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EFFECTS OF DICOUMAROL, INSULIN AND ANOXIA ON RAT DIAPHRAGM—II. PENETRATION OF SUGARS

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Abstract—We have observed that *in vitro* dicoumarol inhibits the uptake of D-glucose, D-fructose, D-galactose and D-2-deoxyglucose by the isolated cut diaphragm of the rat. Dicoumarol has no effect on the accumulation of D-3-oxymethylglucose in intact rat diaphragm, but enhances the accumulation of D-xylose, L-arabinose and α-methyl-D-glucose in intact rat diaphragm. Insulin, as well as anoxia, stimulates the uptake and accumulation of all the sugars tested, but cannot reverse the inhibition produced by dicoumarol in these systems. As an inhibitor, dicoumarol eliminates the effects of insulin and anoxia, while as a stimulus it makes the effects of dicoumarol, anoxia and insulin additive.

KIPNIS and Cori[1] postulated for the first time the possible existence of two systems of sugar transport in muscle. Later Battaglia and Randle,[2] using intact diaphragm concluded that in isolated diaphragm there is more than one system of monosaccharide transport; one for the transport of the competing sugars D-glucose, D-mannose, D-3-oxymethyl glucose, D-xylose, D-arabinose, L-xylose and L-arabinose and another for the transport of those sugars that do not compete with members of this group (D-galactose and D-fructose).

Randle and Smith,[3] presented evidence that, in isolated rat diaphragm, anoxia and substances such as salicylate, sodium cyanide or 2,4-dinitrophenol (uncouplers of oxidative phosphorylation), increase the uptake of D-glucose and D-xylose by accelerating the transfer of these sugars across the muscle cell membrane. These authors concluded that the transfer process for sugars in muscle is inhibited by a substance generated during oxidative phosphorylation, and that insulin activates the transfer process by interfering with the action of this substance.

As shown in our preceding paper† dicoumarol, an uncoupler of oxidative phosphorylation, does not stimulate but inhibits the uptake of D-glucose by the isolated diaphragm. This prompted us to carry out a complete study of the action of this compound on the transport of certain sugars in the cut and intact diaphragm of the rat.

MATERIALS AND METHODS

Incubation medium. All the incubations were carried out in Krebs–Ringer II buffer in a Dubnoff incubator with agitation. The medium was maintained at 37° and

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continuously gassed with $O_2 + CO_2$ (95·5%) or N_2 (100%). Sugars were added at the concentrations stated in the tables.

Animals. Diaphragm muscle was obtained from male and female albino Wistar rats of 100–130 g body wt, fed on our stock laboratory diet. Food was withheld from the intact rats for 18 hr before the experiments. The animals had free access to water at all times.

Cut and intact diaphragm preparations. Both preparations were obtained as described previously.*

Chemicals. D-Glucose and D-fructose were obtained from Merck; D-sorbitol from British Drug Houses; D-xylose, L-arabinose, D-2-deoxyglucose, α-methyl-D-glucoside and vitamin K_3 (Menadione) from Sigma; D-3-oxymethyglucose was from K & K and Dicoumarol from Mann. R.L.; [$U^{14}C$]-D-sorbitol, [$U^{14}C$]-D-xylose and [$U^{14}C$]-α-methyl-D-glucoside were from Radiochemical Centre, Amersham; [^{14}C]-dicoumarol from Junta de Energía Nuclear, Madrid. Crystalline insulin (Lilly) was prepared as previously described.

Analytical methods. Analyses of sugars from the muscle and incubation medium were made after deproteinizing*; D-xylose and L-arabinose by the Roe and Rice method,[4] D-glucose, D-galactose and D-3-oxymethylglucose by the Somogyi[5] modification of Nelson's method;[6] D-fructose by the Dishe method [7] and D-2-deoxyglucose according to Warawdokar.[8] When both D-glucose and D-xylose were in the medium, D-glucose was determined with glucose–oxidase.[9] Labelled sugars were determined as previously described.

Calculations of statistical analysis. These were calculated as described in the previous paper.

RESULTS

Effect of dicoumarol and insulin on the uptake of metabolizable hexoses. Randle and Smith[10] have shown that some uncouplers of oxidative phosphorylation stimulate the uptake of D-glucose by the tissue (50 per cent). Nevertheless the results of our experiments with dicoumarol are not in agreement with these results.

