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STUDIES OF THE HYPOGLYCEMIC EFFECT OF D-AMPHETAMINE IN AGGREGATED MICE*

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Abstract—The hypoglycemia that occurs in aggregated mice after the injection of D-amphetamine sulfate (10 mg/kg) has been investigated. Insulin deficiency as a result of injection with serum containing antibodies to insulin, and which produced hyperglycemia in control mice, was not sufficient to prevent the development of hypoglycemia in aggregated mice given amphetamine. Liver glycogen levels were lower, but liver lipid content was unaltered in the hypoglycemic mice. Pancreas insulin levels were not significantly different from control levels in the aggregated mice which became hypoglycemic after amphetamine injection, but the incorporation of *l*-alanine-^{14}C into glucose by liver slices removed from these animals was increased.

The amount of $^{14}CO_2$ expired from mice injected intravenously with a tracer amount of glucose-U-^{14}C was decreased if the mice were made insulin deficient with anti-insulin serum, but the oxidation of labeled glucose increased if the mice were also injected with amphetamine and isolated. Aggregated mice, injected with anti-insulin serum and amphetamine, oxidized three times the amount of labeled glucose as the control mice given anti-insulin serum alone.

The hypoglycemia that develops in aggregated mice injected with amphetamine is not due to excessive insulin secretion or decreased gluconeogenesis, but is probably the result of the markedly increased glucose utilization during severe motor activity.

THE OBSERVATION, by Gunn and Gurd,[1] that the excitatory effect of sympathomimetic amines on mice was markedly potentiated if the animals were aggregated rather than isolated, led to a series of investigations of the effects of various environmental and pharmacological factors on the toxicity of such drugs. Amphetamine has been the compound most frequently studied in this respect. As a result of his early investigation, Chance[2] showed that the LD_{50} of amphetamine in mice was reduced to one tenth if the mice were kept ten in a cage rather than one per cage, and came to the conclusion that aggregation of treated mice was the most potent factor in increasing the toxicity of amphetamine. Other factors that influence the toxicity of the drug in aggregated or isolated mice include exercise,[3–5] temperature,[2–4, 6–10] noise,[7] body weight,[7] endocrine status,[5, 11, 12] and treatment with certain "tranquilizing" drugs.[4, 5, 12–15] These various studies led to the observation by Moore *et al.*[15] that the death of aggregated amphetamine-treated mice was associated with hypoglycemia and depletion of liver, muscle, and brain glycogen.

The remarkable fact that aggregated amphetamine-treated mice would exercise until they died at least with, if not necessarily as a result of, a low or absent blood sugar, stimulated the present study of the mechanism of this hypoglycemia.

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METHODS

The animals used in the experiments were male albino mice (Cox), weighing 25–30 g, and maintained on laboratory chow and water *ad libitum* until the time of each experiment. *d*-Amphetamine sulfate (10 mg/kg) was administered by i.p. injection in 0·2 ml saline, and the same volume of saline was injected into control mice. To obtain an aggregated effect, 5 to 8 mice were placed in a wire-mesh cage with a floor area of 350 sq cm and a height of 18 cm. Isolated mice were placed in cages of various sizes, but usually about the same capacity as that used for aggregation. Expired $^{14}CO_2$ from injected uniformly labeled glucose (0·5 μc/mouse i.v.) was trapped in CO_2-free sodium hydroxide solution in a system in which CO_2-free air was passed through glass chambers (floor area 320 cm^2, height 12·5 cm) containing six aggregated mice, or a series of six 500-ml Erlenmeyer flasks, each containing one mouse. An aliquot of the sodium hydroxide solution was acidified, the released $^{14}CO_2$ diffused into phenethylamine solution, and the radioactivity counted in a liquid scintillation counter, according to the method of Woeller.[16]

Glucose production and gluconeogenesis by the livers *in vitro* was determined by incubating liver slices for 90 min in Ringer–bicarbonate buffer (pH 7·4) containing *l*-alanine-U-^{14}C (1 mg/ml; sp. act., 0·5 μc/mg). Incorporation of the labeled carbon from *l*-alanine into glucose was measured by preparing the glucosazone on an aliquot of the medium at the end of incubation[17] and counting the radioactivity on a proportional gas-flow counter.

