Effects of Glucagon on General Protein Degradation and Synthesis in Perfused Rat Liver*

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SUMMARY

The direct influence of glucagon on rates of valine incorporation into and release from protein was assessed in the perfused rat liver. Glucagon alone significantly increased net valine release, an effect which was ascribed largely to the stimulation of proteolysis. The magnitude of this increase in absolute terms remained virtually unchanged in the presence of an amino acid mixture that suppressed proteolysis. The increase was also obtained in the presence of sufficient cycloheximide to inhibit valine incorporation by 93%. In addition, free intracellular valine was elevated relative to external values over an 8-fold range of perfusate valine concentration.

Rates of valine incorporation into liver protein were reduced by glucagon alone to a small but significant degree. Of interest, however, was the finding that the inhibition was potentiated by additions of amino acids. In the presence of a complete amino acid mixture, simulating the composition of plasma amino acids at 10 times their normal concentrations, glucagon strongly depressed valine incorporation, and the rate was comparable to the value obtained previously when plasma amino acids at 10 times their normal concentrations, glucagon strongly depressed valine incorporation, and the rate was comparable to the value obtained previously when the amino acid supply was severely limited. These findings suggest that the turnover of liver proteins is regulated by glucagon at sites of both protein degradation and synthesis.

The stimulation of net liver protein catabolism by glucagon, both in the intact animal (2-4) and in isolated tissue preparations (5), is well established. This over-all effect is manifested by increased urea formation (5-8), a reduction of liver protein content (9), and an increase in the net release of amino acids from protein (10). While it is reasonable to assume that processes specifically concerned with protein degradation are involved in this effect of glucagon, the possibility was suggested in earlier studies that general protein synthesis is controlled on a moment to moment basis in liver (11-13). For this reason we have examined this net degradative response in more detail and have attempted to differentiate between regulatory effects either on general protein synthesis or on proteolysis.

In this report we show the existence of two sites of control in the perfused rat liver and conclude that, in addition to stimulating proteolysis, glucagon is capable of inhibiting general protein biosynthesis.

MATERIALS AND METHODS

Animals—Liver donors were male rats of the Lewis strain obtained from Microbiological Associates. They were allowed free access to Purina lab chow and water; lighting was maintained from 7 a.m. until 7 p.m. daily. The weights of the animals at the time of perfusion ranged from 125 to 140 g. In some perfusion experiments liver protein was previously labeled in vivo with L-[1-14C]-valine given intraperitoneally 18 and 4 hours before perfusion (13, 14).

Liver Perfusion—Livers were perfused in situ by a technique described earlier (14-16). The perfusion medium consisted of washed sheep red cells at a concentration of 0.27 (v/v), suspended in a solution of Kreh-Ringer bicarbonate buffer (17) and 4% bovine albumin (Fraction V, Pentex). The initial volume of medium was 45 to 50 ml, of which 5 ml were washed through the liver and lost at the start of perfusion, before the return flow from the liver to the perfusion reservoir was established. In experiments with previously labeled livers, the washout loss was increased to 10 ml. Following perfusion, livers were frozen rapidly between aluminum blocks previously cooled in liquid nitrogen (18). Perfusate plasma and liver samples for analysis were stored at -34°.

On the morning of each day's experiment, glucagon (Lot 238-204 B-167-1, Lilly Research Laboratories) was dissolved in a small volume of dilute HCl containing 0.2% phenol and 1.6% glycerol (pH 2.6). Prior to administration, the glucagon stock solution was further diluted with a solution of 0.85% NaCl and 0.5% bovine albumin (Fraction V, Pentex). Following the addition of a priming dose equivalent to 15 min of infusion, glucagon was delivered into the medium at a rate of 1 or 10 μg (in 0.22 ml) per hour. The two amino acid mixtures employed in this study were reported in detail earlier (15). The first (Mixture 1) contained 14 amino acids and its composition was patterned after an ovalbumin hydrolysate. However, leucine, isoleucine, and valine were not included since these amino acids normally accumulate during perfusion. Furthermore, the absence of added valine made it feasible to assess net alterations in protein during perfusion (13). Tyrosine was also omitted because of its low solubility, but its lack was compensated for by an increase of phenylalanine (15). After the addition of a priming dose of 264 amoles, the mixture was infused into the perfusion medium at a rate of 264 amoles per hour. This infusion rate was shown earlier to inhibit proteolysis maximally (13).

