Studies on Fatal Hypoglycemia in Axenic (Germfree) Piglets.* (29906)

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Spontaneous hypoglycemia has been described in newborn pigs(1). An intense hypoglycemia can be produced by fasting at room temperature for 24-48 hours. Carbohydrate depletion of the liver occurs at time of death. A definite relationship exists between age and time of onset of the acute hypoglycemia in the fasting piglets. There is a progressive increase in ability to tolerate starvation during the first week of life, and after 10 days, survival time is increased to 21 days(2). A decline in the general metabolism of the newborn pig, that is, heart rate, respiration, and body temperature, corresponded with the decline in blood sugar whether induced by fasting or by insulin administration to 48 hour piglets previously fed on the sow. This vital overall dependence upon the blood sugar disappears with age; *e.g.*, the response of weaned pigs to insulin-induced hypoglycemia is confined to neurological symptoms, convulsions(3).

During the course of experiments designed to propagate a strain of human respiratory virus in germfree piglets, a high morbidity and mortality was encountered in the piglets fed a diet consisting of autoclaved, homogenized milk without vitamin and mineral supplementation during the initial 48 hours of life. Survivors after 48 hours showed morbidity extending through the second week of life. Hypoglycemia of an intense degree was seen in animals manifesting a copious diarrhea, unstable body temperature, weakness, ataxia, and convulsions progressing to coma and death. The addition of 25 g of glucose per 1000 ml of autoclaved homogenized milk eliminated all mortality in the germfree piglets. Accordingly, preliminary experiments were undertaken to determine the site of primary failure and to seek an explanation for the failure of utilization of lactose in the diet by the germfree piglets. The general metabolic collapse observed suggested a failure of either glycogenolysis and/or gluconeogenesis.

Materials and methods. Axenic (germfree) piglets. Newborn hybrid piglets were obtained from the sow by hysterotomy between 110-113 days of gestation. Under CO_2 anesthesia, the uterus was extirpated and passed through a germicidal trap into a plastic isolator system designed by Trexler(4). A typical flexible plastic isolator for housing of a germfree pig was fiitted with 2 pairs of 18″ rubber gloves for manipulation of animals. The air filter was fitted with 3 wrappings of Owens-Corning Fiberglas PF 115 filter material, and the plastic structure was partially sustained by air pressure.

Sterility testing of the isolators for detection of bacteria, protozoa, and fungi was performed after the plan described by Wagner (5). All piglets except fasted controls were maintained either on autoclaved homogenized cow's milk or cow's milk with added glucose. In a single experiment, newborn piglets were associated with lactobacilli and fed a diet of sterile cow's milk without glucose supplementation. Tissues for analysis, liver, duodenum, and pancreas were promptly removed and cooled following terminal exsanguination of the animals. Samples of blood for the studies of glucose concentration, blood pH, serum calcium, and electrolytes were collected by internal jugular venipuncture.

Enzyme analyses. Lactase (β-galactosidase) activity was measured in the duodenum after using 0.1 M lactose as substrate(6). The entire duodenum was homogenized in phosphate buffer and the supernatant ana-

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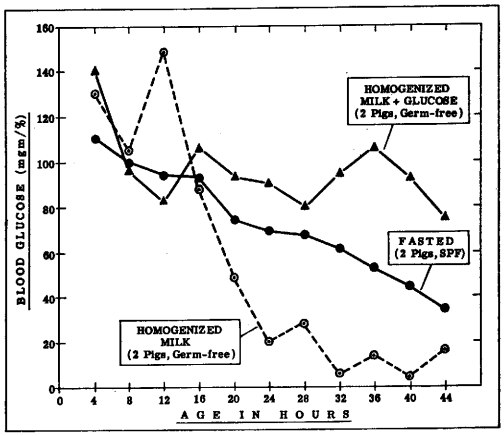
lyzed for enzyme activity. Data are expressed as micromoles lactose hydrolyzed per g wet tissue. Hepatic glucose-6-phosphatase and phosphorylase activities were measured by the phosphate release method(7,8) and units of enzymes have been expressed as micromoles inorganic phosphate released per g liver in 30 minutes.

