INFLUENCE OF AMMONIUM ION, NORVALINE, AND ETHOXYZOLAMIDE*

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The probable involvement of hepatic carbamyl-P in the reciprocal relationship between hepatic ureagenesis and glycogenesis from glucose was explored. Isolated perfused liver preparations from 48-h fasted rats were employed. Moderate (9.2 mm) and relatively high levels of glucose (34 mm) were perfused. Inorganic glycogenesis, glucose-6-P, carbamyl-P, and citrulline levels, hepatic urea formation, and ureagenesis based upon perfusate urea levels were measured. Experimental probes selected to modify hepatic ureagenesis and carbamyl-P production and utilization included: (a) NH₄Cl, maintained at 5 mm by continuous infusion (NH₄⁺ is a substrate for carbamyl-P synthase I and glutamate dehydrogenase); (b) norvaline, an inhibitor of ornithine transcarbamylase which catalyzes the first committed step in the urea cycle; and (c) ethoxyzolamide, an inhibitor of carbamyl anhydrase which produces HCO₃⁻, an essential substrate for carbamyl-P synthase I. NH₄⁺ increased ureagenesis and decreased glycogenesis. The inclusion of norvaline with NH₄⁺ decreased ureagenesis and increased glycogenesis. Ethoxyzolamide with or without NH₄⁺ inhibited both ureagenesis and glycogenesis, and decreased the hepatic glucose-6-P level. Glycogenesis was greater at 34 mm than 9.2 mm glucose, increased in norvaline-containing preparations correlated with increased availability of carbamyl-P, and decreased when carbamyl-P formation was inhibited by ethoxyzolamide. Kinetic analysis indicated a Kₘ,Glue of 31 mm for glucose phosphorylation primary to glycogenesis. Glycogen formation via the "indirect pathway" (i.e., involving extrahepatic glycolysis, transport of lactate to the liver, and glycogen synthesis therefrom) was quantitatively insufficient to account for the observed glycogenesis. Glucokinase is contraindicated by the inverse relationship between hepatic glycogenesis and ATP availability in the ethoxyzolamide-treated preparations. In contrast, carbamyl-P-glucoPhosphotransferase activity of the glucose-6-phosphatase system has the characteristics to bridge hepatic ureagenesis and glycogenesis.

EXPERIMENTAL PROCEDURES

Materials—Fine chemicals of analytical reagent grade were from sources cited earlier (13). Ethoxyzolamide (6-ethoxyzolamide) was from Sigma. Solutions were prepared in doubly distilled water. Reagent solutions used in perfusates were adjusted to pH 7.4 with concentrated NaOH. Male albino rats of Sprague-Dawley strain (obtained from Harlan Sprague Dawley, Madison, WI), 55-60 days old and weighing between 180 and 250 g, served as liver donors. Red blood cell donors (13) were retired male breeders from the same source. Animals were housed in temperature-constant quarters under a 12-h light/12-h dark cycle. They were maintained on tap water and Purina Laboratory Chow, ad libitum. Liver donors were deprived of food for 48 h prior to removal of livers to deplete endogenous hepatic glycogen (14). Livers were removed between 7 and 8 a.m. and immediately perfused.

Liver Perfusions—Isolated livers were perfused at 37 ± 0.5 °C by the recycling system of Alvare and Nordlie (13). The perfusate consisted of rat erythrocytes suspended in Krebs-Ringer bicarbonate buffer (15) pH 7.4.
Livers were perfused as described in the text. The perfusion medium for all four groups contained approximately 9.2 mm glucose (see Table for exact values). As indicated, NH4Cl was maintained at 5 m throughout by initial addition and continuous infusion. Norvaline (10 mm) or ethoxyzolamide (3 mm) was included as indicated. All metabolite concentrations or rates of formation are expressed on a per g liver basis. Mean ± S.E. values are presented. N = 14 livers in Groups 1–3, and 10 livers in Group 4. Other details are given in the text.

### RESULTS

The results of perfusions with 9.2 and 34.0 mm glucose are presented in Tables I and II, respectively. Hepatic glycogen formation was determined in the 44-min period following addition of glucose plus norvaline, or ethoxyzolamide, as indicated. Appearance of urea in the perfusate was determined in the 34-min period following addition of NH4Cl. Hepatic metabolite levels were determined at the conclusion of perfusion.

