BBA 75187

THE ACTION OF CARDIOTONIC STEROIDS ON SUGAR TRANSPORT IN MUSCLE, *IN VITRO**

I. BIHLER**

Department of Pharmacology and Therapeutics, University of Manitoba Faculty of Medicine, Winnipeg (Canada)

(Received May 27th, 1968)

SUMMARY

(1) The membrane transport of sugar in the "intact" rat hemidiaphragm *in vitro* was studied by measuring the distribution of 3-*O*-methyl-D-[^{14}C]glucose into the intracellular water. Transport was significantly increased in the presence of ouabain and several other cardiac glycosides and aglycones at concentrations (about 10^{-5} M) known to inhibit Na^+ transport.

(2) This effect required preincubation of the tissue with the drug and 10 mM glucose and was enhanced by submaximal concentrations of insulin (0.5 munit/ml) in the incubation or preincubation medium. It would seem that a transport and/or binding step is a prerequisite for the action of these drugs.

(3) Stimulation of transport by ouabain was inhibited by phlorizin or *N*-ethylmaleimide and was absent when transport was fully activated by supramaximal concentrations of insulin, indicating that cardiotonic steroids affect the stereospecific, insulin-sensitive sugar transport process.

(4) Stimulation of transport was prevented by anoxia or uncoupling of oxidative phosphorylation, showing that the process affected by the drug depends on respiratory ATP.

(5) Incubation in a K^+-free medium produced a stimulatory effect identical in every manner with that of ouabain. This suggests that the effect of cardiotonic steroids on sugar transport is due to their inhibition of a sodium pump.

(6) It is suggested there exists in muscle a negative feedback from an aerobic sodium pump to sugar transport and that this is part of the regulatory mechanism whereby the Pasteur effect is exerted at the level of membrane transport.

INTRODUCTION

Apart from their ability to increase myocardial contractility and to inhibit the active transport of cations, the cardiotonic steroids are known to have certain other effects. For example, they profoundly affect carbohydrate metabolism in cardiac[1-4]

* A preliminary report of these results was made to the 52nd Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N.J., April 15–20, 1968.

** Associate of the Medical Research Council of Canada.

and skeletal muscle[5–7] and in adipose tissue[8–10], influencing both the levels of various intermediary metabolites and the metabolic fate of radio-labelled glucose. In several recent studies[2–4,8] it was found that ouabain and related compounds increased glucose utilization. As membrane transport of glucose is the major rate-limiting factor in its metabolism[11], it was inferred by some authors[2,8] that the cardiotonic steroids affect glucose transport directly and separately from any effects on glucose metabolism. Direct evidence for such a view is largely lacking.

This paper describes and discusses experiments which show that the transport of a non-metabolized glucose analog in skeletal muscle is stimulated by cardiotonic steroids in concentrations known to inhibit the sodium pump.

METHODS

Fed young male hooded rats, bred in this laboratory were used. They were killed by a blow on the neck and "intact" hemidiaphragms were prepared as described by KONO AND COLOWICK[12]. Each hemidiaphragm was incubated for 30 min at 37° with gentle shaking in 2.0 ml of KREBS–HENSELEIT[13] bicarbonate buffer (pH 7.4), containing 0.8 % bovine serum albumin, 5 mmoles/l of a mixture of ^{14}C-labelled and unlabelled 3-*O*-methyl-D-glucose, tracer amounts of [^{3}H]mannitol and, when indicated, insulin and other additions. For reasons of solubility, some of the cardiac glycosides and aglycones were dissolved in aq. ethanol. The addition of small amounts of ethanol (final concentration 0.25 %) had no effect on sugar transport but it was nevertheless added to paired hemidiaphragms without the drug. All media were equilibrated with 95 % O_2–5 % CO_2 (95 % N_2–5 % CO_2 in anaerobic experiments). In experiments where incubation with 3-methylglucose was preceded by a period of preincubation, the cardiotonic steroids were added to both the incubation and preincubation media.

