STUDIES ON THE STORAGE OF GLYCINE IN TISSUES OF NORMAL AND ADRENALECTOMIZED RATS

by

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INTRODUCTION

Prior to 1938, it was generally assumed that diets rich in carbohydrate were most efficient in promoting the deposition and maintenance of carbohydrate stores in the animal body. In that year, Mirski et al (1) reported that rats fed a high protein diet (meat or 70% casein) for 5 to 12 days exhibited, at the end of a 24 hour fast, liver glycogen levels of one per cent, while rats prefed a high carbohydrate diet showed liver glycogen levels of less than 0.1 per cent. They further showed that the animals prefed a high protein diet were better able to maintain and regain liver glycogen stores when subjected to stresses of various types. This was termed the "protein effect". In the course of their investigations, these workers also showed that the "protein effect" was abolished by adrenalectomy. The effect of prefeeding diets high in protein upon liver glycogen levels following a 24 hour fast has been confirmed by Guest (2) and by Newburgher and Brown (3).

Somewhat later in this laboratory, investigations were undertaken to determine to what extent individual amino acids, fed as part of an otherwise satisfactory synthetic diet, were capable of exerting the "protein effect". It was found (4) that l-leucine and l-glutamic acid produced no "protein effect" under the experimental conditions employed, dl-alanine showed a slight effect, while glycine fed at levels of 10 to 15 per cent exerted a marked "protein effect". For example, rats fed for two days on a diet containing 10 to 15 per cent glycine showed liver glycogen levels exceeding 1 per cent following a 24 hour fast, while rats fed on a diet similar in

composition except for the omission of glycine, had liver glycogen levels of about 0.3 per cent. The "protein effect" of glycine was also abolished by adrenalectomy (4).

Effects such as those just described could be accounted for on the basis of increased glyconeogenesis, decreased glycogenolysis, or a combination of the two. Inasmuch as the data obtained up to that point was insufficient to give any indication as to which mechanism was involved, the reaction of animals prefed on these diets to a different type of stress was studied. A heavy dose of insulin (12 units/kg.) was the stress chosen. Insulin was administered after an 8 hour fast and its action was allowed to continue for 5 hours (5). At the end of that time, the glycine-fed animals showed 2.5 times as much muscle glycogen, over 10 times as much liver glycogen, and a much less marked depression of the blood sugar than did the control-fed animals. This notably higher blood sugar level in the glycine-fed animals following insulin appeared to exclude reduced glycogenolysis as an explanation for the enhanced carbohydrate reserves noted previously.

The extent of body storage of glycine was of interest, then, for two reasons. First, it was necessary to know whether or not a rat could store enough glycine in his body during the feeding period to account for the excess carbohydrate found after insulin or after a 24 hour fast, assuming the glycine to be completely converted to carbohydrate. As a corollary to this, it was desired to know if there were any differences in the storage of glycine in normal and adrenalectomized animals. No references were found in the literature

dealing with this type of investigation on adrenalectomized animals. As for normal animals, one of the earlier pieces of work along this line, using rats as the experimental animal, was carried out by Luck (6). In this work it was shown that glycine administered to rats by gavage increased the amino nitrogen content of the systemic blood, was absorbed to a considerable extent by the liver, and was the only one of the amino acids studied to become concentrated to any appreciable extent in skeletal muscle. Alanine increased the amino nitrogen content of the systemic blood in the same measure as did glycine, but produced no significant change in the amino nitrogen content of liver or muscle. A similar picture with respect to plasma amino nitrogen following administration of glycine and alanine has been demonstrated in rabbits (7,8) and in chicks (9). An increase in the plasma levels of glycine and alanine in humans following ingestion of those amino acids has been demonstrated by Christensen and associates (10). More recently, Friedberg and Greenberg reported a series of investigations on the partition in the blood and tissues of rats of intravenously administered amino acids (11). Their results were essentially the same as those of Luck (6) with, in addition, the demonstration that kidney was also very active in absorbing injected amino acids.

None of these data were applicable to the problem at hand, however, for several reasons. To begin with, in all the investigations cited above, the animals were fasted for variable periods before administration of the amino acid which was given by itself either intravenously or by gavage. In contrast, our dietary regimen involved feeding of the amino acid with the diet over a period of 36

to 48 hours with the fasting period following the last meal. Also, in the previous work, the total dose of glycine per kilo received by their animals was much less than that consumed by rats fed according to our plan. Still a third difference is found in the length of time allowed to elapse between the final dose of amino acid and the time the tissue samples were taken. Johnston and Lewis (8) took blood samples 12 and usually 30 hours after the amino acid was given, but this work was confined to blood levels and rabbits rather than rats were the experimental animal. In the other work mentioned, the maximum time interval allowed between amino acid administration and sacrifice was 6 1/2 hours (7). Our interest lay in glycine levels after fasts of 8 hours or more.

In all these previous studies, except those of Christensen et al (10), the conclusions were based upon increases in the α-amino nitrogen of the tissues after ingestion of a given amino acid, the assumption being that any increase in the α-amino nitrogen of a tissue resulted from accumulation of the amino acid administered. Indications that this may not be a valid assumption are found in the recent work of Hier (12) and of Christensen and associates (10, 13). Using methods of analysis specific for the amino acid in question, Hier (12) was able to show, in dogs, that, although the ingestion of some amino acids simply caused a rise in the plasma level of that particular amino acid, the ingestion of others, such as leucine, isoleucine, and methionine, while causing an increase in the plasma level of the respective amino acid, caused a fall in the plasma levels of certain other amino acids. Still a third effect upon the level of plasma

amino acids was found in the case of phenylalanine. Ingestion of that amino acid resulted in an increase in its own plasma level and simultaneously increased the plasma level of tyrosine.

