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EXPERIMENTAL HYPOGLYCEMIA IN THE RAT

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EXPERIMENTAL HYPOGLYCEMIA IN THE RAT

CHAPTER I

INTRODUCTION

The Hyperinsulinism Theory

From 10 to 30 per cent of the population may suffer from spontaneous, reactive hypoglycemia (Fredericks and Goodman, 1969; Roberts, 1965); yet, this ubiquitous disease remains a mystery. The discoverer of spontaneous hypoglycemia (Harris, 1924) named the disease hyperinsulinism. Although it is now apparent that spontaneous hypoglycemia is a disease with many causes unrelated to insulin secretion (Conn and Seltzer, 1955), hyperinsulinism may cause most cases of reactive hypoglycemia (Portis, 1950; Abrahamson and Pezet, 1954; Roberts, 1965; Fredericks and Goodman, 1969).

Reactive hypoglycemia is hypoglycemia following a meal or a glucose challenge; hence, it is diagnosed by the glucose tolerance test. The typical patient has a history of excessive carbohydrate ingestion, and dietary restriction of carbohydrate may alleviate symptoms. These facts provide a rationale for the etiology of hyperinsulinism: in the person consuming too much carbohydrate, repeated challenges to the pancreas cause hyperplasia of the beta cells and reactive hyperinsulinism. The beta cells respond to glucose ingestion by secreting too much insulin, causing hypoglycemia.

Need for an Experimental Model for Studying Hypoglycemia

The hyperinsulinism theory has a long history of acceptance and offers an attractive explanation for reactive hypoglycemia, but it lacks empirical support. Few studies have revealed abnormally high insulin levels in patients with reactive hypoglycemia. There is no experimental proof that glucose ingestion can cause chronic, reactive hypoglycemia. It is difficult to prove a cause-and-effect relationship between dietary habits and disease: adequate controls are impossible.

An experimental animal with chronic, reactive hypoglycemia is needed. A crucial question in the genesis of such an animal is this: can glucose feeding or exposure of an animal to hyperglycemia by any means (physiological, pharmacological, or pathological) produce chronic hypoglycemia subsequent to withdrawal of the hyperglycemic stimulus? If so, can hyperinsulinism be demonstrated, or is some other control system responsible for the abnormality? These problems can best be solved by choosing a hyperglycemic stimulus most likely to produce hypoglycemia.

Effects of Glucose on Islets

In several mammals, injected or oral glucose in sufficient quantity causes at least transient hyperplasia of islets, but also causes hydropic degeneration of the islets and, if anything, hypofunction of the beta cells (Brown et al, 1952; Wissler et al, 1949; Woerner, 1939). Glucose may cause persistent diabetes in the cat (Dohan and Lukens, 1948) and transient diabetes in the rat (Peterson, 1949). It appears, then, that feeding of glucose is more likely to produce diabetes than hypoglycemia.

The production of experimental hypoglycemia by glucose feeding cannot be ruled out. Frequent occurrence of hypoglycemia in prediabetes in man

suggests that a stage of hyperfunction of beta cells may precede the stage of exhaustion. Paradoxical effects from glucose feeding are likely. Few investigators look for hypoglycemia, and isolated cases might be ignored or merged with normal values. Infusion of the dog's pancreas with glucose causes severe hypoglycemia during the infusion (Brown et al, 1952).

Although production of chronic, experimental hypoglycemia by glucose feeding might be possible, the technique does not look very promising. Ideally, the pancreas should be subjected to abnormally high glucose concentrations to stimulate beta cells, but hyperglycemia is difficult to maintain even by force feeding of glucose (Wissler, 1949). The physiological significance of hyperglycemia obtained by intravenous glucose is uncertain. A technique for easily maintaining and controlling hyperglycemia is needed.

Hypoglycemia in Infants of Diabetic Mothers (IDM)

The fetus in a diabetic mother is constantly exposed to high glucose levels; yet, the pancreas of the fetus is not inherently abnormal. Hypertrophy of islets in infants of human diabetic mothers (IDM) was reported years ago (Dubreuil and Anderodiac, 1920) and has since been confirmed many times (Gray and Feemster, 1926; Bowen and Heilbrun, 1932; Cardell, 1953).

IDM typically exhibit hypoglycemia during the first day or two after birth (Higgons, 1935; Pedersen et al, 1954). Glucose tolerance is abnormal. After glucose there is a rapid rise in plasma insulin associated with a rapid fall in plasma glucose. At the beginning of the glucose tolerance test (GTT), insulin levels are higher in IDM than in normals; later in the GTT, the situation is reversed (Isles et al, 1968; Pedersen et al,

1966). The same relationship holds for infants of gestational diabetic mothers (Pildes et al, 1969). Although most studies employ the radioimmunoassay to detect insulin, hyperinsulinism in IDM has been confirmed by a biological assay for insulin employing rat adipose tissue (Baird and Farquhar, 1962).

The cause of hypoglycemia in IDM might be as follows: (1) The hyperglycemic blood supply to the fetal pancreas causes hyperplasia or hyperfunction of the beta cells. (2) When removed from the hyperglycemic blood supply (at birth), the infant still produces too much insulin, causing hypoglycemia. Also, the beta cells are hypersensitive to a glucose challenge. IDM, then, provide a variation for the hyperinsulinism theory.

Because hypoglycemia in IDM usually abates spontaneously during the first few days of life (Farquhar, 1956), the problem has received scant attention. But carbohydrate metabolism in the neonate is anomalous: confidence that hypoglycemia will not recur is unwarranted. Also, long-term studies to determine possible effects of neonatal hypoglycemia on the mentality of the child are needed.

Offspring of Alloxan-Diabetic Rats (OADR)

The search for a means of producing chronic hypoglycemia in an experimental animal converges on offspring from diabetic mothers for a number of reasons: (1) There is evidence of hypoglycemia and hyperinsulinism in IDM, hence some reason to expect positive results. (2) The hyperglycemic environment can be profound or mild, depending upon the severity of the diabetes. (3) Constant hyperglycemia can easily be maintained. (4) Exposure of the animal (in this case, the fetus) occurs during a stage of development when changes in organ function are most likely to occur.

(5) Because treatment may range from moderate to severe, either positive or negative results are significant. Such a study also promises a wealth of information which might be of importance to IDM. Follow-up studies can be done in a short period of time. The rat, for example, grows from infancy to adulthood in 3 months.

Rats are easy to maintain in large numbers and are easy to handle. Diabetes can easily be produced in the rat by injection of alloxan (Lazarow and Palay, 1946). Offspring of alloxan-diabetic rats (OADR), then, are ideal animals for studying the effects of maternal diabetes on the offspring.

There is reason to suspect that OADR might exhibit neonatal hypoglycemia similar to that observed in IDM. Islet hyperplasia occurs in OADR (Kim et al, 1958; Hellman, 1960), but may be associated with hydropic degeneration (Kim et al, 1960; Carpenter and Lazarow, 1967). Blood sugars of OADR drop rapidly after birth and reach hypoglycemic levels (compared to normals) at 24 hr postpartum (Kim et al, 1960). Serious problems in obtaining viable OADR may be anticipated (Miller, 1947; Lawrence and Contopoulos, 1960), but the grave need for long-term studies of OADR justifies the effort.

CHAPTER II

MATERIALS AND METHODS

Care and Mating of Rats

Adult Rattus norvegicus females of the King-Holtzman strain were fed laboratory chow ad libitum and maintained at 28°C. Water was allowed at all times (even during fasting) except during tests.

The most convenient and dependable method of obtaining and diagnosing pregnancy was as follows: (1) Females were kept in the dark for 36 hr and then were placed back in the regular light cycle at 6:00 AM (day 1). (2) Females were mated on day 3, and vaginal smears were performed on the morning of day 4. (3) Animals in proestrus or early estrus were mated again on day 4, and vaginal smears were performed on the morning of day 5. (4) Males were removed, and non-pregnant females were given at least a 7-day rest before repeating the procedure. The partial synchronization of estrus cycles by changing the light cycle enables one to obtain and diagnose pregnancy with a minimal investment of time. Spermatozoa might be found either on day 4 or on day 5.

Inspection for the vaginal plug is not a dependable procedure for diagnosing pregnancy. Vaginal smears were taken in 0.9% saline by an eyedropper and were inspected immediately with no stain. The day that spermatozoa were observed was taken as day 1 of pregnancy. Pregnancy was confirmed by failure of the rat to enter estrus (as determined by vaginal

smears) during the next 8 days.

Production, Diagnosis, and Treatment of Diabetes

After a 24 hr fast, rats were etherized, and alloxan monohydrate was injected intramuscularly at 100 mg/kg body wt. Food was allowed immediately after alloxan. In treated animals, protamine zinc insulin (1 unit per rat per day) was injected intramuscularly at 4:00 PM beginning 24 hr after alloxan. The insulin dosage was suboptimal. It improved health without abolishing hyperglycemia.

Blood samples were taken at 4:00 PM (before insulin), and plasma glucose was assayed by the ortho-toluidine method. Any rat consistently exhibiting plasma glucose levels above 200 mg/100 ml was considered diabetic. Test-tape measurement of urine glucose proved valuable for on-the-spot diagnosis.

Treatment of diabetic coma was carried out as described in Table 9 (Appendix D). The following techniques were employed in attempts to ameliorate sterility in diabetic rats: (1) intramuscular injection of ascorbic acid at 100 mg per rat per day (Chatterjee, 1964), (2) intramuscular injection of corticosterone at 50 to 500 ug per rat per day (Chatterjee, 1966), and (3) intramuscular injection of FSH at 2 mg per kg body wt per day. Impaired lactation and postpartum calcium depletion (frequently characterized by tail chasing) were treated by intramuscular injection of 3% calcium lactate (0.2 to 0.8 ml as needed).

Treatment and Classification of Rats

A number of control and experimental groups were developed. These groups are listed in Table 10 (Appendix D) for easy reference.

Biological Tests and Measurements

The Glucose Tolerance Test (GTT)

Blood samples were taken from the tail in heparinized capillary tubes. A 24 hr fasting sample was taken, and oral 10% glucose (750 mg/kg body wt) was given by stomach tube at 1:00 PM. Serial samples were taken to 6 hr after glucose. Blood samples were centrifuged immediately after collection, and plasma glucose levels were measured by the glucose-oxidase method.

The Modified Glucose Tolerance Test

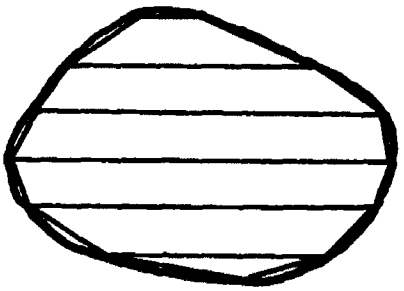
Oral glucose (750 mg/kg body wt) was administered after a 24 hr fast. At 3 hr post glucose, a single blood sample was taken from the tail. In some studies, serial samples were taken at 3 hr post glucose from the tail, from the heart, and again from the tail at approximately 2 min intervals. Blood samples for hematocrit determinations were centrifuged in capillary tubes at 5,500 x g for 10 min.

Histological Techniques

Rats were sacrificed by cervical dislocation. The pancreas was fixed for 10 hr in Helly fluid, washed for 8 hr in tap water, and stored in 70% ethanol (changed periodically). The tissue was dehydrated in tetrahydrofuran, embedded in parafin, and sectioned at 5 μ. Sections were mounted with Haupts solution and stained with a hematoxylin trichrome stain (Lazarus and Volk, 1962). The alpha cells were stained with biebrich scarlet and the beta cells with aldehyde fuchsin.

For islet area measurements, sections of a single islet were traced serially to the largest section. Although choice of sections for area

integrations involved subjective judgements, bias was eliminated by allowing the investigator only a code number to identify tissues. Two independent studies of islet areas were performed. In one study islet areas were determined by planimetry of photomicrographs. In the other study islet areas were determined by component quantitation (Lazarow and Carpenter, 1962). Parallel, linear measurements across islets were made with a timed scanning device on the microscope. The parallel lines were separated by 25 μ. The islet was divided into regular geometric figures.



The area of the islet is the sum of the areas of the individual geometric figures. If the distance between parallel lines is small, the accuracy of this technique is very good. Error is exaggerated in the diagram.

Glucose-Oxidase Assay for Glucose

Glucose-oxidase enzyme was used for plasma glucose determinations requiring maximum precision and accuracy in the normal to hypoglycemic range (e.g. in GTT's and fasting samples). A microanalytical system (Beckman Instruments, Inc.) was employed for all measurements. A 10 μl sample of plasma or glucose standard was placed in test tube A. After

addition of 150 µl of 0.08 N NaOH and 40 µl of 5.0% $ZnSO_4$, the test tube was shaken and allowed to stand for at least 20 min. The test tube was centrifuged for 2 min, and 100 µl of the supernatant fluid was transferred to test tube B. Just before the final incubation, Glucostat vials (Worthington Biochemical Corporation) were dissolved in 75 ml distilled water. This solution contained glucose-oxidase enzyme, peroxidase, and ortho-dianisidine. A 250 µl aliquot of enzyme solution was added to test tube B. The test tube was shaken, and the reaction was incubated for 1 hr at 22°C. The reaction was stopped by addition of 20 µl of 5.0 N HCl. The optical density was read against a blank (100 µl of distilled water, 20 µl HCl, and 250 µl enzyme solution) at 410 mµ. Unknowns, standards, and blanks were processed simultaneously, and the concentration of glucose in the unknowns was determined from the standard curve (Fig. 15 in Appendix A).

The Ortho-Toluidine Assay for Glucose

The ortho-toluidine assay was employed for measurement of plasma glucose levels in normal and diabetic pregnant rats and in all other diabetic rats. The ortho-toluidine reagent was 3.0% ortho-toluidine and 0.35% thiourea in glacial acetic acid. After addition of 350 µl of ortho-toluidine reagent to a 10 µl portion of glucose standard, unknown, or distilled water (blank), the color reaction was accomplished by incubation at 100°C.(boiling water) for 7½ min. The reaction was stopped by plunging the test tube into cold water, and the optical density was read at 630 mµ. Unknowns were read from the standard curve (Fig. 17 in Appendix A).

The Radioimmunoassay of Insulin

After a 24 hr fast, rats received oral 10% glucose (750 mg/kg body wt). Sacrifice was by cervical dislocation at 45 min post glucose, and blood was drawn from the posterior vena cava. Blood was centrifuged immediately. One part of plasma was diluted with 3 parts phosphate buffer (0.04 M, pH 7.4) containing 0.9% NaCl. Samples were stored at -20°C.

A modification of the double antibody technique of Hales and Randle (1963) was employed for the radioimmunoassay of insulin. Materials were obtained in a kit (Schwarz BioResearch), and the assay was performed according to kit instructions. The guinea-pig anti-insulin serum and the rabbit anti-(guinea-pig gamma globulin) serum were incubated by the manufacturer, and the lyophilized anti-insulin complex was supplied in the kit. Unlabelled insulin and I-125 insulin were allowed to compete for binding sites on the anti-insulin complex. Bound insulin was separated from unbound insulin by filtration. Filters were wrapped in foil and counted by scintillation (NaI crystal). Unknowns were determined from the standard curve (Fig. 18, Appendix B). Human insulin standards were used; hence, the rat insulin measurements are expressed in human insulin equivalents.

Statistical Methods

The standard error of the mean (SE) is used in all illustrations showing a measure of variance. All P values are from two-tailed t-tests; the t-table utilized has the following P values: 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, 0.02, 0.01, and 0.001. For simplicity P values are expressed in a shortened form. For example, $P > 0.05$ means that $0.05 < P < 0.1$. $P < 0.01$ means that $0.01 > P > 0.001$.