Serum containing antibodies to insulin (AIS) was prepared in guinea pigs by the method of Robinson and Wright[18] and injected intraperitoneally. Normal guinea pig serum served as control. Mouse pancreas insulin was extracted into an acid–alcohol solution and the concentration determined by adding an excess amount of AIS and assaying the un-neutralized AIS by a modification of the method of Wright and Rivera-Calimlim.[19]

Blood glucose and glucose production by liver slices were measured by the glucose oxidase method,[20] and blood lactate was determined enzymatically with lactic dehydrogenase.[21] Liver glycogen was measured by the procedure of Good *et al.*,[22] except that glucose oxidase was used to estimate the glucose. The long-chain fatty acid content of the livers was measured by the method described by Renold *et al.*[23]

In most experiments, aggregated amphetamine-treated mice were removed for bleeding and excision of other tissues when they reached a moribund state. With experience it was possible to recognize imminent death of an animal by observation of its behavior, particularly the onset of marked depression or convulsions. For the $^{14}CO_2$ production experiments, the collection was terminated and the animals were bled, after a suitable interval (45 min), to avoid death of a mouse within the apparatus, therefore preventing the occurrence of an unequal number of test and control animals.

RESULTS

The behavior of the amphetamine-treated aggregated mice was similar to previous descriptions. As the drug took effect, the motor activity of the animals increased, and pairs often adopted aggressive crouching attitudes toward each other, which were easily disturbed by contact with another mouse, squealing, or extraneous noise. The motor activity tended to be spasmodic in the early stages, taking the form of bursts of

running or jumping with very short rests between. As all the animals became affected, the degree of activity increased so that each animal was in continuous motion, with episodes of acceleration and deceleration. The mice were highly irritable, and contact with a companion usually produced increased activity. The mice did not fight with each other, but occasionally the foot pads were damaged by friction against the wire cages. As the mice reached a stage of depression they remained still on the bottom of the cage, moving only intermittently when stimulated by contact with more active colleagues. This stage was followed by complete immobility, apart from occasional convulsive seizures, and death. Isolated amphetamine-treated animals usually showed greater than normal activity for a few minutes and then settled, but on rare occasions died suddenly after a convulsion. Animals which died in this manner usually had a blood sugar slightly elevated above normal. Control aggregated mice rested quietly, usually in one group.

The mean blood glucose levels and times of survival in a typical experiment are shown in Table 1, in which an isolated amphetamine-treated and an aggregated con-

TABLE 1. BLOOD GLUCOSE LEVELS IN MICE INJECTED WITH AMPHETAMINE

Treatment	Survival time (min)	Blood glucose (mg/100 ml)
Control, aggregated	> 120	122 ± 4
Amphetamine, isolated	> 120	104 ± 4
Amphetamine, aggregated	58 ± 5	26 ± 6
AIS,* Control	> 120	213 ± 8
AIS, Amphetamine, aggregated	52 ± 7	28 ± 6
Normal serum, amphetamine, aggregated	85 ± 10	20 ± 2

* Sufficient AIS to neutralize 0·8 unit of insulin.
Values are means ± S.E.M. of eight observations.

trol animal were sacrificed at the same time that an aggregated amphetamine-treated mouse became moribund. The latter were markedly hypoglycemic. If individual mice were removed from the aggregated amphetamine-treated group for bleeding, a decrease in the activity of the remaining mice was obvious, and if the number of mice was reduced to two or three per cage, the remaining mice occasionally returned to normal behavior which was often accompanied by a recovery in the blood glucose level toward normal.