After incubation, the hemidiaphragms were washed briefly (about 1 sec) with ice-cold buffer solution, trimmed of extraneous tissue, blotted, weighed and homogenized. Samples of incubation media and of tissue homogenates were deproteinized with $Ba(OH)_2$–$ZnSO_4$ (ref. 14) and centrifuged. The radioactivity of the supernatants was determined by double-label liquid scintillation spectrometry as described earlier[15].

3-Methylglucose was a gift of Dr. W. O. GLEN, Ayerst Research Laboratories. Glucagon-free crystalline insulin was kindly supplied by Dr. MARY A. ROOT, Eli Lilly Co. The bovine serum albumin was Cohn Fraction V purchased from Nutritional Biochemicals Inc. (Some other preparations of serum albumin were found to inhibit the stimulation of sugar transport by insulin.)

The results are expressed as per cent penetration of the sugar into the intracellular water space[16], *i.e.* the concentration of sugar in the intracellular water is expressed as a percentage of the final concentration in the incubation medium. The average tissue water content was determined separately and was corrected for the apparent extracellular space, taken to equal the mannitol space measured in each tissue sample. This value depends somewhat on the amount of adhering medium and, therefore, on the handling of the tissue. It is, thus, an operational parameter reflecting accurately the amount of extracellular water in a particular tissue sample rather than the extracellular space *in vivo*. Statistical evaluation of data was done by Student's *t* test applied to paired hemidiaphragms from the same animal.

RESULTS

In these experiments the per cent penetration as defined above is used as a measure of sugar transport across the cell membrane. This is justified since 3-methylglucose enters muscle cells by the same mechanism as glucose but is not metabolized[17]. Also, as the "intact" hemidiaphragm consists of undamaged muscle fibers[12], 3-methylglucose must cross the cell membrane to penetrate the intracellular space. However, sugar transport in muscle is reversible and some sugar efflux from the cell necessarily takes place. The penetration, being derived from the intracellular concentration reached after 30 min of incubation, is a measure of net influx. It will be less than proportional to the unidirectional influx, and the underestimate will become greater as the per cent penetration increases. In this study the effect of a single factor is usually measured in paired hemidiaphragms from a single animal. The difference in the penetration of 3-methylglucose between the treated and the control hemidiaphragm is then a semiquantitative reflection of changes in transport characteristics of the tissue caused by that factor. This experimental arrangement also results in more favorable statistics.

That sugar penetration may serve as an indication of transport rate is illustrated in Fig. 1, showing the expected graded response to insulin, and in Table I which summarizes data from a large number of control hemidiaphragms. The table shows the graded increase in penetration of 3-methylglucose produced by insulin and by anoxia, separately or together, and the results of determinations of water content and mannitol space.

On the basis of these data 0.5 munit/ml insulin was used in subsequent experiments to elicit an appreciable but submaximal stimulatory effect on sugar transport and 25.0 munits/ml were used for a maximal effect.

Fig. 2 shows the stimulation of sugar transport by 10^{-5} g/ml of several cardiac glycosides and aglycones. The bars represent the increase in 3-methylglucose penetration in drug-treated hemidiaphragms when compared with otherwise identically treated paired control hemidiaphragms. With the drugs added only to the glucose-free incubation medium, ouabain or acetylstrophantidin significantly increased sugar

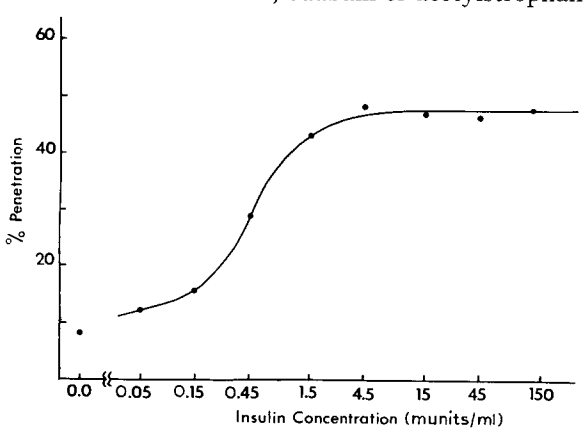

Fig. 1. The effect of graded concentrations of insulin on sugar penetration. The tissues were preincubated for 15 min and incubated for 30 min, as described in METHODS. The insulin concentration is plotted on a log scale. Each point is the mean of three experiments.

penetration in the presence of 0.5 munit/ml insulin but not in its absence (see Fig. 2, "without preincubation"). No significant effect was produced by the other compounds tested in this manner, either in the presence or absence of insulin.

