissues were incubated with labeled inositol and the distribution ratios of both radioactivity and inositol determined. Only with kidney slices could a preferential intracellular concentration of radioactivity be demonstrated (Table I) under the particular conditions employed. At the same time the highest inositol ratio was also found with kidney slices, pointing towards a slow leakage and non-equilibration of intracellular inositol with the medium. Although

TABLE I

DISTRIBUTION RATIOS OF RADIOACTIVITY AND INOSITOL IN DIFFERENT TISSUES

The procedures for preparing slices of brain, heart, liver and spleen and for carrying out the incubations and analyses were identical with those used for kidneys (see METHODS AND MATERIALS). In the experiments with small intestine, a 10-cm segment was removed, starting 5 cm below the pylorus, cut in half lengthwise, rinsed in cold Krebs–Ringer bicarbonate medium and cut into 4 mm × 15 mm rectangles. 4 or 5 of these pieces were used per flask. Each value represents at least duplicate experiments. Inositol concentrations were determined in aqueous extracts of unincubated slices as outlined under METHODS AND MATERIALS.

Source of slices	*Inositol concentration (μg/g fresh weight)*	*Distribution ratio*	
		Radioactivity	*Inositol*
Brain	1036	0.59	28
Heart	37	0.65	26
Kidney	560	7.13	254
Liver	97	0.44	197
Spleen	237	0.74	65
Intestine	137	1.20	—

kidney is relatively high in inositol, all tissues examined contain inositol concentrations greater than those in the circulating blood (*cf.* also ref. 1). In all subsequent experiments only cortical slices from adult male rats were used, and the effects of the conditions of incubation and of the addition of inhibitors on the distribution ratios investigated in this system.

Depletion of endogenous inositol. During the initial search for conditions, which would permit the demonstration of transport into slices, it was felt that partial depletion of the endogenous inositol content might enhance uptake. Consequently, an arbitrary 45-min incubation without added labeled inositol was carried out in medium of identical composition before transfer of the slices into flasks for incubation in medium containing radioactivity. During this "preincubation" about 15 % of the slice inositol (calculated as a percentage of the sum of inositol found at the end of the incubations in the two incubation media and the slice extract) appeared in the medium (Table II). If complete equilibration between the intracellular space and the medium had been reached, more than 95 % of the inositol would have left the cells of the slices. Inclusion of this preincubation period subsequently permitted the observed radioactivity ratios to reach 7–8 rather than 2.64 without it (Table IV). During the second incubation only an additional 7–8 % of inositol leaked into the medium, a maximal figure in view of the radioactive inositol added to the flasks. It appeared that the length of the preincubation period might be critical and several experiments were carried out in which it was varied between 10 and 90 min. However, the results were not consistent and the 45-min span was adopted for routine use.

Effect of the composition of the gas phase and of added energy sources. One indication that an energy-requiring process is involved in the establishment of the observed radioactivity ratios is the reduction of the ratio to 1.00, when the gas phase

TABLE II

INOSITOL LEAKAGE FROM KIDNEY SLICES DURING INCUBATION

Experiments were carried out under standard conditions except as indicated. CO_2, when present, constituted 5 % of the gas mixture. Glucose and ATP additions were present only during incubation. Inositol leakage was calculated as a percentage of the total inositol found in the two incubation media and the aqueous extract of the slices at the end of the experiment. Each value represents at least duplicate experiments.

Gas phase		*Other conditions*	*Inositol in medium*	
Preincubation	*Incubation*		*After preincubation* (%)	*After incubation* (%)
Air	O_2–CO_2	—	15.0	7.2
Air	O_2–CO_2	gassed only once	21.9	8.2
Air	O_2–CO_2	5 mM ATP	17.6	7.4
Air	O_2–CO_2	10 mM glucose	16.2	9.0
Air	Air	—	20.1	13.3
Air	Air	0°	21.7	6.2
O_2–CO_2	O_2–CO_2	—	12.4	8.3
N_2	O_2–CO_2	—	24.2	11.3
N_2	N_2	—	25.0	35.5
N_2–CO_2	N_2–CO_2	—	19.3	18.2
N_2–CO_2	N_2–CO_2	5 mM ATP	17.4	14.6
N_2–CO_2	N_2–CO_2	10 mM glucose	16.7	22.9

