Effect of Anaerobiosis and Cell Poisons on Glucose Uptake of Hemidiaphragms and Epididymal Fat Pads in Vitro

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SUMMARY

An attempt has been made to repeat the observations that anaerobiosis, 2:4-DNP, and the sodium salts of cyanide, arsenite, arsenate, and salicylate all stimulate the glucose uptake of hemidiaphragms in vitro in a way that has been postulated to be analogous to the action of insulin. The glucose uptakes of hemidiaphragms and fat pads have been measured in bicarbonate and phosphate buffers. In no case did anaerobiosis stimulate glucose uptake. Anaerobiosis reduced but did not abolish the effect of insulin.

The effects of the cell poisons were diverse and appeared to be related to both the tissue used and the medium employed. The sodium salts of cyanide, arsenite, and arsenate stimulated the glucose uptake of hemidiaphragms in bicarbonate buffer, whereas, only arsenate was effective in phosphate buffer. When fat pads were incubated in bicarbonate buffer, arsenate and cyanide increased glucose uptake; in phosphate buffer only arsenate was effective. In addition, 2:4-DNP markedly inhibited the glucose uptake of hemidiaphragms in both bicarbonate and phosphate buffers but was without effect on the fat pads. Most of the cell poisons reduced or abolished the effect of added insulin.

These results are discussed with relation to the theory that insulin acts to divert energy away from a process that restricts the entry of glucose into the cell under basal conditions. DIABETES *14*:128-31, March 1965.

In 1958 Randle and Smith[1] reported that the glucose uptake of rat hemidiaphragms in vitro was stimulated not only by insulin but by anaerobiosis and by several agents which uncouple oxidative phosphorylation. These authors suggested that the cell poisons and anaerobiosis have a common action of depriving the muscle cell membranes of energy-rich phosphate important in keeping glucose out of the cell. They concluded "that the entry of glucose into the cells of this preparation is restrained under basal conditions by a process dependent upon a supply of energy-rich phosphate," and that insulin in an analogous way "restricts the access of energy-rich phosphate to the process concerned with the regulation of glucose entry."

While partial confirmation of the observation of the effect of anaerobiosis has been obtained using the isolated, perfused rat heart,[2] to the best of our knowledge the experiments have not been repeated precisely as originally described. The data reported here are the results of attempts to repeat the observations of Randle and Smith[1] on the effect of anaerobiosis and cell poisons on the glucose uptake of hemidiaphragms and extend the observations to the effects of these factors on the glucose uptake of the epididymal fat pads.

METHODS

Incubation Media. Krebs' Ringer bicarbonate buffer and Krebs' Ringer phosphate buffer were prepared as described by Umbreit et al.[3] In some experiments Gey and Gey buffer,[4] a bicarbonate buffered medium, was used and was prepared as described by Randle and Smith.[1] The two bicarbonate media were gassed with 95 per cent O_2 - 5 per cent CO_2 or 95 per cent N_2 - 5 per cent CO_2, and the phosphate medium was gassed with 100 per cent O_2 or 100 per cent N_2. The 100 per cent N_2 and the 95 per cent N_2 - 5 per cent CO_2 were guaranteed by the supplier (National Cylinder Gas Co.) to contain not more than 100 ppm. O_2 with a typical assay giving 25 ppm. The initial *p*H was 7.4 in all cases. Glucose was added to all buffers at a level of 2.5 mg./ml. Insulin,* where indicated, was present at 0.1 U./ml.

Incubation Procedure. Hemidiaphragms and epididymal fat pads were taken from the same animal. All animals were male rats of the Holtzman strain weighing 100 to 160 gm. and were fasted overnight with free access to water. The rats were killed by decapitation. Each

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pair of hemidiaphragms was placed in a beaker containing 3 to 5 milliliter of the appropriately gassed incubation medium for five to ten minutes at room temperature. Each single hemidiaphragm was then transferred with gentle blotting to an Erlenmeyer flask containing 1 ml. of buffer. Each fat pad was dissected free and placed directly into an Erlenmeyer flask containing 1 ml. of incubation medium. Before a tissue was added each flask was gassed with the appropriate gas and stoppered. After placing a tissue in a flask, each flask was regassed and stoppered. In some experiments the stoppers were left in place during the entire incubation. In others the stoppers were removed and the tissues were gassed continuously during the incubation in a Dubnoff metabolic shaker. The method of maintaining the gas phase caused no differences in glucose uptake, and it is assumed that the anaerobiosis achieved was as complete as that reported by others.[1] Hemidiaphragms were incubated for one hour and fat pads for three hours.

A control group of tissues was included in every incubation and frequently served as a control for several experimental groups. The pairs of hemidiaphragms and epididymal fat pads were distributed among the experimental and control groups such that no two pieces from the same animal were in the same group. The incubations were designed such that each incubation could be assessed as an independent observation. As a practical matter, there was little enough variation from one incubation to another to allow the averaging of the various groups and the comparing of the experimental groups to one combined control group.