Abbreviations and Terminology

Codes designating treatments of groups are listed in Table 10 (Appendix D). Other abbreviations and terms are as follows:

OADR -- offspring of alloxan diabetic rats

IDM -- infants of diabetic mothers (human)

FSH -- follicle stimulating hormone

GTT -- glucose tolerance test

islets -- islands of Langerhans

IDI -- immunologically detectable insulin

CHAPTER III

RESULTS

Alloxan Diabetes and Pregnancy

Acute Effects of Alloxan

By 3 days after alloxan injection, rats were seriously ill and weak. Glycosuria, hyperglycemia, and wt loss were observed. Blood was often thin and dark. Hyperglycemia and glycosuria were permanent, but there was usually some improvement in health during the second week after alloxan. Without insulin therapy or with alloxan overdosage, many rats died between the 3rd and 5th days after alloxan. Autopsy revealed distention of stomach with food, dehydration, and hardened masses of feces in the intestine.

Plasma Glucose Levels

Diabetes was characterized by glycosuria, hyperglycemia, and wt loss. Polyuria and increased water consumption were apparent. Plasma glucose levels usually were between 200 and 1000 mg/100 ml. Islets exhibited hydropic degeneration.

Rats consistently exhibiting plasma glucose levels above 200 mg/100 ml were considered diabetic. Definite changes in plasma glucose levels occurred during pregnancy (Fig. 1). Plasma glucose levels dropped slowly between the 9th and 2nd days before parturition and then dropped rapidly

the day before parturition. Plasma glucose levels of normal rats also decreased in late pregnancy, but did not exhibit the precipitous drop on the day before parturition (Fig. 2). Because of diurnal variations in plasma glucose levels, such trends as these are apparent only if blood is taken at the same time each day.

Diurnal variation in plasma glucose levels of insulin-treated, diabetic animals was profound (Fig. 3). Plasma glucose levels dropped rapidly after insulin, remained relatively low until the 8th hr after insulin, increased to the 12th hr, and then leveled off until the next insulin injection. Diurnal plasma glucose levels of an individual rat do not constitute a smooth curve; rather, they tend to be erratic--perhaps because of eating patterns or uneven absorption of insulin.

Complications of Chronic Diabetes

Even with moderate insulin treatment, alloxan diabetes was progressively debilitating. Wt loss was profound. After several months, there was very little muscle and adipose tissue. The abdomen was distended. Movements were slow, and weakness and lethargy were apparent. Cataracts and retinopathy were common. There was an increased incidence of infection. Rats became dehydrated. Water consumption and urine output increased to 10 times their normal values. Estrus cycles stopped, and rats remained in diestrus. There was a large increase in the number of leucocytes in the vaginal smear.

Acidosis was usually preceded by increased weakness and lethargy. Breathing became rapid and shallow, and coma often occurred. An example of the development and treatment of acidosis is given in Table 9 (Appendix D). Death often occurred in acidosis but sometimes occurred in the absence of

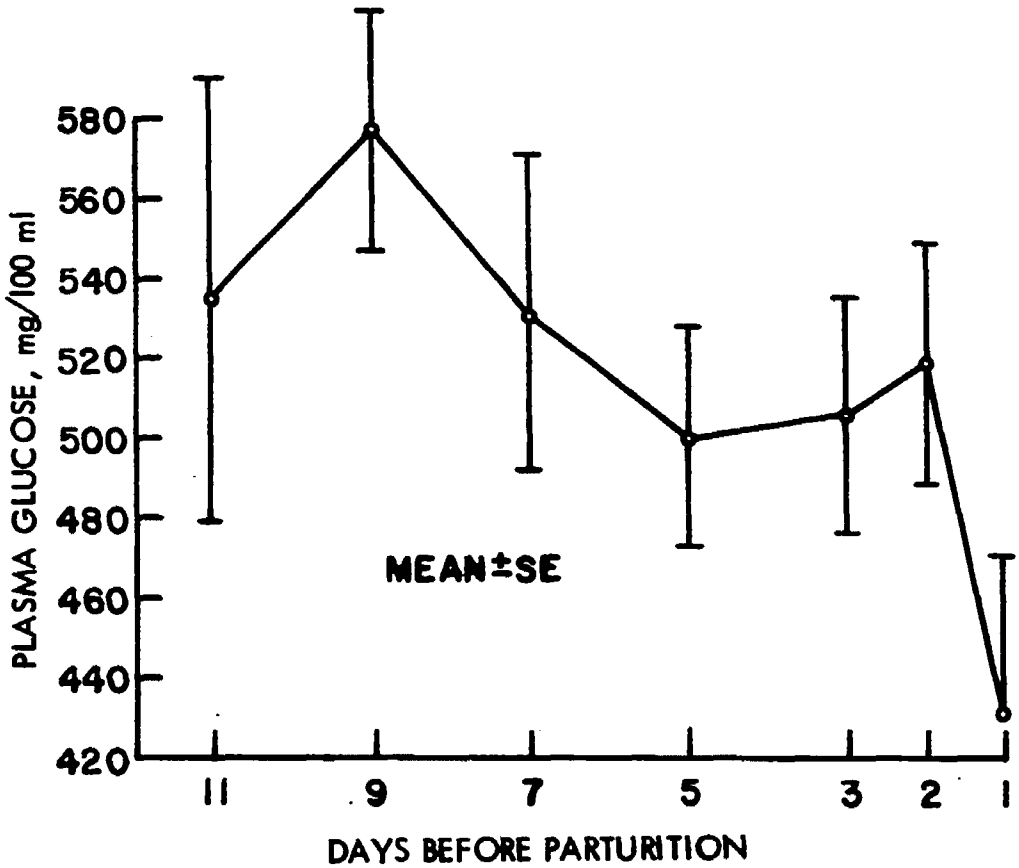


Fig. 1

Plasma glucose levels of insulin-treated, diabetic mothers (group 3E) during pregnancy. Blood samples were taken at 4:00 PM. Each point represents a mean determination from 20 to 30 rats.

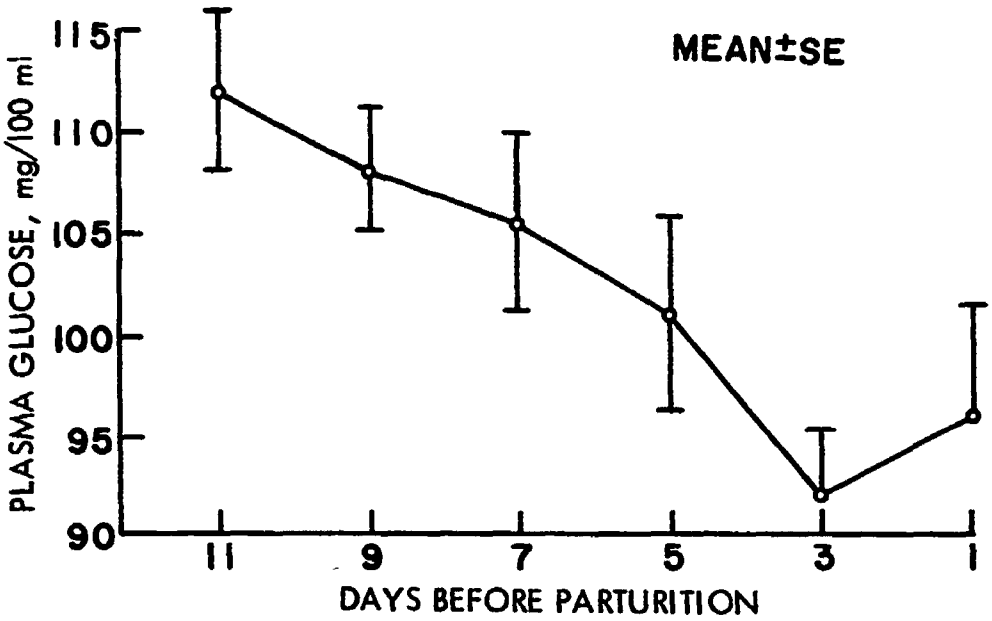


Fig. 2

Plasma glucose levels of normal rats during pregnancy. Blood samples were taken at 4:00 PM. Each point represents a mean determination from 6 to 10 rats.

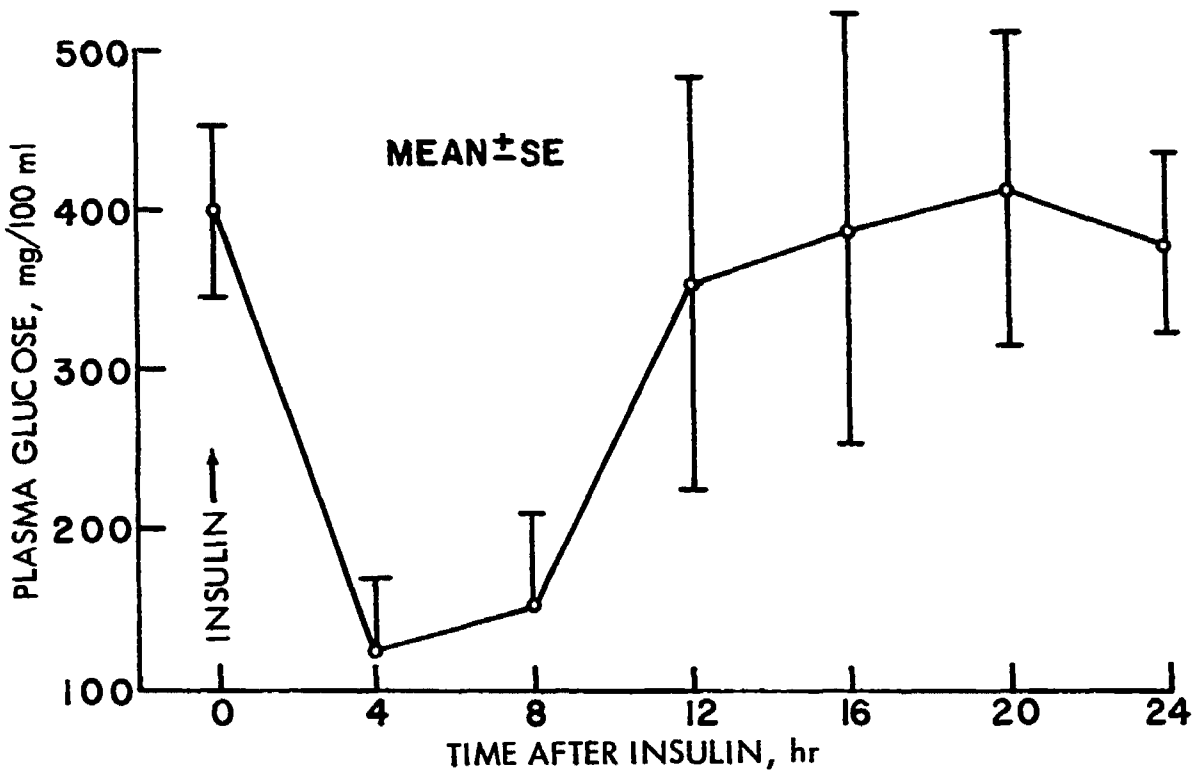


Fig. 3

Diurnal variation in plasma glucose levels in diabetic rats receiving daily injections of protamine zinc insulin. Each point represents a mean determination from four rats. The first blood sample was taken at 4:00 PM (just prior to insulin injection).

acidosis following a period of debilitation and wt loss. Life expectancy was poor. Death was expected after several months of diabetes. Some rats escaped the severe consequences of diabetes and remained healthy in spite of elevated plasma glucose levels. There was poor correlation between the extent of hyperglycemia and the severity of illness.

Evaluation of Alloxan Technique

Alloxan may be injected intravenously (Lazarow and Palay, 1946), but it is difficult, if not impossible, to inject into the tail vein every time. If any or all of the alloxan does not enter the vein, the effect is the same as a variation in dosage. Mortality and failure to induce diabetes are common.

Absolute reliability was needed for production of diabetes in pregnant rats; hence, the intramuscular technique was developed. The animal was etherized, and alloxan (100 mg/kg body wt) was injected into the inside of the thigh near the femoral vein. Mortality from acute effects of alloxan was avoided by beginning insulin treatment 24 hr after alloxan injection.

An independent study with 37 healthy non-pregnant rats was conducted to compare the reliability of this method with that of previous methods. There was no mortality from acute effects of alloxan. One rat exhibited only temporary diabetes. The rest exhibited permanent diabetes (in most cases severe). The mean plasma glucose level for the entire study was 570 mg/100 ml. The study was terminated 2 months after alloxan injection. The reliability of this technique (97.3% success) was far superior to that obtained by intravenous injection.

Problems in Obtaining OADR

A vast expenditure of time and rats is necessary to obtain a single litter of OADR. It would be difficult to exaggerate the hazards facing the prospective OADR. A number of treatments were developed in an effort to circumvent these problems (Table 1). Groups 3A to 3E were developed in the order that they are listed. Treatments of groups are listed in Table 10 (Appendix D). Ideally, one would like to obtain pregnancy in untreated, diabetic rats (group 3A), but the diabetic animals were sterile. Cessation of estrus cycles was associated with cessation of fertility. Fertility was not improved by corticosterone or ascorbic acid. FSH initiated estrus in some rats but had no effect on the incidence of pregnancy. Moderate insulin treatment (group 3B) did not help.

The incidence of mortality in prospective mothers of groups 3A and 3B was associated with the problem of sterility. After months of mating, a number of deaths from chronic diabetes occurred. Also, some of the deaths in groups 3A and 3B were from alloxan overdosage (prior to refinement of technique).

Even when fertilization was accomplished, the fetuses were likely to be aborted, resorbed, or stillborn. Neonates faced the hazards of impaired lactation or absence of maternal behavior in the mother, and idiopathic death was common.

After 6 months of mating (with daily vaginal smears) and a total investment of 108 rats, only 4 viable litters of OADR were obtained from rats that were diabetic before mating. The successful mothers were exceptionally healthy and resistant to the effects of alloxan diabetes. There was no alternative to producing diabetes after conception. At

least the problem of sterility would be circumvented.

Increasing the Probability of Obtaining OADR

Rats receiving alloxan on the 3rd day of pregnancy (group 3C) had high mortality and abortion rates. No viable OADR were produced by this technique. The injection of alloxan on the 10th to 12th day of pregnancy (group 3D) did not increase the percentage of success appreciably (1 viable litter out of 22 attempts). However, when alloxan was injected on the 10th to 12th day of pregnancy and insulin therapy started 24 hr after alloxan (group 3E), the incidence of success jumped to 18%. Also, in group 3E it was possible to obtain offspring from severely diabetic mothers. Because of the technical advantages of working with group 3E, these rats were used for most studies. Unless indicated otherwise, the term OADR will henceforth refer to rats of group 3E.

Both normal and diabetic rats are more likely to be successful mothers if they are not moved from one cage to another either during or after pregnancy. After mating, the prospective mother is moved to a private cage and allowed to remain in the same cage until her litter is weaned. Gentle handling of rats is helpful.

Some pregnant, diabetic rats died mysteriously between the 18th and 20th days of pregnancy. These rats typically appeared very healthy and maintained pregnancy up to the day of death. Usually the rats appeared healthy one evening and were dead the next morning. Autopsy revealed no cause of death. Hypoglycemia is suspected, for a number of hypoglycemic plasma glucose levels were observed in insulin-treated diabetic animals during late pregnancy. It is probably wise to reduce insulin dosages in rats that are heavy and healthy. Insulin dosage should not be in units/kg

TABLE 1

PROBLEMS IN OBTAINING VIABLE OADR: CAUSES OF LITTER FAILURES

Group	Number of Litter Failures Listed Under Cause of Failure				Total Litter Attempts	Total Litter Successes	Percent Success
	Death of Mother	Sterility of Mother	Fetal Abortion, Resorption	Neonatal Mortality Stillborn			
1	1	3	1	3	20	12	60
2A	1	0	2	4	9	4	44
2B	1	0	0	4	10	5	50
2C	0	0	1	0	5	4	80
2D	4	0	0	0	5	1	20
3A	30	29	1	4	66	2	3
3B	18	11	2	9	42	2	5
3C	6	0	6	4	16	0	0
3D	14	0	2	5	22	1	5
3E	3	0	5	6	17	3	18

A litter is considered successful if any littermate lives to the age of 1 month. Some deaths of prospective mothers of groups 3A and 3B are from alloxan overdoses (prior to refinement of technique). Attempts to ameliorate sterility with ascorbic acid, corticosterone, or FSH were not successful.

body wt. If anything, the dosage should be inversely proportional to the body wt. In late pregnancy the vagina often became clogged, preventing urination. Rats which were heavy with pregnancy could not lick the vagina, and it was necessary to keep it clean.