was changed to N_2 (Table III). A concomitant marked drop in the inositol ratio (the result of increased leakage (Table II)) was observed. The highest radioactivity ratios were attained when preincubation was carried out under N_2, followed by incubation under O_2–CO_2. The ratios were much lower where air was used in place of O_2–CO_2 during the incubations, with both uptake and retention of inositol being impaired. The presence of CO_2 in the gas phase presumably serves to control the pH more closely, but its inclusion in an N_2 atmosphere prevented the drop in the values of the distribution ratios only to a small extent. During incubation under N_2 or air for 1 h, the pH of the medium rose to 7.8 or 7.6, respectively.

Despite the pronounced effect of oxygen in the gas phase, no stimulation was achieved by adding potential energy sources either after or without preincubation (Table IV). This is compatible also with the failure of such additions to affect the inositol leakage materially (Table II).

TABLE III

EFFECT OF GAS PHASE ON THE DISTRIBUTION RATIOS IN KIDNEY SLICES

Incubations were under standard conditions except for the modification of the gas phase. CO_2, when present, constituted 5% of the gas mixture. Each value represents at least duplicate experiments.

Gas phase		*Distribution ratio*	
Preincubation	*Incubation*	*Radioactivity*	*Inositol*
N_2	N_2	1.00	34
N_2–CO_2	N_2–CO_2	1.71	80
Air	Air	3.69	104
O_2–CO_2	O_2–CO_2	5.43	209
Air	O_2–CO_2	7.05	237
Air	O_2–CO_2*	7.72	220
N_2	O_2–CO_2	8.49	146

* Gassed only once, at the beginning of the incubation.

TABLE IV

EFFECT OF THE ADDITION OF ENERGY SOURCES ON THE DISTRIBUTION RATIOS

Standard conditions were modified as indicated. During preincubation, the gas phase was either air or, in anaerobic experiments, N_2–CO_2. CO_2, when present, constituted 5% of the gas mixture. Values are averages of from 2 to 6 experiments.

Length of preincubation (min)	*Gas phase during incubation*	*Energy source added*	*Distribution ratio*	
			Radioactivity	*Inositol*
0	O_2–CO_2	—	2.64	63
0	O_2–CO_2	glucose, 10 mM	2.92	69
45	O_2–CO_2	—	7.05	237
45	O_2–CO_2	glucose, 10 mM	6.45	218
45	O_2–CO_2	ATP, 5 mM	6.81	253
45	O_2–CO_2	oxaloacetate, 10 mM	8.46	236
45	N_2–CO_2	—	1.71	80
45	N_2–CO_2	glucose, 10 mM	2.47	59
45	N_2–CO_2	ATP, 5 mM	2.62	108
45	N_2–CO_2	oxaloacetate, 10 mM	2.05	105

Temperature dependence. Although leakage is not materially changed when the incubation is performed at 0° (Table II), no accumulation of radioactivity occurs and the ratio remains well below 1 as previously reported[11]. Thus the observed processes are temperature dependent, as shown also in Fig. 1 which depicts the time course of the change in the radioactivity distribution ratio at two temperatures. The initial rate is more than 3 times as great at 37° than at 27°, and no plateau has been reached even after 2 h.

Michaelis–Menten constant of inositol uptake. Radioactivity accumulation exhibits Michaelis–Menten kinetics with a K_m of $1.57 \cdot 10^{-4}$ M. Since this determination is not based on initial rates, the value is not strictly a K_m, but merely gives an apparent concentration.

TABLE V

EFFECT OF INHIBITORS AND UNCOUPLERS

Preincubations were under standard conditions. During the incubations, the inhibitors were present in the concentrations indicated. Each value represents at least duplicate experiments.