In a companion study on some other aspects of the interaction between insulin

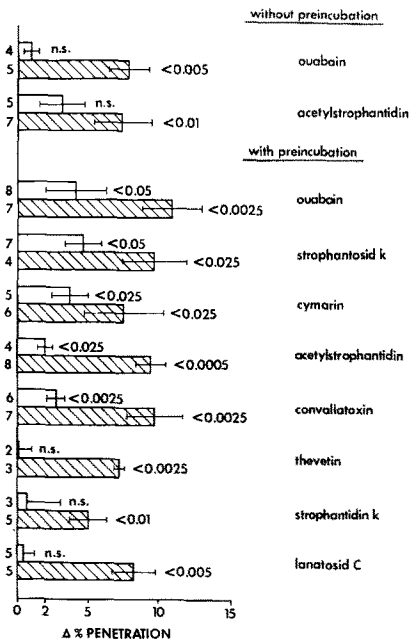

Fig. 2. The effect of cardiotonic steroids on sugar penetration. The tissues were incubated for 30 min with 5 mM 3-methylglucose and, where indicated, preincubated for 15 min with 10 mM glucose. 10^{-5} g/ml of the drugs were added to both incubation and preincubation media. This is equivalent to between $1.04 \cdot 10^{-5}$ and $2.48 \cdot 10^{-5}$ M of the various compounds. The bars represent Δ% penetration, *i.e.* the mean of the differences in per cent penetration between treated and paired control hemidiaphragms. Hatched bars indicate the presence of 0.5 munit/ml insulin in the incubation media (of drug-treated and control tissues). The figures at left refer to the number of hemidiaphragm pairs, those in the center to P calculated by the one-tailed paired t test.

TABLE I

EFFECTS OF INSULIN AND ANOXIA ON SUGAR TRANSPORT

Tissues were preincubated for 15 min in 2.0 ml Krebs bicarbonate buffer and then incubated for 30 min in 2.0 ml fresh medium containing 5 mM 3-methylglucose, tracer amounts of mannitol and the additions listed. For anoxic conditions the medium was equilibrated with 95% N_2–5% CO_2. For other details see METHODS. Figures are means ± S.E. of the per cent of intracellular penetration of 3-methylglucose. The number of single hemidiaphragms in each case is given in parentheses.

Water content after incubation, per cent of wet weight 80.2 ± 0.6 (8). Mannitol space, per cent of total tissue water space 22.4 ± 0.17 (254).

Insulin (munits/ml)	*Per cent penetration*	
	Aerobic	*Anaerobic*
—	10.4 ± 0.6 (45)	30.7 ± 0.8 (25)
0.5	30.2 ± 0.6 (171)	31.8 ± 1.5 (11)
2.0	42.5 ± 1.9 (40)	
25.0	46.1 ± 0.9 (27)	40.8 ± 1.3 (19)

and cardiotonic steroids[18] it was found that these drugs exert their positive inotropic effect *in vitro* only if incubated with the tissue in the presence of glucose or some other substrates of aerobic metabolism. For this reason, the hemidiaphragms were preincubated with the drugs for 15 min in the presence of 10 mM glucose. The paired control hemidiaphragms were also preincubated with glucose, although this treatment by itself did not affect sugar penetration. The drugs, but not glucose, were also present during the subsequent incubation with 3-methylglucose. Fig. 2, "with preincubation" shows that a number of cardiotonic steroids, aglycones as well as partial or native glycosides, now significantly increased the penetration of 3-methylglucose. When 0.5 munit/ml insulin was present during incubation, the effect was much greater and became statistically significant with several compounds which alone had no significant effect.