Table 1 shows the results of the effect of dicoumarol and dicoumarol + insulin on the uptake of D-glucose and D-fructose by the cut diaphragm of rat. As may be observed, the drug, at either of the concentrations used, inhibits the uptake of the two sugars. Insulin stimulates the uptake of the sugars but cannot reverse the inhibition produced by dicoumarol. Figure 1 shows that Vitamin K_3 (menadione) has no effect on the uptake of D-glucose and does not reverse the inhibition produced by dicoumarol. The present results, compared with those previously published (Salinas and Candela, 1972) suggest that the action of dicoumarol on the sugar transport is not related to that of the drug on the mechanism of oxidative phosphorylation. Table 2 shows that the effect of dicoumarol on the uptake of D-galactose and D-2-deoxyglucose is the same as that obtained with D-glucose.

Effect of dicoumarol and insulin on the penetration of nonmetabolizable hexoses into intact rat diaphragm. In this group of experiments we studied the effect of dicoumarol on the penetration of two hexoses: D-3-oxymethyl-glucose and α-methyl-D-glucose

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TABLE 1. EFFECT OF DICOUMAROL AND DICOUMAROL + INSULIN ON THE UPTAKE OF D-GLUCOSE (G) AND D-FRUCTOSE (Fru) BY RAT CUT DIAPHRAGM

No. exp.	Dicoumarol (mM)	Insulin (0·1 U/ml)	Sugar uptake (mg of sugar/ g wet tissue)	Difference ± S.E. of the difference	Significance of the difference (P)
15	None	None	(G) 3·26	1·72 ± 0·13	P < 0·01
15	0·5	None	(G) 1·54		
13	None	None	(G) 2·94	1·21 ± 0·11	P < 0·01
13	0·05	None	(G) 1·73		
11	None	+	(G) 3·37	1·69 ± 0·22	P < 0·01
11	0·05	+	(G) 1·37		
32	None	None	(Fru) 3·11	1·08 ± 0·20	P < 0·01
32	0·5	None	(Fru) 2·02		
26	None	None	(Fru) 3·12	0·63 ± 0·18	P < 0·01
26	0·05	None	(Fru) 2·50		
12	None	+	(Fru) 4·74	2·05 ± 0·50	P < 0·01
12	0·05	+	(Fru) 2·68		

Incubation medium, Krebs–Ringer II, sugar (3 mg/ml). Incubation time, 60 min. Sugars were determined by colorimetric method as described in Materials and Methods.

into rat diaphragm. The extracellular space was not determined as it was already a well-known parameter of intact diaphragm. As seen in Table 3, dicoumarol and insulin produce an increase in the penetration of α-methyl-D-glucose. Dicoumarol has no effect on the penetration of D-3-oxymethyl-glucose, whereas insulin produced an increase in the penetration of this sugar.

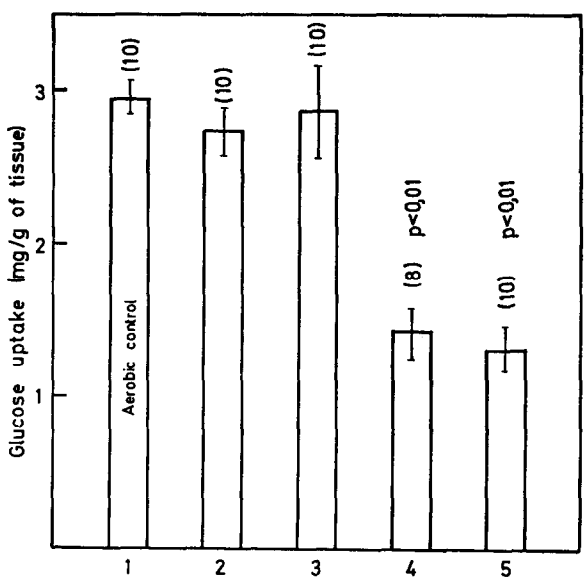

FIG. 1. Effect of vitamin K_3 (menadione) on the uptake of D-glucose by rat cut diaphragm. Incubation medium, Krebs–Ringer II, D-glucose (3 mg/ml); incubation time, 60 min. (1) Aerobic control; (2) sodium bisulphite (0·5 mM); (3) vitamin K_3 (bisulphite salt) (0·5 mM); (4) dicoumarol (0·5 mM); (5) dicoumarol + vitamin K_3 (both 0·5 mM).

