The Use of Asparagine and Glutamine for the Biosynthesis of Casein and Plasma Proteins

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The mammary gland is a system in which the components of the blood stream which are used by body tissues for protein synthesis can be readily investigated (Barry, 1956). Previous work has shown that the mammary gland takes the free essential amino acids from the blood stream to provide the essential amino acids of casein (Barry, 1952; Campbell & Work, 1952; Askonas, Campbell, Godin & Work, 1955), and takes free glutamine and glutamic acid to provide the glutamine and glutamic acid residues respectively of casein (Barry, 1956; Sheldon-Peters & Barry, 1956).

Experiments described in this paper show that the mammary gland of the goat takes free asparagine from the blood stream to provide asparagine, but not aspartic acid, residues of casein. They also show that asparagine and glutamine are used in the biosynthesis of plasma proteins to provide asparagine and glutamine residues. These results provide the first evidence for the function of asparagine in animals, and show that the use of glutamine in protein biosynthesis is not confined to casein synthesis.

In this paper 'casein asparagine' refers to the asparagine residues in casein, 'casein aspartic acid' to the aspartic acid residues in casein, and 'casein aspartic acid (acid hydrolysis)' to the aspartic acid in an acid hydrolysate, which is derived from both the casein asparagine and casein aspartic acid.

EXPERIMENTAL

Isotopic compounds

L-[^{14}C]Aspartic acid (uniformly labelled) and D-[^{14}C]-glucose (uniformly labelled) were bought from the Radiochemical Centre, Amersham, and the [^{15}N]ammonium nitrate from the Atomic Energy Research Establishment, Harwell. DL-[1-^{14}C]Glutamine was synthesized as described by Barry (1956).

DL-[^{14}C]*Asparagine* (*uniformly labelled*). This was synthesized by the method of King & Kidd (1951), with L-[^{14}C]aspartic acid (2·8 g.; 50 μc) substituted for their DL-aspartic acid. Paper chromatography showed that the product contained a trace of aspartic acid which was removed by chromatography on Dowex 2 (acetate form). The crystalline product decomposed at 270–280°, after sintering at 210°, and on paper chromatography gave only one spot in the expected position, which showed the delayed colour production with ninhydrin that is typical of asparagine. A sample prepared from unlabelled L-aspartic acid showed no optical rotation in a polarimeter.

Yield (calculated from L-aspartic acid), 27%.

DL-[amide-^{15}N]*Asparagine*. This was made by the same method but with the following modified preparation of phthaloyl-DL-asparagine. $^{15}NH_4NO_3$ (1·5 g.) was dissolved in a little water and excess of saturated KOH solution added. The NH_3 generated was slowly drawn in a stream of air through a drying tube containing KOH pellets and was trapped in a vessel immersed in liquid O_2. Dry ether (40 ml.), previously cooled in liquid O_2, was then added, followed by a solution of phthaloyl-DL-aspartic anhydride (2·3 g.) in dioxan (40 ml.). The phthaloylasparagine was isolated as before.

Yield (per cent of original ^{15}N recovered in DL-[^{15}N]-asparagine), 36%.

Procedure in experiments on goat

The experiments were performed on a goat which was giving about 1 l. of milk per day, and was in good health. Compounds were injected, and blood and milk samples taken, as described by Barry (1952).

Determination of ^{14}C *specific activities*

All compounds were burnt in a stream of O_2 and the specific activity of the CO_2 measured either directly as the gas, or after conversion into $BaCO_3$ (Barry, 1956). The various compounds were isolated as follows.

Asparagine, aspartic acid, glutamine and glutamic acid of casein and plasma proteins. Casein was isolated and hydrolysed by a pancreas suspension as described before (Barry, 1956). This suspension, when incubated alone at 37° for 24 hr., gave no free asparagine or aspartic acid; when incubated with asparagine it produced no aspartic acid, and with aspartic acid it produced no asparagine. The amino acids were separated from the casein digest by dialysis, the diffusate was freeze-dried, and a crystalline sample of aspartic acid was isolated by chromatography on Dowex 1 (Hirs, Moore & Stein, 1954). The fraction containing asparagine was then hydrolysed with HCl and the resulting aspartic acid isolated in the same way.

