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# Amino-acid Requirements of Amylase Synthesis by Pigeon-Pancreas Slices

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In previous publications (Hokin, 1950, 1951*a*), it was shown that pigeon-pancreas slices synthesize α-amylase when incubated in glucose saline media and that the addition of acid-hydrolysed casein supplemented with tryptophan, or a mixture of twenty amino-acids, increased the rate of synthesis approximately threefold.

The present paper is concerned with the role of the individual amino-acids in α-amylase synthesis. A mixture of ten amino-acids (tryptophan, arginine, threonine, valine, tyrosine, lysine, leucine, histidine, phenylalanine and isoleucine) was found to be necessary and also sufficient for the maximum rate of amylase synthesis.

Part of this work has been communicated to the Biochemical Society (Hokin, 1951*b*).

## EXPERIMENTAL

*Media.* The medium used for the incubation of slices was the bicarbonate saline of Krebs & Henseleit (1932) gassed with 5% $CO_2$ in $O_2$. A similar saline containing only 0·00043 M-bicarbonate not gassed, was used as a suspending medium for slices before incubation. Glucose (0·2%) was added in all experiments.

*Amino-acids.* Commercial amino-acids were used. The standard mixtures of amino-acids and the final concentrations in the medium are shown in Table 1. In earlier experiments mixtures I and III, which contained L-amino-acids, with the exception of DL-serine and DL-threonine, were used. Mixture I contained the ten amino-acids found to be essential for the nutrition of the rat (Rose, 1938) and chick (Almquist, 1947), whilst mixture III contained ten 'non-essential' amino-acids. In the later experiments, after it had been established by paper chromatography that three of the L-amino-acids from natural sources; i.e. leucine, isoleucine and tryptophan were not pure, mixtures II and IV, which contained synthetic racemic amino-acids, with the exception of L-arginine, L-histidine and L-hydroxyproline, were used. Stock solutions of mixtures II and IV were made up by dissolving the amino-acids in water and neutralizing with 1 N-NaOH. Mixtures I and II were added from stock solutions in which the amino-acids were dissolved in bicarbonate saline.

*Ketonic acids.* Indolylpyruvic acid was synthesized from indole aldehyde according to the method of Ellinger & Matsuoka (1920). It was light buff coloured; it darkened at 200° and melted at 205° (uncorr.). Indole aldehyde was

Table 1. *Standard amino-acid mixtures*

(Concentrations are those in incubation vessels in routine experiments.)

| Mixture I | | Mixture II | | Mixture III | | Mixture IV | |
|---|---|---|---|---|---|---|---|
| Amino-acid | Concn. (M) | Amino-acid | Concn. (M) | Amino-acid | Concn. (M) | Amino-acid | Concn. (M) |
| L-Tryptophan | 0·002 | DL-Tryptophan | 0·002 | L-Glutamate | 0·002 | DL-Glutamate | 0·002 |
| L-Arginine | 0·002 | L-Arginine | 0·001 | L-Aspartate | 0·002 | DL-Aspartate | 0·002 |
| DL-Threonine | 0·004 | DL-Threonine | 0·002 | L-Alanine | 0·002 | DL-Alanine | 0·002 |
| L-Lysine | 0·002 | DL-Lysine | 0·002 | L-Glycine | 0·002 | Glycine | 0·001 |
| L-Leucine | 0·002 | DL-Leucine | 0·002 | DL-Serine | 0·004 | DL-Serine | 0·002 |
| L-Valine | 0·002 | DL-Valine | 0·002 | L-Proline | 0·002 | DL-Proline | 0·002 |
| L-Histidine | 0·002 | L-Histidine | 0·001 | L-Hydroxyproline | 0·002 | L-Hydroxyproline | 0·001 |
| L-Isoleucine | 0·002 | DL-Isoleucine | 0·002 | L-Tyrosine | 0·002 | DL-Citrulline | 0·001 |
| L-Phenylalanine | 0·002 | DL-Phenylalanine | 0·002 | L-Cystine | 0·002 | DL-Methionine | 0·001 |
| L-Methionine | 0·002 | DL-Tyrosine | 0·002 | L-Citrulline | 0·002 | L-Citrulline | 0·002 |

synthesized from indole by the method of Boyd & Robson (1935). *p*-Hydroxyphenylpyruvic acid was obtained from Homburg Chemiewerk, Frankfurt a/M. Sodium α-keto *iso*-caproate and sodium α-keto *iso*valerate were kindly given by Dr A. Meister, National Institutes of Health, U.S.A. Indolylpyruvic acid and *p*-hydroxyphenylpyruvic acid were neutralized with 0·16M-$NaHCO_3$.

