group of aspartic acid, giving two configurations, both of which could be expected to have strong metal-chelating properties.

SUMMARY

1. A quantitative study of the inhibition of phosphoglucomutase provides additional evidence that the necessity for a chelating agent is due to the presence of minute traces of inhibitory metals.

2. Zinc is an inhibitor of phosphoglucomutase, competing with Mg^{2+} ions, with dissociation constant $K_{Zn} 3.9 \times 10^{-14} M$. Copper also competes with Mg^{2+} ions, but the inhibition is not purely competitive. The inhibition by Cu^{2+} ions can be explained by competition with the Mg^{2+} ions ($K_{Cu} 2.3 \times 10^{-17} M$), together with non-competitive combination at a second site ($K'_{Cu} 2.5 \times 10^{-16} M$).

3. Three to four SH groups are present in phosphoglucomutase. Although some inhibition is observed with high concentrations of several SH reagents, phosphoglucomutase does not appear to be an enzyme requiring these groups for activity.

4. The effects are discussed in the light of the amino acid sequence in the active centre previously determined.

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REFERENCES

- Barnard, E. A. & Stein, W. D. (1958). *Advanc. Enzymol.* **20**, 51.
 Barron, E. S. G. (1951). *Advanc. Enzymol.* **11**, 201.
 Dixon, M. (1948). *Symp. biochem. Soc.* **2**, 39.
 Fraenkel-Conrat, H. (1957). In *Methods in Enzymology*, vol. 4, p. 247. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Friedman, E., Marrian, D. H. & Simon-Reuss, I. (1949). *Brit. J. Pharmacol.* **4**, 105.
 Gregory, J. D. (1955). *J. Amer. chem. Soc.* **77**, 3922.
 Gundlach, H. G., Stein, W. H. & Moore, S. (1959). *J. biol. Chem.* **234**, 1754.
 Helleman, L., Chinard, F. P. & Deitz, V. R. (1943). *J. biol. Chem.* **147**, 443.
 Jagannathan, V. & Luck, J. M. (1949). *J. biol. Chem.* **179**, 561.
 Ledoux, L. (1954). *Biochim. biophys. Acta*, **13**, 121.
 Lehmann, H. (1939). *Biochem. J.* **33**, 1241.
 McCoy, E. E. & Najjar, V. A. (1959). *J. biol. Chem.* **234**, 3017.
 Maley, L. E. & Mellor, D. P. (1950). *Nature, Lond.*, **165**, 453.
 Malmström, B. C. (1955). *Arch. Biochem. Biophys.* **58**, 381.
 Malmström, B. C. & Rosenberg, A. (1959). *Advanc. Enzymol.* **21**, 131.
 Milstein, C. (1960). *Biochem. biophys. Res. Commun.* **3**, 292.
 Milstein, C. (1961a). *Biochem. J.* **79**, 574.
 Milstein, C. (1961b). *Biochem. J.* **79**, 584.
 Milstein, C. & Sanger, F. (1961). *Biochem. J.* **79**, 456.
 Najjar, V. A. (1948). *J. biol. Chem.* **175**, 281.
 Raaflaub, S. (1956). *Meth. biochem. Anal.* **3**, 301.
 Rabinovitch, M. & Barron, E. S. G. (1955). *Biochim. biophys. Acta*, **18**, 316.
 Smyth, D. G., Nagamatsu, A. & Fruton, J. S. (1960). *J. Amer. chem. Soc.* **82**, 7602.
 Sutherland, E. W. (1949). *J. biol. Chem.* **180**, 1279.
 Thiers, R. E. (1957). *Meth. biochem. Anal.* **5**, 273.

Biochem. J. (1961) **79**, 596

The Effect of Insulin on Amino Acid Metabolism

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In the course of studies on nitrogen metabolism in the alligator, it was observed that glycine, alanine, glutamine and leucine were the main amino acids utilized for the renal synthesis of ammonia (Coulson & Hernandez, 1959). The injection of insulin caused a dramatic decrease in the plasma concentrations of these compounds and as a consequence the renal synthesis of ammonia was virtually abolished.