The plasma proteins, in experiments in which their asparagine and aspartic acid were isolated, were precipitated with 1% (w/v) picric acid by the method of Hamilton & Van Slyke (1943). The precipitate was washed three times with dil. HCl, dissolved in 0·5N-NaOH, neutralized and incubated with the pancreas suspension as before. Picrate ions were removed by passing through a short column of Dowex 2 (chloride form) and aspartic acid and asparagine were isolated as with the digest of casein.

In experiments in which glutamine and glutamic acid of plasma proteins were isolated, the plasma was dialysed overnight against running water to remove the free amino acids, and the proteins were digested in the same way. Glutamic acid, and the glutamic acid produced after acid hydrolysis of glutamine, were isolated on Dowex 1 (Hirs *et al.* 1954).

Aspartic acid (acid hydrolysis) and glutamic acid (acid hydrolysis) of casein and plasma proteins. Casein and plasma proteins were hydrolysed with HCl (Barry, 1952), and crystalline samples of aspartic acid, or glutamic acid, isolated by chromatography on Dowex 1 (Hirs *et al.* 1954).

Free L-asparagine of blood plasma. Since DL-[^{14}C]-asparagine was injected it was necessary to isolate L-asparagine from plasma free from any traces of the D-form. This was done as follows. Blood plasma (50 ml.) was freed from protein by the method of Hamilton & Van Slyke (1943), the excess of picric acid removed with Dowex 2 (Stein & Moore, 1954) and the solution freeze-dried. The residue was dissolved in a little water, brought to pH 4·5, and shaken overnight with glutamic decarboxylase (2 ml.) (Krebs, 1950); this treatment was necessary to remove glutamine, which emerges with asparagine from the columns of Dowex 50 used in the next stage. The supernatant was freeze-dried and eluted from a column of Dowex 50 (100 cm. × 1·15 cm.) as described by Moore & Stein (1951). The fractions containing asparagine (which were proved by paper chromatography to contain no other amino acid) were combined, the asparagine was determined by the ninhydrin method, and a known weight of DL-asparagine added. The solution was then shaken overnight at room temperature with guinea-pig serum (2 ml.) in a dialysis sac. The serum contains an asparaginase (Krebs, 1950) which was found to convert L-asparagine, but not the D-form, into aspartic acid. Salts were removed from the resulting solution (Sheldon-Peters & Barry, 1956) and a crystalline sample of L-aspartic acid was isolated by chromatography on Dowex 1. From the specific activity of this sample the activity of the free L-asparagine of the plasma was calculated, assuming that the D-asparagine of plasma, although possibly detectable by its radioactivity, contributed no significant amount to the asparagine determined by the ninhydrin method. This assumption is justified since the asparagine concentration of plasma was found to be unaffected by the 100 mg. of DL-[^{14}C]asparagine which was injected.

Lactose. This was isolated as described by Reiss & Barry (1953).

Determination of ^{15}N

A known weight of the DL-[^{14}C:*amide*-^{15}N]asparagine used for injection was diluted with a known weight of inactive DL-asparagine and part of this hydrolysed with N-HCl for 2 hr. in the outer chamber of a Conway microdiffusion unit. After evaporation to dryness, saturated KOH solution (2 ml.) was added, and the NH_3 absorbed in 0·25 N-HCl (1 ml.) in the inner chamber. Nitrogen was prepared (Rittenberg, 1946) and the ^{15}N determined in a mass spectrometer. ^{14}C was also determined by combustion of a second sample of asparagine, and the ^{15}N and ^{14}C in the original compound were then calculated.