The second (Mixture 2) contained 20 amino acids and its composition simulated that of rat plasma (19). Mixture 2 was added to the medium as a single dose in a quantity calculated to raise the

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initial perfusate plasma amino acid levels to 10 times those of the normal rat (10).

Cycloheximide was added to the perfusion medium in a solution of 0.85% NaCl. Its final concentration in the medium was 1.8 × 10^{-4} M. We established in separate control studies that this level of cycloheximide inhibited the incorporation of valine into liver protein by 93%.

Analytical Methods—The method for the chromatographic isolation and determination of valine and [14C]valine in samples of perfusate plasma and liver has been detailed earlier (14). For the determination of radioactivity, the aqueous samples were dissolved in a solution of 0.5% 2,5-diphenyloxazole and 10% naphthalene in dioxan. Liver protein was recovered as described earlier (14), dissolved in NCS reagent (Amer sham-Searle) (16), and then added to a toluene-based scintillation mixture (16). Radioactivity of all samples was determined with a Beckman model LS-150 liquid scintillation spectrometer. Results were corrected for quenching by external standards and expressed as disintegrations per min.

Calculations and Expression of Results—The total net accumulation of free valine or [14C]valine during perfusion was calculated by methods described in an earlier report (16). Livers that were previously labeled with L-[l-14C]valine in vivo were utilized in various ways indicated below for measurements of the net release of valine from liver protein, proteolysis, and the over-all rate of protein synthesis. Two general methods were employed for normalizing differences between livers in the amount of radioactivity incorporated during the labeling period. In the first (see Fig. 1 and Table I), the rates of accumulation of free [14C]valine during perfusion were expressed as a percentage of the total quantity of label in liver protein at the start of perfusion. The details of this procedure have been described previously (13, 14). The second, which was dealt with in an earlier paper (16), utilizes the specific radioactivity of released valine as a basis for normalization. This method was used in the experiments of Fig. 2.

The rationale for the second procedure is based on the observation that the specific radioactivity of valine released from previously labeled livers is relatively constant after the first 60 min of perfusion and is equal in intracellular and extracellular valine pools (16). It is thus possible to calculate the net release of valine from liver protein by dividing the rate of accumulation of [14C]valine, expressed as disintegrations per min per 100-g rat, by the specific activity of valine in perfusate plasma at 60 min of perfusion. The addition of unlabeled valine (15 mM) increases the rate of [14C]valine accumulation by interfering competitively with its reincorporation into protein. Since the accumulation of label under these conditions directly reflects protein synthesis, this process can be assessed quantitatively by dividing the rate of [14C]valine accumulation, measured in the presence of excess unlabeled valine (15 mM), by the specific activity of free valine in a perfusate sample taken immediately before the addition of the carrier (16). The difference between the rate of protein synthesis (valine outflow from protein) and the net rate of valine release represents the over-all rate of protein synthesis (valine inflow). Close agreement was found previously between the latter indirect assessment of synthesis and rates of valine incorporation (16).

The rate of valine incorporation into protein, as an estimate of protein synthesis, was determined by dividing the amount of radioactivity which accumulated in liver protein from 60 to 80 min of perfusion by the specific radioactivity of perfusate plasma valine at the end of the experiment. In these experiments L-[l-14C]valine plus sufficient carrier valine to raise the perfusate valine to 15 mM was added at 80 min. This procedure is known to minimize intracellular dilution of the label from perfusion (16). The incorporation of valine into liver protein over a 20-min period reflects total protein synthesis since within this period only negligible amounts of incorporated label are lost from the liver by the secretion of plasma proteins (14).