Substances added	*Concentration*	*Distribution ratio of radioactivity*
None	—	7.05
	(*M*)	
NH_2OH	$1 \cdot 10^{-3}$	7.28
Cu^{2+}	$1 \cdot 10^{-6}$	6.67
Iodoacetamide	$1 \cdot 10^{-3}$	5.77
Iodoacetate	$1 \cdot 10^{-3}$	5.50
NaN_3	$1 \cdot 10^{-3}$	5.00
p-Chloromercuribenzoate	$5 \cdot 10^{-4}$	3.80
$NaAsO_2$	$5 \cdot 10^{-3}$	3.28
2,4-Dinitrophenol	$5 \cdot 10^{-4}$	3.25
NaF	$5 \cdot 10^{-2}$	3.07
Ouabain	$2 \cdot 10^{-3}$	2.76
Phlorizin	$2 \cdot 10^{-3}$	1.50
Hg^{2+}	$1 \cdot 10^{-3}$	1.40
N-Ethylmaleimide	$1 \cdot 10^{-3}$	1.22
NaCN	$2 \cdot 10^{-3}$	1.11
	(*μg/ml*)	
Gramicidin	10	6.65
Oligomycin	10	5.60
Antimycin A	10	4.25

Inhibition studies. A decreased ability to establish normal distribution ratios of radioactivity in the presence of a number of inhibitors and uncouplers such as NaN_3, *p*-chloromercuribenzoate, 2,4-dinitrophenol, NaCN and ouabain has been shown before[11]. The list of agents affecting inositol uptake has now been extended to include several additional substances (Table V). Because of the obvious difficulties in carrying out a bioassay in the presence of metabolic inhibitors, the distribution of inositol has not been determined in these experiments. Hydroxylamine, fluoride, iodoacetate and iodoacetamide are relatively ineffective inhibitors at millimolar concentration, but all other substances tried caused substantial inhibition at this level. Partial inhibition was also produced by several antibiotics such as those interfering with energy

transfer and electron transfer reactions. Inositol ratios were determined in these experiments and ranged from 112 with oligomycin to 177 with gramicidin.

For fluoride, ouabain, 2,4-dinitrophenol and phlorizin, the influence of several different concentrations has been determined (Figs. 2–4). In order for fluoride to exhibit its inhibitory effect, concentrations at least one order of magnitude greater than those for all other agents had to be used (Fig. 2). The effective concentrations of ouabain (Fig. 3) were much larger than those inhibiting either cation transport or the activity of Na^+ and K^+-dependent ATPase, presumably because of the relative insensitivity of the rat, and especially of rat kidney, to the action of cardiac glycosides. The distribution ratio remained at values greater than 2 even at 3 mM, indicating

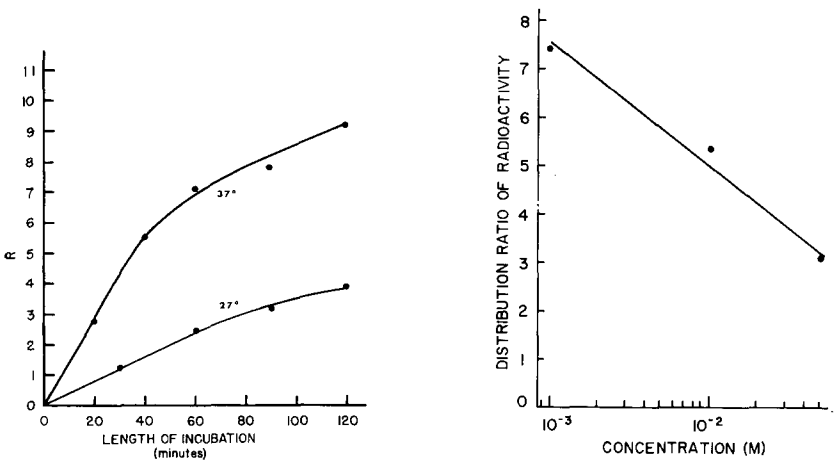

Fig. 1. Time course and temperature dependence of inositol uptake. R = distribution ratio of radioactivity.

Fig. 2. The effect of fluoride on inositol uptake. Preincubation was under standard conditions. The inhibitor was present during the incubation in the concentrations indicated. Each point represents the average of 2 or more experiments.

