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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The gluconeogenic capacity of single cell suspension of hepatocytes isolated from control and S. pneumoniae infected rats was determined. It is shown that (1) gluconeogenesis from pyruvate and lactate is reduced by 35-42% in hepatocytes isolated from agonal rats; (2) addition of ethanol fails to return glucose synthesis to control levels; and (3) the activity of a rate limiting gluconeogenic enzyme, phosphoenolpyruvate carboxykinase is reduced by 47% in livers of agonal rats. It is proposed that the decrease in PEPCK activity, rather than a lack of reducing equivalents may account for the agonal breakdown in glucose synthesis during severe infection.

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CATEGORY: Pathological Physiology

RUNNING TITLE: EFFECT OF INFECTION ON GLUCONEOGENESIS

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the author do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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Introduction. In acute infections complex metabolic adaptations in the host result in sufficient increased hepatic gluconeogenesis so as to maintain near normal levels of plasma glucose in spite of increased utilization of glucose by the body (1,2). In contrast, severe infections are characterized by the development of hypoglycemia during the agonal stages of the disease process as a result of an impaired capacity of the liver to synthesize glucose (3-5).

The search for specific biochemical lesions has yielded a number of proposals to account for the breakdown in glucose sythesis. For example, perfusion studies on isolated livers from S. pneumoniae infected rats led Curnow et al. to conclude that a lack of reducing equivalents (H^+) was the specific point of biochemical failure (6). More recently, an inhibition of the induction of phosphoenolpyruvate carboxykinase (PEPCK) has been proposed to account for the decline of blood glucose levels in mice infected with S. typhimurium (7).

To better delineate the mechanism(s) responsible for the agonal breakdown of carbohydrate synthesis we have determined the gluconeogenic capacity of single cell suspensions of hepatocytes isolated from control and S. pneumoniae infected rats. The use of isolated hepatocytes permits an evaluation of their gluconeogenic capacity in a controlled environment removed from humoral factors. In contrast to in vivo or liver persusion studies, kinetics of glucose synthesis from various substrates and effects of activators or inhibitors can be simultaneously evaluated in isolated hepatocytes by use of aliquots from the same cell population. This approach facilitates comparison and description of possible specific mechanisms. Our results indicate that as infection progresses the capacity of hepatocytes to synthesize carbohydrates

becomes progressively and inherently impaired. This inhibition cannot be reversed by addition of H^+. On the other hand, enzymatic measurements show significantly reduced levels of PEPCK in livers of agonal rats. A fall in the activity of this rate-limiting gluconeogenic enzyme would result in an impaired conversion of pyruvate to phosphoenolpyruvate (PEP) and account for the development of hypoglycemia during the agonal stages of severe infections.

Materials and Methods. Male, Sprague-Dawley rats weighing 200-250 g were housed in temperature- and light-controlled quarters. Rats had access to food and water ad libitum and were acclimatized to a 12-hr day-night cycle for 14 days prior to experimentation. Rectal temperatures were recorded at appropriate intervals with a Yellow Springs telethermometer. Experimental rats were inoculated subcutaneously with 3 to 30 x 10^4 Streptococcus pneumoniae, serotype I, A5 strain organisms. Both control and experimental rats were deprived of food for the 40 hr prior to being sacrificed for the collection of tissues. All rats were killed at 0830 hr, a time corresponding to the midpoint of the night cycle. This required that for temporal studies inoculation of rats with bacteria be made at different times of the day. This procedure was adopted because baseline values for gluconeogenesis were more consistent and reproducible if the time of sacrifice of the rat was uniformly controlled.

For isolation of hepatic parenchymal cells (HPC), the liver of Nembutal (5 mg/100 g body weight) anestetized rats was perfused in situ with a solution of 0.03% collagenase (Type II, Worthington Biochemicals), 1.5% fatty acid free bovine serum albumin (Fraction V,