TABLE 2. EFFECT OF DICOUMAROL AND DICOUMAROL + INSULIN ON THE UPTAKE OF D-2-DEOXYGLUCOSE (DG) AND D-GALACTOSE (Gal) BY RAT CUT DIAPHRAGM

No. exp.	Dicoumarol (mM)	Insulin (0·1 U/m.)	Sugar uptake (mg of sugar/ g wet tissue)	Difference ± S.E. of the difference	Significance of the difference (P)
12	None	None	(Gal) 1·08	0·29 ± 0·09	P < 0·01
12	0·05	None	(Gal) 0·79		
15	None	+	(Gal) 4·24	2·02 ± 0·11	P < 0·01
15	0·05	+	(Gal) 2·22		
20	None	None	(DG) 2·91	1·89 ± 0·02	P < 0·01
20	0·5	None	(DG) 1·02		
10	None	None	(DG) 2·47	1·22 ± 0·15	P < 0·01
10	0·05	None	(DG) 1·25		
11	None	+	(DG) 1·75	0·84 ± 0·10	P < 0·01
11	0·05	+	(DG) 0·91		

Incubation medium, Krebs–Ringer II, sugar (3 mg/ml). Incubation time, 60 min. Sugars were determined by colorimetric method as described in Materials and Methods

Effect of dicoumarol and insulin on the penetration of pentoses into intact diaphragm. According to Kipnis and Cori[1], the penetration of D-xylose into intact diaphragm is proportional to the pentose concentration in the medium during the first 60 min of incubation. We ascertained whether the same results could be obtained in our system.

Figure 2 shows that the extracellular space remains constant when the incubation medium and the tissue are in equilibrium. On the other hand, the penetration of D-xylose is significant during the first 60 min of incubation. The values obtained with

TABLE 3. EFFECT OF DICOUMAROL AND DICOUMAROL + INSULIN ON THE ACCUMULATION OF α-METHYL-D-GLUCOSIDE (α-MG) AND D-3-OXYMETHYLGLUCOSE (3-OMG) IN INTACT RAT DIAPHRAGM

No. exp.	Dicoumarol (mM)	Insulin (0·1 U/ml)	Sugar space (ml/100g of wet tissue)	Significance of the difference with the control (P)*
8	Extracellular space (Sorbitol space)		43·0 ± 1·1	None
12	None	None (α-MG)	52·6 ± 1·3*	None
10	0·5	None (α-MG)	59·4 ± 2·6	P < 0·05
10	None	+ (α-MG)	58·5 ± 1·3	P < 0·02
10	0·5	+ (α-MG)	58·1 ± 2·0	P < 0·05
9	None	None (3-OMG)	45·6 ± 0·7*	None
11	0·5	None (3-OMG)	48·3 ± 1·0	N.S.
11	None	+ (3-OMG)	55·6 ± 1·1	P < 0·01
11	0·5	+ (3-OMG)	51·8 ± 2·0	P < 0·01

Incubation medium, Krebs–Ringer II, D-sorbitol (0·12 mg/ml), sugar (3·5 mg/ml), α-methyl-D-[^{14}C]-glucoside (0·05 μCi/ml).
Incubation time, 60 min.
N.S., not significant.