The amide nitrogen of casein glutamine was isolated and its ^{15}N determined as described by Barry (1956). The amide nitrogen of casein asparagine was isolated as follows. A sample of casein was hydrolysed with the pancreas suspension and the mixture dialysed. The diffusate was brought to pH 7 and heated at 100° for 5 hr., which was found to remove glutamine completely, in agreement with Vickery, Pucher, Clark, Chibnall & Westall (1935). The solution was then subjected to chromatography on a column of Dowex 50 (100 cm. × 1·15 cm.) as described by Moore & Stein (1951), but at room temperature. The fractions containing asparagine were concentrated, and the amide nitrogen was isolated and its ^{15}N concentration determined as described above.

The NH_3 released on acid hydrolysis of casein with 20% (w/v) HCl was also collected in a microdiffusion unit; about 90% is derived from the amide nitrogen of the glutamine and asparagine of casein, and the remainder from the decomposition of other amino acids (Rees, 1946). The remaining nitrogen in the acid hydrolysate was isolated by the Kjeldahl method.

Determination of aspartic acid and asparagine in the blood of guinea pigs

Each guinea pig was killed by decapitation, its blood allowed to drop directly into a weighed amount of 1% (w/v) picric acid, and the weight of blood was found by difference. The free amino acids in the supernatant were separated by chromatography on Dowex 50 exactly as described by Sheldon-Peters & Barry (1956). The fractions containing a mixture of glutamine and asparagine were heated at 100° for 3 hr. with conc. HCl (4 ml.), and the resulting aspartic acid was determined after a second elution from the column. The fractions which contained a mixture of aspartic acid and threonine were desalted (Sheldon-Peters & Barry, 1956), and eluted from the column as before, but with the buffer at pH 4·25; the aspartic acid emerged in a single peak and was determined.

RESULTS

Goat's milk contains about 3% of casein, in which the amino acid concentrations are very similar to those in cow's casein (Davis & Macdonald, 1953). On acid hydrolysis, 100 g. of cow's casein yields 7·1 g. of aspartic acid, which is derived from both the asparagine and aspartic acid residues, the relative proportions of which are not known (Gordon, Semmett, Cable & Morris, 1949). Goat's blood plasma contains about 0·95 mg. of free asparagine/100 ml. (Barry, 1953), and about 0·1–0·2 mg. of free aspartic acid. The asparagine and aspartic acid of casein might therefore be derived from the free asparagine or aspartic acid of plasma, or from plasma proteins, or might be synthesized within the gland from other compounds absorbed from the blood.

The first experiment (Fig. 1) was designed to find whether either the asparagine or aspartic acid of casein is derived from the free asparagine of blood. [^{14}C]Asparagine was injected intravenously into a goat, thus labelling the free asparagine of its blood, and blood and milk samples were taken at intervals after the injection. It can be seen that the specific activity of asparagine in each casein sample

is many times that of aspartic acid, showing that they have different origins. The activity of asparagine in the first sample is over 40 times that of aspartic acid; it is also over 40 times the activity of aspartic acid (acid hydrolysis) from the plasma proteins at 0·33 and 6 hr. It is also seen that the specific activity of the free asparagine of blood at 0·33 hr. is about three times the highest value for casein asparagine, and that the activity at 6 hr. is somewhat lower than the lowest casein asparagine value. From the closeness of their specific activities it is clear that the free asparagine of blood is an important precursor of casein asparagine. If the asparagine residues of casein are derived solely from the free asparagine of blood, then the area under the casein asparagine histogram will equal the area under a curve showing the specific activity of the free asparagine of blood up to 6 hr. (Barry, 1956). Because of the technical difficulty, the specific activity of free L-asparagine was measured in only two blood samples, and a curve cannot be plotted. However, it can be predicted from previous similar experiments in which complete specific activity curves for other free amino acids were obtained with points intermediate between 0·33 and 6 hr., that if the curve were completed the area under it would be approximately equal to that beneath the casein asparagine histogram. It may therefore be concluded that the asparagine residues of casein are largely derived from free asparagine taken from the blood stream by the mammary gland; since a complete curve for free asparagine is lacking, it cannot be concluded with certainty, in the way it could before (Barry, 1952, 1956), that there is no other quantitatively important source of casein asparagine residues. The possibility that free asparagine of blood is converted into a derivative, such as a peptide, before being absorbed by the mammary gland may be excluded for reasons similar to those mentioned before (Barry, 1952, 1956).