The knowledge that plasma amino acids will decrease after the injection of insulin is almost as old as the observation that insulin lowers blood glucose (Luck, Morrison & Wilbur, 1928). For

several reasons, however, there have been few studies of the effect of insulin on individual amino acids of either configuration. Only recently have the pure isomers been available at a reasonable cost, and even now the amount required for experiments on large mammals such as the dog makes such studies exceedingly expensive. When an amino acid is given to a mammal by any route the compound is so quickly absorbed and metabolized or excreted that studies *in vivo* are often impractical. In spite of these limitations Hier (1947) compared the 'tolerances' of several amino acids in the dog. No plasma rise occurred after the oral admini-

stration of DL-tryptophan, L-cystine, DL-phenylalanine and L-tyrosine and very little rise followed the ingestion of L-leucine, DL-isoleucine and L-arginine. DL-Threonine, DL-valine and L-histidine increased the plasma amino acid levels appreciably for a short time and only DL-methionine remained in the plasma in considerable amounts for a period of several hours. The effects of insulin were not studied.

The alligator with a metabolic rate of about 5% of that of man offered an opportunity to study not only the rates at which the individual D- or L-amino acids disappeared from the plasma but also the influence of insulin on those rates. Reactions which are completed in minutes in a mammal last for days or even weeks in the alligator and since the reactions are slow, the quantities of amino acids needed are much less than for comparable studies in mammals. In an effort to determine whether information derived from the alligator experiments could be applied to a mammal, amino acid tolerances were also performed on rats.

It has been suggested that insulin exerts its effects on amino acids by catalysing their conversion into proteins (Lotspeich, 1949; Russell, 1955; Frame & Russell, 1946). The results of the present study suggest that its action is more involved than this.

MATERIALS AND METHODS

Animals. The 200 alligators (*A. mississippiensis*) used in these studies were bought from dealers who collected them from the swamps of Louisiana. They were kept in large concrete tanks with water in a temperature-controlled room (28°). Under laboratory conditions they ate well and were easy to handle. They were fed chopped rats or rabbits once or twice a week. Before each experiment food was denied for at least a week after which time the plasma amino acid level remained fairly constant at about 2–4 m-moles/l. In the majority of experiments animals weighing about 1 kg. were used, but in those experiments with the more expensive and rare amino acids it was necessary to use animals weighing as little as 100 g. In each amino acid tolerance experiment the group receiving insulin was paired with controls of the same weight. Over the period of many months covered by these studies, many of the alligators were used several times since they were not killed in the experiments.

The rats were young adults of both sexes of the Sprague-Dawley strain weighing 100–200 g. They were kept on Purina Laboratory Chow manufactured by Ralston Purina Co., St Louis, Mo.

Blood samples. Blood for the various analyses was obtained by cutting the tip ends of the tails of both the alligators and the rats and gently milking about 0.2–0.8 ml. of blood into heparinized capillary tubes. When larger amounts of blood were required as much as 5 ml. were withdrawn from the heart. The blood was centrifuged and the plasma was separated from the cells and stored in the freezing compartment of a refrigerator until analysed.

In the experiments with rats, blood was removed at about 15 min. intervals after the injection of the amino

acids, and this was continued until the plasma levels had returned to the control values. Owing to the very low metabolic rate of the alligator, blood was obtained at less frequent intervals ranging from 2 to 24 hr., depending upon the rate at which the amino acids were utilized.

For the amino acid analyses protein-free filtrates were prepared by adding 4 vol. of ammonia-free ethanol to plasma in small test tubes. These were then centrifuged and the protein-free supernatants were separated. Samples were used for the various amino acid analyses.

Injections. The insulin used was Iletin, the crystalline zinc insulin of Eli Lilly and Co. The alligators were injected intramuscularly with 100 units/kg. body wt. 1–2 days before the amino acid to be tested to insure a maximum physiological effect. Such a dose was effective for a full 10 days, and after the third day it was necessary to give glucose to prevent hypoglycaemia. The effect of insulin on the rate of amino acid utilization in the rat was not studied.

The amino acids were obtained from Nutritional Biochemicals Inc., Cleveland, Ohio, and in those few instances in which impurities were suspected they were recrystallized before use. Although several experiments on both alligators and rats were performed in which the amino acids were injected intracardially or given by stomach tube, the most consistent results were obtained after the intraperitoneal injection of 10 m-moles/kg. body wt. of an iso-osmotic solution for the soluble compounds, and as an equivalent saturated solution for such sparingly soluble amino acids as leucine, isoleucine, tryptophan and phenylalanine. Aspartic and glutamic acid were injected in the form of their monosodium salts and arginine, histidine, lysine and ornithine in the form of their monohydrochlorides.