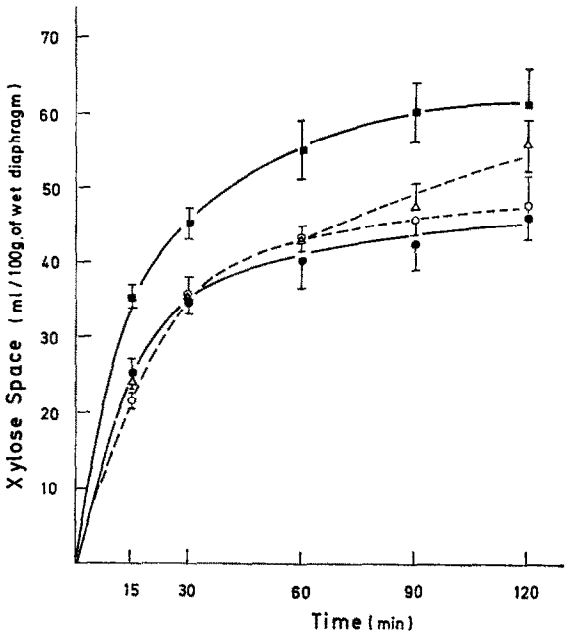

FIG. 2. Comparison between two different methods for the determination of D-xylose. Incubation medium, Krebs–Ringer II, D-xylose (3·5 mg/ml) with or without [^{14}C]-D-xylose (0·05 μCi/ml), D-sorbitol (0·12 mg/ml) with or without [^{14}C]-D-sorbitol (0·05 μCi/ml). ○——○, Sorbital space; △——△ sorbitol space in presence of dicoumarol; ●——●, xylose space; ■——■, [^{14}C]-xylose space. (○ ● △ ■) Mean of five experiments ± S. E. M.

[^{14}C]-D-xylose were higher than those found with D-xylose measured photocolorimetrically. Table 4 shows the effect of dicoumarol on the extracellular space and on the penetration of D-xylose into the tissue. Dicoumarol produces no inhibition, but stimulates the penetration of the pentose into the diaphragm. Both dicoumarol and

TABLE 4. EFFECT OF DICOUMAROL AND DICOUMAROL + INSULIN ON THE SORBITOL AND XYLOSE SPACES OF RAT INTACT DIAGRAM

Addition	Sorbital space (ml/100g of wet tissue) Mean ± S.E.M.	Significance of the difference with the control (P)	Xylose space (ml/100g of wet tissue) Mean ± S.E.M.	Significance of the difference with the control (P)
Aerobic control	43·0 ± 1·1 (8)	None	46·9 ± 1·5 (8)	None
Insulin 0·1 U/ml	44·4 ± 2·2 (8)	N.S.	65·3 ± 3·3 (18)	$P < 0.01$
Dicoumarol 0·5 mM	None	None	53·2 ± 1·6 (9)	$P < 0.05$
Dicoumarol 0·05 mM	43·2 ± 2·2 (7)	N.S.	45·1 ± 2·5 (8)	$P < 0.05$
Dicoumarol 0·05 mM + insulin 0·1 U/ml	47·6 ± 2·0 (5)	N.S.	67·6 ± 2·0 (17)	$P < 0.01$

Incubation medium, Krebs–Ringer II, D-sorbital (0·12 mg/ml), D-sorbitol-^{14} (0·05 μCi/ml), D-xylose (3·5 mg/ml). Incubation time, 60 min.

D-Xylose analyses were by the colorimetric method of Row.

The number of observations are given in parentheses.

N.S., not significant.

insulin stimulate the penetration of the pentose after the first 15 min of incubation, and neither affects the extracellular space. As seen in Table 5, the results obtained with L-arabinose are exactly the same as with D-xylose.

The different effect of dicoumarol on the penetration of hexoses and pentoses suggested a different mechanism of transport for the two groups of sugars. On the other hand, insulin cannot reverse the inhibition produced by dicoumarol on the hexose uptake, whereas the stimulating effect of insulin on the penetration of pentoses adds itself to that of dicoumarol.

TABLE 5. EFFECT OF INSULIN AND INSULIN + DICOUMAROL ON THE DICOUMAROL SPACE OF RAT INTACT DIAPHRAGM INCUBATED IN THE PRESENCE OF D-XYLOSE OR L-ARABINOSE

Addition	Dicoumarol space (ml/100 g of wet tissue) Mean ± S.E.M.	Significance of the difference with the control* (P)	Xylose space (ml/100g of wet tissue) Mean ± S.E.M.	Significance of the difference with the control* (P)
Dicoumarol 0·05 mM	40·5 ± 1·9 (11)*	None	66·5 ± 2·6 (14)	$P < 0.01$
Dicoumarol 0·05 mM + insulin 0·1 Units/ml	38·6 ± 1·9 (11)	N.S.	64·8 ± 3·4 (15)	$P < 0.01$
			Arabinose space	
Dicoumarol 0·05 mM	38·3 ± 2·4 (9)*	None	64·9 ± 5·2 (9)	$P < 0.01$
Dicoumarol 0·05 mM + insulin 0·1 Units/ml	39·0 ± 1·4 (11)	N.S.	66·8 ± 7·9 (10)	$P < 0.01$