#### Glucose

7.4: hematocrit 19–21%, which contained bovine serum albumin (3%, w/v) and heparin (6000 units/100 ml perfusate). This basic perfusate solution was warmed to 37 °C, the liver was attached, and the system was allowed to equilibrate for 10 min. A small (−0.5 g) liver biopsy sample was taken at this point for analysis of basal glycogen content.

Glucose (to 9.2 or 34 mm, along with either norvaline (to 10 mm), ethoxyzolamide (to 3 mm), or the vehicle (isotonic saline), was added and perfusion was continued for 10 min. At this point, a sample of perfusate was taken for basal urea analysis and immediately NH4Cl (as a concentrated solution) was added to the perfusate (to 5 mm), and continuous infusion of NH4Cl solution into the perfusate was begun to maintain the perfusate NH4+ concentration at 5 mm. Perfusion was continued for another 34 min, at which time a sample of perfusate for urea analysis was taken, the perfusion terminated, and the liver immediately removed for metabolite analysis. Citrulline, carbamyl-P, and urea were measured, beginning with frozen liver samples, exactly as described by Cohen et al. (19). The liver samples were homogenized with 300 mm mannitol in 2 mm HEPES buffer, pH 7.4 (19), just prior to assay. Standard recovery curves were run with each assay.

### Glucose

**TABLE I**

| Parameter measured | Group 1, unsupplemented | Group 2, norvaline-supplemented, NH4Cl-infused | Group 3, ethoxyzolamide-supplemented, NH4Cl-infused | Group 4, NH4Cl-infused | Perfusion group |
|-------------------|-------------------------|---------------------------------------------|---------------------------------------------------|-----------------------|----------------|
| Δ Hepatic glycogen (μmol glycogen units/g liver 44 min) | 7.14 ± 0.76^a | 4.45 ± 0.72^b | 0.13 ± 0.58^c | −2.34 ± 0.34^c |  | |
| Hepatic glucose-6-P (μmol/g) | 0.066 ± 0.010 | 0.112 ± 0.011 | 0.120 ± 0.010 | 0.019 ± 0.009 |  | |
| Hepatic urea (μmol/ml/g liver) | 1.84 ± 0.023 | 1.15 ± 0.34^b | 1.18 ± 0.048^b | 0.118 ± 0.021 | 0.118 ± 0.008^c |  | |
|  | | | | | |
| **Effects of Ammonium Ion**—The maintenance of NH4+ concentration at 5 mm by continuous infusion into the perfusate (Tables I and II, Group 2) produced a decrease in glycogenesis from glucose, and an increase in ureagenesis (as indicated by increases in perfusate urea accumulation and in hepatic urea and citrulline, an intermediate of the urea cycle, all compared with control livers (Group 1)). The effects were observed at both concentrations of glucose. Hepatic carbamyl-P was decreased with NH4+ addition (9.2 mm glucose) present. Hepatic glucose-6-P was unchanged. In supplemental studies (results not shown), 5 mm NH4Cl was shown to have no significant effect on glucokinase plus hexokinase or on carbamyl-P-glucose phosphotransferase or glucose-6-P phosphohydrolase activity of glucose-6-phosphatase.

### Norvaline

The effect of norvaline, a non-metabolizable analog of valine (6), was determined in perfused livers with NH4+ infusion into perfusate (Tables I and II, Group 3). Relative to perfused livers with NH4+ infusion (Group 2), norvaline increased glycogenesis and concomitantly decreased ureagenesis as indicated by lowered hepatic urea, hepatic citrulline, and perfusate urea values. Carbamyl-P increased with 34 mm glucose. Glucose-6-P levels were unchanged.

### Glucose Concentration

There was an increase in glycogenesis from glucose and in hepatic glucose-6-P levels.
with 34 mM (Table II) compared to 9.2 mM glucose (Table I) in perfusates with the unsupplemented, NH4Cl-supplemented, and norvaline-treated preparations. Ureagensis with NH4Cl-infusion was higher with 9.2 than 34 mM glucose present. Hepatic carbamyl-P levels were consistently higher with 9.2 than with 34 mM glucose (unsupplemented, NH4Cl-supplemented, and norvaline containing preparations).