A further experiment (Table 1) shows that the free asparagine of blood does not exchange its amide nitrogen when it is incorporated into casein. The asparagine of blood was labelled by injecting [^{14}C:*amide*-^{15}N]asparagine, and casein was isolated from a milk sample taken 3 hr. after the injection. It can be seen that the concentration of ^{15}N in the

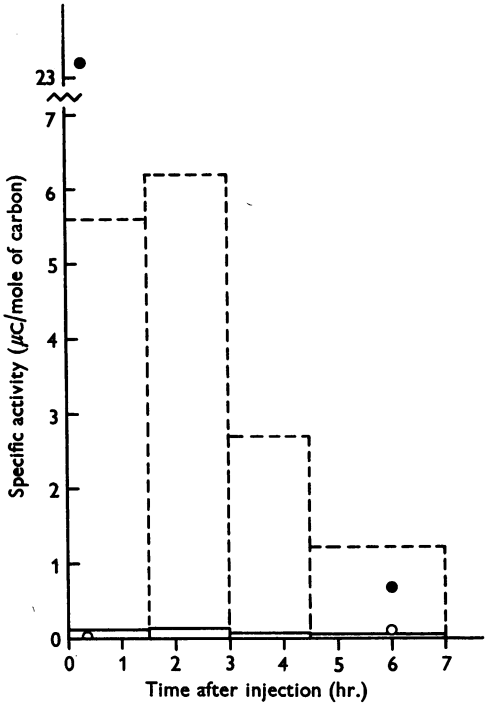

Fig. 1. Use of asparagine for synthesis of casein. DL-[^{14}C]Asparagine (100 mg.; 1·76 μc) was injected intravenously into a lactating goat. The goat was milked immediately before injection, and blood and milk samples were taken at intervals up to 7 hr. after. ●, Free L-asparagine of blood plasma: ○, aspartic acid (acid hydrolysis) of plasma proteins. The histograms show the specific activities of casein asparagine (broken lines) and casein aspartic acid (solid lines) in each milk sample, the width of each rectangle occupying the time during which the milk sample was secreted.

Table 1. *Incorporation of doubly labelled asparagine into casein*

DL-[^{14}C:*amide*-^{15}N]Asparagine (360 mg.; 2·98 μc) injected intravenously into goat and milk taken 3 hr. later.

	Atoms % excess of ^{15}N	Specific activity (μc/mole of carbon)	μc/mole of carbon / Atoms % excess of ^{15}N
Injected asparagine	26·8 (*a*)	273	10·2
Casein asparagine	0·750 (*a*)	8·57	11·4
Casein aspartic acid	—	0·808	—
Casein aspartic acid (acid hydrolysis)	—	5·44	—
Casein glutamine	0·167 (*a*)	—	—
Amide N casein (*b*)	0·418	—	—
Non-amide N of casein (*c*)	0·004	—	—

(*a*) Figures are for amide nitrogen. (*b*) Nitrogen released as NH_3 on hydrolysis with 20% (w/v) HCl. (*c*) Nitrogen not released as NH_3 on hydrolysis with 20% (w/v) HCl.

amide nitrogen of casein asparagine is nearly 200 times that in the non-amide nitrogen of casein, and that the ratio $^{14}C:^{15}N$ is approximately the same in the casein asparagine as in the injected asparagine. From the specific activities of asparagine, aspartic acid and aspartic acid (acid hydrolysis) in Table 1 it can be calculated that 59·8 % of the aspartic acid in the acid hydrolysate of casein came from asparagine residues. This figure may not be exact since it may have accumulated the error in each of the three specific activities, but it suggests that if 100 g. of casein was hydrolysed without decomposition of asparagine it would yield 4·3 g. of asparagine and 2·8 g. of aspartic acid.