Incubation medium, Krebs–Ringer II, D-xylose or L-arabinose (3·5 mg/ml), dicoumarol (0·05 mM, 0·05 μCi/ml). Incubation time, 60 min.
D-Xylose and L-arabinose analyses were by the colorimetric method of Roe.
The number of observations are given in parentheses.
N.S., not significant.

Effect of anoxia on the uptake of D-*glucose and the penetration of* D-*xylose.* According to Levine *et al.*[11] sugar transport is stimulated by muscular work. Candela *et al.*[12] suggested that a factor produced during muscle contraction is responsible for the increase of D-glucose uptake by the tissue. Randle and Smith[10] found that anoxia and some metabolic poisons stimulate sugar transport: which suggests that the stimulation could be due to a decrease in the inhibitor's levels of ATP.

Reports lately published have not made it clear whether anoxia acts at membrane level,[13,14] at phosphorylation level or during the whole process.[15,16] We have carried out a comparative study of the effects of anoxia, dicoumarol and insulin on the uptake of D-glucose and the accumulation of D-xylose in rat diaphragm.

Glucose uptake (Table 6) is higher in anaerobiosis than in aerobiosis, and dicoumarol inhibits the stimulus produced by anoxia. The effect of insulin adds itself to that of anoxia, and in the presence of dicoumarol neither stimulus is produced.

We have previously reported that anoxia produces no variation in the extracellular space of intact diaphragm, but strongly increases the accumulation of D-xylose in the tissue. Insulin increases the accumulation of D-xylose and adds itself to that of anoxia. Dicoumarol also stimulates the penetration of D-xylose into the tissue (Table 7). It may be observed, as in aerobiosis, that the effect of dicoumarol on the transport of hexoses is exactly opposite to the effect on the accumulation of pentoses.

TABLE 6. EFFECT OF DICOUMAROL AND DICOUMAROL + INSULIN ON THE UPTAKE OF D-GLUCOSE BY RAT CUT DIAPHRAGM IN ANAEROBIOSIS

No. exp.	Dicoumarol (mM)	Insulin (0·1 U/ml)	Glucose uptake (mg of glucose/g wet tissue)	Difference ± S.E. of the difference	Significance of the difference (P)
11	None	None	3·18	2·69 ± 0·20	P < 0·01
11	0·5	None	0·87		
10	None	None	3·31	2·03 ± 0·30	P < 0·01
10	0·05	None	1·28		
10	None	None	3·34	0·97 ± 0·19	P < 0·01
10	0·005	None	2·36		
11	None	+	5·44	4·20 ± 0·24	P < 0·01
11	0·05	+	1·24		

Incubation medium, Krebs–Ringer II, D-glucose (3 mg/ml). Incubation time, 60 min. D-Glucose analyses were by the colorimetric method of Somogyi.

Competition between sugars. One of the facts that reveal whether two different sugars are transported by the same system or not, is the competition between the two sugars in that system. Helmreich and Cori[17] with *in vivo* experiments, and Kipnis and Cori[1] with *in vitro* experiments, found no competition between D-glucose and D-xylose. Nevertheless Battaglia and Randle[2] observed that the accumulation of D-xylose decreased in the presence of D-glucose and insulin.

Figure 3 shows that dicoumarol inhibits D-glucose uptake in the presence of D-xylose. The values of glucose uptake after 60 min of incubation are 2·36 mg/g of tissue and 1·42 mg/g of tissue, respectively, in the absence and presence of dicoumarol. Both values are similar to those obtained for the glucose uptake in the absence of D-xylose.