In contrast, ouabain had no effect in the presence of 25.0 munits/ml insulin, a dose which maximally stimulates penetration (see Fig. 1). Under these conditions the mean penetration of 3-methylglucose in 5 pairs of hemidiaphragms was 48.1 and Δ penetration due to ouabain was 0.1 $\pm$ 1.0 (mean $\pm$ S.E.).

The effect of insulin to increase the action of ouabain did not depend on the presence of insulin in the incubation medium from which uptake of 3-methylglucose took place. The first two pairs of bars in Fig. 3A show that preincubation with insulin, followed by a 5-min "wash" in a large volume of insulin-free medium, had no effect on the subsequent uptake of 3-methylglucose, whereas its presence during preincubation with ouabain allowed the drug to exert a clearcut stimulatory effect.

These findings were used to study the specificity of the stimulation of sugar transport by ouabain. It was shown earlier that under appropriate conditions phlorizin[19] and *N*-ethylmaleimide[20] inhibit only the insulin-induced increase in sugar transport without affecting basal uptake, and this is confirmed by the control experi-

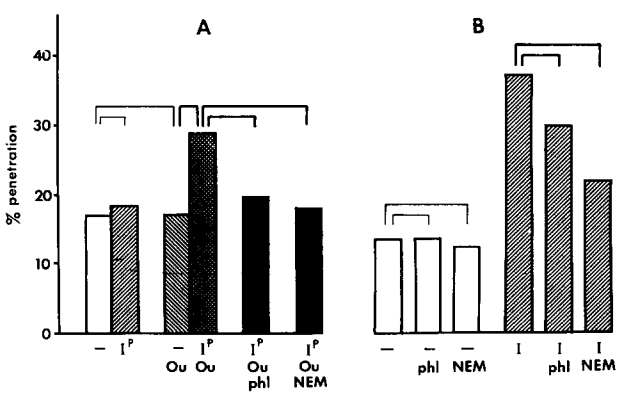

Fig. 3. Effect of phlorizin and *N*-ethylmaleimide on the stimulation of sugar transport by ouabain and insulin. The tissues in Part A were preincubated for 15 min, "washed" for 5 min, as described in the text, and incubated for 30 min with 5 mM 3-methylglucose. The additions are indicated below each bar: I^P, 0.5 munit/ml insulin in preincubation only; Ou, 10^{-5} g/ml ouabain in preincubation, "wash" and incubation; phl, 1 mM phlorizin in incubation only; NEM, 1 mM *N*-ethylmaleimide for 80 sec before the start of final incubation. The tissues in Part B were incubated for 30 min. Where indicated, 0.5 munit/ml insulin and/or 1 mM phlorizin were added to the incubation medium and 1 mM *N*-ethylmaleimide was present for 80 sec prior to incubation. The bars represent per cent penetration of the sugar. Each bracket indicates 5 or more pairs of hemidiaphragms. Heavy brackets show where the mean difference within pairs is significant, $P<0.0025$.

ments shown in Fig. 3B. Accordingly, the tissues were first preincubated with glucose, insulin and ouabain and "washed" as described above, and then either incubated with 3-methylglucose in the presence of 1 mM phlorizin or exposed to 1 mM *N*-ethylmaleimide for 80 sec prior to incubation with 3-methylglucose. The last two bars in Fig. 3A show that the stimulatory effect of ouabain was greatly reduced by phlorizin and *N*-ethylmaleimide. The inhibition of the effect of ouabain by these two specific inhibitors, the failure of ouabain to act if sugar transport was maximally activated by insulin and the failure of ouabain to affect the mannitol space (Δ space = 0.3 ± 0.4 % (54)) all indicate that the cardiotonic steroids enhance sugar transport by specifically activating the insulin-sensitive sugar transport mechanism and not by some nonspecific effect on membrane permeability.