With the exception of the results in Fig. 1 and Table I (see above) all rates of valine flow into and out of liver protein were expressed as micromoles per min per 100 g (body weight) of the liver donor animal. Results are given as means ± 1 S.E., the latter depicted in figures by vertical bars. The significance of error was evaluated by the Student's t distribution of the number of experiments are shown in parentheses.

Chemicals—Crystalline glucagon was kindly supplied by Dr. William W. Bromer of the Lilly Research Laboratories, Indianapolis, Ind. The amino acids were obtained from Schwarz-Mann and Calbiochem; cycloheximide from Nutritional Biochemicals, Inc.; and bovine albumin (Pen tex, Fraction V) from Miles Laboratories, Inc. Resins were obtained from Bio-Rad Laboratories; L-[1-14C]valine (22.4 mCi per mmole) from New England Nuclear Corp. All other reagents were the highest commercial grade obtainable.

RESULTS

Effect of Glucagon and Amino Acids on Net Release of Label from Livers Previously Labeled in Vivo with L-[l-14C]Valine—In earlier studies we have shown that the time-course of accumulation of label in the medium is linear between 60 and 180 min of control perfusion and is not critically dependent on the period of administration of the label before perfusion (14, 16). Since valine is neither synthesized nor degraded appreciably, the released label can be assumed to reflect directly the net loss of valine residues from the pool of valine in peptide linkage (13, 14). As shown in Fig. 1, the continuous administration of glucagon doubled the net rate of [14C]valine release during perfusion of previously labeled livers. This result confirms a number of investigations which have demonstrated stimulatory effects of glucagon on net protein breakdown (9, 10, 20, 21).

It is of interest to note that the onset of this effect was delayed by nearly 20 min. This contrasts rather strikingly with other effects of glucagon, such as the stimulation of glycogenolysis and glucose release (22) which appear rapidly.

In addition to increasing net protein degradation, glucagon also enhances the oxidative utilization of several amino acids which provide carbon for gluconeogenesis and other pathways (10). Since amino acids may inversely affect proteolysis (13), it is possible that a reduction in some amino acid pools might have contributed to the glucagon effect. As is shown in Table I, the coinadministration of an amino acid mixture (Mixture 1, see "Materials and Methods") in an amount known to suppress proteolysis maximally under similar conditions (13) failed to...
rather than explained by a stimulatory action on proteolysis or by an inhibition of protein synthesis (14). Previous experiments have disclosed no effects of the valine pool size on the response to glucagon in terms of the net quantity of valine released. Whether larger quantities of the mixture would have reduced the effect is not known, but it seems reasonable to conclude that the stimulatory effect of glucagon was not mediated directly by a depletion of metabolizable amino acids.

Stimulation of Proteolysis by Glucagon—Since, in the foregoing experiments, it is known that a large fraction of the labeled valine released by protein degradation is reincorporated into protein (14), the effect of glucagon in Fig. 1 can be explained either by a stimulatory action on proteolysis or by an inhibition of over-all protein biosynthesis, or possibly by a combination of the two. To distinguish among these alternatives, proteolysis was independently assessed in previously labeled livers by the addition of a large pool of unlabeled valine (15 mM) which served to dilute the label and thereby minimize its reincorporation (14). Previous studies have disclosed no effects of the valine pool on control rates of protein turnover (16).

The results in Fig. 2 reveal a 140% stimulation by glucagon in the net rate of release of valine from protein (measured in the absence of carrier valine), a finding which substantiates the effect shown in Fig. 1. The method for calculating the rates in Fig. 2 was based on the assumption that the specific activity of valine released during the course of perfusion is constant both in the presence and absence of glucagon (see "Materials and Methods"). This assumption has been verified under control conditions (16), and data in Table II clearly show the lack of effect of glucagon on the specific activity of valine under present conditions of perfusion.