Liver slices were prepared as previously described(7) and approximately 0.5 g of wet liver was incubated in 6.0 ml of a Ringer-bicarbonate buffer containing pyruvate-2-^{14}C, 40 mM. Liver slices were analyzed for glycogen and the medium for glucose after 90 minutes incubation. Glucose, and glycogen after hydrolysis to glucose, were isolated as the phenylosazone for assay of radioactivity. Pyruvate was isolated as the 2,4-dinitrophenyl hydrazone for determination of specific activity. All methods used were identical with those described by Ashmore *et al*(7). Results have been calculated as micromoles of labeled pyruvate recovered as glucose or glycogen(9).

Blood glucose concentration in samples of venous blood was determined by either the Nelson procedure on the Somogyi filtrate(10) or by the glucose oxidase method described by Huggett and Nixon(11). Heparin or oxalate was employed as the anticoagulant. Serum electrolytes were determined by the standard clinical microtechniques employing a Baird flame photometer and the Cotlove aminco chloride titrator. Nonprotein nitrogen determinations were performed by the Butler modification of the method of Folin and Svedberg(12). Blood pH values were determined by the method described by Leifheit(13).

Results. The influence of addition of glucose to the autoclaved cow's milk feedings was striking during the initial 44 hours of life (Fig. 1). The mean blood glucose values of paired germfree (GF) pigs fed sterile cow's milk with supplementary glucose was 95 mg% at 24 hours and 80 mg% at 44 hours of age. In contrast, paired germfree litter mates on feedings with sterile cow's milk with lactose as the carbohydrate source showed mean blood glucose concentrations of 20 mg% at 24 hours and 44 hours of age.



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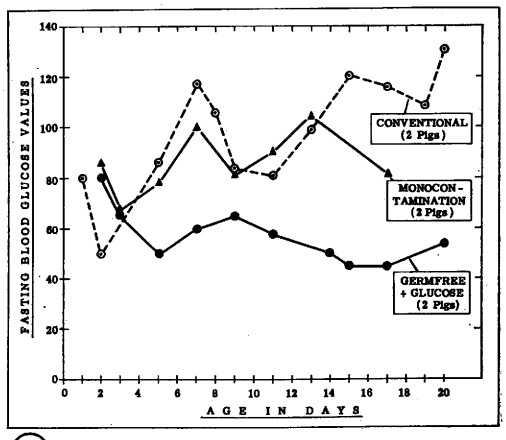

(2)

FIG. 1. Mean blood glucose values in paired animals during initial 44 hr of life.

FIG. 2. Mean blood glucose values in paired animals during initial 3 wk of life (12 hr fasting).

The latter animals showed easily recognized symptoms of hypoglycemia, *e.g.*, unstable body temperature, tachypnea, inability to stand, and seizures progressing to coma and death. Paired specific pathogen-free (SPF) pigs were fasted from birth in a similar environment with a slow, progressive decline in blood glucose concentration to hypoglycemic level (blood glucose less than 50 mg%) observed at 40 hours of age. The delay in onset of hypoglycemia in the fasted SPF animals at 96°F and the abrupt, marked decline in blood glucose concentration in animals fed unmodified milk feedings with lactose as the sole carbohydrate source suggested a failure of utilization of lactose due