Table II shows that preincubation with ouabain and insulin, as in Fig. 2, failed to stimulate sugar transport under anaerobic conditions or in the presence of 2,4-dinitrophenol or 4,6-dinitro-*o*-cresol, two uncouplers of oxidative phosphorylation. The data also show that, as is well known, anoxia or uncoupling of oxidative phosphorylation by themselves cause an increase in sugar penetration but that transport under these conditions could still be activated by insulin. Taken together, these results suggest that the effect of cardiotonic steroids to enhance sugar transport depends on an intact supply of energy derived from oxidative metabolism.

The cardiotonic steroids used in this study are well known as inhibitors of active sodium transport in a wide variety of tissues. More specifically, inhibition of sodium transport in rat diaphragm has been demonstrated with ouabain[21] and strophantin K (ref. 22) at the concentrations used here. If the effect of cardiotonic steroids on sugar transport is related to their inhibition of active cation transport, one would expect a similar stimulation of sugar transport when the Na^+ pump is inhibited by other means. Since the activity of the Na^+ pump in various tissue is greatly depressed in the absence of external K^+ (refs. 23–26), the effect of ouabain was tested in a medium where all K^+ was replaced by choline and where consequently the concentration of

TABLE II

EFFECTS OF ANOXIA AND INHIBITION OF OXIDATIVE METABOLISM ON THE STIMULATION OF SUGAR TRANSPORT BY OUABAIN

Tissues were incubated with 5 mM 3-methylglucose as described in Fig. 2. Where indicated, 10^{-5} g/ml ouabain, 2,4-dinitrophenol and 4,6-dinitro-*o*-cresol were added both to preincubation and incubation media. Figures are means ± S.E. of differences in sugar penetration between paired hemidiaphragms with the number of hemidiaphragm pairs given in parentheses. For other details see Fig. 2.

To both hemidiaphragms	*To one only*	*Δ per cent penetration*	*P*
—	$+N_2$	+20.4 ± 1.5 (6)	<0.0005
N_2 + 0.5 munit/ml insulin	+ouabain	+ 0.2 ± 1.9 (5)	n.s.
N_2	+25 munits/ml insulin	+12.3 ± 0.4 (6)	<0.005
—	+0.1 mM 2,4-dinitrophenol	+10.2 ± 1.4 (7)	<0.0005
0.1 mM 2,4-dinitrophenol +0.5 munit/ml insulin	+ouabain	− 0.4 ± 0.2 (7)	n.s.
0.1 mM 2,4-dinitrophenol	+0.5 munit/ml insulin	+18.9 ± 2.7 (4)	<0.01
0.5 mM 4,6-dinitro-*o*-cresol +0.5 munit/ml insulin	+ouabain	− 0.5 ± 0.8 (6)	n.s.
0.1 mM 4,6-dinitro-*o*-cresol	+0.5 munit/ml insulin	+23.2 ± 1.4 (8)	<0.0005

K^+ in the interstitial water of the tissue is, presumably, very low although not negligible. The data in Table III show that under such conditions a stimulation of sugar transport, analogous to that caused by cardiotonic steroids, could be produced. Low K^+ increased sugar penetration both in the presence of 0.5 munit/ml insulin and in its absence but not in the presence of 25.0 munits/ml insulin. The increase in sugar transport could be blocked by *N*-ethylmaleimide. Insulin and ouabain remained effective in a K^+-free medium. The data show that the effect of ouabain was roughly additive to that of low K^+ and *vice versa*, and suggest that ouabain may be more effective in a K^+-free medium than in a normal one. The effect of 10^{-6} g/ml ouabain in K^+-free medium is about equal to that of 10^{-5} g/ml in normal medium (compare Tables II and III). This increased effect is to be expected from the well known antagonism between extracellular K^+ and cardiotonic steroids.