Since the production of hypoglycemia in aggregated amphetamine-treated mice may have been the result of excessive insulin secretion by these mice, amphetamine was injected simultaneously with an amount of AIS greatly in excess of that required to neutralize the pancreatic insulin in each mouse. The blood glucose values at the time of sacrifice are shown in Table 1. Despite the administration of AIS, which caused the blood glucose in control-treated animals to reach levels over 200 mg/100 ml, the combination of amphetamine and aggregation was sufficient to produce hypoglycemia in these animals. We have noted that even when the AIS was given 30 min before the amphetamine, in order to ensure that the animal was insulin

TABLE 2. BLOOD GLUCOSE AND LACTATE LEVELS, PANCREAS INSULIN CONCENTRATION, LIVER GLYCOGEN AND LIPID CONTENT, AND GLUCOSE PRODUCTION FROM *l*-ALANINE-^{14}C BY LIVER SLICES FROM AMPHETAMINE-TREATED MICE

	Blood		Pancreas insulin (mU/100 mg)	Liver		Liver slices		
							^{14}C-L-Alanine to glucose	
	Glucose (mg/100 ml)	Lactate (mg/100 ml)		Glycogen (μmoles glucose/g)	Long-chain fatty acids (mg/g)	Glucose production (μmoles/g/90 min)	(counts/min/μmole)	(% incorp.)
Control, aggregated	128 ± 7	26·9 ± 2·7	46·5 ± 1·2	107·0 ± 2·0	27·1 ± 1·9	15·8 ± 0·3	560 ± 120	3·8
Amphetamine, isolated	133 ± 23	41·8 ± 4·7	45·8 ± 0·3	87·4 ± 10·6	32·1 ± 2·3	15·8 ± 0·6	490 ± 70	3·3
Amphetamine, aggregated	28 ± 9	28·5 ± 2·6	46·5 ± 0·5	13·5 ± 7·6	30·7 ± 0·9	10·5 ± 1·4	1100 ± 160	4·9

Values are means ± S.E.M. of seven observations.

deficient, and the blood sugar was rising at the start of the increased activity, a hypoglycemic effect was still obtained, though the mice killed in the late part of the experiment showed a recovery with respect to blood glucose levels.

To further exclude the possibility of excessive insulin secretion as a cause of the profound hypoglycemia in these mice, the insulin levels in the pancreases removed at the time of bleeding was not significantly different from the concentrations in the isolated amphetamine-treated and aggregated control injected animals.

The liver glycogen content of the hypoglycemic mice was considerably lower than that of the isolated amphetamine and control animals, and decreased from 33·8 μmoles/g in the first mouse to die, to 0·19 μmole/g in the last (Table 2). The liver lipids, as measured by free and saponifiable fatty acids, were not significantly altered from the control levels in either the aggregated or isolated amphetamine-treated mice.

A failure of gluconeogenesis could conceivably have accounted for progressive hypoglycemia in the glycogen-depleted mice, and this was tested by incubating liver slices removed from the animals when they were in the depressed, hypoglycemic, moribund state. Net glucose production and incorporation of ^{14}C from labeled *l*-alanine into glucose were measured. The results are shown in Table 2. Glucose production by liver slices from the hypoglycemic animals was less than that observed in isolated amphetamine-treated and control mice and probably reflects reduced liver glycogen levels. However, the production of labeled glucose from ^{14}C-labeled *l*-alanine was greatest in the slices from the hypoglycemic, aggregated, amphetamine-treated mice, indicating an increase in gluconeogenesis.

The blood lactate levels of these mice are also shown in Table 2. The hypoglycemic animals had lactate levels which were not different from those of the controls, but the isolated amphetamine-treated mice had increased concentrations of blood lactate.