A further experiment (Fig. 2) shows that there was little synthesis of casein asparagine or aspartic acid from blood glucose within the mammary gland. It has been demonstrated previously that blood glucose is the principal compound used for lactose synthesis by the mammary gland of the goat (Reiss & Barry, 1953). Therefore, after injection of [^{14}C]glucose, the area under the specific activity–time histogram of a compound in milk which is synthesized solely from blood glucose must be at least as large as the area under the lactose histogram (Barry, 1956). In Fig. 2 the area under the casein aspartic acid (acid hydrolysis) histogram is only about one-sixth of that under the lactose histogram; therefore not more than one-sixth of the casein aspartic acid (acid hydrolysis) can have been synthesized in the mammary gland from glucose. In the first casein sample from this experiment the specific activities of asparagine, aspartic acid and aspartic acid (acid hydrolysis) were 3·62, 7·27 and 5·95 μc/mole of carbon respectively, which again shows that casein asparagine and aspartic acid have different origins. It may be concluded that not more than one-sixth of the asparagine residues of casein were synthesized within the mammary gland from blood glucose; in fact the figure is probably less than one-sixth, the activity of casein asparagine being at least partly due to the synthesis of blood asparagine from the injected glucose outside the mammary gland.

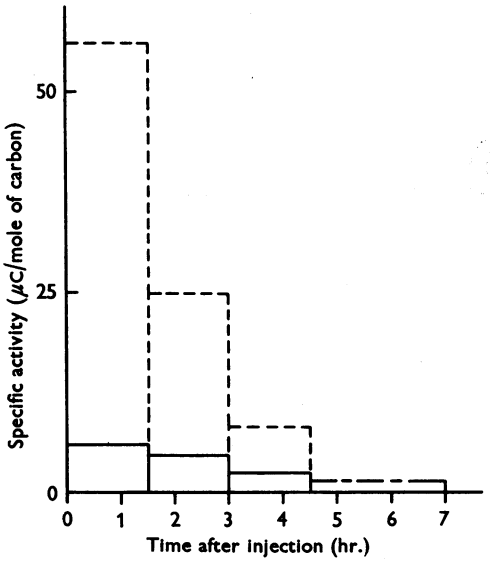

Fig. 2. Synthesis of lactose and casein aspartic acid (acid hydrolysis) from blood glucose. D-[^{14}C]Glucose (uniformly labelled) (224 mg.; 71 μc) was injected intravenously into a lactating goat. The goat was milked immediately before injection and at intervals up to 7 hr. after. The histograms, plotted as in Fig. 1, show the specific activities of lactose (broken lines) and casein aspartic acid (acid hydrolysis) (solid lines) in each milk sample.

The results of this and our earlier work show that the free asparagine and glutamine of blood plasma are used, in the biosynthesis of casein, to provide asparagine and glutamine residues, but no significant portion of the aspartic or glutamic acid residues. The data in Tables 2 and 3 show that the free asparagine and glutamine of blood are used in the same way in the biosynthesis of plasma proteins. The results in Table 2 show that after labelling the free asparagine of the goat's blood with [^{14}C]asparagine, the asparagine of plasma proteins gradually became radioactive, the specific activity in each sample being about ten times that of the aspartic acid. (The possibility that the labelling of plasma proteins is due to physical adsorption of asparagine is excluded by the fact that a plasma-protein sample taken 20 min. after injection, when the activity of the free asparagine was highest, had negligible activity.) Similarly, the results in Table 3 show that after the free glutamine of plasma was labelled the glutamine residues of plasma proteins gradually became active, and that their activity was always about seven times that of the glutamic acid residues.