TABLE III

EFFECT OF K^+-FREE MEDIUM ON SUGAR TRANSPORT

Tissues were incubated with 5 mM 3-methylglucose as described in Fig. 2, "with preincubation". 10^{-6} g/ml ouabain was added both to preincubation and incubation media, where indicated. In the K^+-free medium all K^+ was replaced by choline. Treatment with *N*-ethylmaleimide was done as in Fig. 3. Figures are means ± S.E. of differences in sugar penetration between paired hemidiaphragms. For other details see Fig. 2.

To both hemidiaphragms	*To one only*	*Δ per cent penetration*	*P*
—	K^+-free	+ 8.9 ± 1.8 (7)	<0.0025
0.5 munit/ml insulin	K^+-free	+ 8.7 ± 1.2 (6)	<0.0005
25.0 munits/ml insulin	K^+-free	− 1.4 ± 0.7 (5)	n.s.
N-Ethylmaleimide	K^+-free	+ 0.8 ± 0.4 (5)	n.s.
K^+-free	+ 0.5 munit/ml insulin	+16.9 ± 2.8 (5)	<0.0025
K^+-free	+25.0 munits/ml insulin	+26.1 ± 1.6 (5)	<0.0005
Ouabain	K^+-free	+ 9.1 ± 0.7 (5)	<0.0005
0.5 munit/ml insulin + ouabain	K^+-free	+12.1 ± 3.7 (6)	<0.0125
K^+-free	+ouabain	+ 2.7 ± 0.6 (9)	<0.0025
K^+-free + 0.5 munit/ml insulin	+ouabain	+ 6.2 ± 1.7 (6)	<0.01

DISCUSSION

The increased uptake of glucose under the influence of cardiotonic steroids[2–4,8,9] can be taken as a reflection of increased sugar transport only if the transport of glucose is rate determining for its utilization. However, transport was shown not to be rate limiting in some cases in the presence of insulin[11,27] or 2,4-dinitrophenol[28], and the rate-limiting process in the presence of ouabain is unknown. To avoid these uncertainties, a non-metabolized sugar, 3-methylglucose was used here to measure membrane transport without possible interference from subsequent metabolism. Earlier, KIEN, GOMOLL AND SHERROD[29] used galactose to study the effect of digoxin on sugar transport by the dog heart *in vivo*. Increased uptake of a non-metabolized sugar need not necessarily mean that the cardiotonic steroids affect the sugar carrier directly. Since muscular contraction stimulates sugar transport[28], increased transport in the heart may result from the inotropic effect of these drugs. In the present study, with resting skeletal muscle, this can be excluded. The observed stimulation of sugar transport by cardiotonic steroids is more likely related to their inhibition of active

cation transport, as the same effect is produced by omitting K^+ from the incubation medium. The data also show that stimulation is abolished by anoxia, which is consistent with the dependence of the Na^+ pump in rat diaphragm on respiratory ATP.

Regulation of transport

Sugar transport in muscle is stimulated by anoxia and uncouplers of oxidative phosphorylation and RANDLE AND SMITH[30] concluded that transport is restrained under basal conditions by a process dependent upon the supply of respiratory ATP. RANDLE's[31] suggestion that ATP may restrict transport directly through formation of a less effective, phosphorylated state of the sugar carrier cannot explain the effect of cardiotonic steroids since it was shown[22,23] that 10^{-5} and even 10^{-3} M ouabain —concentrations which strongly inhibit the Na^+ pump and stimulate sugar transport—do not affect overall ATP levels in rat diaphragm. It is possible, however, that only ATP in a particular cellular compartment may affect sugar entry, and TEPPERMAN AND TEPPERMAN[33] suggested that both the metabolic and the membrane effects of insulin could be explained by assuming that the hormone redirects the supply of ATP from a restrictive function at the membrane to synthetic processes within the cell. It should be pointed out that this speculation is consistent with one or several steps intervening between availability of respiratory ATP and restriction of sugar transport. Regardless of the validity of this hypothesis as a whole, the present data provide strong evidence that a Na^+ pump is the link, or one of the links, in the regulation of sugar transport by the availability of respiratory ATP. The data show that sugar transport is increased when the Na^+ pump is inhibited either directly by cardiotonic steroids or a decrease in extracellular K^+, or indirectly by decreasing the supply of respiratory ATP through anoxia or uncoupling of oxidative phosphorylation. Unpublished data from this laboratory also suggest that, conversely, stimulation of the Na^+ pump results in decreased sugar transport. As yet there is no evidence on the mechanism of this apparent negative feedback between the aerobic Na^+ pump and the degree of activation of the sugar transport process. Presumably, it could be mediated by the concentration of a pump-dependent ion in a particular cellular compartment.