In their studies of humans, the Christensen groups showed that the ingestion of glycine or alanine not only increased the plasma concentrations of those amino acids, but also increased the plasma concentrations of other amino acids (10). Christensen and associates then extended their investigation of this subject using guinea pigs as the experimental animal (13). They found that the ingestion of those amino acids which produced a high concentration in the organism resulted in increased concentrations of other amino acids in the extracellular fluid and, in turn, in the plasma. When glutamic acid was fed, however, the plasma amino acids were diminished and the intracellular amino acids increased. The latter results were interpreted by Christensen and associates as indicating that glutamic acid in some way promotes the absorption by the cell of the amino acids presented to it in the extracellular fluid. From these results it becomes apparent that changes in the α-amino nitrogen content of a tissue following the ingestion of an amino acid represents the resultant of the concentration changes of many amino acids, not simply the change in concentration of the one consumed with all the other concentrations remaining constant. Therefore, any conclusions with regard to the storage of a particular amino acid based upon changes in α-amino nitrogen cannot be considered strictly accurate. To get a true picture of the accumulation of a certain amino acid in the various tissues, the quantitative determination must be made by a

method specific for that amino acid.

Most of the work mentioned above dealt with amino acids in the free state only. The possibility that an amino acid consumed might be incorporated into a compound for storage could not be overlooked. Christensen and associates (10) were unable to demonstrate increases in glycine and alanine peptides in the plasma following ingestion of those two amino acids, although an increase in the conjugated amino acid nitrogen of tungstic acid filtrates of plasma followed glycine consumption. In their later work on guinea pigs (13), the Christensen group found tissues, especially muscle, to contain appreciable amounts of non-protein amino acid conjugates other than glutathione. No further statement was made with regard to the composition of these conjugates. There was also the possibility that an amino acid might be stored by incorporation into protein. No reports of studies of this nature were found in the literature. Inasmuch, then, as the previous work on the accumulation of free glycine in the tissues was not applicable to the problem at hand and the data with regard to its incorporation into compounds was very scanty, the work reported here was undertaken.

PROBLEM

Normal, adrenalectomized, and hypophysectomized rats were prefed on diets containing added glycine. After an 8 hour or 24 hour fast, the plasma proteins of blood, and the free glycine, total glycine, and total nitrogen contents of blood, liver, muscle, intestine, and kidney were determined. In short, the storage of glycine in these

tissues in free and combined form was investigated.

EXPERIMENTAL

Animals: Adult rats (200-300 grams) of the Sprague-Dawley strain were the experimental animals. Males were used for the most part. On the few occasions when females were used, no significant deviations from the results found with males were noted. Experiments were carried out on pairs of animals, one receiving control ration and the other glycine ration.

Rations: Colony rats were maintained on Purina Laboratory Chow. The compositions of the control ration and the glycine ration are shown below. It is obvious that in the experimental ration, the

	Control Ration	10% Glycine Ration
Casein	16%	16%
Brewer's Yeast (Squibb)	10%	10%
Salt mixture (14)	5%	5%
Cod liver oil	2%	2%
Wesson oil	5%	5%
Dextrose	8%	8%
Dextrin	54%	44%
Glycine	0%	10%

glycine was substituted for an equal weight of dextrin. All the other constituents of the two diets were present in identical quantities. Thus a 10 per cent glycine diet is one containing 10 per

cent of glycine and only 44 per cent of dextrin. Animals on the control diet were pair-fed with animals on the glycine diet. Food consumption was 10 to 12 grams per animal per day.

Methods: Animals were sacrificed by decapitation. Blood was collected in an oxalated beaker at the time of sacrifice.

Glycine was determined by the method of Alexander, Landwehr, and Seligman (15). A brief description of the techniques follows:

Preparation of tissues for free glycine determination: Free glycine in blood was determined on a 5 ml. aliquot of an iron filtrate (16). Samples of the other tissues were transfered from the animal to a weighed test tube and ground with a modified Potter-Elvehjem tissue homogenizer. After transferring the tissue homogenates to a 50 ml. centrifuge tube, the protein was precipitated by the addition of 2 ml. of 20 per cent trichloracetic acid and the precipitate was removed by centrifugation. The precipitate was resuspended in water, another ml. of 20 per cent trichloracetic acid was added and the suspension was again centrifuged. The supernatants from these two centrifugations were combined in 100 ml. volumetric flasks and the solutions were diluted to the mark with water. Glycine was determined in 5 ml. aliquots of the latter solutions.

Preparation of tissues for total glycine determination: For the determination of total glycine in blood, 0.5 ml. of whole blood was laked in 11.2 ml. of water and 3.3 ml. of concentrated H_2SO_4 were added to make the acidity of the solution about 8N. The other tissues were taken from the animal, minced, and transferred to weighed flasks containing 15 ml. of 8N H_2SO_4. All tissues were then hydro-

FIGURE 1

Stotz type all-glass still used in the determination of glycine.

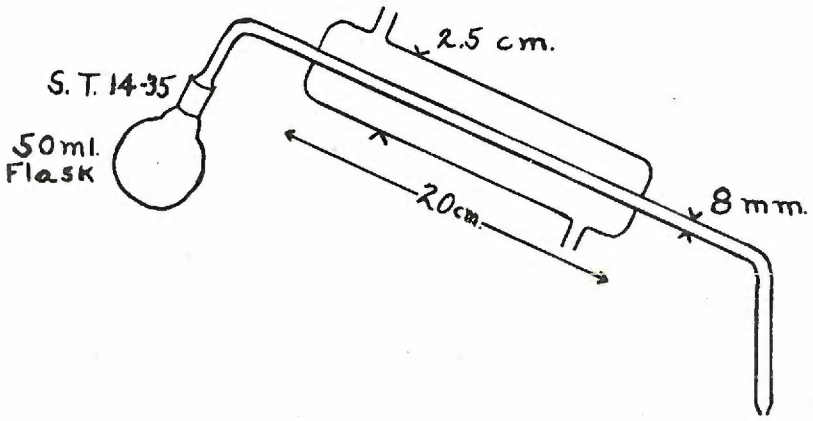

FIG. 1

lyzed by boiling under reflux for 18 to 24 hours. After this procedure, the hydrolyzates gave a negative biuret test. The hydrolyzates were transferred to 200 ml. volumetric flasks and diluted to the mark with water. For the glycine determination, an aliquot of hydrolyzate was transferred to a 50 ml. centrifuge tube containing enough crystalline $Ba(OH)_2$ to neutralize most of the acid in the aliquot, but not enough to render it alkaline. The tubes were fitted with stoppers bearing capillary tubes and heated in a boiling water bath for 1 hour to complete the reaction and to digest the $BaSO_4$. After removal of the $BaSO_4$ precipitate by centrifugation, glycine was determined in a 5 ml. aliquot of the supernatant. When the animals under investigation had been adrenalectomized, the color to be read in the glycine determination was brought into a more suitable range by diluting the hydrolyzate 1:1 in the process of neutralization.