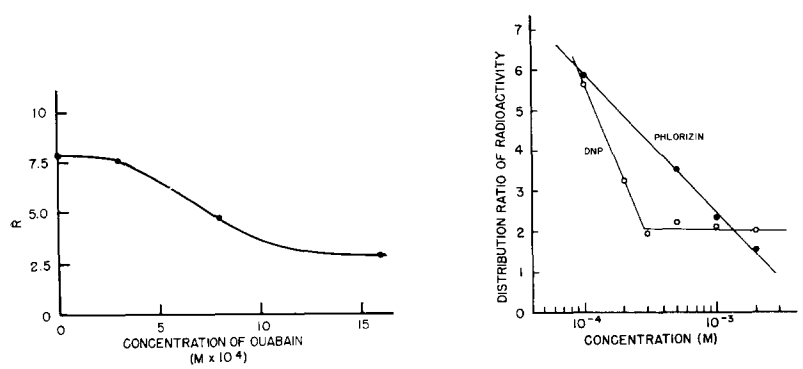

Fig. 3. The effect of ouabain on sodium-dependent inositol uptake. Conditions as in Fig. 2. R = distribution ratio of radioactivity.

Fig. 4. The effect of phlorizin (●—●) and 2,4-dinitrophenol (DNP) (○—○) on inositol uptake. Conditions as in Fig. 2.

that there may be a component of the system which is not sensitive to ouabain. A similar finding was made in the case of 2,4-dinitrophenol. Fig. 4 shows the biphasic nature of the concentration curve, which reaches a plateau at a distribution ratio of about 2 at an inhibitor concentration of 0.3 mM and remains horizontal over a 10-fold increase in 2,4-dinitrophenol concentration. However, iodoacetate added to 2,4-dinitrophenol-containing incubation media, effected a further decrease of the distribution ratio to 1.3.

DISCUSSION

The observation that substantial concentrations of free inositol exist in mammalian organs has been an intriguing and challenging fact for many years. Yet, although effects of inositol on certain metabolic events in mammalian cells have been reported[19–23], no reason can as yet be ascribed to the large variations in inositol concentration from organ to organ nor can its primary biochemical role be pinpointed. Since very little inositol is present in the circulating blood[1], tissues must be capable of either synthesizing or accumulating the inositol which can be isolated from them. Furthermore, a mechanism exists which permits concentration gradients as high as 1000 to be maintained between intracellular and extracellular fluids.

It has previously been pointed out[6,10] that kidney occupies a unique role in the metabolism of inositol and, particularly, contains the enzyme complement involved in the catabolism of this cyclitol. In addition, inositol is conserved by the organism by renal reabsorption, the mechanism for which is analogous to, and may be identical with, that for glucose[24,25]. The special position of kidney with respect to inositol is further emphasized by the finding that slices from none of the other tissues studied yielded a distribution ratio of radioactivity substantially greater than one on incubation with labeled inositol, although the inositol ratios indicate intracellular retention of endogenous inositol in all tissues examined. Apart from kidney cortex slices[10,11], active, Na^+ and K^+-dependent inositol transport has been reported only in Ehrlich ascites cells[26]. This in no way implies that other tissues are not capable of inositol transport and that this could not be demonstrated under suitable conditions. Under our conditions, however, radioactivity was concentrated solely in kidney slices.

Both maintenance of maximal inositol concentrations in and active transport of inositol into slices are energy-dependent processes as shown by the effects of partial or complete oxygen deprivation, temperature and a variety of metabolic inhibitors and uncouplers. These changes in incubation conditions are clearly disruptive of the total metabolism of the slices, and as is becoming more and more recognized, can profoundly affect many of the parameters by which cell integrity is measured. It is, therefore, not surprising that the ability of what may be the membranes of the proximal kidney tubule cells to retain intracellular material on the one hand and to transport actively substances added to the medium on the other is fundamentally altered by the factors mentioned, although we do not yet know the mechanism(s) in which energy is involved in the functioning of the intact membranes. The two parameters studied here—the prevention of passive leakage and the active, possibly carrier-mediated, transport—may depend for their operation on quite different molecular arrangements, which share the feature of requiring energy for their integrity. Yet

although the observed effects on the two phenomena are essentially parallel, very little inositol appeared in the medium at 0° when uptake was completely abolished and leakage might have been expected, since no energy is being supplied to prevent it. Nor did added energy sources increase uptake and reduce leakage under normal conditions or when these were inhibited by anaerobiosis.