The ratio of glycogenesis from glucose with 34 mM glucose to those with 9.2 mM glucose were calculated from data in Tables I and II; i.e. for each of Groups 1-3, the Δ hepatic glycogen values determined with 34 mM glucose (Table II) were divided by Δ hepatic glycogen values determined with 9.2 mM glucose (Table I). Ratios determined in this way were 3.1, 2.2, and 2.2 with unsupplemented, NH4Cl-supplemented, and norvaline-supplemented NH4Cl-infused, respectively. Assuming a "traditional" $K_m$,Glc value of 6.0 mM (25) for glucokinase, we calculate an activity ratio, i.e. glucokinase activity with 34 mM glucose/glucokinase activity with 9.2 mM glucose, of 1.4. The activity ratios calculated from data in Tables I and II, above, are greater than this value, indicating the involvement of an enzyme or enzymes with apparent $K_m$,Glc values considerably greater than 6 mM in hepatic glucose phosphorylation preliminary to glycogenesis. The $K_m$,Glc value for glucose as precursor for glycogenesis was calculated for the condition where carbamyl-P was most available for non-urea cycle functions, i.e. with NH4Cl plus norvaline present. This was done by substituting the two glucose concentration values and corresponding values for glycogenesis in Tables I and II into the simple Michaelis-Menten equation and solving simultaneously the two equations thus generated. A $K_m$,Glc value of 31 mM was obtained.

Comparison of Relative Ureagenic and Glycogenic Values Observed with 9 and 34 mM Glucose—The relative tendency for ureagensis and glycogenesis with 34 compared with 9 mM glucose, respectively, is made clear in Table III. Glycogenic values are from Tables I and II and ureagenic values are based on Δ perfusate urea values from Tables I and II, taking into account that the perfusate volume was 100 ml and adjusting for 44 min rather than 34 min to permit direct comparison with glycogenic rates. Summations of ureagensis plus glycogenesis are also presented in Table III, where ureagenic and glycogenic values, as percent of these summation values, are also included. This treatment presupposes that the major, competing uses of carbamyl-P are for ureagensis and for glucose phosphorylation preliminary to glycogenesis. Furthermore, it is based on the presumed use of one carbamyl-P molecule per turn of the urea cycle and/or one carbamyl-P molecule per glucosyl unit incorporated into glycogen under the influence of carbonic anhydrase (25). The assumption that ureagensis remains linear for at least 44 min in our system. Ureagensis, calculated as the percent of summation values, was consistently lower with 34 mM glucose than with 9.2 mM glucose. With both concentrations of glucose, the percent of summation values for ureagensis increased with NH4Cl infusion and decreased with norvaline, while the inverse responses were seen for glycogenesis. Only with the higher (34 mM) concentration of glucose, with NH4Cl limiting (Group 1), did glycogenesis exceed ureagensis.

Effects of Ethoxyzolamide—Ethoxyzolamide, an inhibitor of carbonic anhydrase (2, 7), was tested in perfused livers with NH4Cl infusion (Tables I and II, Group 4). It inhibited ureagensis as indicated by decreased hepatic urea and citrulline and a decreased perfusate urea accumulation. This is consistent with its role in blocking formation of HCO3- a substrate for carbonic anhydrase. Ethoxyzolamide and norvaline (Tables II, Group 4, and Table IV, Group 5) decreased perfusate urea accumulation. This is consistent with its role in blocking formation of HCO3- a substrate for carbonic anhydrase (25). The net glycogenesis was blocked and the hepatic glucose-6-P level was markedly lowered at both glucose concentrations.

The effects of ethoxyzolamide on net glycogenesis, ureagensis, and hepatic glucose-6-P level also were tested with control livers without NH4Cl infusion (Table IV, Group 5). For comparison, analogous values also are included in Table IV for unsupplemented control (Group 1) and ethoxyzolamide-supplemented NH4Cl-infused livers (Group 4). Ethoxyzolamide by itself markedly lowered net glycogenesis, ureagensis, and hepatic glucose-6-P (compared Table IV, Group 5 with Group 1). As is made clear from data in Table IV, the inclusion of NH4Cl infusion with ethoxyzolamide further decreased net glycogenesis. Glucose-6-P level was not further altered. Ureagensis, which was decreased by ethoxyzolamide by itself (Group 5 versus Group 1, Table IV), was unchanged from the unsupplemented control value when NH4Cl infusion was combined with ethoxyzolamide addition (compare Table IV, Groups 4 and 1). Glucose was initially 34 mM in studies in Table IV. Comparable results were obtained with 9.2 mM glucose (data not shown).
**TABLE III**