Glycine determination: This determination was carried out in a modified Stotz all-glass still (17) which is illustrated in Figure 1. To 2 ml. of phosphate buffer (pH 5.5) and 1 ml. of 1 per cent ninhydrin (triketohydrindene hydrate) solution in the flask of the still were added 5.0 ml. of the amino acid solution. After attaching the condenser, the contents of the flask were distilled rapidly into a test tube calibrated at 10.0 ml. About 7 ml. of distillate were collected and the flask was allowed to cool. When the flask had cooled enough to obviate cracking, it was immersed in a cold water bath to bring it to room temperature more quickly. The condenser was then disengaged, 2 ml. of distilled water were added, and the distillation was continued to dryness. At the end of the distillation, the neck of the

still was gently heated to drive over the few drops of moisture remaining there. The entire distillation was completed in about 15 minutes. Care must be taken not to heat the flask too strongly. Strong heating may drive a red reaction product of ninhydrin over with the distillate and this interferes with the accuracy of the determination. The receiving tube was removed and distilled water was added to bring the total volume to 10.0 ml. Following dilution and thorough mixing of the distillate, 5.0 ml. were pipetted into a Klett colorimeter tube and 4.0 ml. of concentrated H_2SO_4 were added slowly while the tube was being cooled and agitated in an ice bath. When the solution had cooled approximately to room temperature, 3 drops of 5 per cent chromotropic acid (1,8-dihydroxynaphthalene-3,6-disulfonic acid) solution were added. The tube was shaken, the mouth plugged lightly with a small ball of Pyrex glass wool, and placed in a boiling water bath for 30 minutes. After the solution had cooled, the color intensity was read in a Klett photoelectric colorimeter at 560 millimicrons. A blank determination, using 5.0 ml. of distilled water in place of the amino acid solution, was run with each set of determinations in exactly the same way. The color intensities of the unknown solutions were read in a Klett photoelectric colorimeter against the blank determination set at zero.

Plasma proteins: Plasma proteins were determined by the method of Weichselbaum (18). To 4.9 ml. of 0.85 per cent sodium chloride in a Klett colorimeter tube was added 0.1 ml. of blood plasma. After 5.0 ml. of biuret reagent had been added and the solution thoroughly

mixed by whirling, the tube was placed in a water bath at 30°-32°C. for approximately 30 minutes to allow for full color development. A reference blank was prepared simultaneously by the addition of 5.0 ml. of biuret solution to 5.0 ml. of 0.85 per cent sodium chloride in another colorimeter tube and incubating as above. The color intensities of unknown solutions were read in a Klett photoelectric colorimeter at 560 millimicrons, against the reference blank set at zero, as soon as possible after removal from the water bath. The percentage of total plasma protein was read directly from a calibration curve previously constructed by using dilutions of pooled human serum whose protein content had been previously determined by the Kjeldahl method.

Total nitrogen: Total nitrogen determinations were made by the micro-Kjeldahl procedure using aliquots of the hydrolyzates prepared for the total glycine determination. An aliquot equal to 1/10 of the volume to which the hydrolyzate had been diluted was pipetted into a 100 ml. Kjeldahl flask. To this solution were added one or two selenized Hengar granules and 3 ml. of a digestion mixture consisting of sulfuric acid diluted 1:1 with water and saturated with K_2SO_4. Digestion was carried out with the use of a small manifold connected to an aspirator. Boiling was continued for at least an hour after the digest had cleared. After dilution of the digest with about 15 ml. of water, 10 ml. of 40 per cent NaOH were added and the ammonia was distilled into 25.0 ml. of standard N/70 H_2SO_4. The excess of standard H_2SO_4 was back titrated with standard N/70 NaOH using Tashiro's indicator. Multiplication of the titration difference by the factor

0.2 gives the amount of nitrogen in the aliquot in milligrams. The percentage of nitrogen in the sample was calculated from this figure. The figure for total protein was derived by multiplying the figure for total nitrogen by the factor 6.25.

Per cent glycine in tissue proteins: This value was derived by subtracting the figure for free glycine from the figure for total glycine to obtain the amount of bound glycine per 100 grams of wet tissue. Then, since the bound glycine may be assumed to be present chiefly in protein, the per cent glycine was calculated as indicated in the following equation.

$$\frac{\text{Bound glycine per 100 g wet tissue (in grams)}}{\text{Per cent protein in wet tissue}} \times 100$$

Water content: The water contents of kidney and muscle tissues were determined by heating weighed samples in an oven at 100°-105°C. to constant weight.

Plan of a typical experiment:

A. Animals fasted 8 hours: Rats were removed from the colony, weighed, and placed in cages with wire floors to minimize coprophagy. All animals were given the control ration for 24 hours prior to the beginning of the experimental period. This procedure accustoms the rats to synthetic rations and leads to improved food consumption during the experimental period. Following this preliminary period, the animals were weighed and either kept on the control ration or placed on 10 per cent glycine ration for 38 hours. At this time the last meal was given. One to two hours were allowed for the consumption of the last meal and the animals were weighed and sacri-

ficed 8 hours later. In the few instances in which a 15 per cent glycine diet was used as the experimental ration, no significant differences in the results were noted. This is in keeping with the insignificant differences in liver glycogen levels after a 24 hour fast of animals previously fed 10 per cent and 15 per cent glycine diets noted in a previous paper (4).

When adrenalectomized animals were studied, the experiment was carried out as outlined above beginning on the morning of the 5th post-operative day. During the intervening period, the animals were maintained on Purina Laboratory Chow with 1 per cent NaCl solution for drinking water. This procedure is similar to that outlined by Olsen and associates (19) for the preparation of animals to be used in assaying the glycogenic potency of adrenal cortical extracts.

B. Animals fasted 24 hours: The procedure here was essentially the same as that described for the 8 hour fast except that the rats were fed the experimental rations for 48 hours, at which time the last meal was given. One to two hours were allowed for the consumption of the last meal and the animals were sacrificed 24 hours later.