Sigma Chem. Co.), and 50 µg/ml Gentamicin (Schering Corp.) in Ca^{++}-free Krebs-Henseleit bicarbonate (K-H) buffer, pH 7.4. The perfusion and subsequent processing of the liver was according to the method of Berry (8). HPC viability (typically 85-90% viable) was assessed by trypan blue exclusion and the concentration of cells adjusted to 3.5×10^7 HPC/ml in K-H buffer containing 50 µg/ml Gentamicin. For measurements of gluconeogenesis, hepatocytes (3.5×10^6) in 1.5 ml glucose-free K-H buffer were incubated in polypropylene tubes (17 x 100 mm) with either 15 nmoles sodium pyruvate or 15 nmoles lithium lactate and 3 nmoles NH_4Cl. When required ethanol was added to a final concentration of 2 mM. The reaction mixtures were gassed with a mixture of 95% O_2 and 5% CO_2, stoppered and incubated at 37° in a reciprocating water bath (160 oscillations/min). Reactions were stopped every 15 min for up to 60 min by addition of 0.15 ml of ice-cold 3 M $HClO_4$. The mixture was centrifuged at 7,500 RPM for 15 min in a Sorvall RC2B centrifuge and the resulting supernatant neutralized with solid $KHCO_3$. Following centrifugation the final supernatant was assayed for glucose content by the glucose oxidase method (9).

Five percent whole liver homogenates were prepared in 0.2M sucrose containing 20 mM triethanolamine, 1mM glutathione and 1mM EDTA at pH 7.5. Pyruvate carboxylase (PC) and Phosphoenolpyruvate carboxykinase were measured, respectively, on mitochondrial and supernatant fractions isolated by differential centrifugation of homogenates (10). Enzyme activities were measured by a radiochemical assay involving incorporation of (^{14}C)-bicarbonate into oxaloacetate essentially as described by Ballard and Hanson.

(10) except that the concentration of phosphoenolpyruvate in the carboxykinase assay was increased to 7.5mM. Protein concentrations were determined by an automated Lowry procedure using bovine serum albumin as standard (11).

Stock cultures of organisms were stored at -20° in brain heart infusion broth (Difco Laboratories, Detroit, Mich., USA) and subcultured by passage in mice at approximately 3 week intervals. For preparation of challenge inoculum, brain heart infusion broth containing 10% rabbit serum and 1% fresh defibrinated sheep blood were inoculated with stock culture material and incubated at 37° for 18 hr. The cultural growths were harvested, suspended in 0.1% tryptose- 0.5% NaCl solution and the concentration of organisms approximated by adjustment of turbidity, using a spectrophotometer at 400 nm. Viable counts were determined by inoculating blood agar plates with serially diluted portions.

Results. Inoculation of *S. pneumoniae* into rats resulted in a uniformly lethal infection. Infected rats became febrile within 16-20 hr, attained a rectal temperature greater than 39° by 40 hr and died after 54-60 hr.

Glucose production from 10 mM pyruvate or lactate by isolated hepatocytes isolated from control and experimental rats is given in Table I. Gluconeogenesis by hepatocytes from fasted control rats was similar to rates reported by others (12,13) and corresponded to 35 nmoles/min/10^7 cells from pyruvate and 33 nmoles/min/10^7 cells from lactate. The capacity of hepatocytes obtained from agonal rats, 40 hr after inoculation of bacteria, to synthesize glucose from these substrates was meaningfully reduced. The production of glucose from pyruvate and lactate in these cells decreased by 42% and 35%, respectively, when compared with control cells. Hepatocytes obtained from the early stages of the infection, 20 hr post-inoculation, did not differ from fasted controls in their capacity to synthesize glucose. Increasing cellular NADH levels through oxidation of ethanol added to hepatocytes from fasted controls caused a nearly 2 fold increase in glucose formation from pyruvate. In hepatocytes from agonal rats, ethanol failed to return glucose production to control levels. In fact, glucose production from pyruvate in the presence of ethanol in these cells was approximately 300 µg less than that measured in control cells under similar conditions. When lactate was used as substrate, addition of ethanol diminished glucose production by an approximately equivalent amount regardless the source of hepatocytes. This reduction results because conversion of lactate to pyruvate is inhibited due to competition for available NAD with the alcohol dehydrogenase reaction.

The relative specific activity of hepatic pyruvate carboxylase was not altered by infection (Fig. 1). In contrast, PEPCK activity was unchanged with respect to controls 20 hr after bacteria inoculation, but decreased by 47% in agonal rats as compared to uninfected rats fasted for 40 hr.