Amino acid and glucose analyses. The non-protein amino acid composition of alligator plasma was determined by the microbiological method of Steel, Sauberlich, Reynolds & Baumann (1949) and Sauberlich & Baumann (1949) with the exception of glutamine, which was estimated by the glutaminase method of Iber & Bruton (1956).

Depending on the amino acid to be measured microbiologically and the quantity known to be required for optimum growth (Steele *et al.* 1949), 0.05–2.0 ml. of the alcoholic 1:5 protein-free plasma filtrate was put in a culture tube and the alcohol was evaporated in a water bath. Sterile water (1 ml.) and sterile medium (1 ml.) were added and each tube was inoculated with 0.001 ml. of a washed suspension of organisms in 0.9% NaCl solution. The culture tubes were incubated for 3 days at 38° and the lactic acid formed was estimated by titration. The titration values were compared with those obtained with a series of tubes which contained standard amounts of the amino acid to be measured.

In the amino acid tolerance experiments the high plasma concentration of the injected amino acid made it easy to estimate the amino acid microbiologically with accuracy. Plasma from untreated alligators deprived of food for two weeks, however, contained only traces of several of the amino acids and some difficulty was encountered in the estimation of those present in very low amounts. Glutamic acid, in particular, required as much as 2 ml. of plasma to give enough of the protein-free filtrate for satisfactory bacterial growth.

The ninhydrin method of Troll & Cannan (1953) was used for the determination of total amino acids in the amino acid tolerance experiments. A routine was adopted in

Table 1. *Amino acid composition of alligator and human plasma expressed in terms of per cent of the total amino acids determined by microbiological analysis*

'Total amino acids' in this table refers only to the sum of the 18 amino acids which were identified and analysed by microbiological assay. Since only about three-quarters of the total α -amino acids were accounted for in the microbiological assays, the percentage values in the table represent a slight exaggeration. The values for man were calculated from the data of Harper, Hutchin & Kimmel (1952), and those for the alligator represent the values obtained from twelve alligators which had been starved for 2 weeks. The 'total amino acid' concentration of alligator plasma was 1.68 (2.05 by ninhydrin) and that for man was 3.70 mM (ninhydrin total not reported). For abbreviations of amino acids see *Biochem. J.* (1957), 66, 6. Tr, trace.

Amino acid	Alligator		Man		Amino acid	Alligator		Man	
	Mean (%)	Range (%)	Mean (%)	Range (%)		Mean (%)	Range (%)	Mean (%)	Range (%)
Gly	27	(12-32)	11	(3-20)	Thr	4	(2-7)	5	(2-9)
Ala	12	(5-16)	12	(7-23)	Ileu	2	(1-4)	4	(2-8)
Glu-NH ₂	12	(5-30)	14	(9-20)	Cys ₂ S ₂	2	(1-3)	7	(4-11)
Val	8	(7-9)	8	(6-10)	Lys	2	(0-3)	7	(4-11)
Leu	7	(3-14)	5	(2-10)	His	1	(0-2)	4	(4-7)
Ser	6	(5-7)	4	(1-6)	Try	1	(0-2)	2	(1-3)
Pro	5	(0-6)	6	(3-13)	Arg	1	(0-2)	4	(2-5)
Phe	5	(4-7)	4	(2-8)	Met	0.6	(0-0.8)	1	(0.5-2)
Glu	4	(0-8)	2	(0-3)	Tyr	0.6	(0-0.7)	2	(2-4)
					Asp	—	—	Tr	(0-Tr)

Fig. 1. Effect of a single intramuscular injection of 100 units of insulin per kilogram into small alligators. The initial hyperglycaemia is characteristic of the alligator, and, so far as it can be determined, this effect is not due to the contamination of insulin with glucagon. The hypoglycaemia and hypoaminoacidaemia persisted for about 10 days. The perpendicular lines indicate the ranges observed in 10 experimental animals. —, Plasma glucose; --- plasma amino acids.

which the total amino acid concentration was estimated by the ninhydrin method, and occasional samples in each experiment were analysed by microbiological analyses. By this means it was possible to verify the fact that, with rare exceptions, the rise in total plasma amino acids after an injection was due almost entirely to an increase in the amino acid injected. For proline the curves are based solely on microbiological analyses. No satisfactory method was available for the specific analysis of the D-isomers and the tolerance curves reported were derived entirely from the ninhydrin analyses.