TABLE 7. EFFECT OF DICOUMAROL AND DICOUMAROL + INSULIN ON THE SORBITAL AND XYLOSE SPACES OF RAT INTACT DIAPHRAGM IN ANAEROBIOSIS

Addition	Sorbitol space (ml/100g of wet tissue) Mean ± S.E.M.	Significance of the difference with the control (P)	Xylose space (ml/100g of wet tissue) Mean ± S.E.M.	Significance of the difference with the control (P)
Anaerobic control	39·7 ± 1·0 (14)		65·9 ± 2·9 (14)	
Insulin 0·1 U/ml	42·0 ± 2·1 (8)	N.S.	82·9 ± 2·9 (14)	P < 0·01
Dicoumarol 0·5 mM	39·3 ± 1·4 (8)	N.S.	84·3 ± 2·9 (14)	P < 0·01
Dicoumarol 0·05 mM			78·8 ± 3·0 (9)	P < 0·05
Dicoumarol 0·5 mM + insulin 0·1 U/ml	41·0 ± 1·7 (13)	N.S.	84·0 ± 4·2 (7)	P < 0·02

Incubation medium, Krebs–Ringer II, D-sorbitol (0·12 mg/ml), [^{14}C]-D-sorbitol (0·05 μCi/ml), D-xylose (3·5 mg/ml). Incubation time, 60 min.

D-Xylose analyses were by the colorimetric method of Roe.

The number of observations are given in parentheses.

N.S., not significant.

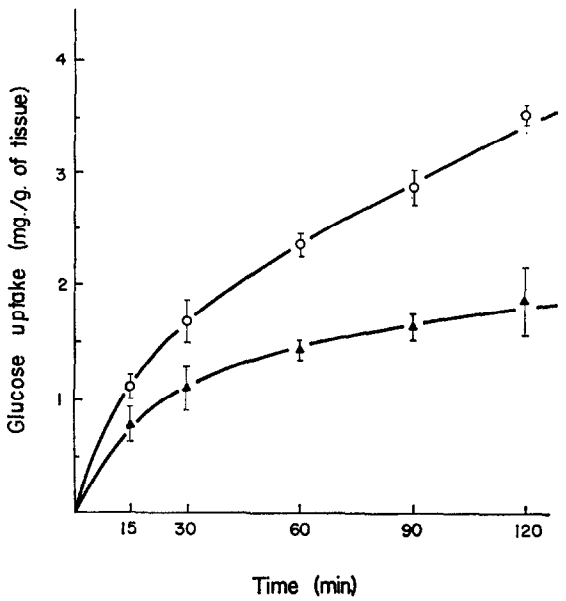

FIG. 3. Effect of dicoumarol on the uptake of D-glucose by rat cut diaphragm incubated in presence of D-xylose. Incubation medium, Krebs–Ringer II, D-sorbital (0·12 mg/ml), D-xylose (3·5 mg/ml), D-glucose (3 mg/ml). ○——○, Control; ▲——▲, dicoumarol (0·5 mM). (○ ▲) Mean of four experiments ± S. E. M.

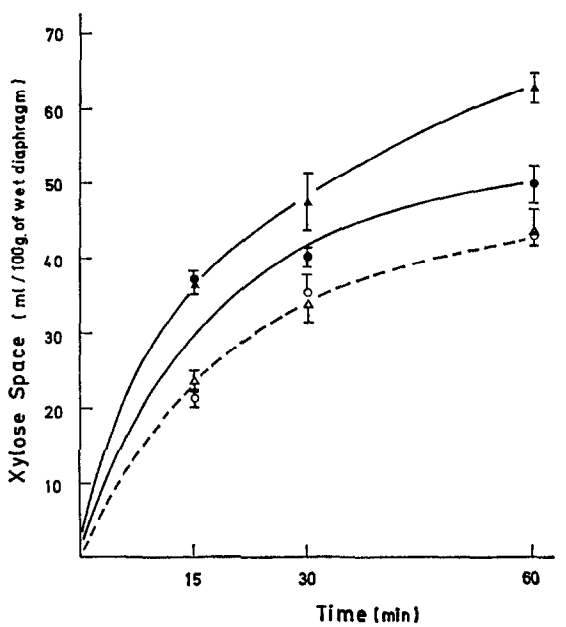

FIG. 4. Effect of dicoumarol on the accumulation of D-xylose in rat intact diaphragm incubated in presence of D-glucose. Incubation medium, Krebs–Ringer II, D-xylose (3·5 mg/ml), [^{14}C]-D-xylose (0·05 μCi/ml), D-sorbital (0·12 mg/ml), D-glucose (3 mg/ml). ○——○, Extracellular space; △——△, extracellular space in presence of dicoumarol (0·5 mM); ●——●, xylose space; ▲——▲, xylose space in presence of dicoumarol (0·5 mM]). (○ △ ● ▲) Mean of three experiments ± S. E. M.