Discussion. The gluconeogenic capacity of hepatocytes isolated from febrile rats during the early stages of pneumococcal infection was not increased in comparison to cells from fasted controls. This observation is in apparent contradiction with *in vivo* studies demonstrating an increased formation of glucose during acute infections (14,15). The difference may be attributed to stimulation of gluconeogenesis *in vivo* by such humoral factors as hormones. On the other hand, the saturating levels of substrates employed in the present study may have obscured differences in gluconeogenic capacity which would have been expressed had lower concentration of substrates been employed. Although these and other possibilities can be experimentally evaluated using isolated hepatocytes, the primary intent of the present study was to identify factors contributing to development of hypoglycemia during the agonal stages of the disease process.

The use of fresh suspensions of isolated hepatocytes and of substrates whose entrance into liver cells are transport independent has served to demonstrate an inherent impairment of hepatocytes from agonal rats to synthesize glucose. Hence, impairment of gluconeogenesis *in vivo* may occur irrespective of possible modulatory effects of hormones or availability of gluconeogenic precursors (16). Since addition of reducing equivalents in the form of ethanol or use of lactate, a more reduced substrate than pyruvate, failed to appreciably alter the gluconeogenic capacity of hepatocytes from agonal rats, it is unlikely that a lack in H^+ is a factor in the impairment of glucose synthesis as previously proposed (6).

Synthesis of carbohydrates from pyruvate proceeds by reversal of several glycolytic reactions, but includes a number of reactions

unique to gluconeogenesis. For example, the conversion of pyruvate to PEP, considered to be a rate limiting step, involves the carboxylation of pyruvate to oxaloacetic acid (OAA) by PC and phosphorylation of OAA by PEPCK. The modulation of PEPCK activity represents an important mechanism for the regulation of gluconeogenesis in the liver by glucocorticoids (17). Hence a diminished rate of OAA conversion to PEP due to a 47% decrease in the PEPCK activity of agonal rats as compared to fasted controls could account for the impairment of gluconeogenesis during severe infections.

The cause of the depressed PEPCK activity, however, is unclear. PEPCK is one of a number of hepatic enzymes whose synthesis is enhanced by glucocorticoids (17). In acute infections an increased secretion, combined with a loss of the diurnal fall in plasma corticosteroids serves to elevate the concentration of plasma glucocorticoids (18). The rise in circulating glucocorticoids promotes a general anabolic response in the liver (19), increasing the activity of various hepatic enzymes and accelerating the rate and synthesis and secretion of acute-phase serum proteins. However, as an infectious illness persists or worsens the liver appears to become refractory to the stimulatory effects of glucocorticoids. The activity of glucocorticoid regulated enzymes such as tryptophan pyrrolase (19), tyrosine transaminase (20), glucose-6-phosphatase (21) and apparently PEPCK decreases despite increasing plasma glucocorticoid concentrations (18).

Factors responsible for the liver's refractoriness to the effect of glucocorticoids are not well defined. Based on studies with endotoxin and other agents active on the reticuloendothelial

system (RES), Agarwal (22) has advanced the concept that hepatic refractoriness of tryptophan pyrrolase and tyrosine transaminase to glucocorticoids induction is mediated by factor(s) released from RES cells in response to injection of microorganisms or noxious agents. In fact, a leukocytic mediator(s) has been purported to be released by activated phagocytes (23). This mediator(s) is thought to modify hepatic RNA and protein synthesis and manifests other aspects of the metabolic sequelae accompanying infections and inflammatory reactions (1). Further studies will be required, however, to determine whether the agonal depression of hepatic PEPCK activity is due to refractoriness to glucocorticoids as a result of mediator(s) secretion from RES cells. In addition, consideration also must be given to the possibility that impairment of gluconeogenesis in severe infection might result from altered kinetics or accumulation of specific inhibitors of PEPCK.

Summary. The gluconeogenic capacity of single cell suspension of hepatocytes idolated from control and *S. pneumoniae* infected rats was determined. It is shown that (1) gluconeogenesis from pyruvate and lactate is reduced by 35-42% in hepatocytes isolated from agonal rats; (2) addition of ethanol fails to return glucose synthesis to control levels; and (3) the activity of a rate limiting gluconeogenic enzyme, phosphoenolpyruvate carboxykinase is reduced by 47% in livers of agonal rats. It is proposed that the decrease in PEPCK activity, rather than a lack of reducing equivalents may account for the agonal breakdown in glucose synthesis during severe infections.