Stock solutions of amino-acids and ketonic acids were stored in the frozen state at approximately −10°.

*Preparation and incubation of tissues.* In previous experiments (Hokin, 1951*a*) the slices were placed in a chilled humidified crystallizing dish before incubation. In the earlier experiments of the present series the slices were suspended in chilled saline as soon as they were cut, where they remained until a sufficient number of slices had been prepared. However, the results were less consistent than with the original procedure (Hokin, 1951*a*), which was therefore re-instituted. Further improvements which increased precision were the rejection of all surface slices and the use of at least 70 mg. of tissue per vessel, derived from the anterior (large) lobe only. These modifications limited the number of parallel incubations from the pancreas of one pigeon. All of the data, except those presented in Table 2, are from experiments in which this improved technique was used.

Slices were incubated at 40° in beakers of about 18 ml. capacity in the metabolic incubator of Dubnoff (1948). Periods of incubation were 2–3 hr.

*Methods of assay.* Amylase activity was assayed by the method of Smith & Roe (1949) with the following modifications. Instead of adding the various ingredients separately to the assay tube, the starch was dissolved in a buffered solution which had been heated to the boiling point in a water bath. This solution contained 0·056M-NaCl and 0·067M-phosphate buffer, pH 7·2. After cooling to room temperature 9 ml. of the buffered starch solution was pipetted into each assay tube. The final volume and concentrations of the constituents in the assay tube were as recommended in the original procedure of Smith & Roe (1949). When enzyme solutions were assayed by the original and modified methods the amylase activities agreed within experimental error.

*Determination of total α-amino nitrogen of pancreas tissue.* Freshly removed pigeon pancreas (about 100–200 mg. tissue per determination) was placed in about 8 vol. water in a boiling-water bath for 2 min. The tissue and boiled extract were transferred to an all-glass homogenizer (Potter & Elvehjem, 1936). After homogenizing for about 10 sec., 1 vol. 1N-$H_2SO_4$ and 1 vol. 10% sodium tungstate were added, and the homogenization was continued for about a minute. The homogenate was transferred to a graduated centrifuge tube, the volume adjusted to 6 ml. and the mixture was centrifuged. Total α-amino N was determined on 5 ml. portions by the ninhydrin method of Van Slyke, Dillon, MacFadyen & Hamilton (1941), as modified by Hamilton & Van Slyke (1943).

*Calculations.* In this paper the difference between the total amylase activity of incubated slices (see Hokin, 1951*a*) and the amylase activity of unincubated slices is referred to as 'amylase units synthesized'. The effect of omitting an amino-acid is expressed by the following quotient: Percentage reduction in synthesis = [('amylase units synthesized' in standard mixture) − ('amylase units synthesized' in deficient mixture)] × 100/('amylase units synthesized' in standard mixture). In experiments in which the early technique was followed (see above) only those experiments were used in which synthesis in the standard mixture during the period of incubation exceeded 50 amylase units/mg. dry wt. (range 56–186 units). As described previously (Hokin, 1951*a*), 1 unit of Smith & Roe (1949) is equivalent to 0·25 μg. crystalline pig α-amylase.

## RESULTS

### *Amino-acid requirements for maximum amylase synthesis*

When the mixture of the twenty amino-acids shown previously to stimulate amylase synthesis (Hokin, 1951*a*) was replaced by one containing only the ten 'essential' amino-acids there was no significant effect on the rate of amylase synthesis. The mean per cent difference between synthesis in the presence of mixtures I plus III and mixture I was 1% for five experiments with a range of −19 to +19% and an S.E.M. of 7·4.

On the other hand, Table 2 shows that when any one 'essential' amino-acid was omitted there was a statistically significant reduction in synthesis, except in the case of methionine.