RESULTS

The effect of anaerobiosis in bicarbonate buffers:

The results of the experiments designed to show the effect of anaerobiosis on the glucose uptake of hemidiaphragms and fat pads are shown in table I. It is clear that in bicarbonate buffer made anaerobic with 95 per cent N_2-5 per cent CO_2 there was no increase of glucose uptake by either hemidiaphragms or fat pads. The action of insulin on glucose uptake was still apparent but was greatly reduced by anaerobiosis. Since Krebs' Ringer bicarbonate buffer differs slightly in its quantitative ionic content from the Gey and Gey buffer which Randle and Smith used, a series of incubations were carried out in this medium. It is apparent that anaerobiosis did not increase glucose uptake. As with the bicarbonate buffer, insulin action was diminished by anaerobiosis. There was no difference in the glucose uptake of hemidiaphragms in either of the bicarbonate buffered media. The final *p*H of these buffers ranged from 7.2 to 7.4.

The effect of anaerobiosis in phosphate buffer:

The glucose uptakes of hemidiaphragms and fat pads in phosphate buffer are also shown in table 1. There was no significant difference between the basal glucose uptakes of either tissues as compared to those in bicarbonate

TABLE 1

The effect of anaerobiosis on the glucose uptake and insulin response of hemidiaphragms and fat pads in bicarbonate and phosphate buffered media (Glucose uptake mg./gm. tissue wet wt./incubation period)

	Hemidiaphragms		Fat Pads	
Gas phase	Control	Insulin 0.1 U./ml.	Control	Insulin 0.1 U./ml.
KRB 95 per cent O_2 - 5 per cent CO_2	4.26±0.18(29)*	7.12±0.26(29)†	1.43±0.07(64)	5.21±0.12(49)†
KRB 95 per cent N_2 - 5 per cent CO_2	4.01±0.13(31)	4.95±0.18(31)†	1.17±0.08(8)	2.99±0.21(8) †
Gey and Gey 95 per cent O_2 - 5 per cent CO_2	4.30±0.15(16)	6.28±0.14(16)†		
Gey and Gey 95 per cent N_2 - 5 per cent CO_2	4.42±0.18(16)	5.18±0.16(16)†		
KRP 100 per cent O_2	4.24±0.20(33)	7.15±0.23(33)†	1.09±0.16(10)	3.67±0.12(10)†
KRP 100 per cent N_2	3.39±0.19(27)	4.94±0.28(27)†	1.51±0.24(5)	2.34±0.16(5) †

*Mean glucose uptake ± SEM. Number in parentheses is number of observations.
†Compares the effect of insulin to its own control group. $p<0.01$.

buffer nor was there any increase in the anaerobic phosphate medium. The action of insulin on the glucose uptake of the hemidiaphragm was identical in bicarbonate and phosphate; however, the action of insulin on the fat pad in phosphate buffer was less than that in bicarbonate buffer. Comparable to the effect seen in bicarbonate buffer, the insulin effect was markedly reduced by anaerobiosis in phosphate buffer. The final *p*H ranged from 6.9 to 7.2.

The effect of cell poisons in bicarbonate buffer:

In table 2 are shown the data from the experiments to determine the effects of cell poisons on glucose uptake. The concentrations used were the same as described by Randle and Smith.[1] In the first series of experiments hemidiaphragms and fat pads were incubated in bicarbonate buffer gassed with 95 per cent O_2 - 5 per cent CO_2. It may be seen that 1 mM. sodium arsenite, 1 mM. sodium arsenate, and 1 mM. sodium cyanide caused a marked stimulation of glucose uptake by the hemidiaphragm. By contrast 5 mM. sodium salicylate caused a slight decrease in glucose uptake, and 0.25 mM. 2:4-DNP (2, 4-dinitrophenol) caused a marked decrease in glucose uptake by the hemidiaphragm. In the presence of these cell poisons insulin did not cause a statistically significant increase in glucose uptake over that of its own control except with salicylate, and even here the response was less than the control group. In the case of the fat pad only sodium arsenate and sodium cyanide caused any stimulation of glucose uptake. Insulin effectively increased glucose uptake in the presence of all of the poisons except cyanide and 2:4-DNP; however, the action of insulin was greatly reduced except in the presence of salicylate.

The effect of cell poisons in phosphate buffer:

When hemidiaphragms were incubated in phosphate buffer gassed with 100 per cent O_2 in the presence of the above cell poisons, 2:4-DNP inhibited, sodium arsenite and sodium cyanide were without effect, and only sodium arsenate stimulated glucose uptake. In this buffer the action of insulin was inhibited by all the poisons except sodium cyanide. In the fat pads all the cell poisons except sodium cyanide stimulated glucose uptake to at least a slight degree. Insulin was without effect except in the presence of arsenate.