In mammalian muscle under normal conditions (aerobiosis, low insulin level) glucose transport is rate-limiting for its utilization. The Pasteur effect *i.e.*, increased glucose utilization during hypoxia must, therefore, involve activation of the transport step, and regulation of sugar transport should play an important physiological role. The present data strongly suggest that the aerobic Na^+ pump serves as a regulatory link between cellular metabolism and the membrane transport of glucose.

The data in Fig. 3 and Table III show that *N*-ethylmaleimide inhibits the sugar carrier which had been activated by pretreatment with ouabain or by low K^+. Hence, *N*-ethylmaleimide is able to act in the absence of insulin. These data do not support earlier views[34] that *N*-ethylmaleimide prevents activation of the sugar carrier by interfering with the binding of insulin to SH-groups at the cell membrane. They are consistent with more recent observations[20] that *N*-ethylmaleimide does not inhibit the effect of insulin on glycogen synthesis in rat diaphragm while blocking the effect on sugar transport. Thus, *N*-ethylmaleimide may act by attacking SH-groups involved in the activation of the carrier itself, rather than by affecting the binding of insulin to a receptor site.

Relation of effects on transport and on metabolism

In isolated hearts the metabolic effects of ouabain may reasonably be attributed to the increased contraction caused by the drug and the consequent increase in glucose transport[3]. However, in the diaphragm[5,6] and in adipose tissue[9,10] ouabain (and omission of K^+) alters the pattern of glucose metabolism and inhibits lipolysis in a typical insulin-like manner. In common with other antilipolytic agents ouabain inhibits adenyl cyclase in adipocytes, and Ho *et al.*[10] suggested that alterations in cationic concentrations at an intracellular site may affect the activation of this enzyme. Thus, there is a striking parallelism between the effects of the Na^+ pump on metabolism and on membrane transport of glucose. Since there is no evidence for an effect of the adenyl cyclase system on the membrane transport of sugar, one is led to speculate whether, perhaps, changes in ionic concentration at a particular site or sites in the cell may affect simultaneously but separately, both transport and metabolism.

Effectiveness of cardiotonic steroids

The data presented here provide some evidence to suggest that a transport or binding step is a prerequisite for the action of these drugs on sugar transport. First, the degrees of effectiveness of the various cardiotonic steroids (Fig. 2) are at variance with lipid solubility alone governing the drugs' access to their site of action. Second, insulin enhances the effect of the drugs (Fig. 2), even when it is present only during preincubation and has no effect of its own on sugar transport (Fig. 3). Third, the strong influence of preincubation with glucose is also consistent with an energy-requiring transport or binding step. These questions are presently being further explored. MARKS and coworkers[35] have recently presented evidence for a membrane transport step for cardiac glycosides in heart muscle.

ACKNOWLEDGEMENTS

I thank Drs. L. E. BAILEY and P. E. DRESEL for valuable discussions and Mr. P. C. SAWH and Mrs. B. COOK for able technical assistance. This work was supported by grants from the Medical Research Council of Canada and the Muscular Dystrophy Association of Canada.