A further point is the connection of inositol uptake with the maintenance of Na^+ and K^+ gradients across the membrane, which is emphasized by the requirement for Na^+ in essentially physiological concentration for both uptake and retention[11]. However, an attempt to prevent the failure of these two functions by maintaining intracellular Na^+ and K^+ levels through the addition of oxaloacetate during anaerobic incubation[27] (Table IV) was unsuccessful. The Na^+ requirement, analogous to that shown for other uncharged molecules by a number of investigators, points to the cotransport of sodium and inositol, conceivably by a mobile carrier system similar to that proposed by CRANE[28] for glucose in intestine. The inability of other monovalent ions to replace Na^+ and the role of other cations is discussed in the following paper[29]. The problem of coupling of the transport of cations and metabolites is discussed lucidly in the recent monograph by STEIN[30] (see also the review on Biochemical Aspects of Active Transport by ALBERS[31]) and the model is examined in detail by CRANE, FORSTNER AND EICHHOLZ[32]. The present findings are entirely in harmony with its features.

Sugar transport in renal slices, which is dependent on metabolism, sensitive to phlorizin and requires sodium (and is consequently inhibited by cardiac glycosides) has been studied by KRANE AND CRANE[33] and KLEINZELLER and coworkers[34–36] and appears to proceed by the same mechanism and conceivably the same carrier for several monosaccharides (D-glucose, D-galactose, D-fructose, α-methyl-D-glucoside) and inositol. Presumably the active uptake occurs from the lumen of the kidney tubules across the luminal face of the membrane into the cells. As in the case of glucose, the study of inositol is complicated by the ability of kidney tissue to oxidize the substrate[10,37] as well as to utilize it for the biosynthesis of phosphoinositides[38–40]. Oxidation of inositol to CO_2 and water has been found to be affected in all cases where it was measured in parallel with the reduction of inositol uptake by metabolic inhibitors (G. HAUSER, unpublished observations). However, of the inhibitors which have the greatest effect on the inositol-cleaving enzyme (NaN_3, Cu^{2+}, NH_2OH and Hg^{2+}, 94–100% inhibition)[41], only Hg^{2+} had a similar influence on inositol uptake. A direct connection between the activity of this enzyme and the inositol uptake would, therefore, seem unlikely.

There is the additional difficulty of ascertaining the specificity of action of any other changes made in the incubation conditions. Apparently the substitution of choline for sodium in incubations of kidney cortex slices not only renders the sodium pump inactive and makes the interaction of sodium with the carrier (which according to CRANE's hypothesis is necessary for the transport of nonelectrolytes[28,32]) impossible, but has also profound effects on respiration, glucose metabolism and water uptake[42]. Similar effects are noted when ouabain is used at the levels required for the inhibition of the active transport of sugars and sodium[43]. In view of the interconnections between metabolic events, extreme caution must be exercised in ascribing an effect on a transport system to the direct action of the agent in question.

ACKNOWLEDGEMENTS

These studies were supported by U.S. Public Health Service Grant NB-06399 from the National Institute of Neurological Diseases and Blindness, and by a grant from the Ford Foundation for the study of The Dynamic Biochemistry of Brain Structural Components.

The excellent technical assistance of Mrs. MARY E. GEROCH and Mrs. CARMELA BROOKS is gratefully acknowledged.