Table 2. *Effect of omission of essential amino-acids on α-amylase synthesis*

(Bicarbonate saline; 0·2% glucose; standard mixtures I plus III in four experiments and I alone in the remainder of experiments; 40°; incubation period 2–3 hr.; slices suspended in saline before incubation.)

| Amino-acid omitted | Reduction in synthesis (%) Mean | Range | S.E.M. | *P* | No. of experiments |
|---|---|---|---|---|---|
| L-Methionine | −4·9 | +25 to −29 | 6·7 | 0·450 | 8 |
| L-Phenylalanine | −13 | +7 to −29 | 4·6 | 0·020 | 9 |
| L-Isoleucine | −18 | +15 to −46 | 6·4 | 0·020 | 10 |
| L-Histidine | −22 | −3 to −55 | 5·4 | 0·004 | 9 |
| L-Valine | −29 | −9 to −46 | 5·4 | 0·002 | 7 |
| L-Leucine | −32 | +6 to −58 | 9·2 | 0·012 | 6 |
| L-Lysine | −42 | −15 to −69 | 7·3 | 0·002 | 6 |
| L-Threonine | −55 | −43 to −74 | 3·2 | <0·001 | 6 |
| L-Arginine | −59 | −39 to −66 | 4·7 | <0·001 | 6 |
| L-Tryptophan | −66 | −50 to −75 | 4·2 | <0·001 | 6 |
| All | −60 | −20 to −76 | 6·2 | <0·001 | 8 |

Subsequently it was found by paper chromatography that certain samples of the L-amino-acids contained other amino-acids as impurities. The L-tryptophan (L. Light and Co. Ltd., Wraysbury, Bucks) contained tyrosine; the L-leucine (Light's) contained tyrosine, methionine and cystine. The L-isoleucine (F. Hoffman-La Roche and Co., Basel, Switzerland) contained valine. The amounts of contaminating amino-acids were judged to be no more than 1 %. The remaining natural amino-acids were pure. Experiments on the omission of the amino-acids which were present as contaminants were therefore repeated using mixtures made up predominantly of synthetic amino-acids (mixtures II and IV). With twenty amino-acids added the rate of amylase synthesis was the same either in the presence of predominantly DL-amino-acids or in the presence of L-amino-acids; thus, the D compounds were not inhibitory.

Results from experiments using the 'purer' mixtures (II and IV) are summarized in Table 3. The omission of tyrosine now produced a mean reduction in synthesis of 53 %. The small amounts of tyrosine present as contaminant in the experiments recorded in Table 2 were thus sufficient to meet the needs of maximum amylase synthesis under the conditions of these experiments. Whilst in 'impure' mixtures the 'omission' of valine had caused a mean reduction of 29 % (Table 2) its complete omission from mixtures II and IV produced a mean reduction of 67 %.

Eight 'non-essential' amino-acids plus DL-methionine (mixture IV) still had no statistically significant effect on synthesis when added to the nine remaining 'essential' amino-acids plus tyrosine (mixture II). Thus methionine, although essential for the rat (Rose, 1938), chick (Almquist, 1947) and human (Rose, Johnson & Haines, 1950) was not required under the conditions of these experiments. This is consistent with the recent observation that methionine is the only 'essential' amino-acid not present in crystalline α-amylase (Boissonnas, 1950). The addition of L-cystine to mixtures II plus IV produced in three experiments a mean reduction in synthesis of 12 % (range 10–15 %; S.E.M. 2·2 %), which is significant. This inhibition was not affected by the omission of serine or its addition in larger quantities indicating that the metabolism of serine was not inhibited competitively by cystine.

Table 3 also shows the results of experiments in which the omission of phenylalanine and isoleucine was studied by the improved technique described in the experimental section. Lack of phenylalanine or isoleucine produced a mean reduction of 21 and 15 % respectively, as compared to 13 and 18 % in the experiments recorded in Table 2. The higher degree of precision of this technique, as compared to the earlier one, is borne out by the far greater degree of statistical significance achieved in about half as many experiments.

It will be noted in Tables 2 and 3 that when either tryptophan, arginine, threonine or valine was omitted, synthesis was about the same as in the complete absence of added amino-acids.

