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INHIBITION OF THE HYPERGLYCEMIC EFFECT OF cAMP BY INTRAVENOUS INJECTION TO MICE OF CARBONIC ANHYDRASE ENTRAPPED IN LIPOSOMES

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SUMMARY

Mice treated with acetazolamide or cAMP received intravenously liposomes containing carbonic anhydrase. The hyperglycemic effect of both these substances was suppressed by carbonic anhydrase entrapped in liposomes. The later also abolished the glycogenolytic action of cAMP on liver.

INTRODUCTION

Previous work from our laboratory has already shown (1-3) that the *in vivo* administration of acetazolamide, an inhibitor of carbonic anhydrase (EC 4.2.1.1) (4, 5) produced in mice some of the main metabolic effects of cAMP or of its dibutyryl derivative, e.g. : glycogenolysis, hyperglycemia (6-8), and inhibition of lipogenesis (9-11). It was also observed that insulin was able to suppress the inhibitory effect of acetazolamide on lipogenesis and hyperglycemia (1). It is well known that insulin must be incubated in the presence of a bicarbonate buffer to be able to act on adipose tissue (12). To test whether the actions of cAMP, insulin and carbonic anhydrase were closely related we used the technique of introducing enzymes into cells by liposomes (13-15). Liver is the most efficient organ in removing liposomes from circulation (16, 17) and we therefore expected that an increased concentration of this enzyme in hepatic cells would follow injection of carbonic anhydrase entrapped in liposomes.

It was interesting to establish whether this carbonic anhydrase entrapped in liposomes and injected was biologically active and would suppress the

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hyperglycemic effect of acetazolamide injected 30 min later, and if this were so, whether liposomes containing entrapped carbonic anhydrase would abolish the hyperglycemic effect and the glycogenolytic action of cAMP on liver.

MATERIALS and METHODS

Treatment of animals.-

Exp. No 1 : Swiss female albinos mice (20-25 g) were divided into five groups : the "control group" in which mice received no injection of liposomes. Two other groups of animals were intravenously injected with liposomes with or without carbonic anhydrase of bovine erythrocytes (2000 I.U./mg) (Boehringer, Mannheim, Germany). Half of the animals in each of these last groups was intravenously injected with 10 mg of acetazolamide (Lederle, American Cyanide Company, Pearl River, N.Y.) 30 min later. The animals were killed 1 hour after the administration of liposomes.

Exp. No 2 : The animals were treated as described above except that 6 mg of 3'5' cAMP (Boehringer) were administered intraperitoneally to each mouse instead of acetazolamide. This experiment was repeated twice with similar results.

Preparation of liposomes : Liposomes were prepared according to the method of Steger and Desnick (15). 147 mg of synthetic dipalmitoyl-DL-α-phosphatidyl-choline, 22 mg of cholesterol and 20 mg phosphatidic acid (Sigma Chemical Co., Saint Louis, USA) were dissolved in chloroform/methanol 2 v/1 v and mixed (vol final : 10 ml) in round bottom flask. The solvent was evaporated to dryness in a rotatory evaporator under reduced pressure at 37°C. The remaining thin film was dispersed by adding 4 ml of 3.3 mM KH_2PO_4 buffer pH 7.2 with or without 1.2 mg/ml of carbonic anhydrase. The flask was vortexed at 37°C till the majority of lipids was removed from the flask. Dispersion of the resulting suspension was carried out using the microprobe of a Branson sonifier setting at 6 for 10 min. To prevent excessive heating, the tube was cooled during sonication in ice at intervals of 30 sec. The suspension was centrifuged at 100,000 g for 2.5 h. The liposomal pellet was suspended in 4 ml of 0.9 % NaCl containing 20 mM sodium phosphate buffer pH : 7.4. 0.3 ml of this suspension was injected to mice. The percentage of total entrapment of carbonic anhydrase was 41 % and 48 % for the first and second experiment respectively.

The concentration of protein entrapped in liposomes was measured according to the method of Bollum (18).

Determination of liver glycogen and blood glucose concentrations : Liver glycogen was measured following the method of Holt and Buhring (19) after **extraction** of the organ with boiling KOH, precipitation with ethanol and washing the precipitate with ethanol/ether 2 v/1 v.