We have shown that asparagine is used in protein biosynthesis in the goat, and therefore probably also in other animals, such as man and the cat, whose blood plasma contains similar concentrations of free asparagine (Stein & Moore, 1954; Tallan, Moore & Stein, 1954). The blood plasma of guinea pigs, unlike that of other animals which have been studied, contains an asparaginase (Krebs, 1950), and we were interested to see whether it also contained asparagine. Three guinea pigs were killed by decapitation, the blood of each was allowed to drop directly into picric acid solution, and asparagine and aspartic acid were determined in each protein-free supernatant. The concentrations were too low to be accurately measured, but each animal had about 0·1 mg. of asparagine/100 ml. of whole blood, and about 0·3 mg. of

Table 2. *Incorporation of labelled asparagine into plasma proteins*

Specific activities of amino acids in plasma-protein samples taken at intervals after injecting DL-[^{14}C]asparagine (uniformly labelled) (250 mg.; 4·4 μc) intravenously into goat.

Time of sampling (hr.)		0·33	1·5	4·0	6·0
Specific activity of total carbon (μmc/mole of carbon)	Asparagine	—	154	351	475
	Aspartic acid	—	20	29	33
	Aspartic acid (acid hydrolysis)	<20	93	211	276

Table 3. *Incorporation of labelled glutamine into plasma proteins*

Specific activities of amino acids in plasma-protein samples taken at intervals after injecting DL-[1-^{14}C]glutamine (101 mg.; 9·04 μc) intravenously into goat.

Time of sampling (hr.)		0·33	1·5	2·5	4·0
Specific activity of total carbon (μmc/mole of carbon)	Glutamine	—	92	101	132
	Glutamic acid	—	12	19	22
	Glutamic acid (acid hydrolysis)	<10	53	74	97

aspartic acid/100 ml. The asparagine concentration is thus about one-tenth of that in the blood of man, the cat, the goat (Stein & Moore, 1954; Tallan *et al.* 1954; Barry, 1953) and the cow (unpublished work).

The blood of guinea pigs was also found to contain a compound which reacts with ninhydrin, and which is not found in the blood of man, the goat or the cow. It was eluted from the columns of Dowex 50 by the buffer of pH 3·42 together with threonine and aspartic acid, but emerged before them with buffer of pH 4·25. It gave a blue ninhydrin colour, equivalent to about 0·6 mg. of aspartic acid/100 ml. of whole blood, but was not identified.

DISCUSSION

We have shown that the mammary gland takes free asparagine from the blood stream to provide asparagine, but not aspartic acid, residues of casein, and that free asparagine also provides asparagine residues of plasma proteins. The compound or compounds that are taken from the blood stream by the mammary gland to provide the aspartic acid residues of casein have not yet been discovered. Asparagine has been found in animal tissues by Krebs (1950) and also occurs in urine and blood plasma (Ågren & Nilsson, 1949; Stein, 1953; Stein & Moore, 1954; Barry, 1953), but there has previously been no evidence for its function. Waelsch (1952) tentatively suggested that β-aspartyl peptides formed from asparagine are intermediates in protein synthesis. Our results (Fig. 1, Tables 1 and 2) suggest that asparagine does not form these intermediate peptides when it is incorporated into casein and plasma proteins, for reasons similar to those mentioned with reference to glutamine (Barry, 1956).

The evidence that no significant part of the casein asparagine was synthesized from blood glucose is consistent with the conclusion that casein asparagine comes directly from the blood, for, if it had been synthesized within the gland, the glucose, lactate or propionate of the blood would probably have been used to make it. [Mammals apparently cannot use acetic acid for the net synthesis of C_4 compounds, although Kornberg & Krebs (1957) suggest that animals on fatty diets may, like bacteria, become adapted to do this. It is conceivable that ruminants become adapted since their metabolism is in many ways like that of animals fed on large amounts of fat. The difficulty that propionate is the only product of digestion which is known to be able to provide any of the large carbohydrate output of a lactating ruminant would then disappear.]