As may be seen in Fig. 4, dicoumarol increases the accumulation of D-xylose in the presence of D-glucose, and the values obtained are not statistically lower than those obtained in the absence of the hexose. In view of the previous results we assume that there is no competition between D-glucose and D-xylose for a single system of transport.

DISCUSSION

We conclude from the experiments described here that insulin and anoxia increase the uptake of all the sugars tested, whereas the action of dicoumarol may be summarized as follows: (1) Dicoumarol inhibits the uptake of aldohexoses and ketohexoses, with a free or unfree hydroxyl group, on C-2 of the pyranose ring. (2) Dicoumarol stimulates the accumulation of pentoses and α-methyl-D-glucoside. (3) Dicoumarol has no effect on the accumulation of D-3-oxymethylglucose. (4) As an inhibitor, dicoumarol eliminates the effects of insulin and anoxia. When the drug acts as a stimulus, this effects adds itself to that of insulin and anoxia.

Randle[14] observed that the effect of phlorizin on the transport of D-xylose and D-galactose is different from that of the uncouplers of oxidative phosphorylation. Randle attributes this apparently anomalous effect of phlorizin to its inability to penetrate significantly into the muscle cell. We cannot come to the same conclusion. We have proved that dicoumarol penetrates into the cell.

On the other hand, the fact that dicoumarol inhibits the uptake of D-galactose by rat muscle and that D-galactose is not phosphorylated in this muscle, suggests that the effect of dicoumarol on the sugar uptake takes place at membrane level.

Several authors,[3,18] have reported that D-3-oxymethylglucose is transported by the same system as D-glucose (carrier-mediated transport). But Morgan, from experiments performed with avian erythrocytes, deduces that the movement of this sugar across the membrane involves two systems: (a) carrier-mediated transport and (b) a first-order process, presumably diffusion. The fact that dicoumarol does not inhibit the uptake of D-3-oxymethyl glucose makes us think that a free hydroxyl group on the C-3 of the pyranose ring may be somehow connected with the mechanism of inhibition by the drug.

Finally the only hexose in which dicoumarol acts as a stimulus is α-methyl-D-glucoside. Randle reported that this sugar penetrates into the tissue by free diffusion, which suggests to us that D-xylose, L-arabinose and α-methyl-D-glucoside are transported in muscle by a different system than hexoses or by free diffusion.

Competition experiments support the former theory. We found no competition between D-glucose and D-xylose for the same system of transport. On the other hand, dicoumarol inhibits the uptake of D-glucose and stimulates the accumulation of D-xylose in the presence of both sugars in the medium.

Our results on the uptake of D-glucose in anaerobiosis are in agreement with those of Lotspeich[13] and Morgan,[14] but contradict those of Randle,[10] who reported that the lack of response to anaerobiosis in phosphate buffer appears to be related to the marked fall in pH which occurs during incubation. Nevertheless, the pH in our experiments remained constant throughout the incubation time.

Concentrations of phlorizin (1 mM) inhibit the effect of insulin and anoxia on sugar transport,[13] but a minimum concentration of 10 mM is necessary to obtain

inhibition of the transport. On the contrary, a concentration of dicoumarol 0·5 mM inhibits both the transport and the stimulating effect of insulin and anoxia.

In aerobiosis the stimulus produced by dicoumarol for the accumulation of pentoses adds itself to that of insulin. Nevertheless, in anaerobiosis the effects of dicoumarol and insulin are the same as those of dicoumarol plus insulin. This is perhaps due to saturation of the system.

The biochemical and physiological effects of vitamins and hormones in relation to the control of membrane permeability may open up new active centres or increase the mobility of the "substrate–carrier" complex. Cuatrecasas[20] supports this theory, finding that insulin molecules can retain transport and antilipolytic activities while coupled to a large polymer of sepharose that cannot enter the cell, with concentrations of insulin–sepharose that are nearly as low as those of native insulin.

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