Some diabetic rats carried the young beyond the normal term. Caesarian delivery saved the mothers but not the young, for the young were stillborn.

In group 3E, the problem of sterility was eliminated by giving alloxan after conception, and other problems were at least partially eliminated by insulin therapy. The emphasis was shifted from trying to obtain and maintain pregnancy to keeping neonates alive once they arrived. Lactation and maternal behavior were impaired. Injection of 3% calcium lactate (0.2 to 0.8 ml) improved both behavior and lactation (especially in rats chasing their tails). Beef pituitary extract was of some value in inducing maternal behavior. When available, a normal foster mother (with young less than 1 wk old) was the best solution to the problems of impaired lactation and lack of maternal behavior. Usually either all or none of the littermates died. Some died for no apparent reason. Idiopathic death in OADR may or may not resemble hyaline membrane disease in IDM.

OADR

Birth Weights

Although fetal gigantism in IDM is firmly established, the effect of diabetes on birth wt in the rat is uncertain. Most investigators report increased birth wt, but some report decreased birth wt in OADR. The 1-day increase in length of gestation in diabetic rats allows more than normal time for fetal growth, but does not adequately explain existing discrepancies.

Neonates in groups 3A, 3C, and 3D were significantly heavier than normal neonates (Table 2). Neonates in group 3E were significantly lighter than normal, but only because the mean value was depressed by the light wts of a single litter of OADR. If the abnormal litter in group 3E is omitted, the mean birth wt is the same as that for normals. Birth wts in group 3B were not significantly different from normal. A pattern emerges: groups whose mothers received insulin have normal birth wts, but OADR whose mothers were not treated exhibit fetal gigantism. Occasionally, a diabetic mother may give birth to a litter whose wts are decidedly below normal.

Low Body Weight in Young OADR

Many OADR exhibited depressed body wts, particularly during the first month of life (Fig. 4). Ironically, this depression of body wt was most pronounced in OADR whose mothers received insulin (groups 3B and 3E). An explanation is possible by putting a few facts together.

A glance at the standard errors in Fig. 4 shows that the depression of the body wts is not consistent. One litter of OADR may exhibit extremely low body wts; yet, another litter of OADR may exhibit normal body wts. OADR exhibiting severe hypoglycemia had very low body wts, and at first it was thought that low body wt was a part of the hypoglycemic syndrome; however, it now appears likely that low body wt and hypoglycemia are independent maladies, both of which are more likely to occur when the mother of the OADR is severely diabetic.

More light is shed on the problem of depressed body wt by a control group than by the OADR. A litter in group 2C, whose mother received alloxan on day 19 of pregnancy, was split. Three males were reared by their

TABLE 2

BIRTH WEIGHTS OF OADR AND CONTROLS
COMPARED TO NORMALS

	Birth Weight, g			
Group	Mean	SE	N	P (vs Norm)
1	5.45	0.090	97	-
2A	5.43	0.049	23	>0.5
3A	5.70	0.046	17	<0.01
3B	5.46	0.137	18	>0.5
3C	6.33	0.142	10	<0.001
3D	6.20	0.037	10	<0.001
3E*	5.28	0.078	43	<0.02

*One litter in group 3E exhibits very low birth weights. If this litter is omitted, the mean weight is 5.46 and P>0.5. OADR whose mothers received no insulin exhibit fetal gigantism, but OADR whose mothers received insulin exhibit normal or subnormal birth weights.

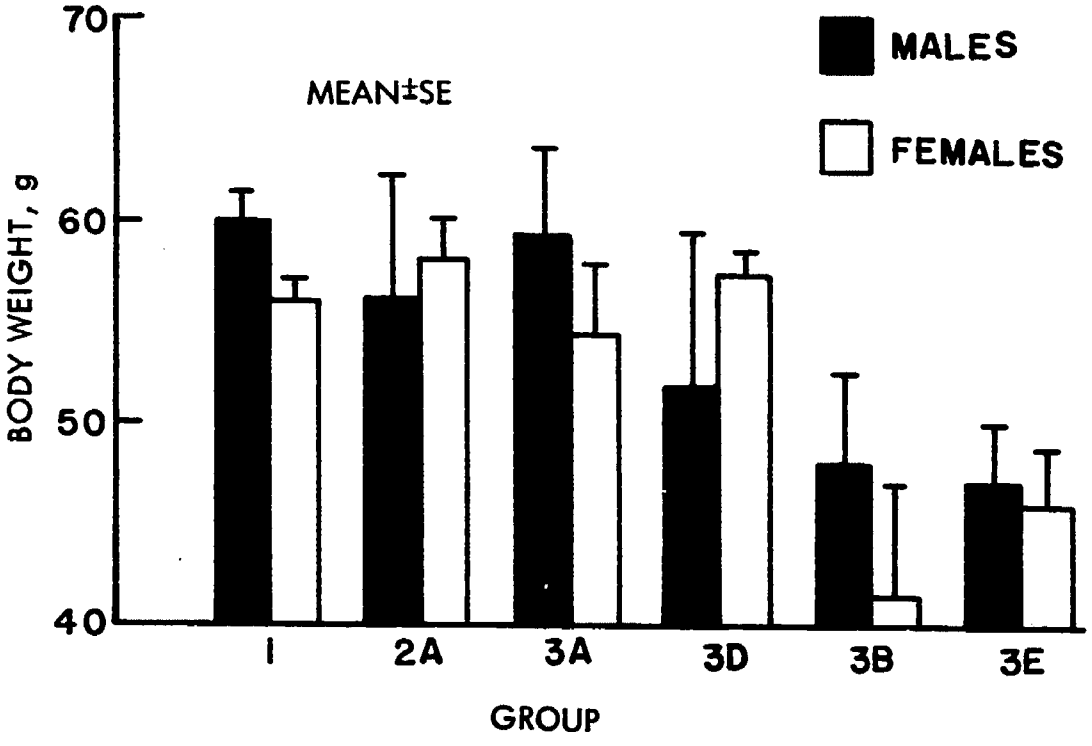


Fig. 4

Body weights of offspring at 1 month of age. Diabetic mothers of groups 3B and 3E received insulin. Diabetic mothers of groups 3A and 3D did not receive insulin.

diabetic mother, and three females were reared by a normal foster mother (separation of sexes was coincidental). At 1 month of age the mean body wt of the rats nursed by the foster mother was 73.0 g, and the mean body wt of the rats nursed by the diabetic mother was 24.0 g. The difference was highly significant ($P < 0.001$). None of the rats in this litter exhibited hypoglycemia at the 1 month GTT. Apparently, the depression of body wt in many litters of OADR is caused by impaired lactation in the mother, and the impaired lactation is correlated with the severity of diabetes.

Only relatively healthy animals in groups 3A and 3D (no insulin) were able to have litters. Insulin therapy (groups 3B and 3E) enabled rats with severe diabetes to have litters. Insulin therapy (perhaps unfortunately) was frequently omitted after OADR reached the age of 1 wk, and lactation probably was impaired in the mothers. In summary, some lactating mothers in groups 3B and 3E were severely diabetic; whereas, lactating mothers of groups 3A and 3D were healthy.

Plasma Glucose Levels

Factors influencing plasma glucose levels. Hypoglycemia is not as easily diagnosed as diabetes. Small differences in plasma glucose levels may be important; therefore, stringent control of experimental conditions is necessary. Several studies of factors influencing plasma glucose levels demonstrated the importance of keeping all controllable conditions constant. To avoid bulk in this section, these studies are included in Appendix C.

It is difficult to get reliable plasma glucose levels in neonates for two reasons: (1) Carbohydrate metabolism in the neonate is anomalous,

and plasma glucose levels are influenced by factors not important in the adult. (2) It is difficult to control conditions of the newborn animal (e.g. length of fasting, feeding habits of mother just prior to parturition). Figure 19 (Appendix C) shows the effect of fasting temperature on plasma glucose levels of the neonate. This anomaly was discovered by serendipity; it might be related to poikilothermy in the newborn rat.

Any type of stress may affect plasma glucose levels. Plasma glucose levels are higher if blood samples are taken 15 min apart in a GTT than if they are taken 1 hr apart. Anesthesia causes erratic and elevated plasma glucose levels (Fig. 20, Appendix C). Oral 0.9% saline (control for GTT) causes a transient rise in plasma glucose (Fig. 21, Appendix C).

Plasma glucose levels vary with both age and sex (Table 7, Appendix C). The effect of age is considerable (especially during fasting), and comparisons between different aged rats should not be made. But the effect of sex is small (about 3 mg/100 ml), and for most purposes, the sexes can be lumped. The effect of sex on the GTT is apparent only if variance is reduced by constant experimental conditions (e.g. time of day, room temperature, handling techniques, fasting times, and age).

Also, there are factors influencing plasma glucose levels that cannot be controlled. Within the same group, there is variation among litters.

Glucose tolerance and hypoglycemia in OADR. Since the primary purpose of the present investigation is to study long-term effects of alloxan diabetes on the offspring, emphasis will be on glucose tolerance of rats at least 1 month old.

There was no evidence of permanent diabetes in OADR from any group. A few OADR had slightly depressed glucose tolerance at 2 wks of age, but

all evidence of diabetes disappeared by the 1-month GTT. No diabetes was found in rats of control group 2C, whose mothers received alloxan on day 19 of pregnancy.

Many OADR had normal GTT's. There was evidence of hypoglycemia in some OADR (Fig. 5). Some individuals exhibited severe hypoglycemia (Fig. 6). This abnormality will be called the "hypoglycemic syndrome." The hypoglycemic syndrome afflicted two litters. With one exception, all rats in the two litters exhibited hypoglycemia. Body wts were very low. The GTT lacked the characteristic peak following glucose ingestion. Blood samples became increasingly hard to get as hypoglycemia progressed. Indeed, it became impossible to obtain blood from the tail. The blood supply to the tail was exactly like that of a rat in severe shock, but the rat was not prostrate. The hypoglycemic syndrome is a chronic disease (Fig. 7), but hypoglycemia is not quite as severe at 2 months of age as at 1 month of age.

Some OADR exhibited only slight depression of plasma glucose levels. The diagnosis of hypoglycemia is difficult. Certainly, fasting plasma glucose levels (as one might expect) are too variable to be very useful and, furthermore, would not be useful in diagnosing reactive hypoglycemia. The choice of a cut-off point below which hypoglycemia should be diagnosed is arbitrary. Inspection of normal GTT's suggests that a plasma glucose level below 70 mg/100 ml at any time after the glucose challenge is abnormal. Using this criterion to diagnose hypoglycemia, the percentage of hypoglycemia in each group was calculated (Table 3). No hypoglycemia was diagnosed in normals and controls, with 2 minor exceptions in group 2A. Hypoglycemia was present in all groups of OADR except group 3D in both

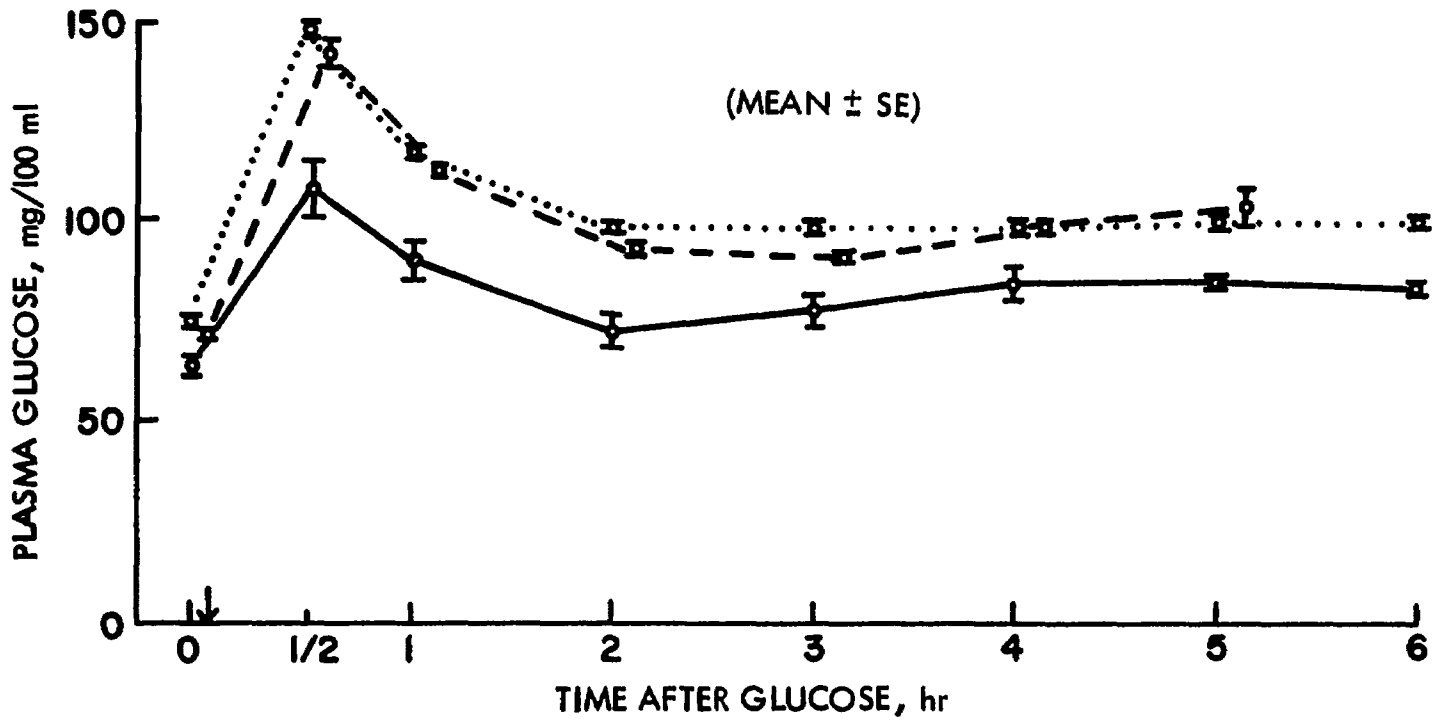


Fig. 5

Lumped 1-month GTT's. Normals (N=56) are represented by the dotted line, control group 2B (N=24) by the broken line, and OADR group 3E (N=13) by the solid line.

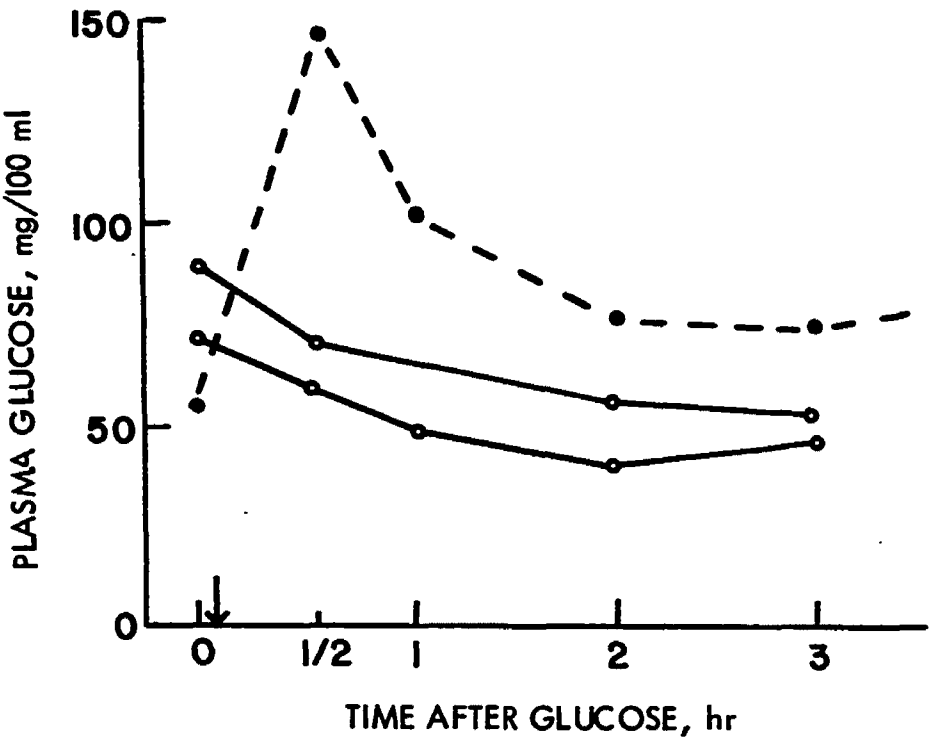


Fig. 6

GTT's of two OADR (group 3E) exhibiting the hypoglycemic syndrome at 1 month of age (solid lines). For comparison, the broken line represents the lowest GTT in any of the 56 normal rats. The GTT's of the two OADR were terminated at the 3rd hr because of difficulty in obtaining blood samples.