The results of this and previous work (Barry, 1956) show that free glutamine provides the glutamine residues, but no significant portion of the glutamic acid residues, of both casein and plasma proteins. It is clear that the glutamine, glutamic acid, asparagine and aspartic acid of casein and plasma proteins must be considered as four independent amino acids which are not interconverted during or after their incorporation into the protein. Glutamine and asparagine residues occur in most animal and plant proteins, and it seems likely that they are in general derived from free glutamine and asparagine of the tissues. This idea is supported by the following evidence that cells in tissue culture use the free glutamine of their medium to provide the glutamine residues of new protein. Rabinovitz, Olson & Greenberg (1956) found that, if glutamine formation by Ehrlich ascites cells was inhibited, the incorporation of other amino acids into protein was also inhibited; and if labelled glutamine was added to the medium of growing cells, its carbon was incorporated into protein. Also, human uterine carcinoma cells of the strain HeLa, when in tissue

culture, need glutamine for growth. Levintow (1957) labelled the culture medium of these cells with [^{14}C:*amide*-^{15}N]glutamine, and showed that the glutamine residues of new protein were derived from the medium without change in the ^{14}C:^{15}N ratio.

It seems therefore that glutamine and glutamic acid, and asparagine and aspartic acid, should be considered as distinct from one another as are any other non-essential amino acids. It is true that the free glutamine of blood is continually synthesized from glutamic acid (Barry, 1956), but many other non-essential amino acids, such as serine and glycine, are also rapidly interconverted. This distinction may have been overlooked, because of the conversion of asparagine and glutamine into aspartic and glutamic acids which occurs when proteins are hydrolysed with acid, particularly in interpreting experiments of two types:

(i) The first type is that in which conclusions are drawn from the concentrations of isotope in glutamic and aspartic acid in acid hydrolysates of protein. For example, in the experiment of Steinberg & Anfinsen (1952), crystalline ovalbumin, isolated from hen oviducts which had been incubated with $^{14}CO_2$, was partially hydrolysed enzymically, and the resulting fractions of low and high molecular weight were separated and hydrolysed with acid. The specific activities of glutamic and aspartic acid in one hydrolysate were found to differ from those in the other, and it was concluded that both the glutamic and aspartic acid residues of ovalbumin had different activities in different parts of the molecule. This conclusion would be justified only if glutamic and aspartic acid had been isolated after enzymic digestion of the two fractions under conditions in which glutamine and asparagine are not hydrolysed, since the results could equally well be explained by the amides and acids having different specific activities, and being in different proportions in the two fractions. (These authors have, however, unequivocal evidence that the alanine residues of ovalbumin, isolated after incubating oviducts with [^{14}C]alanine, do not have identical activities.)

(ii) Experiments of the second type are those in which requirements of cells for glutamine or asparagine have been found (see review by Waelsch, 1952). The possibility that these are normal amino acid requirements for protein synthesis has rarely been considered, and much energy has often been spent, with little success, in attempting to find special functions of glutamine and asparagine in metabolism. These functions certainly exist, examples being the use of glutamine in purine biosynthesis (Hartman, Levenberg & Buchanan, 1956), and glucosamine biosynthesis (Leloir & Cardini, 1953), but the fact that asparagine and glutamine are protein amino acids should be remembered. As mentioned above, this fact has recently been found to explain the earlier discovery of a requirement of cells in tissue culture for glutamine.

SUMMARY

1. From experiments with asparagine labelled with ^{14}C and ^{15}N it is concluded that the lactating mammary gland of the goat takes free asparagine from the blood stream to provide asparagine, but not aspartic acid, residues of casein.

2. This conclusion agrees with the finding that there is little synthesis of casein asparagine from glucose within the mammary gland.

3. It is also shown that asparagine and glutamine are used in the biosynthesis of plasma proteins, to provide asparagine and glutamine residues.

4. It is clear that glutamine and glutamic acid, and asparagine and aspartic acid, are not interconverted during or after their incorporation into casein or plasma proteins. It seems therefore that they should be considered as four distinct non-essential amino acids.