**Comparison of ureagenesis with glycogenesis**

Net glycogenesis values are \( \Delta \) hepatic glycogen values transposed from Tables I and II. Net urea formation values have been calculated from "\( \Delta \) perfusate urea" values in Tables I and II, as described in the text. These values are added together to give the Summation values. Values are expressed as micromole of urea formed per g 44 min or micromole of glucosyl units incorporated in the glycogen per g 44 min. The numbers in parentheses pertain to individual values immediately above, calculated as percent of the comparable summation value. These percent of summation values serve as an index of the relative tendency for ureagenesis or glycogenesis to occur under the defined condition. Mean \( x \) values are presented.

| Parameter | Perfusate glucose concentration |
|-----------|---------------------------------|
|           | 9.2 mm                          | 34 mm                          |
| Net urea formation |                                  |                                |
| Without NH\(_4\) infusion (Group 1) | 23.81 ± 2.98                    | 10.74 ± 2.71                   |
| With NH\(_4\) infusion (Group 2) | 68.97 ± 6.21                    | 44.13 ± 3.11                   |
| With NH\(_4\) infusion (Group 3) | 15.27 ± 2.71                    | 18.37 ± 5.82                   |
| With norvaline (Group 3) | 62 ± 13%                        | (62 ± 13%)                     |
| Net glycogenesis |                                  |                                |
| Without NH\(_4\) infusion (Group 1) | 7.14 ± 0.76 \( ^c \)            | 22.15 ± 1.79 \( ^c \)          |
| With NH\(_4\) infusion (Group 2) | 4.45 ± 0.72 \( ^c \)            | 9.61 ± 1.18 \( ^c \)           |
| With NH\(_4\) infusion (Group 3) | 9.13 ± 0.98 \( ^c \)            | 20.21 ± 2.71 \( ^c \)          |
| With norvaline (Group 3) | (57 ± 6%) \( ^c \)              | (52 ± 11%) \( ^c \)            |

\( ^a \) \( p < 0.05 \) for indicated value compared with value immediately to left.

\( ^b \) \( p < 0.05 \) for indicated value compared with comparable value immediately above.

\( ^c \) \( p < 0.05 \) for indicated glycogenic value compared with ureagenic value for the identical group (i.e. Group 1, Group 2, Group 3). Statistical analysis from other relevant comparisons between ureagenesis and glycogenesis among Groups 1, 2, and 3, and between these values with 9.2 mm compared with 34 mm glucose, have been made in Table I and II, and are not repeated here.

**TABLE IV**

**Effects of ethoxyzolamide, and of ethoxyzolamide plus ammonium ion, on glycogenesis, ureagenesis, and hepatic glucose-6-P in isolated livers from 48-h fasted rats perfused with 34 mm D-glucose**

Livers were perfused as described in the text. In Group 5, ethoxyzolamide (3 mm) was included. Data for Group 1 (unsupplemented) and Group 4 (ethoxyzolamide-supplemented, NH\(_4\)Cl-infused) are from Table II and are included here for direct comparative purpose. Mean ± S.E. values are presented. \( n = 8 \) livers for Groups 1 and 4 and 9 livers for Group 5. Initial glucose concentrations (mM) in perfusates were 34.71 ± 1.24, 35.42 ± 0.51, and 33.99 ± 0.36 for Groups 1, 5, and 4, respectively. Other details are as in Tables II and I.

| Parameter measured\( ^a \) | Perfusate group |
|-----------------------------|-----------------|
|                             | Group 1, unsupplemented | Group 5, ethoxyzolamide-supplemented | Group 4, ethoxyzolamide-supplemented, NH\(_4\)Cl-infused |
| \( \Delta \) Hepatic glycogen (\( \mu \)M glucosyl units/g 44 min) | 22.15 ± 1.79 | 3.72 ± 0.39 \( ^b \) | -1.50 ± 0.47 \( ^b,c \) |
| Hepatic glucose-6-P (\( \mu \)mol/g) | 0.172 ± 0.016 | 0.024 ± 0.003 \( ^b \) | 0.027 ± 0.005 \( ^b \) |
| \( \Delta \) Perfusate urea (\( \mu \)mol/ml 44 min) | 0.083 ± 0.021 | 0.032 ± 0.011 \( ^b \) | 0.060 ± 0.013 |

\( ^a \) Defined as in Footnote a, Table I.