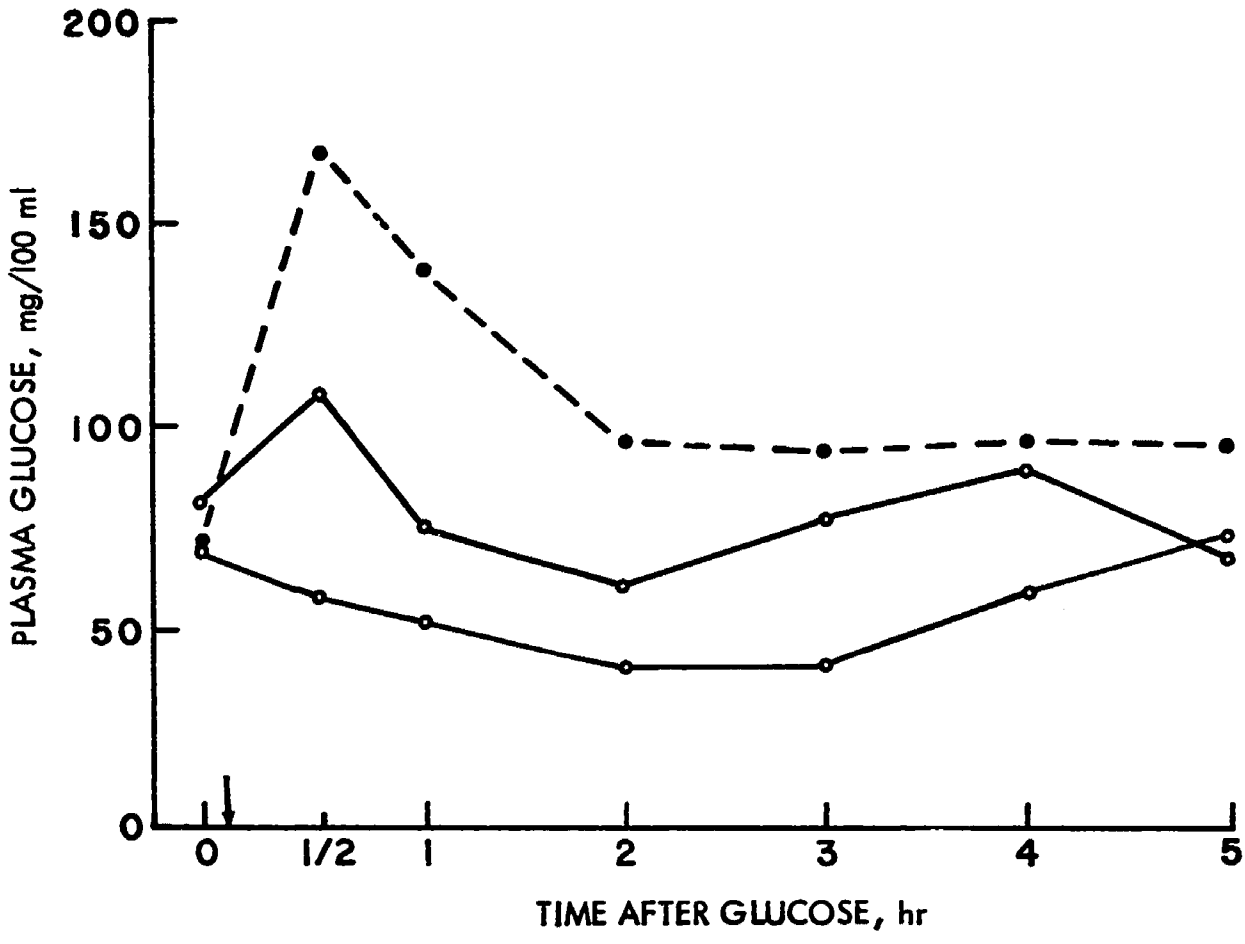


Fig. 7

Two-month GTT's of same rats shown in Fig. 6 confirming hypoglycemia in the OADR (solid lines) and demonstrating that the syndrome is chronic.

TABLE 3

PERCENTAGE OF RATS FROM EACH GROUP EXHIBITING HYPOGLYCEMIA* DURING THE SIX-HOUR ORAL GLUCOSE TOLERANCE TEST

	One Month Old		Two Months Old	
Group	No Rats in Group	Percent Hypoglycemia	No Rats in Group	Percent Hypoglycemia
		Normal and Control Groups		
1	56	0	52	0
2A	25	8	4	0
2B	24	0	13	0
2C	10	0	8	0
2D	3	0	3	0
		OADR		
3A	12	25	9	22
3B	8	25	6	33
3D	6	0	5	0
3E	14	36	7	86

*An animal with a plasma glucose level below 70 mg/100 ml at any time after oral glucose is considered hypoglycemic. Actual hypoglycemic measurements in control group 2A at 1 month of age are 67 and 65 mg/100 ml.

Actual hypoglycemic measurements in 1 month OADR are 69, 69, 69, 68, 67, 66, 63, 61, 61, 61, 60, 59, 58, 57, 54, 53, 52, 48, 46, 39, 36, and 34 mg/100 ml. Actual hypoglycemic measurements in 2 month OADR are 69, 68, 67, 63, 61, 59, 58, 57, 56, 56, 53, 51, 39, and 39 mg/100 ml.

1-month and 2-month GTT's.

Testing for artifacts. A number of questions are raised by these data. Why do some but not all of the OADR exhibit hypoglycemia? In animals with the hypoglycemic syndrome, why do plasma glucose levels fail to rise--at least initially--after glucose? What is the significance of the association of hypoglycemia with hard-to-get blood samples? Could the hypoglycemic syndrome be an artifact, produced by dilution of blood with tissue fluids while squeezing the tail in hard-to-get blood samples?

Unfortunately, rats with the hypoglycemic syndrome were dead before adequate testing was done. The first litter with the syndrome died mysteriously within 3 days of the 1-month GTT. Indirect methods were devised to test the possibility that the hypoglycemic syndrome is an artifact. A question that can be answered empirically is this: is the mild hypoglycemia in OADR an artifact? The modified GTT was devised to test this possibility. At 3 hr post glucose blood was taken from the tail, from the heart, and again from the tail (Fig. 8). If hypoglycemia were an artifact of the sampling technique, it should disappear in the heart sample. Hypoglycemia was present in the OADR in all 3 samples. Also, if samples from OADR were diluted with tissue fluids, the hematocrits should be lower. But the hematocrits of OADR were normal (Fig. 9).

Another approach is to determine whether hard-to-get blood samples are likely to be diluted with tissue fluids. A number of the samples from the modified GTT were hard to get. Some animals went into shock after the cardiac punctures. Hematocrits from hard-to-get blood samples were not lower than hematocrits from easy-to-get blood samples (Fig. 10). There was no correlation between hard-to-get samples in the modified GTT

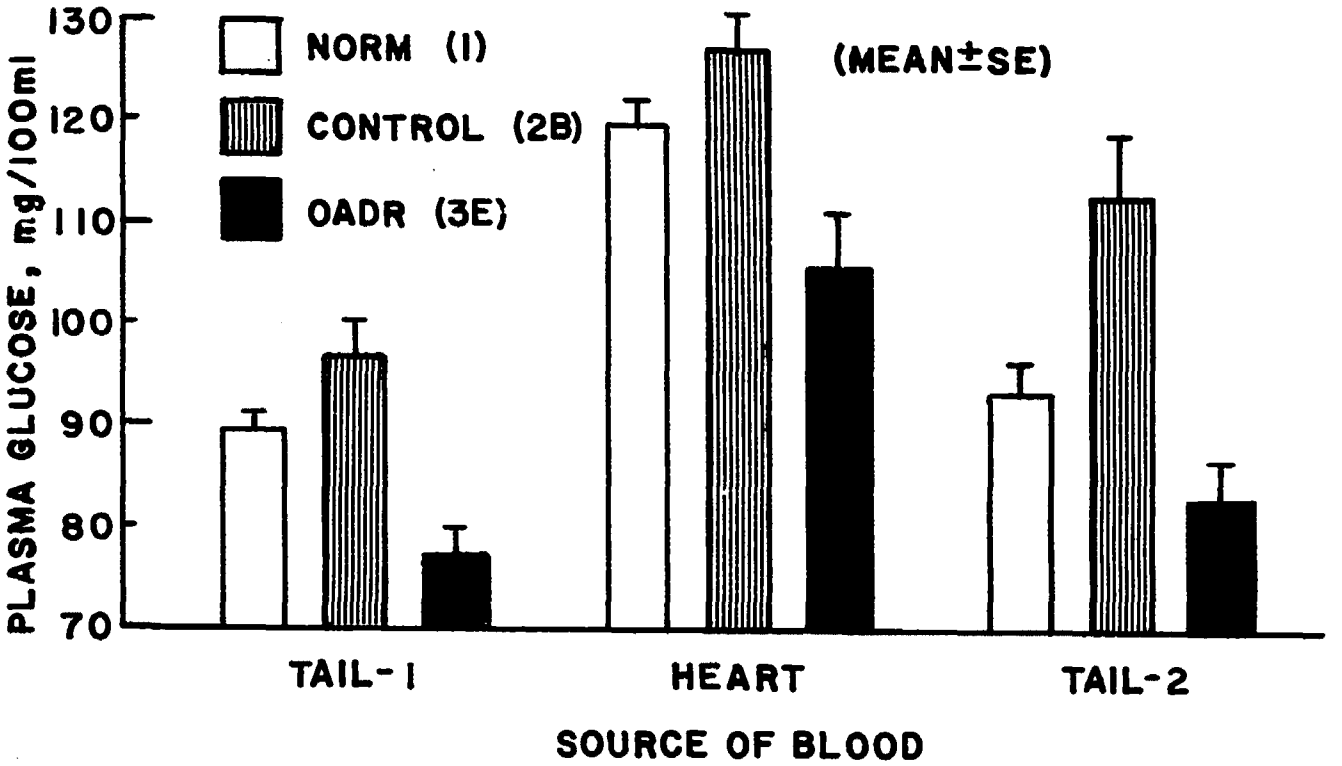


Fig. 8

The modified GTT: plasma glucose levels in 2-month-old rats at 3 hr post glucose. Blood was taken from the tail, from the heart, and again from the tail at approximately 2 min intervals.

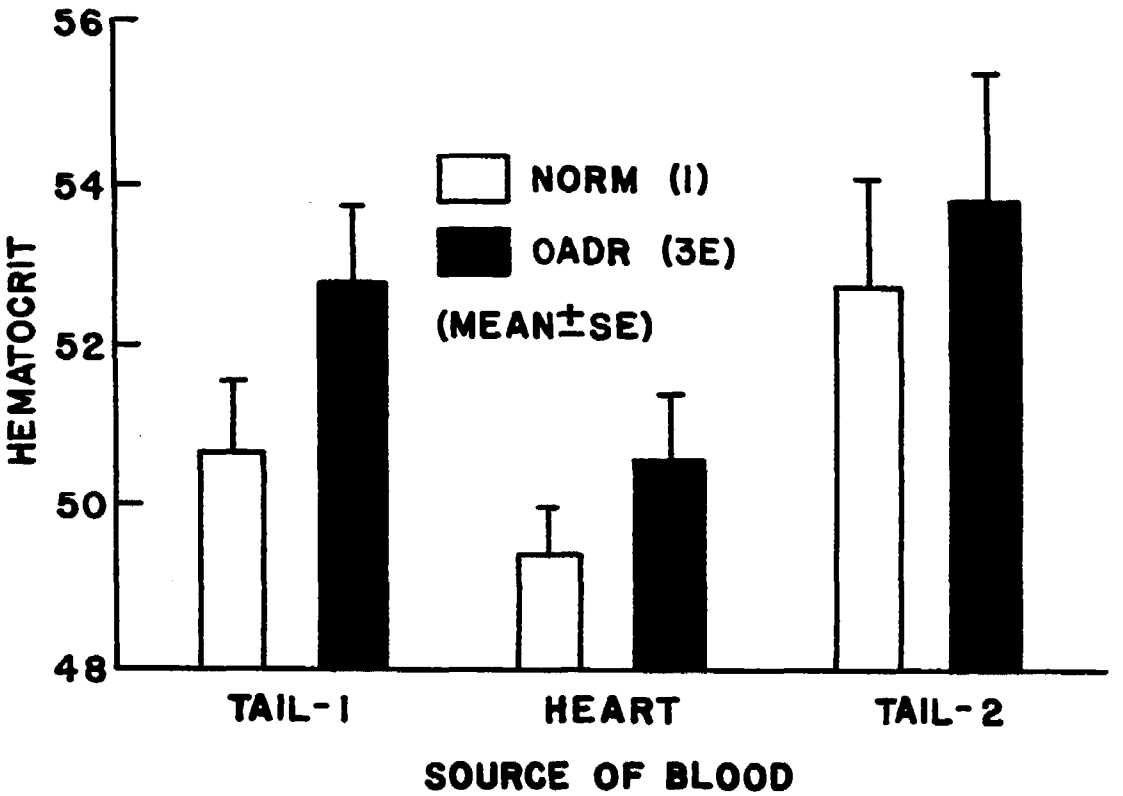


Fig. 9

Hematocrits in 2-month-old rats at 3 hr post glucose. Blood was taken from the tail, from the heart, and again from the tail at approximately 2 min intervals.

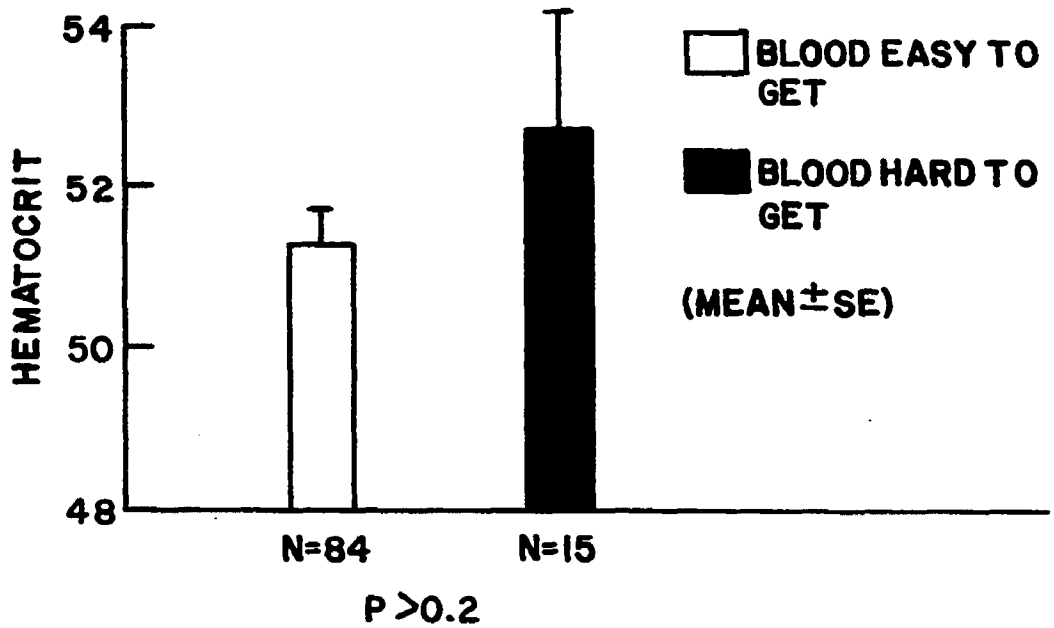


Fig. 10

Comparison of hematocrits from blood samples which were hard to get with hematocrits from blood samples which were easy to get. All tail samples from the modified GTT were classified subjectively as either hard to get or easy to get, regardless of the group.

and hypoglycemia. Also, when the tail of a normal rat was bruised deliberately to make blood hard to get, there was no hypoglycemia.