Plasma glucose of the alligator was estimated by the glucose oxidase method of Keston (1956) with the commercial reagents and the modifications recommended by the Worthington Biochemical Corp., Freehold, N.J. This very convenient procedure required only 0.1 ml. of plasma. When it was discovered that the method of Folin & Wu (1920) gave identical results for the alligator, this method was then employed instead.

RESULTS

Amino acid composition of alligator plasma

As a preliminary to the proposed tolerance tests 9 ml. of plasma from each of 12 control alligators deprived of food for 14 days was assayed for 18 amino acids. The results are shown in Table 1 along with those obtained by Harper, Hutchin & Kimmel (1952) for a group of fasting men. Curiously enough the composition of alligator blood is quite similar to that of man. In both species 50 % or more of the total identified α -amino nitrogen was in the form of glycine, alanine, glutamine, valine and leucine. Of these five amino acids, all but valine proved to be precursors of urine ammonia. None of the other amino acids, which were present in lesser concentrations, contributed greatly to renal ammonia synthesis in either man or the alligator (Coulson & Hernandez, 1959).

When insulin was injected into alligators (Fig. 1) there was a slow decline from a value of about 4 m-moles of total amino acid/l. to a level approaching zero. It was not possible to determine whether there was any change in the relative ratio of these amino acids with respect to each other since their concentrations fell too low to be measured by our methods of analysis. In this experiment glucose concentrations were also measured and it is apparent that, apart from the phenomenon of an initial hyperglycaemia phase (Coulson & Hernandez, 1953), both glucose and amino acid concentrations decreased at about the same rate (Fig. 1). In this regard, at least, the effect of insulin on amino acids was as marked as it was on glucose.

L-Amino acid tolerance in the alligator

In the series of experiments illustrated in Fig. 2A and Fig. 2B the changes in the plasma amino acid levels were followed after the injection of amino acids into alligators with and without insulin. Where the amino acids disappeared very slowly

from the plasma, the results of only the first 2 or 3 days appear in the figure owing to space limitations. Several methods of assessing the relative rates of utilization were tried and the most satisfactory index of metabolic activity was obtained by comparing the reciprocals of the respective areas under the curves with that of L-serine, the amino acid which disappeared at the fastest rate. If the area beneath the tolerance curve of L-serine is given an arbitrary value of 100 %, then a tolerance curve for another amino acid which had twice the area of that of L-serine would be given a metabolic index value of 50 %. Although this is a rough measure at best, it is still possible to say that L-serine was utilized many times as fast as L-methionine or L-glutamic acid.

D-Amino acid tolerance in the alligator

Although several of the D-amino acids were removed much more slowly than their respective natural isomers (Figs. 2A, 2B), about half were metabolized at the same rate and it was considered possible that the animals could have converted some of the D-forms into the L-isomers. To test this, eight of the slowly metabolized D-amino acids were injected into alligators and blood for analysis was removed 48 hr. later. The results of the microbiological assays of the L-amino acids in the plasma appear in Table 2. Injection of D-isoleucine and D-leucine did not lead to an increase, whereas each of the other six amino acids caused significant increases in the respective L-isomers. There is a problem of interpretation involved. Since an assay of all of the amino acid components was not attempted, it is possible that some of the other amino acids may have increased as well and that the magnitude of the small rise in the L-forms of valine, threonine and tryptophan may not have exceeded that of some of the others which were not determined. Although the results suggest isomerization, more experiments are needed for conclusive proof.

Cell permeability may also be an important factor in that in order to detect an increase in the L-isomer it must first be synthesized in the cell and then released into the plasma where it can be detected. The evidence does not permit the conclusion that the eight D-amino acids in Table 2 were selectively prevented from entering the cells since each of these compounds was distributed in about the same volume of water as its enantiomorph (Figs. 2A, 2B).