REFERENCES

1 R. M. C. DAWSON AND N. FREINKEL, *Biochem. J.*, 78 (1961) 606.
2 F. EISENBERG, JR. AND A. H. BOLDEN, *Nature*, 202 (1964) 599.
3 W. H. DAUGHADAY, J. LARNER AND C. HARTNETT, *J. Biol. Chem.*, 212 (1955) 869.
4 J. HALLIDAY AND L. ANDERSON, *J. Biol. Chem.*, 217 (1955) 797.
5 N. FREINKEL AND R. M. C. DAWSON, *Biochem. J.*, 81 (1961) 250.
6 G. HAUSER, *Biochim. Biophys. Acta*, 70 (1963) 278.
7 Y. IMAI, *J. Biochem. (Tokyo)*, 53 (1963) 50.
8 T. POSTERNAK, W. H. SCHOPFER, B. KAUFMANN-BOETSCH AND S. EDWARDS, *Helv. Chim. Acta*, 46 (1963) 2676.
9 G. HAUSER AND V. N. FINELLI, *J. Biol. Chem.*, 238 (1963) 3224.
10 C. F. HOWARD, JR. AND L. ANDERSON, *Arch. Biochem. Biophys.*, 118 (1967) 332.
11 G. HAUSER, *Biochem. Biophys. Res. Commun.*, 19 (1965) 696.
12 M. SOMOGYI, *J. Biol. Chem.*, 160 (1945) 69.
13 K. L. MANCHESTER AND F. G. YOUNG, *Biochem. J.*, 75 (1960) 487.
14 J. E. SILBERT, *J. Biol. Chem.*, 239 (1964) 1310.
15 J. D. CAMPLING AND D. A. NIXON, *J. Physiol. (London)*, 126 (1954) 71.
16 M. FOX, S. THIER, L. ROSENBERG AND S. SEGAL, *Biochim. Biophys. Acta*, 79 (1964) 167.
17 R. WHITTAM, *J. Physiol. (London)*, 131 (1956) 542.
18 L. E. ROSENBERG, S. J. DOWNING AND S. SEGAL, *Am. J. Physiol.*, 202 (1962) 800.
19 F. CHARALAMPOUS, M. WAHL AND L. FERGUSON, *J. Biol. Chem.*, 236 (1961) 2552.
20 S. TSUKAGOSHI, K. LEMBACH AND F. C. CHARALAMPOUS, *J. Biol. Chem.*, 241 (1966) 388.
21 K. LEMBACH AND F. C. CHARALAMPOUS, *J. Biol. Chem.*, 241 (1966) 395.
22 K. LEMBACH AND F. C. CHARALAMPOUS, *J. Biol. Chem.*, 242 (1967) 2599.
23 K. LEMBACH AND F. C. CHARALAMPOUS, *J. Biol. Chem.*, 242 (1967) 2606.
24 N. FREINKEL, C. J. GOODNER AND R. M. C. DAWSON, *Clin. Res.*, 8 (1960) 240.
25 R. PERLÈS, M.-C. COLAS AND M.-C. BLAYO, *Rev. Franç. Etudes Clin. Biol.*, V (1960) 31.
26 R. M. JOHNSTONE AND C.-P. SUNG, *Biochim. Biophys. Acta*, 135 (1967) 1052.
27 J. CASCARANO AND I. SEIDMAN, *Biochim. Biophys. Acta*, 100 (1965) 301.
28 R. K. CRANE, *Federation Proc.*, 24 (1965) 1000.
29 G. HAUSER, *Biochim. Biophys. Acta*, 173 (1969) 267.
30 W. D. STEIN, *The Movement of Molecules across Cell Membranes*, Academic Press, New York, 1967.
31 R. W. ALBERS, *Ann. Rev. Biochem.*, 36 (1967) 727.
32 R. K. CRANE, G. FORSTNER AND A. EICHHOLZ, *Biochim. Biophys. Acta*, 109 (1965) 467.
33 S. M. KRANE AND R. K. CRANE, *J. Biol. Chem.*, 234 (1959) 211.
34 A. KLEINZELLER AND A. KOTYK, *Biochim. Biophys. Acta*, 54 (1961) 367.
35 A. KLEINZELLER, J. KOLÍNSKÁ AND I. BENEŠ, *Biochem. J.*, 104 (1967) 843.
36 A. KLEINZELLER, J. KOLÍNSKÁ AND I. BENEŠ, *Biochem. J.*, 104 (1967) 852.
37 F. C. CHARALAMPOUS AND C. LYRAS, *J. Biol. Chem.*, 228 (1957) 1.
38 B. W. AGRANOFF, R. M. BRADLEY AND R. O. BRADY, *J. Biol. Chem.*, 233 (1958) 1077.
39 H. PAULUS AND E. P. KENNEDY, *J. Biol. Chem.*, 235 (1960) 1303.
40 W. THOMPSON, K. P. STRICKLAND AND R. J. ROSSITER, *Biochem. J.*, 87 (1963) 136.
41 F. C. CHARALAMPOUS, *J. Biol. Chem.*, 234 (1959) 220.
42 E. E. GORDON, *Biochim. Biophys. Acta*, 124 (1966) 50.
43 E. E. GORDON, *Biochim. Biophys. Acta*, 104 (1965) 606.