All tests for the possibility of an artifact were negative. Also, if an artifact is responsible for the hypoglycemic syndrome, it is an artifact that recurs in the same animals. It appears that it is indeed hypoglycemia that is being measured, but the problem of the association of hypoglycemia with hard-to-get blood samples is unresolved.

The modified GTT--a more sensitive test. Prior to the studies involving the modified GTT, it was known that stress of blood sampling affects plasma glucose levels. If blood samples are taken frequently, plasma glucose levels are higher than if blood samples are taken infrequently. The first tail sample in the modified GTT is taken under conditions of minimal stress. The rat has not been disturbed for 3 hr (since the administration of glucose). During a GTT, where samples are taken serially, the mean plasma glucose level for 2-month-old normal rats at 3 hr post glucose was 98 to 99 mg/100 ml (Table 7 in Appendix C). The corresponding value in the modified GTT was about 90 mg/100 ml (Fig. 8). In the second tail sample (Fig. 8), taken 4 min after the first tail sample, plasma glucose levels were higher than in the first tail sample in all groups. Stress increases variance and masks hypoglycemia. A group of OADR with essentially normal 1-month GTT's exhibited significant hypoglycemia at the first tail sample in the 2-month, modified GTT.

Islet Histology

The gross appearance of islets from OADR was not much different from normal. Occasionally, there was evidence of hydropic degeneration. There

was enough variance in islet sizes to make subjective evaluation of the presence or absence of hyperplasia worthless. Measurement of the area of the largest section of each islet provides a simple method for quantitating results. Variance is large, but at least some indication of relative quantities of islet tissue may be obtained.

Areas were measured by planimetry and by component quantitator (Fig. 11). Both methods produced similar results. There is a tendency for islets from OADR to be larger than those from normals, but the difference is not significant. Perhaps a more refined technique would demonstrate islet hyperplasia in OADR, but a more crucial question in the present study involves function of the beta cells. On the basis of islet histology (hydropic degeneration in some islets, possible hyperplasia in others), one might hypothesize either hypo- or hyperfunction of beta cells.

Plasma Insulin Levels

The presence of anti-insulin antibodies in the offspring of an insulin-treated mother might cause a false elevation of immunologically detectable insulin (IDI), but the antibodies probably disappear within a few days after birth (Isles and Farquhar, 1967). Possible effects of insulin treatment on IDI of OADR were controlled for by group 2B, whose mothers received insulin during pregnancy.

In OADR plasma IDI at 45 min post glucose was significantly below normal but was not significantly different from control group 2B (Fig. 12). Variance is large and tends to obscure differences. Whether or not the insulin treatment of the mother depresses IDI is not clear. Although the results are not firm in most respects, one point seems clear: OADR do

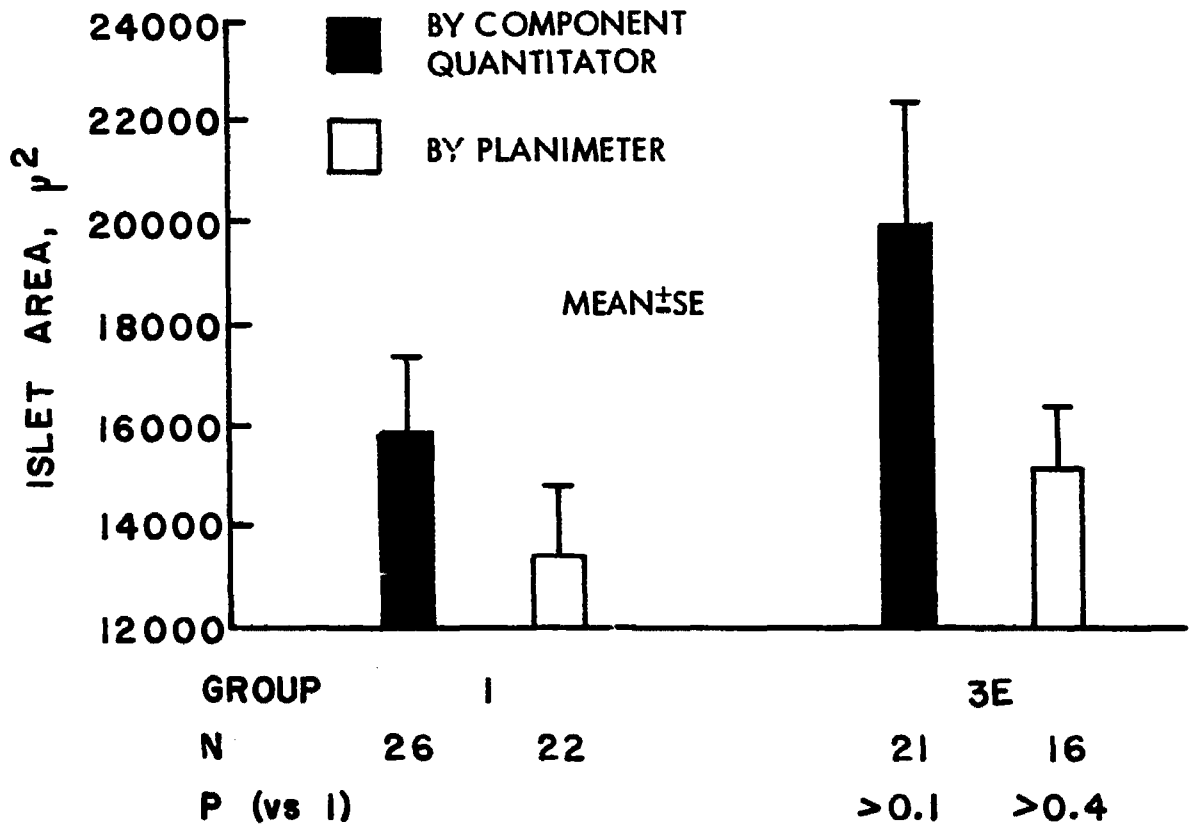


Fig. 11

Islet areas in 2-month-old OADR and normals. Islet sections were traced serially to the section containing the largest area, and the area of that section was determined. Two independent studies of islet areas were performed: one by component quantitator and one by planimeter. OADR are compared with normals by t-tests.

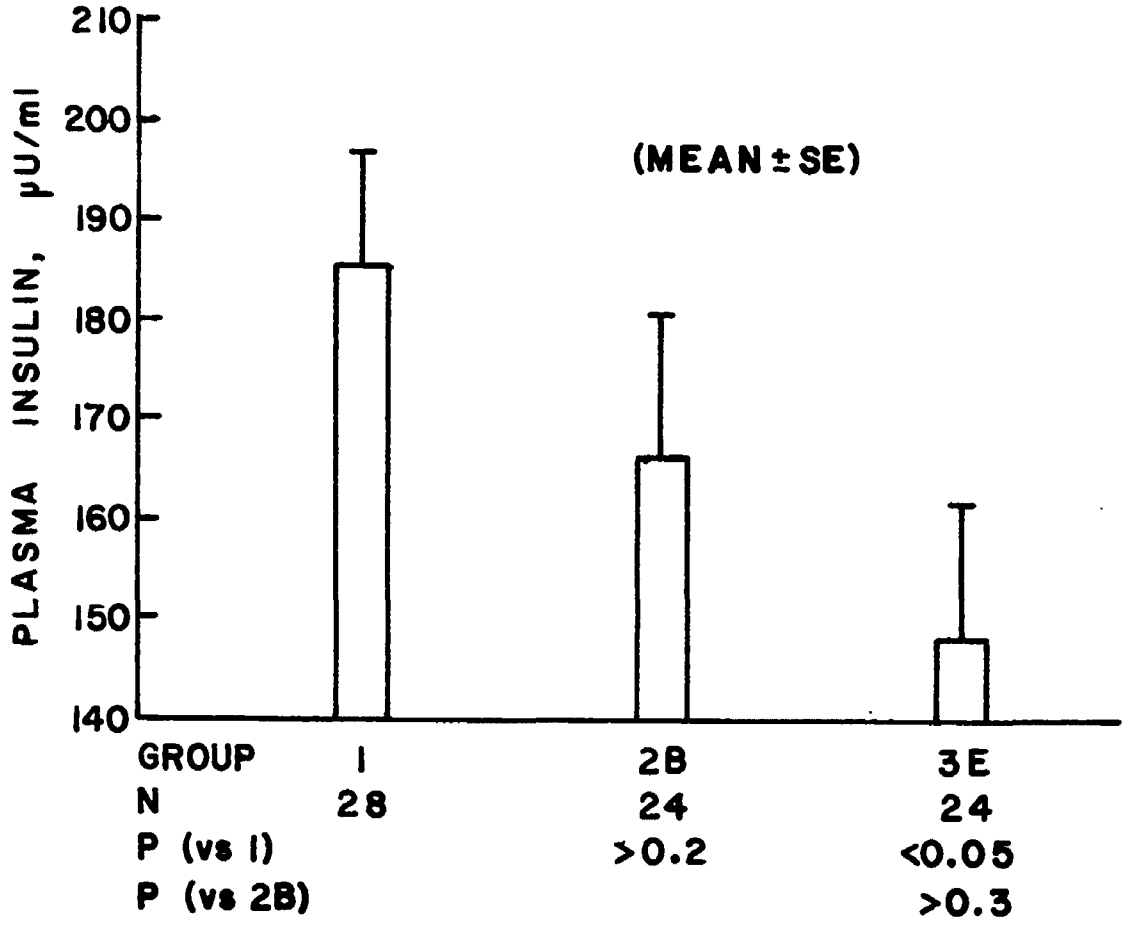


Fig. 12

Plasma insulin levels in 2-month-old rats. Animals were sacrificed at 45 min post glucose, and blood was taken from the posterior vena cava.

not have elevated IDI. Hyperinsulinism is not indicated by the radioimmunoassay. If anything, plasma insulin levels are below normal in OADR.

These data are not actually surprising. The hypoglycemic syndrome does not resemble hypoglycemia caused by exogenous insulin. After injection of insulin, the blood supply to the tail is increased, and blood flows profusely when the tail is cut. This effect may be caused by the response of the adrenal medulla to hypoglycemia; injected epinephrine produces a similar effect. In OADR with the hypoglycemic syndrome, hypoglycemia is associated with decreased blood supply to the tail--just the reverse of the effect observed after insulin injection. The hypoglycemic syndrome, then, might be characterized by a decrease in insulin resistance rather than by hyperinsulinism.

Attempts to Reproduce and Characterize the Hypoglycemic Syndrome

Difficulty of repeating results. The hypoglycemic syndrome has not, thus far, been reproduced. It takes many months to obtain a single litter of OADR, and idiopathic death is common. The poor health of OADR with the hypoglycemic syndrome suggests the possibility that rats that would have had the syndrome are the ones that die. Even if the hypoglycemic syndrome should prove to be a useful model for studying hypoglycemia, the time investment necessary to obtain the syndrome is prohibitive. Adequate testing of the syndrome might take years.

If the hypoglycemic syndrome (or anything remotely similar) could be reproduced by a short-circuit technique, a number of problems might be solved. A "shotgun" approach to the problem was employed. Negative re-

sults might have little value, but positive results would be exceedingly valuable.

Special diets. Although there is little precedent for inducing hypoglycemia by special diets, the development of a sensitive and reliable test for hypoglycemia (the modified GTT) makes the problem worthy of investigation. Rats maintained on special diets (Nutritional Biochemicals Corporation) from 1 month of age were tested at 2 months of age by the modified GTT (Fig. 13). The results constitute a dramatic demonstration of the ability of stress to mask hypoglycemia.

Rats maintained on a high protein diet were not significantly different from normal. In the first tail sample, rats maintained on the high carbohydrate diet were significantly hypoglycemic compared to normals ($P<0.02$), and rats maintained on the high fat diet exhibited severe hypoglycemia ($P<0.001$). In the heart sample, hypoglycemia in rats on the high carbohydrate diet disappeared, and in the second tail sample, even rats on the high fat diet were not significantly different from normal ($P>0.3$). Four min of stress totally masked even severe hypoglycemia. Hypoglycemia encountered in rats on special diets does not resemble the hypoglycemic syndrome. Hypoglycemia in rats on the high carbohydrate diet is mild. Blood samples are easy to get in rats on the high fat diet, and hypoglycemia is confirmed in a fasting sample (Table 4). Ironically, rats on the high fat diet are significantly hyperglycemic compared to normals if blood samples are taken while food is allowed. The hypoglycemia in rats on special diets deserves attention, but it does not shed light on the hypoglycemic syndrome in OADR.

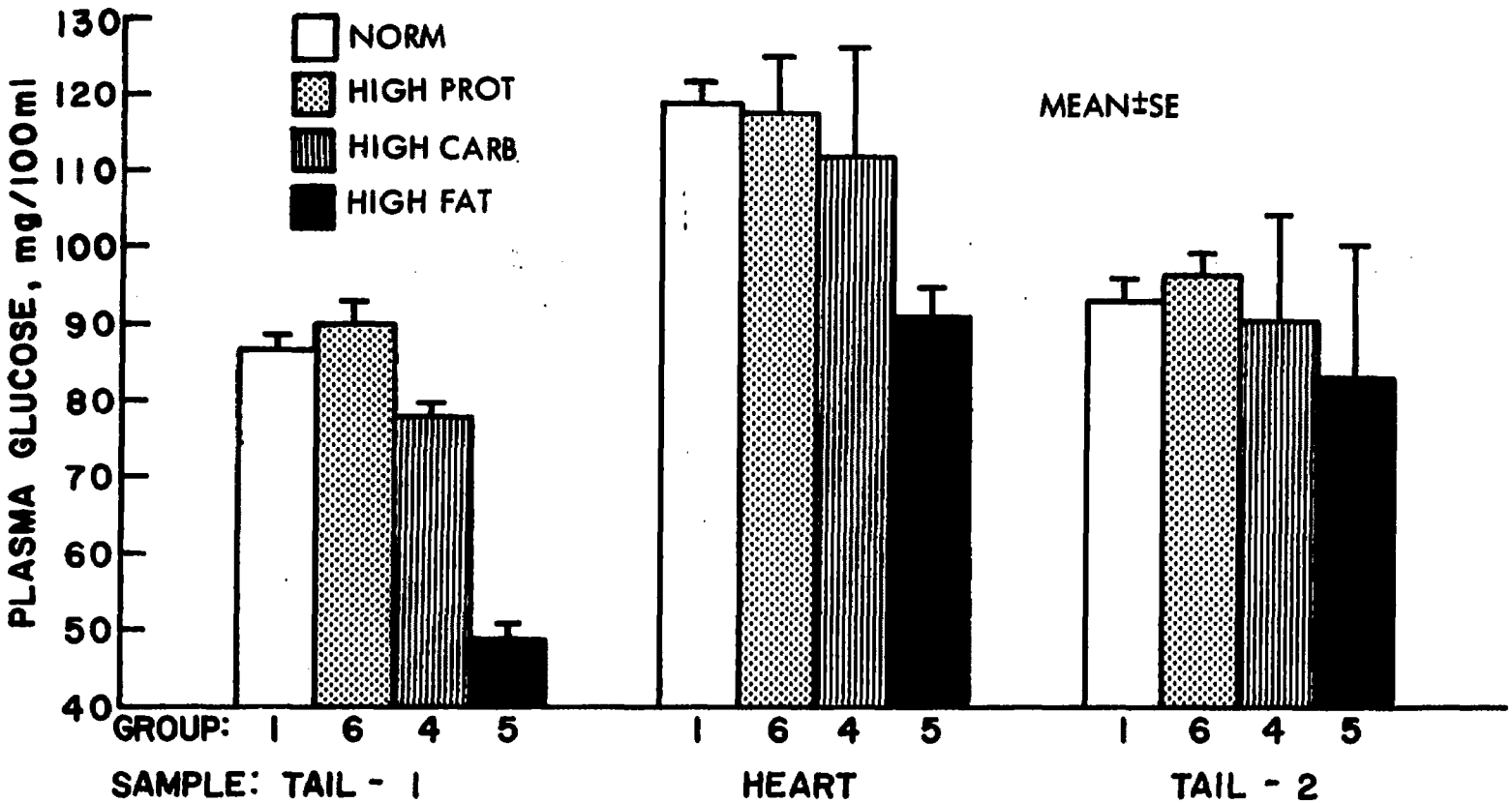


Fig. 13

Modified GTT in animals on high protein (group 6), high carbohydrate (group 4), and high fat (group 5) diets. At 3 hr post glucose, samples were taken from the tail, from the heart, and again from the tail at approximately 2 min intervals. Each group is compared to normal (group 1) by t-test. In the first tail sample, groups 4 and 5 are significantly different from group 1. In the heart sample, only group 5 is significantly different; and in the second tail sample, none of the groups are significantly different from normal.