Effect of insulin on amino acid tolerance in the alligator

Although most of the amino acids of both isomers disappeared more rapidly when insulin was injected, there were a number of exceptions (Figs. 2A, 2B). It is difficult to formulate any logical rule

which would apply to the entire group. Some of the D-isomers were not as susceptible to the action of this hormone as their natural derivatives and in other cases insulin catalysed the removal of both isomers at the same rate. Valine was the only amino acid in which insulin affected the D-form significantly more than it did the L-form.

Insulin appeared to increase the toxicity of many of the amino acids whether the animals received glucose or not. This was a serious problem in the tryptophan experiments where the addition of insulin invariably killed the alligators within 48 hr. In the entire series of experiments thirty alligators died as a result of the amino acid injections and all

Fig. 2A. For legend see facing page.

Fig. 2B

Figs. 2A and 2B. Amino acid tolerances in the alligator. Each amino acid (10 m-moles/kg. body wt.) was injected intraperitoneally into all animals. Four or more alligators were used in each of the groups. ●, Tolerance curves for the control groups; ○, tolerance curves for alligators treated with insulin. The perpendicular bars in the graph refer to the ranges of values encountered. As D-tryptophan was toxic and no alligator receiving insulin and D-tryptophan lived beyond 48 hr., the significance of these results is questionable. The first number in the upper right corner of each tolerance curve refers to the rate of utilization of each amino acid relative to that of L-serine, which was given an arbitrary value of 100% (see text). The second number refers to the rate found after injection of insulin. For example, L-glutamic acid without insulin was utilized at 8% of the rate of L-serine and insulin increased the rate to 17%. To conserve space, in some cases only, the values for the first 2-3 days were plotted. Several D-amino acids were unavailable and it was necessary to substitute the racemic mixtures.

but two were in the groups which received insulin. With the exception of D-serine no apparent specific difference was seen in the nature of the toxic symptoms occurring after different amino acids. The alligators vomited occasionally, gradually became torpid and died from causes which were not due to hypoglycaemia but were otherwise undetermined. The deaths were distributed at random

among both D- and L-isomers of tryptophan, isoleucine, threonine, valine, citrulline, histidine, leucine, arginine, ornithine and serine. D-Serine always produced renal failure characterized by an initial anuria followed by death or by slow recovery over a period of months. None of the other amino acids were toxic enough to interfere with the amino acid tolerances.

Table 2. Mean plasma concentration of L-amino acids 48 hr. after the injection of 10 m-moles/kg. of the corresponding D-isomers

Three alligators were used in each experiment. The control values represent an average for 12 alligators deprived of food for two weeks. The plasma concentrations of the natural isomers were determined microbiologically.

D-Amino acid injected	L-Isomer (m-moles/l.) (Expt.)		L-Isomer (m-moles/l.) (Controls)	
	Mean	Range	Mean	Range
Glu	4.51	(1.80-8.20)	0.07	(0.02-0.10)
Met	1.12	(0.71-1.92)	0.01	(Trace-0.02)
His	0.62	(0.48-0.79)	0.02	(0.01-0.03)
Val	0.40	(0.32-0.42)	0.13	(0.10-0.15)
Thr	0.20	(0.10-0.26)	0.07	(0.04-0.10)
Try	0.11	(0.07-0.19)	0.02	(0.01-0.02)
Ileu	0.06	(0.02-0.08)	0.04	(0.02-0.09)
Leu	Trace	(0.0-0.02)*	0.11	(0.05-0.15)

* The D-isomer may have interfered with the growth of the assay organism.

Amino acid tolerance in the rat

The difficulties encountered in performing amino acid tolerances in the rat were many. Perhaps the most serious one was the variability of the results seen in replicate experiments. Fig. 3 is derived from the areas underneath the respective tolerance curves in the rat for some D- and L-amino acids. With L-serine receiving an arbitrary value of 100, the bar graphs compare the respective reciprocals of the areas derived from the amino acid tolerances and hence they are an index of the relative ease with which each amino acid was utilized by the rat. The rat metabolizes amino acids so much more rapidly than the alligator that it is difficult to compare their tolerances on the same scale. Some indication of the relative metabolic rates may be seen in Fig. 4 in which representative alligator and rat glutamic acid tolerance curves are compared. Since the area of the alligator curve is about 50 times that of the rat, the rat utilizes glutamic acid some 50 times as fast as the alligator. In general this ratio

Fig. 3. Relative rates of utilization of L- and D-amino acids by the rat. The reciprocals of the areas of the rat tolerance curves are indicated by the heights of the rectangles. Since L-serine disappeared in the shortest time, and since this tolerance curve had the least area, it was given an arbitrary value of 100%. The perpendicular lines indicate the range. There were four or more rats in each experiment. Each rat received 10 m-moles of amino acid/kg. body wt.

prevailed for the other amino acids as well. The rapid removal of amino acids from rat plasma prevented an accurate assessment of their distribution in body fluids.