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Effect of pH on the Activity of Eel Esterase towards Different Substrates

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Much fundamental information about the structure of the active centre in cholinesterases has been derived from studies on changes of activity with pH. These enzymes show a maximum activity at about pH 7–8. On the acid side, activity falls off in approximately the same manner for all substrates studied. However, in the alkaline range great differences were observed between different esters (Bergmann, Segal, Shimoni & Wurzel, 1956). The bell-shaped form of the pH–activity curves has been interpreted to indicate two different components of the esteratic site, one of which (the nucleophilic group G_1) is inactivated competitively by H^+ ions and the other (the electrophilic group G_2) by OH^- ions. Such an interpretation permits the calculation of the respective dissociation constants, pK_a and pK_b. The determination of the dissociation constants, in turn, leads to some speculation about the possible nature of these two groups (Bergmann *et al.* 1956).

A new argument about the influence of pH on esterase activity has been raised recently by Kistiakowsky & Mangelsdorf (1956). These authors observed that hydrolysis of methyl butyrate by horse-liver esterase varies according to reaction conditions. If reaction rates were measured immediately after addition of the enzyme to the alkaline buffer and for short periods, no decrease in rate in the alkaline range (beyond pH 9) was found. If, however, a pre-incubation period of about 20–60 min. preceded the addition of substrate to the enzyme–buffer mixture, the usual decrease in activity with increasing pH became apparent. The authors concluded from their results that the 'true' activity does not change, but that the enzyme protein undergoes slow changes in the alkaline range, which at first are reversible but progressively become irreversible. These changes are assumed to simulate the competition of substrate and OH^- ion for the active surface, which forms the basis of the above-mentioned interpretation of the pH–activity curve.

In the following experiments we have tried to elucidate this problem and to test our previous assumptions about the role of the electrophilic group G_2 in the hydrolytic mechanism.

MATERIALS AND METHODS

Substrates. Phenyl acetate, *p*-methoxyphenyl acetate and *p*-nitrophenyl acetate were prepared by refluxing the appropriate phenol with excess of acetic anhydride and subsequent distillation. Standard solutions of these esters were prepared in water, except for *p*-nitrophenyl acetate, which shows appreciable hydrolysis in aqueous solution. Therefore an M-solution in dioxan was diluted 1:2500 with water, immediately before use, to give a final concentration of 0·4 mM. The dioxan present was found to be devoid of any inhibitory effect in such dilutions.

Acetylthiocholine (AThCh) was the commercial product of Hoffmann-LaRoche, Basel, Switzerland.

Enzyme. Eel esterase was prepared from the electric organ of *Electrophorus electricus* according to the method of Nachmansohn & Rothenberg (1945). The standard preparation, diluted 1:7500, with 4 mM-acetylcholine (ACh) as substrate, hydrolysed 8 μmoles/hr./ml. at 37°.

Measurement of activity. The following buffers were used to cover the pH range required: 0·1 M-sodium acetate for pH 5·0–6·5; 0·1 M-sodium phosphate for pH 6·5–8; 0·1 M-sodium pyrophosphate for pH 8·0–9·5; aq. NH_3 soln.–ammonium acetate for pH values above 9·5.

For manometric measurements, a buffer of the following composition was used: NaCl, 0·1 M; $NaHCO_3$, 0·025 M; $MgCl_2$, 0·04 M. The pH was adjusted to 7·4. The gas phase was CO_2 + air (5:95, v/v).

Hydrolysis of AThCh was measured either by the Warburg manometric method (Ammon, 1933) or colorimetrically under conditions which are suitable for the determination of ACh (Hestrin, 1949). Non-enzymic hydrolysis of AThCh is negligible up to pH 10. Therefore this ester is the substrate of choice for the standardization of cholinesterase preparations. The p*S*–activity curve of AThCh, shown in Fig. 1, is different from that of ACh, although the maximum appears in the same concentration range.