TABLE 4

FASTING AND REACTIVE HYPOGLYCEMIA IN RATS MAINTAINED ON HIGH FAT DIET

Group	Plasma Glucose Measurements			
	Mean	SE	N	P
Fed				
Normal	106.3	3.00	12	<0.01
High Fat	119.5	2.55	23	
Fasted				
Normal	75.9	1.33	56	<0.001
High Fat	54.6	3.55	25	
3 Hr Post Glucose				
Normal	87.6	1.79	31	<0.001
High Fat	49.4	1.93	22	

Fed animals received food ad libitum, and their blood samples were taken at 4:00 PM. Fasting samples and samples at 3 hr post glucose were taken under conditions identical to those in the modified GTT.

If food is allowed, rats maintained on a high fat diet are significantly hyperglycemic compared to normals. Fasted or at 3 hr post glucose, animals on a high fat diet exhibit hypoglycemia.

Injection of alloxan into the sella turcica. The injection of alloxan into the sella turcica was decided upon because of reports that alloxan decreases growth hormone titers (Lawrence et al, 1958). Perhaps alloxan crosses the placenta and permanently damages the pituitary of the fetus. Actually, control group 2C (alloxan on day 19 of pregnancy) constitutes a reasonable test for this hypothesis. No pretense is made that the present experiment constitutes hypothesis testing: it is one of many attempts to find a simple solution to a complex problem.

Alloxan was injected via the ear canal into the sella turcica. Overdosage caused death. Injection of 0.7 ml of 3% alloxan monohydrate into the sella turcica of a 1-month-old rat caused coma, followed by severe pain. Hind legs were temporarily paralyzed, and growth of long bones was arrested. The rat became obese. Glucose tolerance was normal.

Adrenalectomy. Because the adrenal is involved in maintaining blood pressure and blood glucose levels, there is reason to suspect that adrenal damage might be involved in the hypoglycemic syndrome. Adrenalectomies were performed to investigate possible similarities between adrenalectomized rats and rats with the hypoglycemic syndrome.

Two rats were adrenalectomized at 1 month of age. A 1% saline solution was substituted for the drinking water, and a high carbohydrate diet was allowed in addition to standard laboratory chow. The 2-month GTT's were characterized by progressive hypoglycemia associated with hard-to-get blood samples (Fig. 14). The rats were ill at the fasting sample, but they improved after oral glucose. As hypoglycemia progressed, the rats became very ill; one rat died in convulsions just after the 5 hr sample. The illness, hard-to-get blood, and hypoglycemia suggest a similarity between

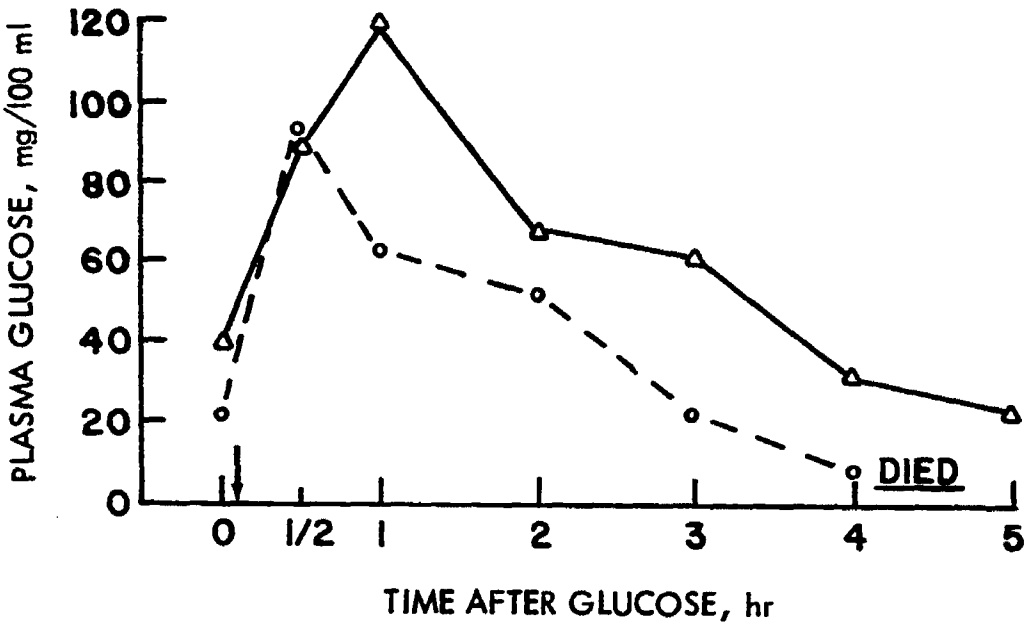


Fig. 14

GTT's in 2-month-old adrenalectomized rats. Each line represents a single rat. As hypoglycemia progressed, blood samples became increasingly hard to get (as in OADR with the hypoglycemic syndrome).

the adrenalectomized rats and rats with the hypoglycemic syndrome. Unlike rats with the hypoglycemic syndrome, the adrenalectomized rats exhibited increased plasma glucose levels after the glucose challenge.

CHAPTER IV

DISCUSSION

The Road Less Traveled By

Basic research (involving diets, studies of islet histology, offspring of diabetic animals, and glucose tolerance) has been applied to the problem of diabetes, but the problem of reactive hypoglycemia has been largely ignored. Hypoglycemia is harder to detect than diabetes. Even in severe hypoglycemia, the deviations of plasma glucose levels from normal are small compared to the deviations observed in diabetes: accuracy, specificity, and precision of the glucose assay are important. Sensitive and precise biological tests are necessary for diagnosis of hypoglycemia, and all conditions must be controlled. Stress, anesthesia, and age are of little consequence in the diagnosis of diabetes; yet, all of these factors are important in the diagnosis of hypoglycemia.

In the present investigation, hypoglycemia was found to result from a number of common treatments; yet, previous studies of these treatments have not demonstrated hypoglycemia. The failure of other investigators to find hypoglycemia is not surprising. Their assay systems and techniques were designed to detect diabetes--not hypoglycemia. Even moderate hypoglycemia was apparent in the present investigation because the tests were designed to detect hypoglycemia.

Hypoglycemia in Rats Fed High Fat Diet

Hypoglycemia in rats fed high fat diets has not been reported, but hyperglycemia has frequently been reported (Roberts and Samuels, 1949; Blazquez and Quijada, 1968; Zaragoza and Felber, 1970). In each case failure to detect hypoglycemia may be accounted for by some combination of the following factors: (1) Blood samples were taken only from fed rats. (2) Rats were anesthetized for blood sampling. (3) Rats were sacrificed for blood sampling.

I confirmed hyperglycemia in rats maintained on high fat diets when blood samples were taken in rats receiving food ad libitum, but the hyperglycemia was moderate (mean=119.5 mg/100 ml as compared to 106.3 mg/100 ml in normals, $P<0.01$). Blazquez and Quijada (1968) reported a mean plasma glucose level of 210 mg/100 ml in rats on the high fat diet and 156 mg/100 ml in normals. Probably the rats were severely stressed, and I have demonstrated that rats on the high fat diet are more sensitive to stress than normals (Fig. 13). Blazquez and Quijada did not specify the blood-sampling technique, but in a similar study by the same investigators (Blazquez and Quijada, 1970), rats were decapitated, and blood was collected from the neck veins. I have measured the response of plasma glucose levels to the stress of sacrificing, and the response is rapid.

Although hypoglycemia in animals on the high fat diet is profound in the fasting state (mean=54.6 mg/100 ml compared to 75.9 mg/100 ml in normals, $P<0.001$), this fact has been overlooked because of inadequacy of the tests for detecting hypoglycemia. Blazquez and Quijada (1968) reported a mean fasting plasma glucose level in rats on high fat diet of 86.7 mg/100 ml compared with 115.8 mg/100 ml in normals. The significance of this difference was obscured by the variance. In the studies of Blazquez

and Quijada (as in many studies), hypoglycemia was masked by the effects of stress.

Significance of the Hypoglycemic Syndrome

The hypoglycemic syndrome in some OADR provides support for the theory that a hyperglycemic stimulus can, under appropriate conditions, permanently modify glycemic control systems, causing chronic hypoglycemia. It cannot be proved that hyperglycemia is responsible for the syndrome, but other possible causes are unlikely. Groups 2A and 2B provide adequate controls for effects of fasting, ether, and insulin injections. Apparently, alloxan is necessary in the genesis of the hypoglycemic syndrome. Whether alloxan might cross the placenta and cause hypoglycemia directly is a more complex problem, but there are some indications that it does not. When alloxan is given on day 19 of pregnancy, one should expect to observe the effects of alloxan on the fetuses but not the effects of diabetes (at most, one day of diabetes is present before birth). Alloxan on day 19 of pregnancy does not cause hypoglycemia in the offspring. The problem with this type of control is that alloxan is given at a late stage of pregnancy, and one can never be sure that the effects are the same as they would be earlier in pregnancy. If the hypoglycemic syndrome could be demonstrated in offspring of rats given alloxan before conception, the possibility of direct effects of alloxan could be eliminated. Although the hypoglycemic syndrome was not observed in rats given alloxan before pregnancy, mild hypoglycemia was observed. Failure to obtain the hypoglycemic syndrome in these rats may be explained by the failure to obtain OADR from severely diabetic mothers in these groups. These facts, together with the precedent for anticipating hypoglycemia in offspring of diabetic

animals (see Introduction) implicate diabetes as the cause of the hypoglycemic syndrome. Whether the cause involves hyperglycemia or some other metabolic abnormality of diabetes is open to speculation.

The occurrence of the hypoglycemic syndrome in 1- and 2-month-old OADR suggests that maternal diabetes may produce chronic effects on the offspring. There is, at present, no reason to believe that similar abnormalities will be found in IDM; but, certainly, extended studies are warranted.

The hypoglycemic syndrome seems to provide a model for studying chronic, reactive hypoglycemia, but a number of reservations are noteworthy: (1) It is never safe to presume that one species reacts in the same way as another species. (2) The hypoglycemic syndrome results from a highly specific treatment, and there is no reason to believe that it is a generalized response. (3) The hypoglycemic syndrome is associated with poor health, low body wt, and circulatory problems. (4) The hypoglycemic syndrome involves an abnormal response not usually observed even in reactive hypoglycemia: total absence of the usual increase in plasma glucose levels following a glucose challenge. With these reservations, the hypoglycemic syndrome might be used advantageously in the study of reactive hypoglycemia.

It is significant that a glucose challenge depresses plasma glucose levels in rats with the hypoglycemic syndrome. Reactive hypoglycemia is present, but there is no evidence of hyperinsulinism. Apparently, insulin resistance is depressed, and the same amount of insulin that is secreted in the normal rat has an exaggerated effect. Such findings do not invalidate the theory of hyperinsulinism, but it should not be assumed that

hyperinsulinism is the cause of hypoglycemia merely because the hypoglycemia is exaggerated by a glucose challenge.

The Road Not Taken

There are many reasons for suspecting that the adrenal is involved in the hypoglycemic syndrome. Both the adrenal cortex and the adrenal medulla are involved in insulin resistance, and both are involved in maintaining blood pressure. Failure to maintain blood pressure might account for the association of hypoglycemia with hard-to-get blood samples. Since epinephrine causes vasodilation in the tail (Weathers, unpublished), a lack of epinephrine might cause vasoconstriction--another possible explanation for hard-to-get blood samples. The adrenal is necessary for meeting the demands of stress. OADR with the hypoglycemic syndrome often die after stress (e.g. after fasting and GTT). Also, adrenal failure might explain idiopathic death in OADR. In a comprehensive study of organ wts in newborn OADR, Angervall (1959) reported hypertrophy in almost all organs (attributed to hyperinsulinism) with one notable exception: the adrenal was atrophied. Although the GTT's of adrenalectomized rats are different from those of OADR with the hypoglycemic syndrome, the similarities are remarkable enough to suggest some correlation between the two abnormalities.

It is interesting to speculate with regard to the effects of maternal diabetes on the fetal adrenal. The adrenal of the fetus is bathed in hyperglycemic blood. Certainly, the adrenal has no cause to resist hypoglycemia. As a result of disuse, the adrenal atrophies. The adrenal of the diabetic mother is hypertrophied, and there is a 38% increase in maternal steroid production (Devecerski and Frawley, 1963). The steroids

cross the placenta, inhibiting ACTH production in the fetus. The result, once again, is atrophy of the adrenal cortex.

The extent of adrenal atrophy in the fetus is probably correlated with the severity of diabetes. Adrenal atrophy is more likely to occur when OADR have been obtained from mothers with severe diabetes (e.g. group 3E). Most of the neonates with severe adrenal atrophy die from adrenal failure soon after birth (idiopathic death). Occasionally, OADR with severe atrophy of the adrenal live and exhibit the hypoglycemic syndrome. Techniques are being devised to test this hypothesis.

Many questions concerning the effects of maternal diabetes on offspring are unanswered. The theory that hyperinsulinism accounts for fetal gigantism, increased organ wt, and hypoglycemia (Pedersen, 1967) is attractive, but it is an oversimplification. The metabolic and physiological abnormalities of diabetes are many, and a clear understanding of the effects on offspring will be possible only when the pancreas, insulin, and blood sugars are viewed as only a small part of a very complicated cybernetic system.

CHAPTER V

SUMMARY

A reliable technique for producing alloxan diabetes with virtually no mortality was developed. Maternal plasma glucose levels were found to decrease during the latter half of pregnancy in both normal and diabetic rats.

Problems in obtaining viable offspring from alloxan-diabetic rats (OADR) were encountered, and some solutions to the problems were found. Fetal gigantism was found in OADR from untreated mothers but not from insulin-treated mothers. OADR, under appropriate conditions, exhibited depressed body wts, poor health, hypoglycemia, and circulatory abnormalities. Islets of OADR exhibited mild hyperplasia in some areas and hydropic degeneration in others, making interpretation difficult. Plasma insulin levels in OADR from insulin-treated mothers were significantly lower than normal, but were not significantly different from those of controls whose mothers were treated with insulin.

Effects of age, sex, temperature, stress, anesthesia, diurnal rhythms, and drugs on plasma glucose levels were noted. Techniques for evaluating possible artifacts in plasma glucose determinations were developed. A modified GTT for detecting reactive hypoglycemia was developed.

Moderate reactive hypoglycemia was found in rats on a high carbohydrate diet. Severe hypoglycemia was found in rats on a high fat diet

in blood samples taken during fasting or at 3 hr post glucose, but mild hyperglycemia was found if blood samples were taken in rats receiving food ad libitum.