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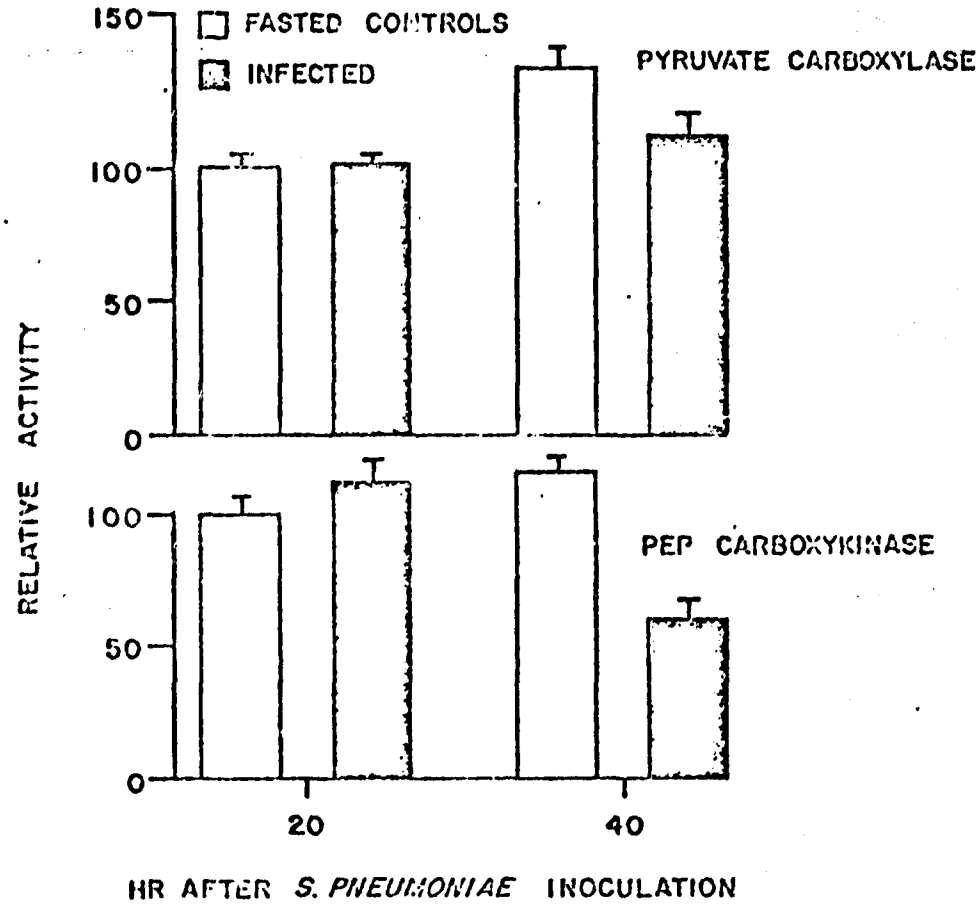
TABLE VI: GLUCONEOGENESIS FROM PYRUVATE OR LACTATE IN ISOLATED SUSPENSIONS OF HEPATOCYTES OBTAINED FROM CONTROL AND S. PNEUMOCOCCAL INFECTED RATS AFTER 40 HOURS OF FASTING

Substrate	μg GLUCOSE FORMED/45 MIN/10^7 CELLS (MEAN + S.E.)					
			Infected			
	Control	[N]	20 Hr	[N]	40 Hr	[N]
Pyruvate (10 mM)	284 ± 40	[5]	254 ± 22	[4]	164 ± 22	[6]
Pyruvate + ETOH (2 mM)	528 ± 45	[5]	373 ± 28	[4]	237 ± 32	[6]
Lactate[a] (10 mM)	264 ± 12	[8]	267 ± 13	[3]	171 ± 14	[5]
Lactate[a] + ETOH (2 mM)	182 ± 18	[6]	170 ± 22	[3]	73 ± 19	[5]

[a] 2 mM NH_4Cl added

Legends to Figures

Figure 1. Relative specific activity of liver pyruvate carboxylase and phosphenol pyruvate carboxykinase of S. pneumoniae infected and pair-fasted control rats. Values shown are the mean of six livers per group. Bars indicate the standard error of the mean for each group.


FASTED CONTROLS
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PEP CARBOXYKINASE
RELATIVE ACTIVITY
150
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HR AFTER S. PNEUMONIAE INOCULATION