The stimulation of the rate of valine release in Fig. 2 appears to be a consequence largely of an increase in the rate of proteolysis. Virtually no difference was noted between the control and glucagon-treated groups in rates of valine inflow or over-all protein biosynthesis. It should be emphasized, however, that, since these estimates were computed from the mean differences between rates of net valine release and proteolysis, small effects might be obscured.

Effect of Glucagon on Intracellular and Extracellular Valine Pools—In earlier experiments the relationship between intracellular and extracellular valine concentration was shown to be linear over an external concentration range of 0.3 to 15 mM (16).

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Table I

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<tr>
<th>Additions</th>
<th>Net % Initial 14C-Labeled Valine Release</th>
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<tbody>
<tr>
<td>A. None</td>
<td>0.03 ± 0.15 (6)</td>
</tr>
<tr>
<td>B. Glucagon (1 μg/hr)</td>
<td>3.04 ± 0.33 (6)</td>
</tr>
<tr>
<td>C. Amino acid Mixture 1</td>
<td>0.19 ± 0.08 (4)</td>
</tr>
<tr>
<td>D. Glucagon (1 μg/hr) + amino acid Mixture 1</td>
<td>1.06 ± 0.08 (4)</td>
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* Tests of significance: B versus A, p < 0.025; D versus C, p < 0.001.
valine is raised, the net inward transport of valine increases, and the ratio of intracellular to extracellular specific activity approaches unity. Thus the diluting effect of proteolysis on the specific activity of the total intracellular pool becomes smaller as transport is increased.

From the observed effect of glucagon on proteolysis, we expected the ratios of valine specific activity in the glucagon experiments to be lower than corresponding control values. By using equations from an earlier report (16), we calculated, for example, that the specific activity ratio at 0.6 mm valine would have been reduced 28% by glucagon. However, the ratios of intracellular to extracellular specific activity were similar in glucagon-treated and control livers over the valine concentration range shown in Fig. 3. This might be explained in part by the small size of the expected effect. It should also be pointed out, however, that glucagon stimulates the inward transport of α-aminoisobutyric acid and 1-aminoacycloheptaneacryboxylic acid in liver slices (23), the perfused rat liver (10, 24), and the rat liver in vivo (25). If valine transport is similarly affected by glucagon, the effect of the increase in proteolysis on the specific activity ratio would be diminished.

Effects of Glucagon, Cycloheximide, and Epinephrine on Valine Release—The direct stimulation of proteolysis by glucagon was independently confirmed in experiments in which valine incorporation was inhibited 93% by the addition of cycloheximide at a concentration of 1.8 × 10^{-6} M. Owing to this inhibition, we expected valine release to increase to 0.278 pmole per min in the presence of cycloheximide. This was particularly marked in those experiments which received complete amino acid mixtures (Mixture 2). The increase in valine incorporation over the mean control value with Mixture 2 alone has been reported previously and appears to represent the correction of a moderate deficiency in the availability of amino acids (13).

We have no explanation at present for the reduction in the rate of valine incorporation with the administration of glucagon and Mixture 2 to values significantly lower than those obtained with glucagon alone. The observed rate of 0.134 pmole per min is comparable to rates obtained earlier, when the initial supply of amino acids was severely limited (13). In these experiments,
Glucagon and amino acids were administered to the perfused livers beginning 30 min after the start of perfusion. L-Valine was added, together with L-[1-14C]valine, at 60 min to bring the perfusate concentration to 15 mM. The livers were frozen at 80 min for analysis of valine incorporation into protein, and the specific activity of medium valine was also determined. The results are followed by the standard error of the mean, and the number of experiments is shown in parentheses.