The probability that inadequate control of stress and sampling techniques has masked hypoglycemia in many previous studies was discussed. Adrenal failure was proposed as the cause of severe hypoglycemia in OADR. Studies of OADR provided valuable information concerning reactive hypoglycemia and suggested that long-term studies of infants of human diabetic mothers are warranted.

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APPENDIX A

GLUCOSE ASSAYS

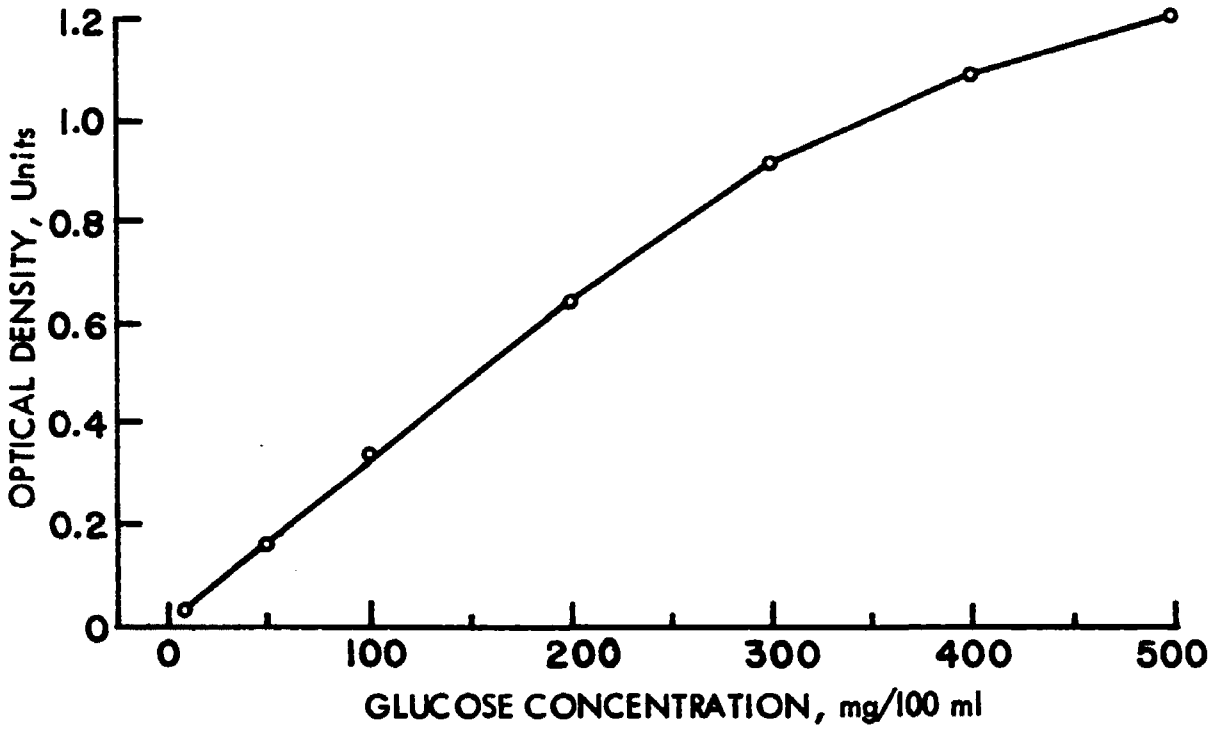

Fig. 15

Standard curve for glucose-oxidase assay for glucose. Each point represents the mean value from five determinations.

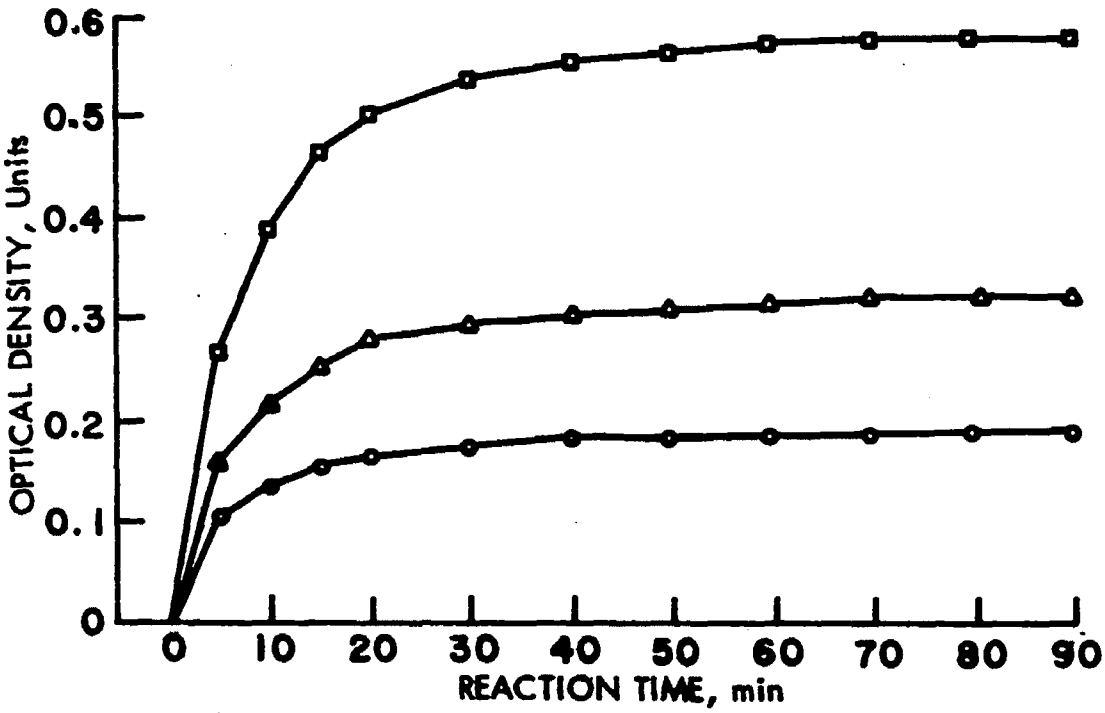

Fig. 16

Timed reaction for the glucose-oxidase assay for glucose. Three concentrations of glucose were employed: 50 mg/100 ml (o), 100 mg/100 ml (Δ), and 200 mg/100 ml (□). Each line represents a series of measurements from the same test tube.

TABLE 5

PRECISION OF THE GLUCOSE-OXIDASE ASSAY FOR GLUCOSE

Glucose Concentration (mg/100 ml)	Optical Density Units (Replicates)					Mean	Percent Error in Precision
10	0.037	0.037	0.037	0.037	0.037	0.0370	0
50	0.164	0.164	0.165	0.166	0.162	0.1641	0.61
100	0.339	0.340	0.342	0.342	0.342	0.3409	0.35
200	0.650	0.650	0.650	0.645	0.655	0.6499	0.32
300	0.90	0.92	0.93	0.93	0.93	0.922	1.04
400	1.05	1.10	1.10	1.05	1.15	1.090	2.94
500	1.25	1.21	1.18	1.18	1.25	1.214	2.48

A replicate is a single measurement from one test tube.

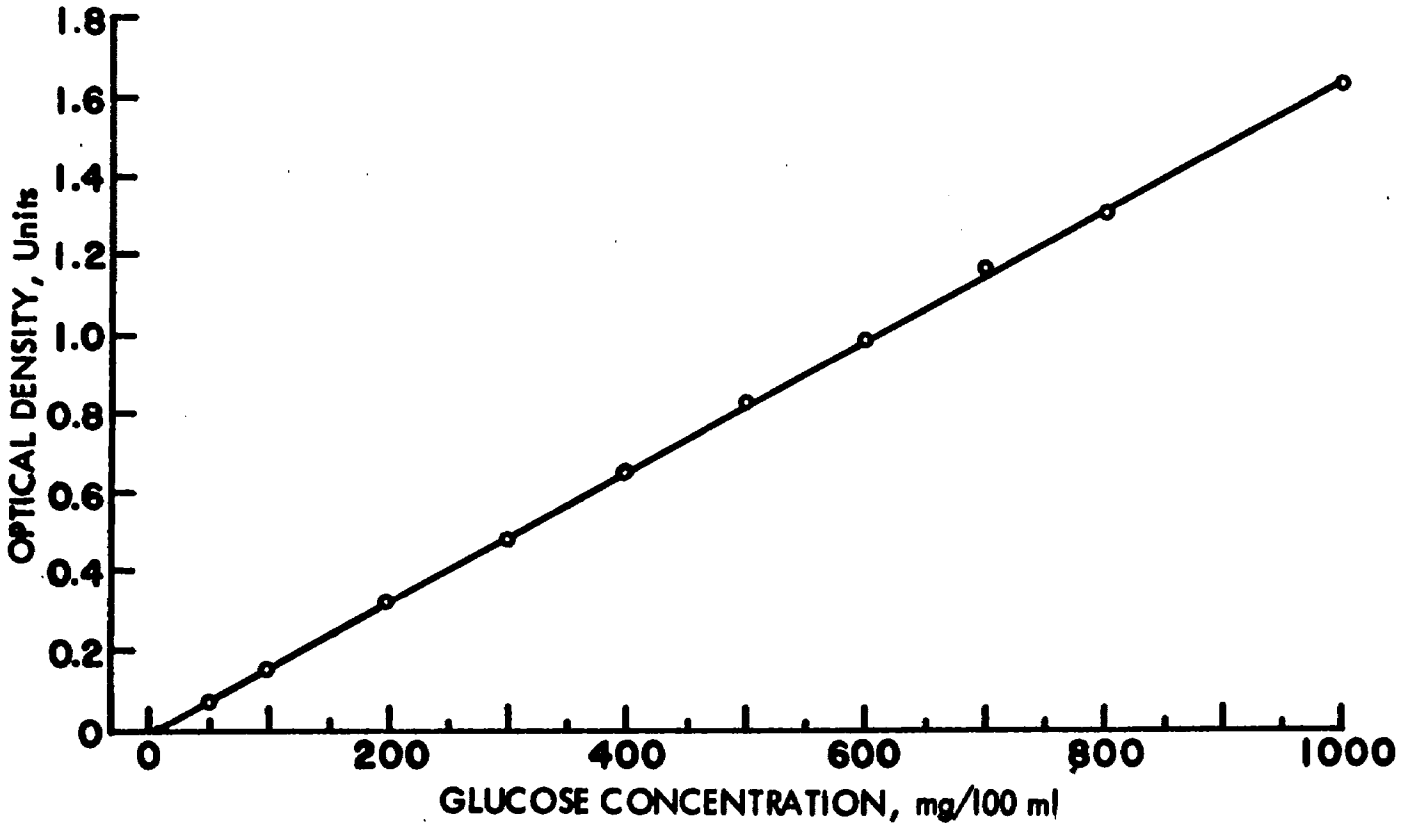

Fig. 17

Standard curve for the ortho-toluidine assay for glucose. Each point represents the mean value from five determinations.

APPENDIX B

INSULIN ASSAY

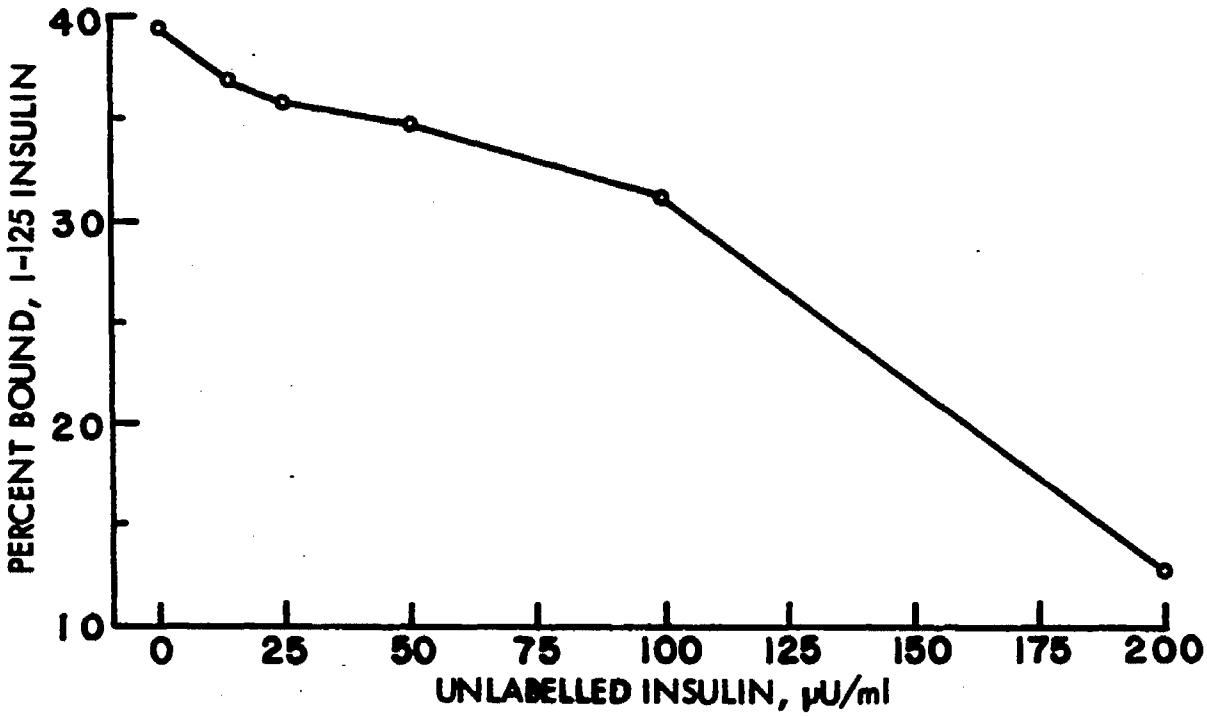

Fig. 18

Standard curve for the radioimmunoassay of insulin: human insulin standards. Each point represents a mean value from five determinations.

TABLE 6

PRECISION OF THE RADIOIMMUNOASSAY FOR INSULIN

Unlabelled Insulin (μU/ml)	CPM in Precipitate (Replicates)					Mean	Percent Error in Precision
0	7999	7694	7617	7322	7373	7571	2.76
12.5	7025	7229	7284	6877	7140	7081	1.89
25.0	6987	6955	6975	7079	6732	6915	1.50
50.0	6824	6785	6711	6667	6552	6678	1.27
100.0	6135	6006	6179	6101	5996	6053	1.19
200.0	2546	2503	2514	2520	2493	2515	0.56

A replicate is a single measurement from one test tube.