\( ^b \) \( p < 0.05 \) for indicated group with control value.

\( ^c \) \( p < 0.05 \) for indicated compared with ethoxyzolamide value.

**Effects of Ethoxyzolamide on Glucokinase plus Hexokinase, Carbamyl-P-Glucose Phosphotransferase, and Glucose-6-P Phosphohydrolase**—The effects of 3 mm ethoxyzolamide upon the enzymes of ATP-dependent glucose phosphorylation (glucokinase plus hexokinase), carbamyl-P-dependent glucose phosphorylation (carbamyl-P-glucose phosphotransferase), and glucose-6-P phosphohydrolase, were studied, in vitro (Table V). No significant effect was noted with the glucokinase plus hexokinase, or with glucose-6-P phosphophydrolysis activity of the glucose-6-phosphatase system. Carbamyl-P-glucose phosphotransferase activity of the latter system was inhibited less than 10%.

**Potential Contributions to Glycogenesis by the "Indirect Pathway"**—Current concepts of hepatic glycogenesis (25, 26) include both the "direct" formation of glycogen from glucose through a series of enzyme-catalyzed reactions in the liver, and formation via the indirect pathway involving glucose phosphorylation in some peripheral tissue, glycolysis to lactate there, return of lactate to the liver, and ultimate formation of glycogen via hepatic glycogenogenesis from this lactate. The compound 3-mercaptopicolinic acid inhibits this process by inhibiting the enzyme phosphoenolpyruvate carboxykinase which is integral to the glycogenogenesis process (1, 25, 26). Here, the possible contribution to glycogenesis from glucose via the indirect pathway must be considered, because 3-mercaptopicolinic acid was not included (1, 25), and a glycolytic tissue, erythrocytes, was present. This was done using Sukalski and Nordlie’s (28) estimate of the maximum rate of glycolysis by red blood cells of 0.76 \( \mu \)mol of glucose/min/100 ml of perfusate with the perfusion system unattached to livers, independent of glucose concentration. Making the extreme assumption that the entire amount of glucose fluxing through the red blood cells is used for glycogen synthesis via glycogenogenesis and the indirect pathway, it follows that 0.76 \( \mu \)mol of glucose/min × 44 min (the period of perfusion used here), i.e. a maximum of 33.4 \( \mu \)mol of glucosyl units/44 min-perfusate system, could be due to the indirect pathway.

Glycogenic values from Tables I and II were multiplied by
mean weights of livers, in grams, in each perfusion group to express the data on a "μmol of glucosyl groups/44 min-perfusate system." The maximum contribution, as percentage of total net glycogen formation which may be explained based on the indirect pathway, was calculated by dividing 33.4 μmol of glucosyl units/44 min-perfusate system by the individual values for net glycogen formation expressed "per 44 min-perfusate system" and multiplying by 100 to convert to percent.

From the analysis (shown in Table VI), it is apparent that the maximum percent contribution of the indirect pathway to total net glycogen formation is considerably lower with 34 mM glucose than with 9.2 mM glucose. Also, at either glucose concentration, the addition of NH₄Cl increases the percent maximum contribution by the indirect pathway to glycogen formation, and the addition of norvaline to NH₄Cl-infused preparations lowers the percentage of possible contribution of the indirect pathway to total net glycogen formation.

**DISCUSSION**

Here, ureagenesis and glycogenesis respond inversely to NH₄Cl; without and with norvaline, in the isolated perfused liver. Similar inverse responses were seen previously with glutamine and proline (1). We have suggested that the inverse character of these responses is due to the competitive requirements for carbamyl-P in the biosynthesis of urea and glycogen (1). A further analysis of this, and other possible mechanisms underlying this reciprocity of response of ureagenesis and glycogenesis, is given here. A metabolic scheme depicting the various pathways and effects of the varied parameters considered is presented in Fig. 1.