For measurement of glycemia, immediately after decapitation of the mice, the blood was collected in a beaker containing NaF, 0.25 ml of the blood were mixed with 3 ml of distilled water. Deproteinization was performed using 0.5 ml each of previously titrated 4 % $ZnSO_4$ and 0.3 N $Ba(OH)_2$ and the suspension was then centrifuged at 500 g for 5 min (20). The deproteinized supernatant was used to measure the glucose concentration by the ortho-toluidine method (21, 22).

Table 1

Effect of carbonic anhydrase entrapped in liposomes on glycemia of mice treated with or without acetazolamide

(LIPOSOMES)	(LIPOSOMES) + CARBONIC ANHYDRASE)	(LIPOSOMES + CARBONIC ANHYDRASE) + ACETAZOLAMIDE	(LIPOSOMES) + ACETAZOLAMIDE	CONTROL
n = 6	n = 5	n = 5	n = 6	n = 5
114.2 (± 8.2)	116.4 (± 6.9)	107 (± 4.35)	187 (± 12.6)	120 (± 9.1)
n.s.	n.s.	p<0.001	0.001<p<0.005	
n.s.				
p<0.001				
n.s.				

n = number of animals.
p = probabilities according to Student "t" test.
n.s. = not significant (p>0.05).
Other conditions : cf "Materials and Methods".

RESULTS

The intravenous administration of liposomes with or without carbonic anhydrase did not modify the glycemia of mice (Table 1) . Acetazolamide injected intravenously 30 min before sacrifice of animals increased glycemia by about 64 %. This hyperglycemic effect was suppressed by injection of liposomes containing carbonic anhydrase.

Intraperitoneal injection of cAMP (Table 2) increased glycemia by about 50 % relative to controls, i.e. to animals which received only liposomes. Again, this hyperglycemic effect was found to be prevented by administration of liposomes containing carbonic anhydrase. The later also inhibited the hepatic glycogenolytic effect of cAMP.

DISCUSSION

The fact that carbonic anhydrase is able to suppress the effect of acetazolamide on glycemia strongly suggests that this sulphonamide exerts its hyperglycemic effect by its inhibitory action on carbonic anhydrase. This preliminary assay was necessary since it has been found that the action of the drug on carbonic anhydrase is not so specific as had been thought. For

Table 2

Effect of carbonic anhydrase entrapped in liposomes on glycemia and liver glycogen of mice treated with or without cAMP

GLYCEMIA (mg/100 ml)			LIVER GLYCOGEN (weight mg/g of liver)			
(LIPOSOMES)	(LIPOSOMES + CARBONIC ANHYDRASE) + cAMP	(LIPOSOMES) + cAMP	(LIPOSOMES + CARBONIC ANHYDRASE) + cAMP	(LIPOSOMES) + cAMP	(LIPOSOMES)	(CONTROL)
n = 4	n = 6	n = 5	n = 4	n = 5	n = 5	n = 5
227.5 (± 25.2)	220.7 (± 12.2)	321.0 (± 21.6)	98.4 (± 15)	16.9 (± 3.9)	92.2 (± 21)	94.6 (± 13)
n.s.	0.001<p<0.005		0.001<p<0.005	0.001<p<0.005	n.s.	

n = number of animals.

p = probabilities according to Student "t" test.

n.s. = not significant (p>0.05).

Other conditions : cf "Materials and Methods".

example, it is also capable to inhibit *in vitro* acetyl CoA carboxylase (EC 6.4.1.2) (23). In addition, this experiment allowed us to verify the efficiency of carbonic anhydrase entrapped in liposomes inside the cell.

A relation between cAMP and carbonic anhydrase in the renal cortex had been proposed by other workers (24), but this conclusion has been disputed (25). Thus, acetazolamide, an inhibitor of carbonic anhydrase, would increase the urinary excretion of cAMP in rats by activation of adenylcyclase (26) and inversely cAMP would inhibit carbonic anhydrase (27). These actions could explain why in our assay carbonic anhydrase was able to suppress the effect of cAMP on glycogenolysis and glycemia. Similar results were obtained when mice were injected with dibutyryl cAMP instead of cAMP (data not shown). The observation that the increased concentration of CO_2 in the incubation medium at constant pH caused a large increase in glycogen synthesized from glucose by rat liver slices (28) supports the theory that the action of cAMP on the concentration of hepatic glycogen is mediated by carbonic anhydrase. Since insulin can suppress the effect of acetazolamide on glycemia and fatty acid synthesis (1) an action of this hormone on carbonic anhydrase might also be envisaged.

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