APPENDIX C

FACTORS AFFECTING PLASMA GLUCOSE LEVELS

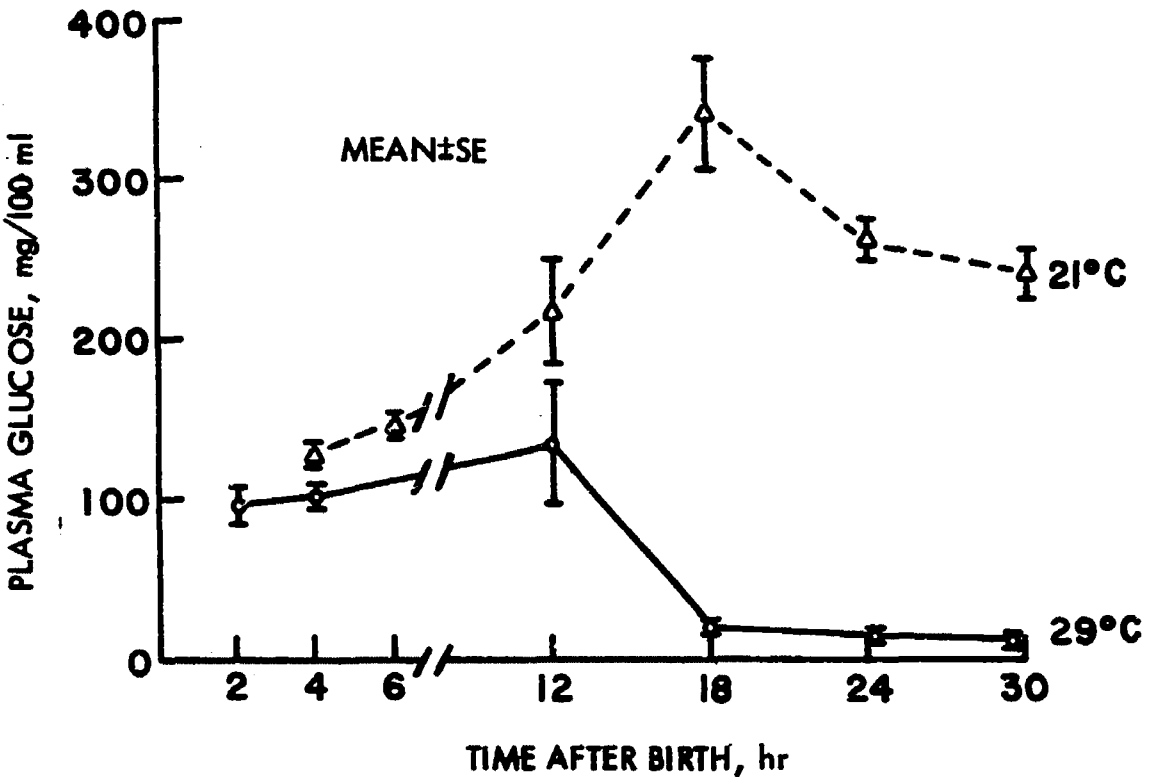

Fig. 19

Effect of temperature on fasting plasma glucose levels in newborn rats. Normal litters were split at birth and fasted at 21°C and 29°C, and blood samples were taken serially (from different rats) to 30 hr after birth.

Fig. 20

Effect of anesthesia during a GTT. The rat was etherized before the glucose feeding, and anesthesia was maintained with sodium pentabarbital (dosage determined empirically).

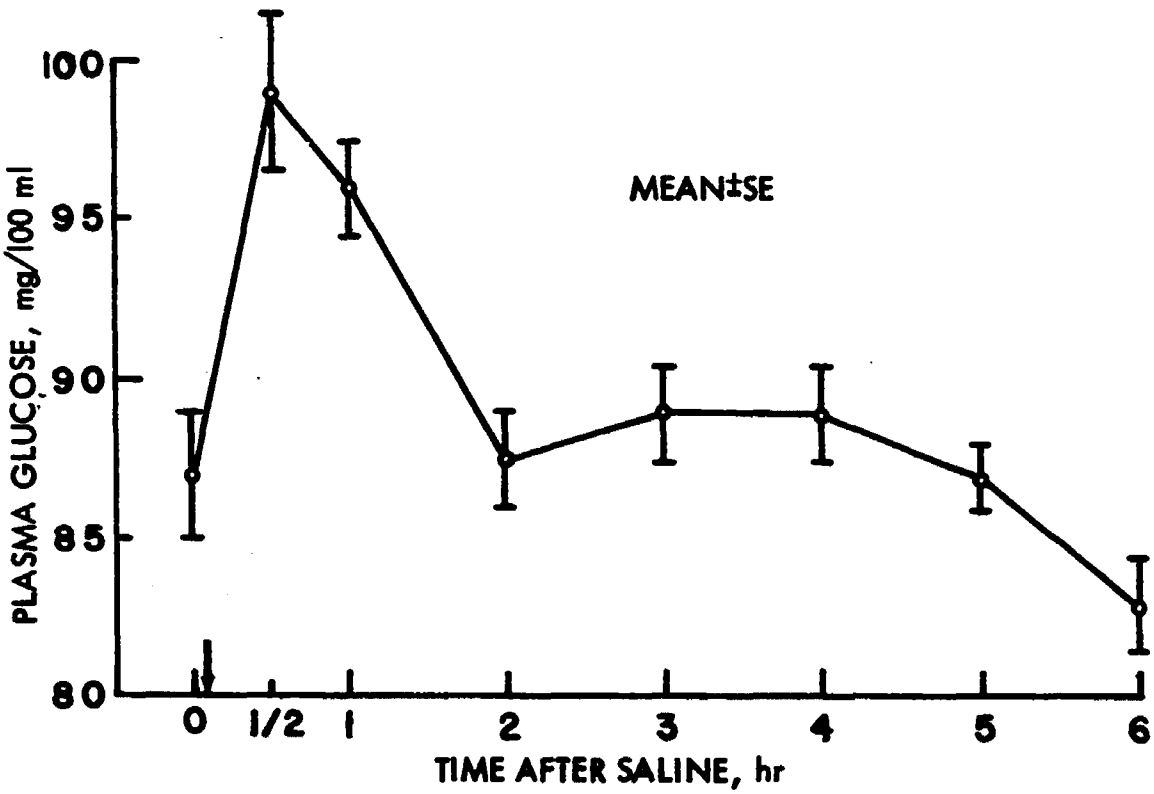

Fig. 21

Plasma glucose levels after oral feeding of 0.9% saline (control for GTT). All conditions are identical to those in the GTT except that saline is used instead of glucose.

TABLE 7

EFFECTS OF AGE AND SEX ON PLASMA GLUCOSE OF NORMAL RATS DURING A GTT

Hours After Glucose	Sex	Plasma Glucose (mg/100 ml) 1 Month Old Mean	SE	2 Months Old Mean	SE	3 Months Old Mean	SE
0	M	77.0	2.12	92.0	2.95	97.3	2.59
	F	75.0	1.68	93.2	3.16	94.4	2.91
1/2	M	150.5	3.88	154.1	2.70	133.7	1.94
	F	145.3	2.99	146.4	2.92	138.6	2.58
1	M	118.7	3.37	131.6	2.04	128.4	1.35
	F	113.7	1.94	123.0	3.54	122.9	3.41
2	M	99.4	3.04	99.3	1.19	94.9	1.90
	F	96.2	1.37	97.9	2.07	98.3	2.13
3	M	100.0	2.50	98.8	1.24	93.8	1.79
	F	97.6	1.35	97.9	1.51	95.8	2.17
4	M	99.5	2.40	101.4	1.46	95.9	1.61
	F	97.2	1.45	99.1	1.41	94.8	2.11
5	M	101.4	2.42	101.5	1.20	95.8	1.61
	F	98.9	1.75	97.8	1.52	93.2	2.23
6	M	100.5	2.29	99.4	1.17	95.3	1.66
	F	98.1	1.54	97.5	1.82	92.0	2.46

Large sample sizes (N=24 to 30 for each measurement), careful control of conditions surrounding the GTT, and the excellent precision of the glucose-oxidase assay (Table 5) make it possible to demonstrate that plasma glucose levels in females are consistently lower (by approximately 3 mg/100 ml) than in males. Differences in plasma glucose levels at different ages are also apparent.

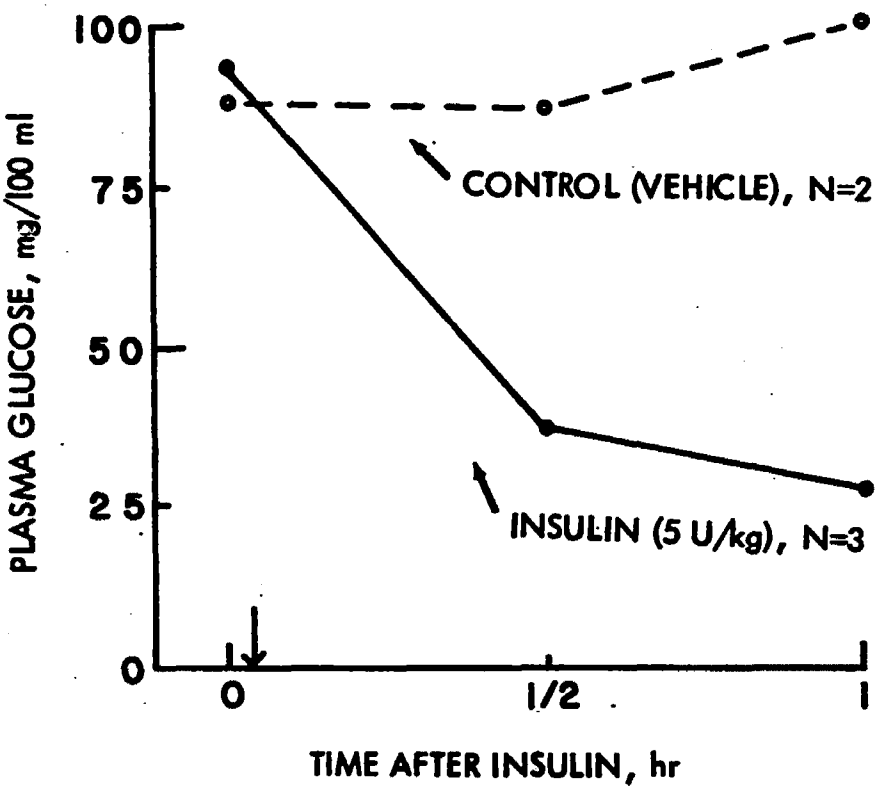

Fig. 22

Effect of regular insulin on plasma glucose levels in fasted rats.

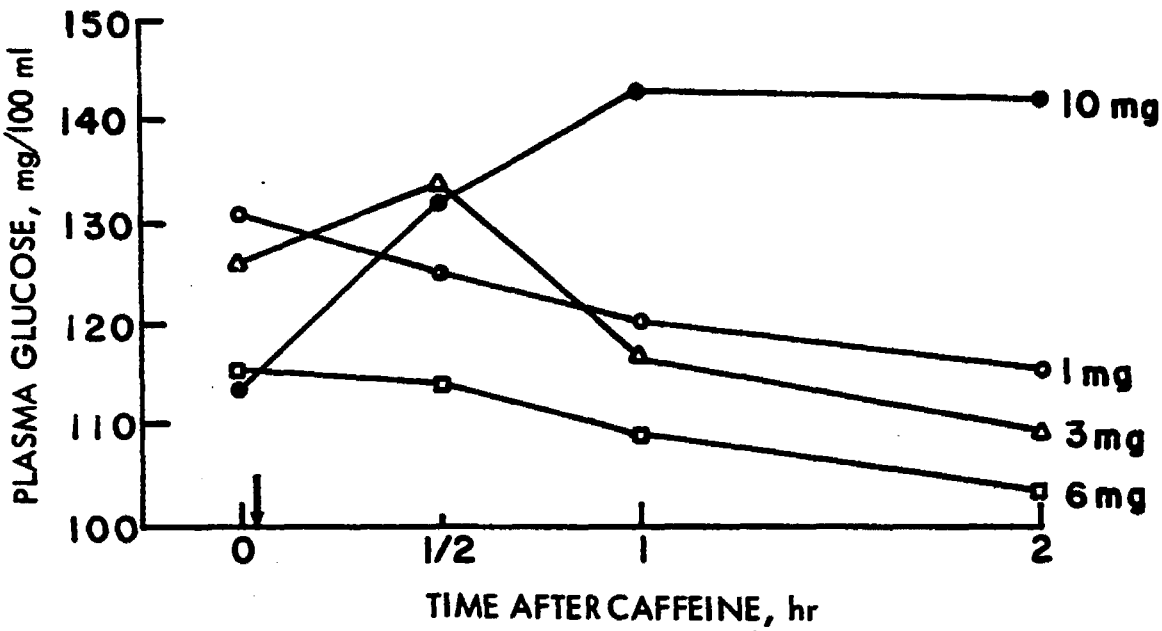

Fig. 23

Plasma glucose levels in fed rats after intraperitoneal injection of caffeine.

APPENDIX D

MISCELLANEOUS

TABLE 8

BODY WEIGHTS OF NORMAL RATS
FROM BIRTH TO 4 MONTHS

		Body Weight, g		
Age	Sex	Mean	SE	N
Birth	?	5.45	0.090	97
1 Mo	M	59.9	1.39	20
	F	55.9	1.10	22
2 Mo	M	179.5	3.27	22
	F	133.5	2.40	25
3 Mo	M	253.8	6.29	17
	F	172.9	2.32	21
4 Mo	M	277.8	7.92	12
	F	191.5	3.28	14

TABLE 9

TREATMENT OF DIABETIC COMA-INSULIN SHOCK CRISIS (SAMPLE)

Solutions: sol'n A--hypotonic bicarbonate (100 meq sodium, 60 meq chloride, 40 meq bicarbonate). sol'n B--5% glucose, 30 meq potassium as KCl.
Rat no 45: group 3A, rec. alloxan 1-16-69, rec. ascorbic acid (100 mg/kg, i.m.) daily since 2-1-69, mean plasma glucose level since alloxan is 440 mg/100 ml.

Date	Time	Description	Plasma Glucose (mg/100 ml)	Treatment
2-10-69	8:30 AM	gasping, ataxic, bloody nose and eyes, wt 85 g		
	4:00 PM	coma, gasping, near death		1.0 ml sol'n A; 1½ U insulin (protamine zinc), i.m.
	6:30 PM	much improved, conscious, not gasping for breath		
	7:00 PM	unconscious, convulsions, bulging eyes	64	1.0 ml sol'n B, i.m.
	7:40 PM	unconscious, breathing regular, no glucose in urine	35	2.0 ml sol'n B, i.m.
	8:40 PM	gasping, convulsions, eyes glassy, feet cold		0.5 ml 30% glucose, oral; 0.3 ml 30% glucose, i.m.
	9:15 PM	conscious, pawing with feet		

TABLE 9--Continued

Date	Time	Description	Plasma Glucose (mg/100 ml)	Treatment
2-10-69	9:30 PM	active during blood sample; dark, viscous blood	230	
	10:40 PM	unconscious, convulsions, stopped breathing		tube-to-mouth rescuscitation; 0.5 ml 30% glucose, oral; 0.5 ml 30% glucose, i.m. (breathing resumed)
	10:55 PM	conscious, much improved for the next hour		
2-11-69	1:00 AM	unconscious, glassy eyes		0.5 ml 30% glucose, oral; 0.5 ml 30% glucose, i.m.
	1:05 AM	conscious, much improved		
	3:30 AM	still conscious and well		
	6:30 AM	not breathing, cold feet, appears dead, but faint heartbeat		tube-to-mouth rescuscitation; 0.5 ml 30% glucose, oral; 1.0 ml 30% glucose, i.m.
	7:00 AM	breathing resumed after continuous rescitation		
	8:00 AM	still unconscious		0.5 ml 30% glucose, oral; 1.0 ml sol'n B, i.m.
	11:30 AM	deep coma, renal failure (no urine passed), condition hopeless, further fluid will drown		

TABLE 9--Continued

Date	Time	Description	Plasma Glucose (mg/100 ml)	Treatment
2-11-69	12:02 PM	shallow respiration, weak, near death, wt 81 g		½ U insulin (protamine-zinc); 1.0 ml sol'n A, i.m.; 0.8 ml 30% glucose, oral
	12:20 PM	semiconscious, breathing rapidly, may be drowning		
	2:10 PM	drop of urine--dark yellow, contains glucose, breathing shallow and fast, convulsions		
	2:55 PM	death: spleen atrophied and hyperemic, heart darkened on one side, lungs edematous		

TABLE 10

DESIGNATIONS AND TREATMENTS OF GROUPS

OADR and their Controls

Group Code	Group Class	Treatment of Mother
1	Normal	none
2A	Control	fasted, etherized, and injected with 0.9% saline on day 10 to 12 of pregnancy
2B	Control	same as 2A but with insulin treatment
2C	Control	alloxan on day 19 of pregnancy
2D	Control	alloxan after parturition
3A	OADR	alloxan before pregnancy
3B	OADR	same as 3A but with insulin treatment
3C	OADR	alloxan on day 3 of pregnancy
3D	OADR	alloxan on day 10 to 12 of pregnancy
3E	OADR	same as 3D but with insulin treatment

The same codes are used to designate both mothers and offspring. Unless mothers are specified, codes designate the offspring.

Special Diets

Group Code	Diet
4	high carbohydrate diet
5	high fat diet
6	high protein diet

Rats were weaned and placed on diets at 1 month of age.