No satisfactory corrections for undoubted urinary losses of the D-isomers (Berg, 1953) in the rat were possible without the necessity for the complete isolation of these components in the urine. It was not possible to measure the unnatural isomers by microbiological assay, and ninhydrin reacts not only with the D-isomers but with the other α -amino metabolic derivatives as well. Fortunately urinary losses were not a problem in the alligator owing to the very low excretory rate of the kidney (Coulson & Hernandez, 1955). The inability of the alligator to excrete urine more concentrated than the plasma (280 m-osmoles/l.) prevented the excretion of amino acids in any quantity. For example, if the urine volume were 10 ml./kg./day and if half the solids in the urine were due to amino acids, the total excreted would be only a little over 1 m-mole/day or 10% of the amount injected. The true values were so much lower than this quantity that they were of no consequence in these experiments.

Conversion of histidine into glutamic acid

For a number of reasons experiments *in vivo* are unsuitable for determining the extent to which one compound is converted into another. For example, if a large amount of the injected glycine were converted into alanine, the increase in plasma alanine would necessarily be very small owing to the fact that it is also metabolized rapidly. However, if one

Fig. 4. Rate of disappearance of 10 m-moles of L-glutamic acid/kg. in the rat (—) and in the alligator (---). There were ten animals in each group. The sodium salt was injected in an iso-osmotic solution into the peritoneum.

amino acid were converted into a different amino acid which was utilized slowly, it might then be possible to detect a significant increase in the plasma concentration of the new derivative. Table 3 illustrates one such interconversion. It appears that both D- and L-histidine were converted into L-glutamic acid. In this instance at least, it was possible to show that a reaction well known in mammalian liver-slice studies also occurs in the intact alligator.

DISCUSSION

When the height of the individual peaks in the amino acid tolerance curves are considered they fall into two general classes: those which reached a plasma concentration of about 20 m-moles/l. and those which reached a level of about 12 m-moles/l. It is logical to assume that for a brief time at least the class with the lower peaks must have been distributed in about twice the body-water volume of the class that reached the higher concentrations. An alligator of the size used in these experiments has an extracellular-fluid volume of about 400 ml./kg. body wt. and a total body-water volume of about 700 ml./kg. (Coulson & Hernandez, 1953). The injection of 10 m-moles of any non-utilizable substance would result in the attainment of a plasma concentration of about 14 m-moles/l. if it were distributed evenly in the body water, and of about 25 m-moles/l. if it were restricted to the extracellular spaces. Although absolute proof is lacking, the evidence would indicate that histidine, aspartic acid and glutamic acid are largely confined for a time to the extracellular fluids and that most of the others are more or less distributed evenly throughout the body water.

The rate at which any amino acid may be burned by an alligator is limited by the metabolic rate. On an average the complete oxidation of about 10 m-moles of an amino acid by a 1 kg. alligator would furnish all of the energy required for 1 day (Coulson & Hernandez, 1959). From the fact that several amino acids disappeared in about half a day, it must be assumed that either some form of storage occurred or that the amino acids were only partially degraded to carbon dioxide, water and uric acid. The slowly utilized compounds such as methionine

Table 3. *Conversion of D- and L-histidine into L-glutamic acid in the alligator*

Plasma was analysed 96 hr. after the injection of 10 m-moles of histidine/kg. body wt. There were four alligators in each experiment and four controls. All values are expressed as m-moles/l. of plasma.

Amino acid injected	Total amino acids (ninhydrin)		L-Glu (Microbiol.) (Expt.)		L-Glu (Microbiol.) (Controls)	
	Mean	Range	Mean	Range	Mean	Range
L-His	2.7	(2.0-3.3)	1.60	(1.38-1.83)	0.04	(0.02-0.10)
D-His	3.3	(1.8-5.1)	0.41	(0.29-0.65)	—	—

and glutamic acid could neither have functioned as efficient immediate energy sources nor as ready sources of raw material for protein synthesis in these experiments since the rate of their disposal was too low.