REFERENCES

1 A. WOLLENBERGER, *Ciba Found. Symp. Enzymes and Drug Action*, J. and A. Churchill, London, 1962, p. 127.
2 G. E. KIEN AND T. S. SHERROD, *Circulation Res.*, 8 (1960) 188.
3 R. A. KREISBERG AND J. R. WILLIAMSON, *Am. J. Physiol.*, 207 (1964) 347.
4 P. ARESE, A. BOSIA AND L. ROSSINI, *Biochem. Biophys. Res. Commun.*, 27 (1967) 138.
5 T. CLAUSEN, *Biochim. Biophys. Acta*, 109 (1965) 164.
6 T. CLAUSEN, *Biochim. Biophys. Acta*, 120 (1966) 361.
7 C. CRONE, *Acta Physiol. Scand.*, 68 (1966) 105.
8 J. LETARTE AND A. E. RENOLD, *Nature*, 215 (1967) 961.
9 R. J. HO AND B. JEANRENAUD, *Biochim. Biophys. Acta*, 144 (1967) 61.
10 R. J. HO, B. JEANRENAUD, TH. POSTERNAK AND A. E. RENOLD, *Biochim. Biophys. Acta*, 144 (1967) 74.
11 H. E. MORGAN, E. CADENAS, D. M. REGEN AND C. R. PARK, *J. Biol. Chem.*, 236 (1961) 262.
12 T. KONO AND S. P. COLOWICK, *Arch. Biochem. Biophys.*, 93 (1961) 514.
13 H. A. KREBS AND K. HENSELEIT, *Z. Physiol. Chem.*, 210 (1932) 33.

14 M. ŠOMOGYI, *J. Biol. Chem.*, 160 (1945) 69.
15 S. ADAMIČ AND I. BIHLER, *Mol. Pharmacol.*, 3 (1967) 188.
16 I. BIHLER, H. M. CAVERT AND R. B. FISHER, *J. Physiol. London*, 180 (1965) 157.
17 H. T. NARAHARA AND P. ÖZAND, *J. Biol. Chem.*, 238 (1963) 40.
18 L. E. BAILEY, *Proc. Can. Federation Biol. Soc.*, 11 (1968) 118.
19 I. BIHLER, H. M. CAVERT AND R. B. FISHER, *J. Physiol. London*, 180 (1965) 168.
20 D. EBOUE-BONIS, A. M. CHAMBAUT, P. WOLFIN AND H. CLAUSER, *Bull. Soc. Chim. Biol.*, 49 (1967) 415.
21 A. KLEINZELLER AND A. KNOTKOVA, *Physiol. Bohemoslov.*, 13 (1964) 317.
22 J. E. PARRISH AND D. M. KIPNIS, *J. Clin. Invest.*, 43 (1964) 1994.
23 I. M. GLYNN, *J. Physiol. London*, 136 (1957) 148.
24 A. L. HODGKIN AND R. D. KEYNES, *J. Physiol. London*, 128 (1955) 28.
25 G. VOGEL AND U. TERVOOREN, *Arch. Ges. Physiol.*, 284 (1965) 103.
26 R. D. KEYNES, *Proc. Roy. Soc. London, Ser. B*, 142 (1954) 359.
27 O. B. CROFFORD AND A. E. RENOLD, *J. Biol. Chem.*, 240 (1965) 14.
28 P. J. RANDLE AND H. E. MORGAN, *Vitamins Hormones*, 20 (1962) 199.
29 G. A. KIEN, A. W. GOMOLL AND T. R. SHERROD, *Proc. Soc. Exptl. Biol. Med.*, 103 (1960) 682.
30 P. J. RANDLE AND G. H. SMITH, *Biochem. J.*, 70 (1958) 490, 501.
31 P. J. RANDLE, in G. PINCUS, K. V. THIMANN AND E. B. ASTWOOD, *The Hormones*, Vol. 4, Academic Press, New York, 1964, p. 481.
32 J. L. KOSTYO AND J. E. SCHMIDT, *Am. J. Physiol.*, 204 (1963) 1031.
33 J. TEPPERMAN AND H. M. TEPPERMAN, *Pharmacol. Rev.*, 12 (1960) 301.
34 E. CADENAS, H. KAJI, C. R. PARK AND H. RASMUSSEN, *J. Biol. Chem.*, 236 (1961) PC64.
35 S. DUTTA, J. O. LINDOWER AND B. H. MARKS, *Federation Proc.*, 27 (1968) 347.