RESULTS

Results are summarized in Tables I, II, III, IV and V.

Blood: The level of free glycine in the blood of control-fed animals is essentially the same in 8 hour fasted and 24 hour fasted normal animals and in adrenalectomized animals fasted 8 hours. These figures could be interpreted as representing the normal fasting blood

TABLE I

Summary of Data Obtained from Analysis of Tissues Taken
From Normal Animals Following a 24-Hour Fast

Expressed as wet weight of tissue

Tissue	Free Glycine Mgs. % Animals Pre-fed		Total Glycine Mgs. % Animals Pre-fed		Plasma Protein % Animals Pre-fed		Total Nitrogen % Animals Pre-fed		Total Protein % Animals Pre-fed	
	Control Diet	Glycine Diet	Control Diet	Glycine Diet	Control Diet	Glycine Diet	Control Diet	Glycine Diet	Control Diet	Glycine Diet
Blood	4.1 (4)	4.6 (4)	282 (5)	334 (5)	6.7 (4)	6.6 (4)	3.38 (2)	3.41 (2)	21.2 (2)	21.3 (2)
Kidney	80 (2)	77 (2)	455 (4)	424 (4)	—	—	3.72 (2)	3.16 (2)	23.3 (2)	19.7 (2)
Intestine	80 (2)	90 (2)	428 (5)	453 (5)	—	—	2.76 (2)	2.76 (2)	17.2 (2)	17.2 (2)
Liver	70 (2)	66 (2)	462 (5)	478 (5)	—	—	3.72 (2)	3.83 (2)	23.5 (2)	23.9 (2)
Muscle	40 (2)	59 (2)	426 (5)	441 (5)	—	—	3.43 (2)	3.57 (2)	21.5 (2)	22.3 (2)

Values are averages for the numbers of animals shown in parentheses.

TABLE II

Summary of Data Obtained from Analysis of Tissues of Hypophysectomized Animals Following a 24-Hour Fast

Expressed as wet weight of tissue

Tissue	Free Glycine Mgs. % Animals Pre-fed		Total Glycine Mgs. % Animals Pre-fed		Total Nitrogen % Animals Pre-fed		Total Protein % Animals Pre-fed	
	Control Diet	Glycine Diet	Control Diet	Glycine Diet	Control Diet	Glycine Diet	Control Diet	Glycine Diet
Blood	----	----	456 (1)	480 (1)	3.32 (1)	3.36 (1)	20.8 (1)	21.0 (1)
Kidney	110 (1)	113 (1)	469 (1)	475 (1)	2.83 (1)	3.06 (1)	17.7 (1)	19.2 (1)
Intestine	99 (1)	145 (1)	439 (1)	431 (1)	2.56 (1)	2.30 (1)	16.0 (1)	14.4 (1)
Liver	51 (1)	56 (1)	519 (1)	343 (1)	3.92 (1)	3.97 (1)	24.4 (1)	24.8 (1)
Muscle	63 (1)	56 (1)	434 (1)	458 (1)	3.27 (1)	3.48 (1)	20.4 (1)	21.7 (1)

Values are averages for the numbers of animals shown in parentheses.

TABLE III

Summary of Data Obtained by Analysis of Tissues Taken
from Normal Animals Following an 8-Hour Fast

Expressed as wet weight of tissue

Tissue	Free Glycine Mgs. % Animals Pre-fed		Total Glycine Mgs. % Animals Pre-fed		Plasma Protein % Animals Pre-fed		Total Nitrogen % Animals Pre-fed		Total Protein % Animals Pre-fed		Water Content % Animals Pre-fed	
	Control Diet	Glycine Diet	Control Diet	Glycine Diet	Control Diet	Glycine Diet	Control Diet	Glycine Diet	Control Diet	Glycine Diet	Control Diet	Glycine Diet
Blood	3.7 (6)	8.1 (6)	483 (9)	533 (9)	7.7 (6)	7.6 (7)	3.01 (7)	3.14 (8)	18.8 (7)	19.6 (8)	—	—
Kidney	77 (6)	79 (3)	434 (11)	439 (9)	—	—	3.04 (8)	2.75 (6)	19.0 (8)	17.2 (6)	76.3 (7)	77.6 (7)
Intestine	76 (6)	103 (6)	396 (10)	444 (11)	—	—	2.44 (9)	2.45 (9)	15.2 (9)	15.3 (9)	—	—
Liver	57 (6)	77 (4)	459 (11)	476 (11)	—	—	3.21 (9)	3.15 (9)	20.0 (9)	19.7 (9)	—	—
Muscle	55 (6)	133 (5)	390 (11)	471 (11)	—	—	3.35 (9)	3.31 (9)	20.9 (9)	20.7 (9)	77.6 (7)	78.1 (7)

Values are averages for the numbers of animals shown in parentheses.

TABLE IV

Summary of Data Obtained from Analysis of Tissue of Adrenalectomized Animals Following an 8-Hour Fast

Expressed as wet weight of tissue

Tissue	Free Glycine Mgs. % Animals Pre-fed		Total Glycine Mgs. % Animals Pre-fed		Plasma Protein % Animals Pre-fed		Total Nitrogen % Animals Pre-fed		Total Protein % Animals Pre-fed		Water Content % Animals Pre-fed	
	Control Diet	Glycine Diet	Control Diet	Glycine Diet	Control Diet	Glycine Diet	Control Diet	Glycine Diet	Control Diet	Glycine Diet	Control Diet	Glycine Diet
Blood	3.5 (2)	6.8 (5)	377 (3)	416 (6)	6.0 (3)	6.1 (5)	2.60 (3)	2.78 (6)	16.3 (3)	17.4 (6)	—	—
Kidney	82 (2)	98 (5)	538 (2)	569 (5)	—	—	2.63 (3)	2.66 (6)	16.4 (3)	16.6 (6)	78.7 (1)	78.1 (3)
Intestine	86 (2)	95 (5)	492 (2)	573 (5)	—	—	2.31 (3)	2.49 (6)	14.4 (3)	15.6 (6)	—	—
Liver	58 (1)	71 (5)	652 (2)	717 (5)	—	—	3.43 (3)	3.40 (5)	21.4 (3)	21.3 (5)	—	—
Muscle	45 (2)	126 (4)	585 (2)	650 (5)	—	—	3.12 (3)	3.29 (5)	19.5 (3)	20.6 (5)	79.1 (2)	79.8 (5)

Values are averages for the numbers of animals shown in parentheses.