<table>
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<tr>
<th>Conditions</th>
<th>Valine incorporation (µmoles/min/100-g rat)</th>
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<tbody>
<tr>
<td>A. Control (no additions)</td>
<td>0.187 ± 0.004 (7)</td>
</tr>
<tr>
<td>B. Glucagon</td>
<td>0.167 ± 0.001 (4)</td>
</tr>
<tr>
<td>C. Amino acid Mixture 1</td>
<td>0.186 ± 0.004 (5)</td>
</tr>
<tr>
<td>D. Amino acid Mixture 1 + glucagon</td>
<td>0.136 ± 0.006 (5)</td>
</tr>
<tr>
<td>E. Amino acid Mixture 2</td>
<td>0.211 ± 0.004 (7)</td>
</tr>
<tr>
<td>F. Amino acid Mixture 2 + glucagon</td>
<td>0.134 ± 0.008 (7)</td>
</tr>
</tbody>
</table>

Tests of significance: B versus A, p < 0.005; D versus C, p < 0.005; F versus E, p < 0.001; B versus F, p < 0.005.

The incorporation of valine, measured during the first 20 min of perfusion in the absence of added amino acids, averaged 0.144 µmole per min. The mean rate increased spontaneously to 0.181 µmole per min by 60 to 80 min as additional amino acids became available from the breakdown of endogenous protein (13).

**DISCUSSION**

**Measurement of Protein Degradation and Synthesis**—The release of [14C]valine from livers previously labeled with L-[1-14C]valine in vivo appears to offer a useful method for the measurement of proteolysis in isolated liver experiments. As we mentioned earlier, this mode of labeling provides an intracellular source of free valine of relatively constant specific activity (13). Since the total quantity of free valine in the system is small at the start of the perfusion, the specific activity of free valine that accumulates intracellularly and extracellularly during the course of perfusion will be nearly the same, and rates of proteolysis can be calculated conveniently from the specific activity of perfusate valine determined prior to the addition of 15 mM valine carrier (16). It follows from these considerations that any differences between animals in the quantity of labeling will affect both the quantity of label released and the specific activity of valine to the same degree.

We have assumed that the addition of 15 mM valine dilutes the newly appearing label generated by proteolysis sufficiently so that its reutilization by protein synthesis is negligible. The same assumption applies in a reverse manner to the direct assessment of valine incorporation into liver protein, where the addition of labeled 15 mM valine would reduce dilution by unlabeled valine arising from proteolysis. The assumption is reasonable, we believe, in view of the agreement that we obtained earlier in estimates of over-all protein turnover between the present method (16) and the guanidyl arginine labeling method of Swick and Handa (32). Reutilization of the guanidyl carbon of arginine is thought to be small, owing to the rapid loss of this carbon by the action of arginase. The additional possibility that the high concentration of carrier valine altered rates of proteolysis in some unknown way seems unlikely, since net rates of leucine and isoleucine accumulation from 60 to 180 min of perfusion were unaffected by the addition of 15 mM valine (16).

**Regulation of Proteolysis**—The results of the present study, together with earlier reports (13, 14) indicate that general protein degradation in the perfused rat liver can be increased in two ways. First, it has been shown to increase spontaneously shortly after the onset of perfusion in the absence of any additions to the medium. This elevation can be suppressed either by the administration of insulin (14) or mixtures of amino acids (13), and it occurs in the apparent absence of an increase in the tissue level of adenosine 3':5'-monophosphate (33). It is probable that this effect is mediated by a reduction or loss of the inhibition normally supplied by amino acids and insulin in vivo (13, 14). The second mode of stimulation is represented by the effects of glucagon and epinephrine in this study. These were undoubtedly associated with an increase in the tissue level of the cyclic nucleotide and were probably mediated by it.