**Table V**

Effects of ethoxylzolamide on glucokinase plus hexokinase, carbamyl-P-glucose, phosphotransferase, and glucose-6-P phosphohydrolase activities, in vitro

| Enzyme activity measured | Observed activity | Without inhibitor | Plus 3 mM ethoxylzolamide | Enzyme activity measured | Observed activity | Without inhibitor | Plus 3 mM ethoxylzolamide |
|---------------------------|-------------------|-------------------|---------------------------|---------------------------|-------------------|-------------------|---------------------------|
| Glucokinase + hexokinase | nmol/min/mg cytosolic protein | 11.00 ± 0.83 | 9.73 ± 0.55 |
| p = 0.25 | | | |
| Carbamyl-P-glucose phosphotransferase, p < 0.05 | nmol/min/mg microsomal protein | 7.98 ± 0.16 | 7.20 ± 0.14 |
| Glucose-6-P phosphohydrolase, p > 0.5 | | 66.8 ± 2.0 | 66.7 ± 0.5 |

**Table VI**

Maximum contributions of "indirect pathway" to glycogen formation

| Parameter | Group 1, un-supplemented | Group 2, NH₄Cl-infused | Group 3, NH₄Cl-infused, norvaline supplemented |
|-----------|--------------------------|------------------------|---------------------------------------------|
| 9.2 mM Glucose | | | |
| Glycogenesis (μmol of glucosyl units/g-44 min) | 7.14 ± 0.76 | 4.45 ± 0.72 | 9.13 ± 0.98 |
| Liver weight (g) | 7.00 ± 0.21 | 6.84 ± 0.16 | 6.79 ± 0.18 |
| Glycogenesis (μmol of glucosyl units/perfusion system-44 min) | 49.98 | 30.44 | 61.99 |
| Relative maximum contribution by indirect pathway (%) | 67% | 100% | 54% |
| 34 mM Glucose | | | |
| Glycogenesis (μmol of glucosyl units/g-44 min) | 22.15 ± 1.79 | 9.61 ± 1.18 | 20.21 ± 2.71 |
| Liver weight (g) | 5.73 ± 0.26 | 5.83 ± 0.15 | 6.12 ± 0.19 |
| Glycogenesis (μmol of glucosyl units/perfusion system-44 min) | 126.9 | 55.06 | 123.7 |
| Relative maximum contribution by indirect pathway (%) | 26% | 61% | 27% |
**NH₄⁺, Norvaline, Ethoxyzolamide, and Hepatic Glycogenesis**

![Diagram of metabolic pathways](image)

**Fig. 1. Some metabolic pathways of carbamyl-P synthesis, ureagenesis, and glycogenesis in liver, with an indication of sites of action of NH₄⁺, norvaline, and ethoxyzolamide. Bold numbers in braces identify specific reactions. The stippled bar represents the inner mitochondrial membrane. Enzymes and reactions above the membrane are intramitochondrial (27-31). Enzymes and reactions of the urea cycle below the membrane are cytosolic (25); carbamyl-P:glucose phosphotransferase activity of the glucose-6-phosphatase system is associated with the endoplasmic reticulum (9). NH₄Cl supplied to the system provides the nitrogen directly for carbamyl-P synthesis via carbamyl-P synthase I (11). Glutamate may be produced from NH₄⁺ and Krebs/trichloroacetic acid cycle intermediates (i.e. α-ketoglutarate/oxaloacetate) via mitochondrial glutamate dehydrogenase (4), (2). Glutamate thus produced may serve as substrate for aspartate formation via transamination (4), (5), (12). Resultant L-aspartate functions as a substrate in the argininosuccinate synthase-catalyzed reaction, (14), thus accelerating ureagenesis and hence enhanced utilization of carbamyl-P (32). The addition of norvaline in the presence of NH₄⁺ inhibits (-1 the utilization of carbamyl-P by the ornithine transcarbamylase-catalyzed reaction (5, 6), (5), thus making carbamyl-P more available for other reactions, e.g. carbamyl-P:glucose phosphotransferase activity of the glucose-6-phosphatase system, (6). Ethoxyzolamide inhibits (-) carbonic anhydrase (2, 7), (7), leading to diminished production of HCO₃⁻ (2, 7), an essential substrate for carbamyl-P synthase I (3) (see (11)). As a result of this diminution of carbamyl-P, both ureagenesis (see (15)) and glycogenesis from glucose (see (16)) are lowered.