*Replacement of tryptophan, tyrosine, valine and leucine by their respective ketonic acids in the synthesis of α-amylase*

In order to test whether certain amino-acids necessary for maximum synthesis can be replaced by their corresponding α-ketonic acids, indolylpyruvate, *p*-hydroxyphenylpyruvate, α-keto*iso*caproate and α-keto*iso*valerate were substituted for tryptophan, tyrosine, valine and leucine, respectively, as shown in Table 4. Addition of ketonic acids was found to be as effective, or almost as effective, as their respective amino-acids in supporting maximum amylase synthesis. This suggests that the ketonic acids were aminated, presumably by transamination. The recent work of Cammarata & Cohen (1950) indicates that transamination reactions in the tissues studied by these authors cover a wider range of amino-acids than was thought previously (Cohen & McGilvery, 1949).

*Transamination in pigeon pancreas.* To test whether certain 'non-essential' amino-acids can arise by transamination of intermediates of carbohydrate metabolism—homogenates of pigeon pancreas were incubated with α-ketoglutarate and various amino-acids. Considerable quantities of glutamate (determined by the method of Gale (1945) as modified by Krebs, 1948) were formed from alanine and aspartate. On the other hand, no significant quantities of glutamate were formed from

Table 3. *Omission of amino-acids from 'pure' amino-acid mixtures*

(Bicarbonate saline; 0·2 % glucose; standard amino-acid mixtures consisted of mixtures II plus IV; 40°; incubation period 3 hr.)

| Amino-acid omitted | Reduction in synthesis (%) Mean | Range | S.E.M. | *P* | No. of experiments |
|---|---|---|---|---|---|
| Mixture IV | +2·8 | +13 to −14 | 5·9 | 0·644 | 5 |
| Isoleucine | −15 | −5 to −27 | 3·4 | 0·007 | 6 |
| Phenylalanine | −21 | −14 to −26 | 2·1 | <0·001 | 5 |
| Tyrosine | −53 | −34 to −71 | 8·2 | <0·001 | 4 |
| Valine | −67 | −64 to −72 | 2·4 | <0·001 | 3 |

Table 4. *Replacement of tryptophan, tyrosine, leucine and valine by their respective ketonic acids*

(Bicarbonate saline; 0·2% glucose; 40°; incubation period 3 hr.)

| Exp. no. | Amino-acid omitted | Ketonic acid added | 'Amylase units synthesised' (units of Smith & Roe (1949) per mg. dry wt.) |
|---|---|---|---|
| 1 | None | None | 68* |
| | DL-Tryptophan | None | 26 |
| | DL-Tryptophan | Indolylpyruvic acid | 62 |
| 2 | None | None | 71* |
| | DL-Tyrosine | None | 52 |
| | DL-Tyrosine | *p*-Hydroxyphenylpyruvic acid | 68 |
| 3 | None | None | 83 |
| | DL-Leucine | None | 50 |
| | DL-Leucine | α-Keto*iso*caproic acid | 84 |
| | DL-Valine | None | 23 |
| | DL-Valine | α-Keto*iso*valeric acid | 87 |

* Mean of duplicate slices.

glycine, DL-serine, DL-tyrosine, DL-tryptophan, DL-phenylalanine, or DL-proline. These data indicate that aspartic acid and alanine can arise in pigeon pancreas by transamination of intermediates of the tricarboxylic acid cycle.

The failure of tryptophan and tyrosine and possibly other amino-acids to form significant quantities of glutamate is probably due to the relatively insensitive technique of detection used (see Cammerata & Cohen, 1950).

*Total α-amino nitrogen of pigeon pancreas.* The mean free α-amino nitrogen of the pancreas of four pigeons was 36 mg. N/100 g. tissue, range, 28–45, which is of the same order as the values found by Hamilton (1945) for various tissues of the dog.

## DISCUSSION

*Amino-acids and protein synthesis.* In preliminary experiments Boissonnas (1950) has found by paper chromatography the following amino-acids in crystalline α-amylase in decreasing order of concentration: aspartic acid, alanine, glycine, leucine, and isoleucine, valine and serine, phenylalanine, glutamic acid, threonine, lysine, arginine, proline, histidine, tyrosine and tryptophan. It is of considerable interest that all of the 'essential' amino-acids and only one 'non-essential' amino-acid (tyrosine) are needed for maximum synthesis. The fact that tyrosine is necessary indicates that pigeon pancreas is unable to convert phenylalanine to tyrosine.