Some of the D-derivatives disappeared at a much slower rate than their respective natural isomers. The decreased ability to utilize the unnatural forms must have been associated with a decreased ability of the alligator to deaminate them. If the proper D-amino acid oxidases were present, either they were not in contact with the substrate, or they were present in too low a concentration to be effective. The existence of considerable concentrations of seemingly useless D-amino acid oxidases in the liver and kidney of several species of animals has long been a curiosity. These studies *in vivo* have not established the existence of such functional enzymes in the intact alligator.

The rate at which any amino acid of either configuration disappears from alligator plasma must be associated in some way with the rate at which it is used for protein synthesis, the rate at which it can be deaminated, or both. Those amino acids that remain in the extracellular fluid for days could not have entered rapidly into any deamination or transamination reactions. If the amino group had been removed, microbiological assay would have indicated the disappearance of the compound injected. That this did not occur was indicated by the fact that the ninhydrin-reacting substance found in the plasma 4 days after the injection of L-methionine was identified as unchanged methionine by microbiological assay.

There was a striking difference between the rate of removal of the essential amino acids and that of the non-essential amino acids. If one considers that arginine is not essential, then it can be said that of the essential compounds only lysine was removed rapidly by the alligator and only leucine by the rat. Conversely all of the dispensable amino acids were utilized very quickly by both species with the exception of glutamic acid and aspartic acid, which were metabolized with difficulty. This difference between the two types of amino acids probably illustrates a physiological adaptation in which the dispensable ones are used as a ready source of energy in contrast with the indispensable ones which are reserved for protein synthesis.

The relative rates of metabolism of various amino acids in the intact rat were in general similar to those in the alligator, which suggests that the metabolic pathways involved are similar for both species. It is generally assumed (although almost never stated) that transaminases and L-amino acid oxidases are ordinarily utilized *in vivo* in the catabolism of the various amino acids subject to their actions. When the activity of rat-kidney L-amino acid oxidase was tested by the successive addition

of different amino acids *in vitro*, oxidative deamination occurred most rapidly with L-leucine as the substrate (Blanchard, Green, Nocito & Ratner, 1944). The decreasing order of activity was leucine, methionine, proline, isoleucine, tryptophan, valine, alanine etc. We found no correlation between the results of these studies *in vitro* and the rate at which the amino acids disappeared from the plasma of either the intact rat or the alligator. The compounds deaminated most readily *in vitro* usually had the lowest metabolic rate *in vivo*.

If it is assumed that the use of L-amino oxidases represented only a minor pathway of metabolism, and that the mechanism of transamination is the ordinary means by which these compounds are degraded, new theoretical problems arise. If most amino acids transfer their amino groups to such acceptors as α -oxoglutarate and oxaloacetate, the net result would be the formation of glutamate and aspartate. Since neither of these substances is metabolized rapidly *in vivo*, this would represent a rate-limiting step. It is difficult to conceive how large amounts of many of the non-essential amino acids could disappear in such a short time by such a route.

In general there appears to be very little correlation between the structure of an amino acid and the amount of influence insulin has upon its rate of removal. The absence of any effect of insulin on valine, leucine and isoleucine may indicate that this hormone is not active in accelerating the metabolism of certain branched-chain compounds. It is very difficult to visualize the effect of insulin in terms of the activation of any single enzyme system and it is also difficult to understand its effects solely in terms of increased cell permeability. For example, threonine is distributed in body water and glutamic acid is restricted to the extracellular fluid. It might be expected that insulin would drive glutamic acid across the cell barrier and hence that its effects would be much greater than on threonine, which is already available to the cell. The reverse was true.

Sugars which are metabolized more rapidly under the influence of insulin have the same configuration with respect to the first three carbon atoms (Levine & Goldstein, 1955). This and other similar observations inclined many observers toward the view that the classical 'lock and key' principle is involved. According to many, the observed effects of insulin on fats and proteins are most easily explained on the basis of their direct effects on carbohydrate metabolism. Although such theories have the advantage of simplicity it is difficult to apply them to explain the observed effects of insulin on all of the different amino acids of both configurations. It is possible that insulin affects directly more types of compounds than is commonly believed.