to either faulty absorption or lack of hydrolysis of the carbohydrate. Another variable factor between the animals was the presence of bacteria in the gastrointestinal tract of the SPF animals. Conceivably, the presence of bacteria in the gastrointestinal tract of the newborn pig contributed favorably to the "homeostasis" of the blood glucose level. This hypothesis was tested in an *in vivo* experiment with litter mates of the same sex (males). Paired animals were maintained in the laboratory under germfree conditions, a germfree environment with the exception of association of the piglets with lactobacillus acidophilus at 4 hours of age, and standard laboratory conditions. All animals were fed sterile homogenized cow's milk supplemented with 25 g sterile glucose per 1000 ml milk. Animals were observed for an interval of 14-21 days. Blood glucose determinations were performed on alternate days after a 12-hour fasting period. The SPF pigs and the germfree animals associated with lactobacilli showed similar normoglycemic curves with instability of the blood glucose concentration during the initial 48 hours of life (Fig. 2). In contrast, the blood glucose values in the germfree pigs were persistently low and values in the upper range of hypoglycemia were frequently observed and correlated with the clinical symptoms of tremors and tachypnea.

Subsequently, *in vitro* experiments were designed for determination of the adequacy of liver glycogenolysis and intestinal lactase (galactosidase) in both the normoglycemic (SPF) and the hypoglycemic (GF) baby pigs. Hepatic glucose-6-phosphatase activity was measured and similar values were observed between SPF and GF animals. The mean glucose-6-phosphatase activity measured in 5 normoglycemic pigs was 372 $\pm$ 23 and 471 $\pm$ 94 in 6 hypoglycemic pigs. The activity of hepatic phosphorylase was found to be essentially the same in the normoglycemic and hypoglycemic pigs and in the same range as that observed in normal rat liver. Units of enzymes are reported as micromoles of inorganic phosphate released per g wet liver in 30 minutes.

A significant difference in intestinal lactase (galactosidase) activity was observed between normoglycemic and hypoglycemic pigs. Intestinal lactase activity was 23 micromoles/g/hour in normoglycemic (SPF) pigs and compares with a value of 3.4 micromoles/g/hour observed in the hypoglycemic (GF) pigs. Values represent the average of 3 determinations.

Comparison of the blood glucose values between the SPF and GF animals demonstrated the intense degree of hypoglycemia encountered in the newborn pigs. The range of blood glucose values in the germfree pigs was 2.5-34.2 mg% with a mean value of 11.65 mg% in 12 animals. In contrast, the mean blood glucose values in 11 SPF animals was 104.45 mg% with a range from 82 to 131 mg%.

Diarrhea with malnutrition was frequently observed in the germfree pigs between 2-7 days of age fed sterile cow's milk with lactose as the sole carbohydrate source. The symptom was reversed with addition of glucose to the milk feedings. Dehydration was severe in some animals. Serum electrolytes determinations including Na, K, CO_2, Cl and pH were done. The mean serum Na and Cl values were 148.2 and 106 mEq/l in the hypoglycemic animals fed unmodified sterile cow's milk (Table I). Normoglycemic SPF animals showed a serum Na of 135.9 mEq/l and Cl of 102.2 mEq/l. Urine and serum acetone determinations were negative. Stool analyses for lactic acid and volatile fatty acids were not done.

Hepatic metabolism was studied in liver slices from germfree glucose pigs, exhibiting normal blood sugar levels (88 mg/100 ml), fasted SPF pigs also normoglycemic (71 mg/100 ml) and hypoglycemic germfree milk fed pigs (blood sugar 27 mg/100 ml). Liver glycogen values in glucose fed pigs averaged 4.3% while in fasted and hypoglycemic pigs glycogen values were reduced to 0.2 to 0.3%. Net glucose production observed with liver slices reflects primarily the glucogen content of the liver (Table II). No significant difference in glucose production was observed between livers from normoglycemic fasted SPF pigs and hypoglycemic germfree pigs. Pyruvate incorporation into glucose and glycogen was used as a measure of gluconeo-

TABLE I. Mean Values of Serum Electrolytes, Calcium and Blood pH.

	No. of obs	Milliequivalents/liter					
		Na	K	CO_2	Cl	Ca*	pH
Hypoglycemia (fed)	7	148.2	6.7	22.3	106	4.9	7.3
Normal	8	135.9	7.1	25.4	102.2	—	7.45
Fasted (hypoglycemic)	2	120.0	4.5	20.0	81	4.5	7.25

* Leifheit method *J. Lab. & Clin. Med.*, 1956, 47:625.