TABLE V

Percentage of Glycine in Various Tissue Proteins in Animals Subjected to Different Experimental Procedures

Tissue	Duration of Fast	Condition of Animal	% Glycine in Tissue Protein Animals Pre-fed	
			Control Diet	Glycine Diet
Blood	24 Hrs.	Normal	1.31	1.54
	24 Hrs.	Hypophysectomized	2.20	2.28
	8 Hrs.	Normal	2.54	2.68
	8 Hrs.	Adrenalectomized	2.29	2.35
Kidney	24 Hrs.	Normal	1.61	1.76
	24 Hrs.	Hypophysectomized	2.03	1.89
	8 Hrs.	Normal	1.88	2.09
	8 Hrs.	Adrenalectomized	2.78	2.84
Intestine	24 Hrs.	Normal	2.02	2.11
	24 Hrs.	Hypophysectomized	2.12	1.99
	8 Hrs.	Normal	2.10	2.22
	8 Hrs.	Adrenalectomized	2.82	3.06
Liver	24 Hrs.	Normal	1.67	1.72
	24 Hrs.	Hypophysectomized	1.92	1.16
	8 Hrs.	Normal	2.01	2.02
	8 Hrs.	Adrenalectomized	2.78	3.03
Muscle	24 Hrs.	Normal	1.80	1.71
	24 Hrs.	Hypophysectomized	1.82	1.86
	8 Hrs.	Normal	1.60	1.63
	8 Hrs.	Adrenalectomized	2.77	2.54

level of this amino acid. In animals fed on the glycine diet, the picture is quite different. Unoperated, glycine-fed animals show more than twice as much free glycine in their blood after an 8 hour fast as do control-fed animals. When adrenalectomized animals were fed the glycine ration and fasted 8 hours, the levels of free glycine in their blood was nearly twice that of control-fed, adrenalectomized animals. Upon extension of the fasting period of normal animals to 24 hours, it was found that the blood level of free glycine in glycine-fed animals had declined almost to that of the control-fed group. Insufficient blood was available for free glycine determinations in the case of the one pair of hypophysectomized animals used.

Comparison of the values for total glycine shows that the total glycine content of the blood of glycine-fed animals is higher in all cases. In unoperated animals, the differences between control-fed and glycine-fed rats are almost exactly the same following either an 8 hour or a 24 hour fast. In adrenalectomized animals fasted 8 hours and hypophysectomized animals fasted 24 hours, the differences are somewhat smaller. In no case does free glycine account for a large proportion of the excess total glycine found in glycine-fed animals. Almost all of the excess must therefore be present in combined form.

The total nitrogen, and hence the total protein, contents of the blood of glycine-fed animals are a little higher than those of the controls, although in some cases the differences are probably insignificant. Differences in plasma protein levels are so small that they cannot be considered significant.

Although the absolute values for plasma protein, total nitrogen,

and total protein vary a little in animals receiving different treatments, it will be noted (Table V) that the percentage of glycine in the total blood proteins are very similar in both glycine-fed and control-fed animals receiving the same treatment and that these values for the different groups fall within a relatively narrow range except for normal animals fasted 24 hours. No satisfactory explanation for the decreased total glycine content in the presence of a normal or slightly elevated protein content seen in normal animals fasted 24 hours has been found, but further investigation of this problem is planned.

Kidney: The amounts of free glycine in the kidneys of control-fed animals is almost identical in all animals except those which were hypophysectomized, in which case the content of free glycine was appreciably elevated. This could be attributable to the inability of an animal deprived of its nitrogen retaining hormones by hypophysectomy to incorporate amino acids into its tissue proteins or to convert them to carbohydrate in the absence of the glyconeogenetic hormones of the adrenal cortex. In glycine-fed animals, the content of free glycine in kidney tissue was almost exactly the same as in control-fed animals except in the case of adrenalectomized animals. There, the amount of free glycine is a little greater in the glycine-fed animals. This again may be a reflection of the animals' inability to convert protein or amino acids to carbohydrate in the absence of the cortical hormones.

Total glycine is very little higher in the kidneys of glycine-fed rats in all cases except that of normal animals fasted 24 hours.

About 50 per cent of the excess can be accounted for as free glycine in all instances. The reduced total glycine content in normal, glycine-fed animals after a 24 hour fast is probably a reflection of the lower protein content of the kidneys of those animals. It should be noted that the values for total glycine in the kidneys of adrenalectomized animals are appreciably higher than those for the other groups of animals, regardless of the experimental diet consumed.

Total nitrogen and total protein are lower in normal glycine-fed animals after both 8 hour and 24 hour fasts, and higher in glycine-fed hypophysectomized and adrenalectomized animals after 24 hour and 8 hour fasts respectively. The difference in protein content in normal animals fasted 8 hours largely disappears when allowance is made for the increased water content of the kidneys of glycine-fed animals. Unfortunately, the water contents of the kidneys of normal animals after a 24 hour fast were not determined so it is not known whether or not the same situation exists after a 24 hour fast as holds after an 8 hour fast. Data of this type are not available for hypophysectomized animals either, but adrenalectomized animals show only small differences in the water contents of the kidneys of control-fed and glycine-fed animals.

If the per cent glycine in the kidney protein is calculated in each case, it is found that this figure is very similar for control-fed and glycine-fed animals of each group (see Table V). In normal and hypophysecomized animals, these values fall within a rather narrow range while adrenalectomized animals show a noticeably higher percentage of glycine in their kidney proteins than do the other

groups of animals. Some of the implications of this very interesting observation, and some other observations of a similar nature will be considered later.

Intestine: In this tissue, the levels of free glycine in control-fed animals are quite comparable in all groups except for the hypophysectomized animals where it is somewhat higher. In all instances, glycine-fed animals show more free glycine than do control-fed animals, the differences being rather small in normal animals fasted 24 hours and adrenalectomized animals fasted 8 hours, larger in normal animals fasted 8 hours and largest of all in hypophysectomized animals fasted 24 hours. The situation existing in this respect in hypophysectomized animals may again result from the animals' lack of nitrogen retaining and glyconeogenetic hormones.