Although the forgoing considerations implicate the relative availability of carbamyl-P to, and utilization by, the ureagenic and glycogenic processes, they do not by themselves unequivocally establish carbamyl-P:glucose phosphotransferase activity as a major determinant in hepatic glycogenesis from glucose. Because 2 ATP molecules are required for the synthesis of one carbamyl-P molecule via carbamyl-P synthase I (Reaction 1, Fig. 1), an enhanced synthesis and utilization of carbamyl-P in response to added NH₄⁺ could result in a lessened glycogenesis from glucose (as observed) if glucokinase were a major determinant even under 48-h fasted conditions where the level of this enzyme was diminished by more than two-thirds (13). The noted effects of norvaline in increasing net glycogenesis from glucose thus also might be explained on this same basis. Norvaline inhibits ornithine transcarbamylase and hence ureagenesis, lowering the utilization of carbamyl-P by the urea cycle. This reduced utilization of carbamyl-P in turn would place a lessened demand on ATP for its synthesis, making more ATP available for glucose phosphorylation via residual glucokinase thus leading to increased glycogenesis from glucose. However, considerations of varied glucose concentrations and studies with ethoxyzolamide serve to obviate this contention (see below).
**Effects of Varied Glucose Concentration**—Because of possible ambiguities of interpretation of data to this point, responses of the various parameters measured with 34 mM glucose were compared with those same parameters determined with 9.2 mM glucose (Table II compared with Table I). Consistent with our hypothesis that carbamyl-P-glucose phosphotransferase activity of the glucose-6-phosphatase system contributes importantly to glycolysis from glucose are the observations that carbamyl-P levels were consistently lower in the livers perfused with 34 mM glucose than with 9.2 mM glucose in the un-supplemented, NH4Cl-infused, and norvaline-treated, NH4Cl-infused livers. These observations correlate with the direct utilization of carbamyl-P as a substrate for glucose phosphorylation by the relatively high \( K_m, \text{Glc} \) (about 50 mM; Ref. 37); phosphotransferase activity of the glucose-6-phosphatase system in hepatic glucose phosphorylation was preliminary to glycolysis. More carbamyl-P would be used for this purpose when the glucose concentration is high.

The second approach, also consistent with this hypothesis, involves a more formal kinetic analysis. With livers continuously infused with a source of nitrogen, i.e., NH4Cl, in the presence of the urea cycle inhibitor norvaline, an apparent \( K_m, \text{Glc} \) value of 31 mM was determined for glucose as a precursor of glycogen. This value is consistent with the apparent \( K_m, \text{Glc} \) value for carbamyl-P-glucose phosphotransferase activity of the glucose-6-phosphatase system determined at low near-physiological carbamyl-P concentration (50 mM; Ref. 37), and with the value of 30 mM recently observed for glucose as precursor of glycogen in isolated perfused livers with 3-mercaptopicolinic acid added to inhibit glucose-6-P rehydrolysis (38). The \( K_m, \text{Glc} \) values determined with intact livers no doubt contain input not only from carbamyl-P-glucose phosphotransferase, but from residual glucokinase and hexokinase also. The \( K_m, \text{Glc} \) values for these last two activities are considerably lower than the carbamyl-P-glucose phosphotransferase.

The recent discovery by van Schaftingen (39) of a regulatory protein affecting glucokinase3 complicates the interpretation of observed \( K_m, \text{Glc} \) values, however. The present authors concur with van Schaftingen (39) that "it is presently impossible to calculate the precise contribution of glucokinase to the formation of glucose-6-P from measurements of this enzyme made in cell-free systems." We must thus conclude that any interpretation from the apparent \( K_m, \text{Glc} \) determined here is equivocal.

The comparative rates of ureagenesis and glycolysis, relative to one another, are made clear both by the absolute values and percent of summation values in Table III. Glycogen is formed at the expense of urea synthesis by increasing the level of glucose from 9 to 34 mM, and by inhibiting (with norvaline) the intramitochondrial usage of carbamyl-P in the ornithine transcarbamylase reaction. Ureagenesis is markedly favored at the expense of glycolysis when NH4Cl is infused. The summation of ureagenesis plus glycolysis is very similar with 9 and 34 mM glucose, without additions to the system (Control, Group 1). However, when norvaline is present to inhibit the use of carbamyl-P in the ornithine transcarbamylase reaction, the total glycolysis plus residual ureagenesis is much higher with 34 than with 9 mM glucose, consistent with an increase in glycolysis concentration-dependent use of available carbamyl-P (e.g., for glucose phosphorylation preliminary to glycolysis) under these conditions.