TABLE II. Labeled Pyruvate Metabolism by Liver Slices from Normal (S.P.F.) and Hypoglycemic (Germfree) Piglets.

(Liver slices were incubated in a Ringer-bicarbonate medium with pyruvate-2-^{14}C 40mM. All values are recorded as micromoles per g wet liver per 90 min incubation.)

Animals	No. of obs	Net glucose produced	Pyruvate-2-^{14}C to	
			Glucose	Glycogen
Germfree glucose fed	3	90 ± 2*	34 ± 2	4.2 ± .7
Normal (S.P.F.) fasted	3	11.2 ± 1.5	11 ± 2.2	4.0 ± .5
Hypoglycemic (germfree)	6	10.5 ± 2.1	5.1 ± 1.7	1.2 ± .4

* Mean ± S.E.

genesis. The amount of pyruvate carbon incorporated into carbohydrate was reduced in the fasted and hypoglycemic pigs as compared with glucose fed animals. A further reduction was observed between fasted and hypoglycemic animals. These data would indicate that the hypoglycemic animals are capable of producing glucose, but perhaps at a reduced rate.

Discussion. Numerous theories have emerged to explain the hypoglycemia that occurs in the initial hours of life. These theories include the withdrawal from maternal hormones with delayed response to epinephrine, altered liver threshold for glucose, exhaustion of carbohydrate reserve, and insulin hypersensitivity and insulin hypersecretion.

Since glucose concentration in blood depends upon the balance between entry, hepatic and/or alimentary, and removal, utilization or storage, a comprehensive study of hypoglycemic syndrome necessarily involves several parameters. Our preliminary studies suggest a failure of alimentary entry in germfree piglets due to failure of hydrolysis of the lactose contained in the sterile cow's milk. Glucose feeding eliminated the mortality in the neonatal period. Preliminary studies also indicate a reduction in liver glycogen and reduced hepatic carbohydrate synthesis in hypoglycemic pigs. This would suggest that during the first 48 hours of life, the liver is unable to supply adequate glucose for maintenance of normoglycemic levels in the absence of glucose in the diet. Whether glucose feeding or lactobacillus contamination supplies factors other than adequate ingestible carbohydrate is not known.

The finding of reduced β-galactosidase activity in the duodenum of the germfree piglet at 48 hours is in variance with the observations of other investigators(14,15). However, the previous studies were performed with newborn piglets farrowed in the field and which were not maintained in a germfree environment. The rate of enzyme maturation in a newborn animal is a major consideration in the study of an animal devoid of stimulation from a microbial flora in the gut tract. Obviously, a developmental study of intestinal lactase activity is essential in germfree piglets for elucidation of the role of microorganisms in the induction and utilization of carbohydrates.

Furthermore, defects in lactose absorption resulting in diarrhea and malnutrition have been described in human infants and children(16-19).

Summary. 1. The newborn, germfree pig-

let soon develops a state of severe hypoglycemia with a corresponding decline in general metabolism unless glucose is present in the available diet. Our finding of a virtual absence of intestinal lactase activity (β-galactosidase) in the small intestine of axenic piglets suggests a failure of hydrolysis of lactose in the sterile, homogenized cow's milk feedings. 2. Our preliminary studies with germfree piglets reveal similar hepatic glucose-6-phosphatase activities between the normoglycemic (SPF) and hypoglycemic (GF) pigs. The activity of hepatic phosphorylase was also essentially the same for the normoglycemic (SPF) and hypoglycemic (GF) piglets and was in the range of activity usually found in normal rat liver. 3. Since blood glucose concentration depends upon the balance between entry, alimentary or hepatic, and removal, utilization and storage, a comprehensive study of the hypoglycemic syndrome in the piglet involves several parameters. Rate of glucose utilization and failure of hepatic glucose formation await further study.

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