The total glycine of intestine is higher in glycine-fed animals than in control-fed animals in all cases, except that of hypophysectomized animals. In that case, the apparently anomalous situation of a lower total glycine content in the presence of a higher free glycine content in the glycine-fed animal is probably reflecting the somewhat lower protein content of the intestine of the glycine-fed animal. Here again, it is found that the total glycine content of the intestines of adrenalectomized animals is considerably higher than in the other groups of animals. In normal and adrenalectomized animals, there is no instance in which the excess total glycine found in the intestines of glycine-fed animals is entirely accounted for as free glycine. The maximum accountable as free glycine is about 56 per cent in the case of 8 hour fasted normal animals. The remaining glycine must,

of course, be bound in some manner.

In normal animals fasted 24 hours, glycine-fed and control-fed animals show identical levels of total nitrogen and total protein. The figures for total nitrogen and total protein are almost identical for control-fed and glycine-fed rats after an 8 hour fast. With adrenalectomized rats, glycine-fed animals show more nitrogen and protein than do control-fed animals, while with hypophysectomized animals glycine-fed rats show less total nitrogen and total protein. This rather complicated picture is again simplified considerably by calculating the per cent of glycine in the tissue protein. As may be seen in Table V, these values for normal and hypophysectomized animals fall within a narrow range, while those for adrenalectomized animals are, again, markedly higher

Liver: Free glycine in the livers of glycine-fed rats is appreciably higher than in control-fed rats in both normal and adrenalectomized animals after an 8 hour fast. When the animals were fasted 24 hours, the level of free glycine in normal glycine-fed animals was slightly lower than in normal control-fed animals, while the level in hypophysectomized glycine-fed animals was slightly higher than in control-fed hypophysectomized animals. In both the latter cases, the difference is very small and probably of doubtful significance.

Total glycine is higher in the glycine-fed animals in all groups except the hypophysectomized animals. In that instance, the total glycine content of the liver of the glycine-fed animal is a great deal lower than in the control-fed animal. There is no obvious explanation for this very marked apparent decrease and,

although no error in the process of analysis is known to have occurred, in view of the well-maintained protein level in the liver of this animal, it seems fruitless to seek an explanation until the results on this one pair of animals have been confirmed or repudiated by further determinations of the same nature. As for the other groups of animals, the slightly increased total glycine content in the livers of normal glycine-fed rats after a 24 hour fast probably reflects the slightly greater percentage of protein in the livers of those animals. In normal animals after an 8 hour fast, the excess total glycine found in the livers of glycine-fed animals is all accounted for as free glycine. When adrenalectomized animals are fasted 8 hours, the difference in total glycine content (in favor of the glycine-fed rats) is much increased and only 20 per cent of the excess can be accounted for as free glycine. The levels of total glycine in the livers of both control-fed and glycine-fed adrenalectomized animals are again significantly elevated.

Total nitrogen and total protein contents of the livers of control-fed and glycine-fed animals are very similar in each group. A glance at Table V shows that the percentage of glycine in the liver proteins is pretty much the same in control-fed and glycine-fed animals except in the case of the hypophysectomized animals in which case the validity of the figure for total glycine in the liver of the glycine-fed animal is in some doubt. Again the percentage of glycine in the tissue protein of both control-fed and glycine-fed adrenalectomized animals is markedly elevated.

Muscle: As might be expected from the observations of earlier

workers (6, 9) considerable accumulation of glycine in this tissue was demonstrated. The level of free glycine in muscle is higher in the glycine-fed animals in all cases except that of the hypophysectomized rats. There the level of free glycine is somewhat lower in the glycine-fed rat but the difference is so small that it is of doubtful significance.

Total glycine in muscle is greater in the glycine-fed animals in all cases, and in every instance except that of the hypophysectomized pair, the excess total glycine can be accounted for as free glycine. The muscles of adrenalectomized animals also show an increased total glycine content compared to the other groups.

Levels of total nitrogen and total protein are very similar in unoperated animals after both 8 hour and 24 hour fasts. In both hypophysectomized and adrenalectomized animals, total nitrogen and total protein are somewhat higher in glycine-fed animals. The water content of the muscle of both normal and adrenalectomized animals after an 8 hour fast is nearly the same for control-fed and glycine-fed rats.

If the per cent glycine in muscle protein is calculated, it is again found (Table V) that these figures are very similar in control-fed and glycine-fed animals subjected to the same experimental procedure. It is also found that the figures for normal and hypophysectomized animals fall within a narrow range, while the values found for adrenalectomized animals are strikingly higher.

DISCUSSION

This work was undertaken in an attempt to discover whether or not a rat pre-fed on a diet containing added glycine could store enough of that amino acid in his body to account for the enhanced carbohydrate stores found in such a rat after a 24 hour fast or after insulin, provided the stored glycine was completely converted to carbohydrate. In addition, it was desired to know if there was any difference in the storage of glycine in normal animals and adrenalectomized animals.

The calculations to be outlined here show that there is insufficient glycine stored in the body of a glycine-fed rat after an 8 hour fast to account for the enhanced carbohydrate stores found in the body of such an animal subjected to an 8 hour fast and 5 hours of insulin action. Carbohydrate stores of glycine-fed and control-fed rats receiving such treatment were calculated from the data given in Table VI which was adapted from the paper of Cunningham et al (5). The figures for excess glycine found in glycine-fed rats were derived from the values for total glycine presented in Table III. Table VII summarizes the series of calculations made to find the extent of glycine storage in a glycine-fed rat after an 8 hour fast and the extra carbohydrate present in the body of such an animal after an 8 hour fast and 5 hour insulin action.

TABLE VI

The Effect of Prefeeding Extra Glycine on the Response of Rats to 12 Units of Insulin/Kg. as Measured by Alterations in Blood Sugar, and Muscle and Liver Glycogen. Adapted from Cunningham, Barnes and Todd (5).