It is also of interest that methionine, which is the only 'essential' amino-acid not present in α-amylase (Boissonnas, 1950) is not needed for amylase synthesis.

A comparison of the total α-amino nitrogen of pigeon pancreas with the data of Krebs, Eggleston & Hems (1949) indicates that glutamic acid plus glutamine comprise about 50% of the total amino nitrogen of pigeon pancreas. These compounds, by transamination of intermediates of carbohydrate metabolism, may give rise to alanine and aspartic acid in sufficient quantities to satisfy the needs of amylase synthesis. The source of the remaining 'non-essential' amino-acids, i.e. glycine, serine and proline, which are present in α-amylase, but which are not needed for its synthesis under the conditions of these experiments is not known. These three amino-acids may be formed in pancreas from non-protein sources or they may exist in concentrations sufficiently high to satisfy the requirements of amylase synthesis. The data of Wiss (1949) indicate that glycine and serine, but not proline, exist in relatively high concentrations in liver. Preliminary experiments indicate that considerable proteolysis occurs during the incubation of pancreas slices. This would obviously be another source of amino-acids, but the data presented here indicate that this process cannot supply adequate amounts of 'essential' amino-acids.

Peters & Anfinsen (1950) have recently reported a net synthesis of serum albumin by chicken-liver slices, but no stimulation of albumin synthesis was observed when a mixture of amino-acids was added. The fact that amino-acids do not stimulate albumin synthesis in chicken-liver slices may be due to the fact that the rate of protein synthesis in this system is below that rate which is likely to be limited by the tissue stores of free amino-acids. It has also been observed by Borsook, Deasy, Haagen-Smit, Keighly & Lowy (1950) that the *in vitro* incorporation of labelled amino-acids into tissue proteins of guinea pig liver homogenate is not stimulated by the addition of mixtures of non-labelled amino-acids. Greenberg, Friedberg, Schulman & Winnick (1949), on the other hand, have observed a twofold stimulation of glycine-1 $^{14}C$ uptake by the addition of a mixture of unlabelled amino-acids to adult rat-liver

homogenates but not to homogenates of foetal liver. Later, Winnick (1950), by dialysing foetal-liver homogenates, obtained a stimulation of glycine-1 $^{14}C$ uptake by the addition of an unlabelled amino-acid mixture, and he suggested that the previous failure to obtain a stimulation of glycine-$^{14}C$ uptake in foetal liver homogenates was probably due to relatively higher amino-acid concentrations prevailing in embryonic tissues.

The variable effects of added amino-acid mixtures on the *in vitro* synthesis of specific proteins and on the incorporation of labelled amino-acids into proteins may be accounted for by several factors. It is likely that the *in vitro* incorporation of labelled amino-acids into tissue proteins is a resultant of both net protein synthesis and a 'dynamic state' in which no additional protein is formed. The relative contribution of each of these factors to the total amino-acid turnover will depend on the particular system employed. The *in vitro* stimulation of either the synthesis of specific proteins or the incorporation of labelled amino-acids into proteins by added amino-acid mixtures would probably depend on the amount of new protein being formed and the concentrations of endogenous amino-acids.

## SUMMARY

1. Of the sixteen amino-acids found in crystalline α-amylase (Boissonnas, 1950) ten have been found necessary and also sufficient for maximum synthesis of the enzyme by pigeon-pancreas slices. These amino-acids are: tryptophan, arginine, threonine, valine, tyrosine, lysine, leucine, histidine, isoleucine and phenylalanine. They include every 'essential' amino-acid present in crystalline α-amylase, and one 'non-essential' amino-acid, i.e. tyrosine.

2. In the synthesis of amylase, tryptophan, tyrosine, valine and leucine can be replaced 80–100 % by their respective ketonic acids.

3. The origin of the 'non-essential' amino-acids is discussed. Glutamate plus glutamine comprise about 50 % of the total free amino nitrogen of pigeon pancreas. These compounds by transamination of intermediates of carbohydrate metabolism, may give rise to aspartic acid and alanine.

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