The mechanism of proteolysis is unknown at present. However, a few clues have emerged which suggest that the lysosomal system may be involved in the regulatory effects of glucagon, insulin, and amino acids (34–36). We have noted that lysosomes from control perfused livers are more sensitive to osmotic shock and exhibit increased density on equilibrium density centrifugation, alterations which were interpreted as indicative of lysosomal enlargement (35–37). These physical changes were prevented or reversed during perfusion by the administration of insulin or amino acids, or both (35, 36), and appear to correlate with rates of proteolysis measured either in the intact perfused rat liver or in 0.325 m sucrose homogenates prepared from perfused livers (34).

The fact that glucagon is a potent inducer of autophagic vacuoles in rat liver (38) and is also known to enhance the sensitivity of lysosomes to osmotic and mechanical shock (37) may be of significance in connection with the foregoing observations. It is conceivable that the lysosomal system directly participates in the final phase of endogenous protein breakdown and that the above regulatory effects of glucagon, insulin and amino acids on general proteolysis simply reflect this participation. This notion, of course, does not take into account such important questions as how protein substrates are selected for degradation or how the association between the substrates and the lysosomal proteases is obtained. It is also possible that the participation of the lysosomal system is but a single component of over-all proteolysis as measured by valine turnover in this and earlier studies.

**Effects of Glucagon on Liver Protein Synthesis**—The inhibition of valine incorporation by glucagon in the present study contrasts with a number of inductive or stimulatory effects of glucagon on liver enzymes (39–45). It is our belief that this difference represents two separate effects. This belief is supported in part by the fact that the inhibitory effect of glucagon is strongly potentiated by additions of physiological mixtures of amino acids. Such a potentiation of an inhibitory effect certainly would not explain increases of specific enzymes by glucagon, except perhaps when expressed on a basis of total cellular protein. Thus, if the synthesis of many proteins were depressed by glucagon, unaffected specific enzymes might appear to increase as total liver protein decreased. However, this effect would take considerable time to develop, and would not explain the rapid increases of certain liver enzymes following glucagon treatment in the perfused rat liver (see for example Ref. 41). Some of these specific effects may be explained by absolute increases in the rates of enzyme synthesis. We do not know the additional quantity of valine involved in the induction of these enzymes, but it is reasonable to assume that it was obscured by...
larger inhibitory effects on other proteins. While this report was in preparation, Tavill et al. (46) reported that glucagon inhibited albumin synthesis in the perfused rat liver.

Any explanation for the reduction in synthesis by glucagon would likely fall into one of two general categories: (a) a diminution in the availability of amino acid substrate and (b) inhibition at one or more steps in the sequence of amino acid activation and peptide formation. Although glucagon is known to decrease some pools of amino acids such as alanine, glutamate, glycine, and phenylalanine as a consequence of the enhancement of gluconeogenesis (10), it is doubtful that the decreases were rate-limiting for protein synthesis since the additions of amino acids reported here did not ameliorate the inhibition; rather, they intensified the effect.

Consideration of Category b raises an important question concerning a possible regulatory effect of amino acids on protein synthesis (11, 47). In one study by Jefferson and Korner (11), which utilized the perfused rat liver, polysomes were reported to disaggregate into subunits spontaneously in the absence of added amino acids. Mixtures similar to our Mixture 2 prevented their disaggregation and increased the rate of incorporation of [3H]phenylalanine into protein, both in the intact liver and in isolated ribosomes. By using absorbance measurements for monitoring RNA, we confirmed the stabilizing effect of Mixture 2 reported by Jefferson and Korner (11) and have shown further that Mixture 1 is considerably less effective, although not devoid of activity. Of interest was the additional observation that glucagon failed to reverse the stabilizing effect of Mixture 2. The present findings thus suggest that conditions which are optimal for valine incorporation are also necessary for the full expression of the inhibitory effect of glucagon on protein synthesis. If true, the locus of inhibition may reside at steps beyond the initiation and may involve factors concerned with the elongation or termination of peptide chains.

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