	Glycogen (% Wet Wt.)				Blood Sugar Mgs. %	
	Control Ration		Glycine Ration		Control Ration	Glycine Ration
	Muscle	Liver	Muscle	Liver		
Before Insulin	0.43	3.89	0.63	3.51	114	133
After Insulin	0.21	0.13	0.52	1.36	66	106

TABLE VII

Summary of Calculations Showing 1) Excess Glycine Stored in the Body of a Glycine-fed Rat After 8 Hours Fast, and 2) the Excess Carbohydrate Found in the Body of a Glycine-fed Rat After 8 Hours Fast and 5 Hours of Insulin Action. Calculations Are for a 200 Gm. Rat

1. Tissue	2. Body Weight	3. Tissue Wt. in a 200 Gm. Rat	4. Excess Total Glycine in Glycine-fed Rat 8 Hrs. Fast[1]	5. Column 4 Calculated to a 200 Gm. Rat	6. Excess Carbohydrate in Glycine-fed Rat After Insulin[2]	7. Column 6 Calculated to a 200 Gm. Rat
	%	Gms.	Mgs. %	Mgs.	Mgs. %	Mgs.
Blood	6.7[3]	13.4	50	6.7	40	5.4
Kidney	0.9[4]	1.8	5	0.1	—	—
Intestine	4.0[4]	8.0	48	3.8	—	—
Liver	5.0[4]	10.0	17	1.7	1230	123
Muscle	45.0[4]	90.0	81	72.9	310	279
Totals				85.2		407.4

[1]From Table III.
[2]From Table VI.
[3]From Griffith (20).
[4]Approximate values calculated from the data of Donaldson (21).

Suppose, now, that all the carbon atoms of glycine were converted to carbohydrate as illustrated in the theoretical equation given below with glycolaldehyde polymerizing to form larger carbohydrate molecules. It can be deduced from this that the weight of carbohydrate

H_2NCH_2COOH
Glycine
M.W. = 75

$HOCH_2-C(H)=O$
Glycolaldehyde
M.W. = 60

so formed could be no more than about 60/75 of the weight of glycine available. (This is purely a theoretical assumption. It should not be inferred that such a mechanism is postulated.) According to this, then, if the extra glycine found in the body of a glycine-fed rat after an 8 hour fast (Table VII) were completely converted to carbohydrate during 5 hours of insulin action, 60/75 x 85 or about 68 mgs. of extra carbohydrate could be accounted for. But it was found experimentally that after 8 hours fast and 5 hours insulin action, the glycine-fed rat has in his body an excess of some 407 mgs. of carbohydrate over that found in the control-fed rat. This is very nearly 6 times the amount of carbohydrate that could be formed by complete conversion of the extra glycine carbon atoms shown to be present after 8 hours fast. Thus it seems evident that the "protein effect" of glycine must be accounted for by some other mechanism (such as glyconeogenesis) than direct conversion of this amino acid to carbohydrate.

From examination of Table VI it might be argued that the glycine-fed rat has more carbohydrate in his body after an 8 hour fast and before the action of insulin than the control-fed rat due to the higher level of muscle glycogen in the glycine-fed animal and that

this extra carbohydrate gives the glycine-fed rat an advantage in resisting the action of insulin. This fact does not refute the theory of a stimulation of glyconeogenesis by feeding glycine, however. It must be remembered that the glycine supplemented diet contains less carbohydrate than the control diet. Therefore, if the animal's body stores of carbohydrate were simply a reflection of the amount of carbohydrate ingested in the diet, the glycine-fed animal should have less carbohydrate in his body. It seems, then, that in the glycine-fed animal there is an increased rate of glyconeogenesis as compared with the rate of carbohydrate utilization. To approach this problem from another angle, calculation of the excess carbohydrate found in the body of a 200 gram glycine-fed rat before the action of insulin has been made (Table VIII) and was found to amount to 144 mgs. If the value for excess carbohydrate in a glycine-fed rat following insulin is then corrected for this pre-insulin excess by subtracting 144 from 407, there is a net excess of 263 mgs. of carbohydrate in the body of the glycine-fed rat, which is still nearly 4 times as much as can be accounted for by complete conversion of the extra glycine present to carbohydrate.

Although not all the body tissues were examined for glycine, those which have been studied comprise about 60 per cent of the body weight and it seems unlikely that analyses of other tissues would reveal a reservoir of this amino acid sufficient to account for the differences noted. On the other hand, neither have all the body tissues been examined for carbohydrate. Such studies might reasonably be expected to add something to the figures for total carbohydrate

TABLE VIII

Summary of Calculations Showing the Excess Carbohydrate Found in the Body of a Glycine-Fed Rat After 8 Hours Fast

1. Tissue	2. Tissue Wt. in a 200 G. Rat[1] Gms.	3. Excess Carbohydrate in Glycine-Fed Rat Before Insulin[2] Mgs. %	4. Column 3 Calculated to a 200 G. Rat Mgs.
Blood	13.4	19	2.5
Liver	10.0	-380	-38
Muscle	90.0	200	180
Total			144.5

[1]From Table VII.
[2]From Table VI.

thus offsetting, to a greater or less extent, any extra stored glycine found.

Since these calculations seem effectively to rule out direct conversion of stored glycine to carbohydrate as the mechanism of production of the "protein effect" of this amino acid, and since reduced glycogenolysis was ruled out by the observation of Cunningham et al (5) that the blood sugar level of glycine-fed animals after insulin is higher than that of control-fed animals after insulin, a stimulation of glyconeogenesis by the feeding of glycine is postulated. The previously noted fact that the "protein effect" of glycine is eliminated by adrenalectomy (4) indicates that such a stimulation might be mediated by the adrenals. The adrenal cortical hormones are known to be concerned in the conversion of protein to carbohydrate in the animal body (22), so perhaps the feeding of glycine supplemented diets in some way potentiates the action of the adrenal cortex so that the animal is better able to maintain his carbohydrate reserves when placed under some kind of stress. It is unfortunate that no studies of the nitrogen content of lymphoid tissues were carried out in connection with this work, inasmuch as White and Dougherty (23) have shown that the adrenal cortex is concerned in the mobilization of nitrogen from such tissues in fasting. In any case, it is obvious from Tables IV and V that the adrenalectomized animal handles this amino acid differently than the unoperated animal.

Other workers have postulated a stimulation of glyconeogenesis from other molecules by glycine (24, 25) but they advance no theories with regard to a possible mechanism for such an action.