**Effects of Ethoxyzolamide**—Ethoxyzolamide and related compounds have been shown (2, 7) to inhibit liver carbonic anhydrase V, thereby lowering the production of HCO3−, an essential substrate for carbamyl-P synthase I, resulting in lowered rates of ureagenesis (7). Data presented in Tables I, II, and IV confirm these earlier findings. Furthermore, if carbamyl-P is important for hepatic glucose phosphorylation preliminary to glycolysis, then addition of ethoxyzolamide to NH4Cl-perfused livers should also diminish glycolysis. Both hepatic glucose-6-P concentration and glycolysis were markedly lowered by ethoxyzolamide included either with (Tables I and II) or without NH4Cl infusion (Table IV). Because HCO3− is required for the essential gluconeogenic enzyme pyruvate carboxylase (2, 42), a part of the inhibitory effect of ethoxyzolamide on hepatic glycolysis could be through inhibition of the indirect pathway. Note, however, that maximally the indirect pathway may contribute only a portion of observed net glycolysis, since the second pathway for glycogen formation (Table VI). The marked decrease in hepatic glucose-6-P level seen with ethoxyzolamide (Tables I, II, and IV) also is inconsistent with an effect of ethoxyzolamide manifest exclusively upon the indirect pathway of glycolysis.

The decrease in hepatic glucose-6-P concentration as well as decreased glycolysis from glucose in ethoxyzolamide-perfused livers supports carbamyl-P-dependent glucose phosphorylation as an important determinant in hepatic glycolysis from glucose. Both decrease, respectively, with an ethoxyzolamide-induced decrease in carbamyl-P production as evidenced also by reduced ureagenesis (Tables I and II). Further consistent with these observations in Table IV are the observations showing that: (a) ethoxyzolamide added to un-supplemented control livers significantly lowers rates of both net glycolysis and ureagenesis, as well as reduces levels of hepatic glucose-6-P; and (b) the presence of NH4Cl, which enhances ureagenesis and lowers net glycolysis when included by itself, further lowered net glycolysis and abolished the ethoxyzolamide-related decrease in ureagenesis when included with ethoxyzolamide. We interpret these observations to indicate that ethoxyzolamide, through its inhibition of bicarbonate production by carbonic anhydrase, inhibits glycolysis by carbamyl-P-glucose phosphotransferase-initiated glucose phosphorylation and by the indirect pathway, and that the addition of NH4Cl further lowers net glycolysis by directing any residual carbamyl-P to the urea cycle at the expense of glucose phosphorylation by carbamyl-P-glucose phosphotransferase activity.

The ambiguity introduced in the earlier interpretation of our data by the possible impact of altered carbamyl-P synthesis upon availability of ATP for glucose phosphorylation by glucokinase is obviated by our observations with ethoxyzolamide. Here, carbamyl-P synthesis is lowered as demonstrated by markedly reduced ureagenesis. Under this circumstance, using the previous line of reasoning, there would be a much lesser drain on cellular ATP for carbamyl-P synthesis. Yet, while ATP will be more available, glycolysis from glucose is markedly reduced.

Had an effect of ethoxyzolamide been to inhibit glycogen synthase or modify one of its regulatory enzymes, or to activate glycogen phosphorylase or modify one of its regulatory enzymes (any of which could explain the ethoxyzolamide-related decrease in net glycolysis), then the hepatic glucose-6-P level should have increased in response to ethoxyzolamide. In fact, the hepatic glucose-6-P level dropped extensively in ethoxyzolamide-treated livers. This observed decrease in liver glucose-6-P thus logically resulted from an ethoxyzolamide-associated
decrease in hepatic glucose phosphorylation.

Levels of carbonic anhydrase V (2, 43), carbamyl-P synthase I (44), and carbamyl-P-glucose phosphotransferase (13) all increase in fasting while glucokinase decreases (13). Carbamyl-P-glucose phosphotransferase was but little affected and residual glucokinase plus hexokinase was unaffected by ethoxyzolamide (Table V). It follows, then, that inhibition of glycogenesis from glucose by ethoxyzolamide results in a significant part from the reduced availability of carbamyl-P for glucose phosphorylation. These observations, and the generally observed, positive correlation of glycogenesis with the availability of hepatic carbamyl-P for non-ureagenic processes described above and elsewhere (1), support carbamyl-P-glucose phosphotransferase activity of the glucose-6-phosphatase system (5, 9, 37) as a participant in hepatic glycogenesis, under our exaggerated conditions. The feasibility of function of this activity in situ appears to be thus established. Further studies will be necessary to verify the significance of these observations under more physiologic conditions.

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