TABLE 3. EXPIRED $^{14}CO_2$ AFTER INTRAVENOUS GLUCOSE-U-^{14}C

Treatment	Blood glucose	$^{14}CO_2$ Production (counts/min $\times$ 10^{-3}/6 mice/45 min)	(% injected ^{14}C)
Normal serum, aggregated	132 ± 12	411 ± 16	13·7
AIS,* aggregated	247 ± 26	342 ± 2	11·4
AIS, amphetamine, isolated	250 ± 35	520 ± 14	17·3
AIS, amphetamine, aggregated	24 ± 5	976 ± 19	32·5

* Sufficient AIS to neutralize 0·8 units of insulin.
Values are means ± S.E.M. of six observations.

The amounts of expired $^{14}CO_2$ from labeled glucose injected into mice treated with AIS or normal serum are shown in Table 3. Glucose oxidation by the insulin-deficient animals was less than that of the controls, but injection of amphetamine into AIS-treated mice in isolation increased $^{14}CO_2$ as in the AIS-treated controls during the same period of collection.

DISCUSSION

The mechanism of the central stimulant action of amphetamine, first described by Alles in 1933,[24] is not completely understood, but available evidence points toward a direct effect on the reticular formation. How this effect is converted into the remarkably increased motor activity and toxicity when several amphetamine-treated mice are placed in the same cage is unknown, but many factors have been shown to influence this response. Lasagna and McCann[13] have indicated that aggregated amphetamine-treated mice were protected by certain sedative drugs, and Burn and Hobbs[14] have devised a test for tranquilizing drugs, using mice treated in this manner. The hormonal state of the mice also has a considerable influence on amphetamine toxicity in aggregated animals. Corticotropin was shown to increase the toxicity in isolated and aggregated mice, whereas adrenalectomy decreased toxicity. Moore[25, 26] has proposed that the release of endogenous stores of norepinephrine plays a role in the toxicity of amphetamine in aggregated mice and has recently shown that amphetamine reduces the norepinephrine stores in hyperthyroid mice.[12] Depletion of norepinephrine stores with reserpine or α-methyl-*m*-tyrosine also reduces the toxicity of amphetamine in aggregated mice.[26]

The depletion of glycogen stores and fall in the blood glucose to levels that may be fatal has been observed in aggregated mice given amphetamine, and it has been shown that many of the drugs that protect these mice also prevent the development of hypoglycemia.[15] The combined effects of amphetamine administration and aggregation may be regarded as a form of stress, but from the observation of Hardinge and Peterson[3] it appears that muscular activity in these animals is an essential factor in the production of hypoglycemia.

We have shown in our experiments that death of the aggregated amphetamine-treated animals was usually, but not invariably, associated with a low blood glucose concentration. The fall in blood sugar cannot be attributed to an increased release of insulin from the pancreas or a failure in hepatic gluconeogenesis. The present experiments indicate that the fall in blood glucose is associated with an increase in glucose oxidation and that this increased burning of carbohydrate occurs in the absence of insulin. The lack of accumulation of lactate in the blood probably indicates that the glucose is completely metabolized to CO_2 and water, whereas the isolated amphetamine-treated mice, whose degree of activity was less, tended to allow lactate to accumulate. When the expired labeled CO_2 is collected from mice injected with glucose-U-^{14}C and either AIS or normal serum, it is apparent that the insulin-deficient animals oxidize the glucose at a rate some 20 per cent below that of the mice with normal blood sugars. This agrees with previous findings on effects of AIS on the glucose oxidation in rats.[27] However, AIS-treated aggregated mice given amphetamine were able to oxidize glucose at a rate three times that of the controls. The most likely explanation for the hypoglycemia observed is that it is due to increased muscular activity. Increased oxidation of labeled glucose has been observed in man during periods of muscular work.[28] Studies in experimental animals also suggest that glucose utilization is increased by exercise[29] and that insulin is not required for this process.[30]

Whatever the mechanism of the hypoglycemic effect of muscular exercise may be, it is apparent that the greatly increased motor activity of aggregated amphetamine-treated mice is responsible for glycogen depletion, increased glucose oxidation, and

hypoglycemia which is not the result of excessive insulin secretion or depressed gluconeogenesis, and can occur in insulin-deficient animals.

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