SUMMARY

1. By taking advantage of the low metabolic rate of the alligator, it was possible to study the effect of insulin on the rate at which each of a large group of amino acids was utilized *in vivo*.

2. 'Amino acid tolerances' of both isomers of amino acids were determined in the alligator and in the rat by the injection of 10 m-moles/kg. followed by analyses of plasma amino acids at frequent intervals. The rat metabolized amino acids from 50 to 100 times as fast as the alligator. In general, the non-essential amino acids were utilized much more rapidly than the essential ones by both species.

3. Each amino acid had its characteristic tolerance curve.

4. In the alligator, insulin increased the rate of metabolism of most of the exogenous amino acids of both isomers.

5. Insulin appears to enhance the metabolism of compounds which differ greatly in chemical structure.

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REFERENCES

- Berg, C. P. (1953). *Physiol. Rev.* **33**, 145.
 Blanchard, M., Green, D. E., Nocito, V. & Ratner, S. (1944). *J. biol. Chem.* **155**, 421.
 Coulson, R. A. & Hernandez, T. (1953). *Endocrinology*, **53**, 311.
 Coulson, R. A. & Hernandez, T. (1955). *Proc. Soc. exp. Biol., N.Y.*, **88**, 682.
 Coulson, R. A. & Hernandez, T. (1959). *Amer. J. Physiol.* **197**, 873.
 Folin, O. & Wu, H. (1920). *J. biol. Chem.* **41**, 367.
 Frame, E. G. & Russell, J. A. (1946). *Endocrinology*, **39**, 420.
 Harper, H. A., Hutchin, M. E. & Kimmel, J. R. (1952). *Proc. Soc. exp. Biol., N.Y.*, **80**, 768.
 Hier, S. W. (1947). *J. biol. Chem.* **171**, 813.
 Iber, F. L. & Bruton, J. (1956). *Clin. Chem.* **2**, 413.
 Keston, A. S. (1956). *Abstr. Pap. Amer. Chem. Soc., Div. Biol. Chem.*, April, 31c.
 Levine, R. & Goldstein, M. S. (1955). *Recent Progr. Hormone Res.* **11**, 343.
 Lotspeich, W. D. (1949). *J. biol. Chem.* **179**, 175.
 Luck, J. M., Morrison, G. & Wilbur, L. F. (1928). *J. biol. Chem.* **77**, 151.
 Russell, J. A. (1955). *Fed. Proc.* **14**, 696.
 Sauberlich, H. E. & Baumann, C. A. (1949). *J. biol. Chem.* **177**, 545.
 Steel, B. F., Sauberlich, H. E., Reynolds, M. S. & Baumann, C. A. (1949). *J. biol. Chem.* **177**, 533.
 Troll, W. & Cannan, R. K. (1953). *J. biol. Chem.* **200**, 803.

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The Effect of Testicular Hyaluronidase on the Hyaluronic Acid Complexes of Human Synovial Fluid

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It has been shown that the electrophoretic mobility of the human synovial-fluid proteins is affected by the viscosity of the solution (Pigman, Patton & Platt, 1957). This effect was interpreted as a formation of a complex of the viscous component of synovial fluid, hyaluronic acid, with the proteins. Evidence for this formation has been given by a number of investigators (Ropes, Robertson, Rossmeisl, Peabody & Bauer, 1947; Ogston & Stanier, 1950, 1952; Curtain, 1955; Blumberg & Ogston, 1957; Pigman *et al.* 1957; Hamerman & Sandson, 1959; Pigman, Gramling, Platt & Holley, 1959). A component moving with

a mobility between those for hyaluronic acid and albumin was found in many normal and post-mortem human synovial fluids (Platt, Pigman, Holley & Patton, 1956; Pigman *et al.* 1957). This component has been named the π -peak (Platt *et al.* 1956) and was believed to be a stable complex between hyaluronic acid and protein (Platt, 1957). The present investigation was undertaken to ascertain whether the π -peak is a non-dissociable complex between hyaluronic acid and protein, and whether the increase in the electrophoretic mobility of the synovial proteins is solely the result of an increase in viscosity.