DISCUSSION

The observations of Randle and Smith[1] that anaerobiosis, cell poisons and insulin all act in an analogous way in stimulating the glucose uptake of the hemidiaphragm are important because of the interpretation of these data in terms of the mechanism of insulin action. Randle and

TABLE 2

The effect of cell poisons on the glucose uptake and insulin response of hemidiaphragms and fat pads in bicarbonate and phosphate buffers
(Glucose uptake mg./gm. tissue wet wt./incubation period)

	KRB (95 per cent O_2 - 5 per cent CO_2)		KRP (100 per cent O_2)	
	Control	Insulin 0.1 U./ml.	Control	Insulin 0.1 U./ml.
Hemidiaphragms				
Control	3.98±0.13(69)*	5.86±0.10(49)<0.01†	4.07±0.23(20)	5.17±0.19(20)<0.01†
0.25 mM. DNP	1.56±0.12(10) <0.01‡	1.44±0.08(10)	1.73±0.33(10) <0.01	1.24±0.21(10)
1 mM. arsenite	5.34±0.22(15) <0.01	5.33±0.21(15)	4.40±0.33(15)	4.97±0.23(15)
1 mM. arsenate	6.09±0.29(35) <0.01	6.63±0.39(15)	6.01±0.26(20) <0.01	6.48±0.31(20)
1 mM. NaCN	6.69±0.42(23) <0.01	7.70±0.55(23)	3.44±0.33(10)	5.66±0.48(10)<0.01
5 mM. salicylate	3.38±0.14(20) <0.05	4.38±0.23(4)<0.01		
Fat pads				
Control	1.43±0.07(64)	5.21±0.12(49)<0.01†	0.96±0.07(25)	3.83±0.33(25)<0.01
0.25 mM. DNP	1.21±0.03(20)	1.43±0.08(10)<0.01	1.32±0.21(10)	1.29±0.18(10)
1 mM. arsenite	1.24±0.05(30)	1.97±0.06(15)<0.01	1.26±0.29(15)	1.43±0.19(15)
1 mM. arsenate	3.98±0.28(30) <0.01	5.07±0.32(15)<0.01	3.29±0.35(20) <0.01	5.55±0.48(20)<0.01
1 mM. NaCN	2.03±0.22(38) <0.01	2.78±0.46(18)	0.86±0.16(10)	1.32±0.15(10)
5 mM. salicylate	1.40±0.10(15)	5.39±0.39(5)<0.01		

*Mean glucose uptake ± SEM. Number in parentheses is number of observations.
†Compares the effect of insulin to its control group. $p<0.01$.
‡Compares the effect of cell poison to the control group.

Smith have concluded that the entry of glucose into muscle cells in basal conditions is restrained by a process dependent upon a supply of energy-rich phosphate and that insulin in common with anaerobiosis and cell poisons acts to deprive the cell membrane of the energy-rich phosphate necessary to prevent the entry of glucose. This theory has been termed the "keeper-out-ase" theory by Tepperman[7] and has already appeared in two text books.[8,9]

It would seem reasonable to expect that if this hypothesis is acceptable at least one criterion should be fulfilled. Anaerobiosis and cell poisons should be, at least qualitatively, effective in promoting glucose uptake in the same circumstances in which insulin is effective. In addition, unless the response of the hemidiaphragm to insulin is fundamentally different from that of the fat pad, it could be expected that both tissues would respond equally well to the cell poisons, anaerobiosis and insulin if the underlying mechanism of these agents is to deprive or divert energy-rich phosphate away from the process which restricts glucose entry.

Randle and Smith found that anaerobiosis and the cell poisons tested were effective in stimulating the glucose uptake of hemidiaphragms in bicarbonate but not in phosphate buffer. It has been demonstrated repeatedly in many laboratories that insulin stimulates glucose uptake in phosphate buffer. In the experiments reported here insulin was equally effective in promoting the glucose uptake of hemidiaphragms in both bicarbonate and phosphate buffers. Insulin was not as effective in stimulating the glucose uptake of the fat pad in phosphate buffer but still caused a significant increase.

There are major conflicts between our data and those of Randle and Smith.[1] Neither in bicarbonate nor in phosphate buffered media did anaerobiosis stimulate the glucose uptake of either hemidiaphragms or fat pads. Others also[10,11] have failed to note any stimulation of the glucose uptake of hemidiaphragms in anaerobic phosphate buffer. The cell poisons used have in common the property of preventing the formation of high energy phosphate bonds. In contrast to Randle and Smith we have observed no consistent effect of these poisons on the glucose uptake of the hemidiaphragms in bicarbonate buffer. Indeed, the effect of the poisons appears to be altered by changing from the bicarbonate to phosphate buffer. It is concluded from these data that anaerobiosis, cell poisons and insulin do not necessarily have any common effect on the glucose uptake of hemidiaphragms or fat pads.

The variability in the effect of the cell poisons on the glucose uptake in bicarbonate medium compared to phosphate medium or in hemidiaphragms compared to fat pads cannot be discussed on the basis of these data. The variability may reflect the influence of the buffer media per se on the utilization of glucose, but this question cannot be answered until comparative studies on the fate of the substrate have been done.

In the data reported here anaerobiosis markedly reduced but did not abolish the action of insulin in either fat pads or hemidiaphragms. This was seen consistently regardless of whether bicarbonate or phosphate buffer was used. This may indicate a biphasic action of insulin, one oxygen-dependent and the other independent of the presence of oxygen.

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