Figures for muscle glycogen and blood sugar of glycine-fed and control-fed rats after a 24-hour fast are not available, so a mathematical comparison similar to the one outlined above cannot be made. However, it is interesting to note that the differences in glycine contents of the tissues are smaller after a 24-hour fast than after an 8-hour fast. This is especially true in the case of muscle where the difference drops from 81 mgs. per cent after an 8-hour fast to 15 mgs. per cent after a 24-hour fast. Since muscle comprises such a large portion of the body weight, this is a sizable decrease in the amount of glycine stored. These data are in keeping with the previous observation (26) that the "protein effect" of glycine drops off rapidly when the fast is prolonged beyond 24 hours. The rapid disappearance of the "protein effect" of glycine after more than 24 hours fast is interpreted as indicating, not an exhaustion of the supply of glycine available for conversion to carbohydrate, but rather a removal of the stimulus for glyconeogenesis with the disappearance of the extra glycine.

Studies of the glycine contents of tissues taken from adrenalectomized animals revealed a different picture from that seen in normal animals or even in hypophysectomized animals. All of these tissues, with the exception of blood, showed appreciably higher total glycine contents than were found in normal or hypophysectomized animals (see Tables I, II, III, and IV). As might be expected from this fact, the percentage of glycine in the tissue proteins, again with the exception of blood, was found to be markedly higher in adrenalectomized animals than in the other types of animals studied. The

same general picture was observed even though the animal had not been pre-fed the glycine ration. From this it appears that the effects noted are attributable to the absence of the adrenal glands rather than to the type of diet received during the experimental period. The increased amount of glycine found in the tissues of animals fed the control ration during the experimental period may have been derived from the diet consumed by the animal during the period between adrenalectomy and the beginning of the experiment.

These results are puzzling and difficult to explain. The failure of the blood proteins of adrenalectomized animals to show an elevation in glycine content comparable to that shown by the other tissues may result from the effort of the animal body to maintain its internal environment in as nearly constant a state as possible. If, as the evidence indicates, the adrenals are required for the metabolism of glycine, it may be that an animal deprived of these organs, when fed this amino acid, stores in his tissues whatever he is unable to utilize or excrete in an effort to keep his body fluids normal in composition. With respect to the other tissues, the increased percentage of glycine found in adrenalectomized animals points toward two possible explanations. Such results may reflect an alteration in the amino acid composition of the proteins of adrenalectomized animals or an increased binding of glycine to the tissue proteins. The former, of course, is a very radical departure from the views generally held regarding the amino acid composition of proteins, but the data presented here points toward such a possibility. The latter alternative is an equally plausible explanation for the results noted here. The binding of

various substances to proteins is a part of the physical chemistry of tissues about which little is yet known.

Another interesting aspect of this situation is revealed by comparing the figures for per cent glycine in the tissues of adrenalectomized animals with those for hypophysectomized animals. It will be noted that these figures for hypophysectomized animals are very close to those for normal animals and quite different from those for adrenalectomized animals (see Table V). If one thinks only of the control exercised by the anterior pituitary over the adrenal cortex, disregarding the other endocrine organs controlled by the anterior pituitary, one would expect the tissues of hypophysectomized animals to present the same picture as those of adrenalectomized animals. This, however, did not prove to be the case. A question might be raised as to whether or not the adrenals of the hypophysectomized animals were actually atrophic. Because of the anterior pituitary-adrenal cortical relationship, it seemed reasonable to assume that the "protein effect" of glycine could not be demonstrated in hypophysectomized animals. This assumption was proved to be correct by experiment. Since the animals whose tissues were studied were taken from the experimental group with which this fact was demonstrated, and since the evidence points toward the adrenal cortex as the mediator of the "protein effect" of glycine, it seems reasonable to assume that the adrenal cortices of these animals were atrophic.

How, then, might the differences in tissue glycine in adrenalectomized and hypophysectomized animals be explained? It seems possible that this picture might be the result of hormonal imbalance

in adrenalectomized animals. Normal metabolism is known to involve rather delicate hormonal interrelationships. Removal of the adrenals, depriving the animal of the secretions of those glands, but leaving the other endocrine glands intact and functional, may so upset the normal hormonal equilibrium as to result in the apparently bizarre picture noted. Ablation of the hypophysis, on the other hand, results in the almost simultaneous atrophy of all its associated endocrine organs, leaving the animal essentially devoid of the hormones secreted by all these glands. The animal is thus deprived, practically simultaneously, of its most important mediators for both the synthesis and breakdown of body proteins. The body proteins of such an animal, then, might be thought of as having fallen into a relatively stagnant state, thus retaining the composition found in the normal animal. This is an interesting hypothesis and seems to explain the results noted, but, of course, would require a great deal more work for substantiation.

It should be pointed out that the techniques used in these investigations were designed only to indicate how much glycine was present in the free state and how much in combined form without regard for the size or type of molecule in which it was bound. However, the differences between normal and adrenalectomized animals seemed large enough to warrant some speculation on the subject and to stimulate further investigations into the amino acid composition of the proteins of adrenalectomized animals. The methods employed in such investigations must, of course, include purification of the proteins preliminary to the determination of their amino acid contents.

SUMMARY

The storage of glycine in various body tissues of rats pre-fed on diets containing added glycine and of rats pre-fed on diets similar in composition except for the substitution of an equal amount of carbohydrate in lieu of glycine has been studied. Calculations are outlined which show that complete conversion to carbohydrate of the excess stored glycine present in the body of a glycine-fed rat fails to account for the extra carbohydrate found in the body of a glycine-fed rat after 8 hours fast and 5 hours insulin action (5). These results are interpreted as indicating a stimulation of glyconeogenesis by the ingestion of glycine with the diet, possibly as a result of increased adrenal cortical activity.

Similar studies of the storage of glycine in the tissues of adrenalectomized and hypophysectomized rats have been made. Evidence pointing toward a possible alteration in the amino acid composition of the body proteins of adrenalectomized animals is presented. Inasmuch as the glycine picture in hypophysectomized animals is more like that of normal animals than that of adrenalectomized animals, it is suggested that the situation found in adrenalectomized animals may be due to hormonal imbalance.

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