EFFECT OF ANIONS ON THE NON-ENZYMATIC DESAMIDATION OF GLUTAMINE

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Glutaminase is the designation given the enzyme which catalyzes the hydrolysis of glutamine at the amide bond to yield as products ammonia and glutamic acid (1). Highly specific preparations of this enzyme from dog and beef kidney have been prepared (2). The enzymatic desamination of glutamine in aqueous extracts of rat and mouse tissues is considerably accelerated by added phosphate, arsenate, and sulfate (3-6). The enzyme which is activated by these anions, and which is relatively heat-labile, acid-labile, and sedimentable on high speed centrifugation of the extract, has been given the designation of glutaminase I (5, 6).1 Under the conditions of the enzymatic reaction (1 hour incubation at 37° at pH 8.0 in the presence of 14 μM of glutamine, 330 mg. of fresh tissue, and 0.01 to 0.20 M phosphate), whereby the substrate is nearly completely desamidated, less than 5 per cent of the substrate is hydrolyzed when the fresh tissue is replaced by boiled tissue.2

The amide group of glutamine is unusually labile at higher temperatures, and numerous methods for the determination of this compound in biological materials have been based upon this property (7-11). Thus, solutions of glutamine heated at 100° for several hours readily yield ammonia and pyrrolidonecarboxylic acid (7-10). Hamilton has shown that this conversion occurs more rapidly in the presence of phosphate than of citrate, acetate, or lactate solutions at the same molar concentration (11).

In view of this observation of Hamilton's, the question naturally arises as to whether the enzyme simply accelerates still further a desamination reaction already catalyzed by phosphate. It was therefore considered desirable to investigate the effect of several anions on the non-enzymatic desamination of glutamine, including both those anions which accelerate and those which do not accelerate the enzymatic desamination of glutamine.

1 The designation of glutaminase II has been given to the soluble, hepatic system which hydrolyzes glutamine only in the presence of α-keto acids (5, 14).

2 Phosphate-activated glutaminase activity is demonstrated not only in tissue extracts but in slices as well (15).
EXPERIMENTAL

The glutamine employed gave no test for ammonia. On boiling in 1 N HCl for 1 minute it yielded 90 per cent of the theoretical amount of amide nitrogen as ammonia. The non-enzymatic digests consisted of 1 cc. of glutamine at the stated concentration, 2 cc. of veronal acetate buffer (0.028 M with respect to veronal), and 1 cc. of either water or the anion at the stated concentration. The pH of each digest was carefully adjusted, and initial and final pH values were nearly identical. The enzymatic digests consisted of 1 cc. of fresh aqueous rat liver extract equivalent to 330 mg. of tissue, 2 cc. of the veronal buffer with and without added anion, and 1 cc. of either water or 0.014 M glutamine solution. At the end of the reaction, the digests were alkalized with saturated K₂CO₃ and the ammonia aerated into dilute sulfuric acid traps and subsequently nesslerized. The glutamine was entirely stable under the aeration conditions. The enzymatic data were corrected for the extract blanks. Determinations of carboxyl nitrogen were made by the gasometric procedure with ninhydrin (12).

The anions were furnished by the following sodium salts: phosphate, phosphite, arsenate, methyl arsenate, bicarbonate, sulfate, borate, pyruvate, acetate, chloride, and nitrate.

Time Course of Non-Enzymatic Desamidation of Glutamine—The very considerable effect of phosphate on the non-enzymatic desamidation of glutamine is revealed in Fig. 1. After 48 hours of incubation, when nearly 100 per cent of the glutamine in the presence of phosphate was desamidated, only about 30 per cent of the amide in the absence of this anion was desamidated.

Effect of Various Anions on Non-Enzymatic Desamidation of Glutamine—The effect of increasing amounts of various sodium salts on the liberation of amide nitrogen from glutamine is revealed in Fig. 2. The values given are corrected for the ammonia evolved from the digests in the absence of added salt. At equimolar concentrations, phosphate was more effective than arsenate, and arsenate more so than methyl arsenate. Phosphite had a relatively weak effect. Borate accelerated the non-enzymatic desamidation of glutamine, but this salt was too insoluble to be used much above 0.05 M final concentration. Chloride, sulfate, nitrate, acetate, and pyruvate up to 0.20 M final concentration possessed no measurable effect on the desamidation of glutamine. Digests containing both sulfate and phosphate yielded the same results as those with phosphate alone.

Bicarbonate was very effective in accelerating the non-enzymatic desamidation of glutamine, but the pH of digests containing this salt was invariably higher than 8.0. At pH 8.4 ± 0.1, digests of glutamine with various salts at 0.2 M final concentration yielded the following amounts of
Fig. 1. Time course of non-enzymatic desamidation of glutamine. ○, no added phosphate; X, with added phosphate. The mixtures consisted of 1 cc. of 0.025 M glutamine, 2 cc. of 0.08 M veronal acetate buffer at pH 8.0, and 1 cc. of either water or 0.8 M phosphate at pH 8.0. The initial and final pH of each mixture was 8.0 ± 0.05. Temperature 47°. Complete desamidation yields 22.5 μM of ammonia.

Fig. 2. Effect of various sodium salts on the non-enzymatic desamidation of glutamine. □, phosphate, ○, arsenate, ●, methyl arsenate, Δ, borate, ■, phosphate. The mixtures consisted of 1 cc. of 0.1 M glutamine, 2 cc. of 0.08 M veronal buffer at pH 8.0, and 1 cc. of water or salt solution at pH 8.0. The values were corrected for water blanks. The initial and final pH of each mixture was 8.0 ± 0.05. Incubation period, 8 hours at 37°.
amide nitrogen as micromoles of ammonia over the blanks: phosphate 18.1, arsenate 15.8, chloride 0, phosphite 1.7, and bicarbonate 12.1. The experimental conditions, other than those stated, were the same as those described in Fig. 2.

**Effect of Phosphate Concentration at Different Concentrations of Glutamine** The data in Fig. 3 indicate that for a given concentration of phosphate the amount of desamidation is proportional to the concentration of glutamine up to about 30 per cent desamidation.

![Graph showing the effect of phosphate concentration on glutamine desamidation](image)

**Fig. 3.** Non-enzymatic desamidation of glutamine at two different concentrations. X, 0.05 M; O, 0.025 M glutamine. The mixtures consisted of 1 cc. of glutamine solution, 2 cc. of 0.08 M veronal buffer at pH 8.0, and 1 cc. of phosphate solution at pH 8.0. The initial and final pH of each mixture was 8.0 ± 0.05. The values were corrected for water blanks. Incubation period, 12 hours at 47°.

**Effect of pH**—The data in Fig. 4 demonstrate that a striking acceleration of the non-enzymatic desamidation of glutamine by phosphate occurs progressively with increasingly alkaline pH. In acid pH ranges, on the other hand, the desamidation is little affected by phosphate. The essential stability of glutamine at pH 4 to 7 in the absence of phosphate is revealed by Fig. 4. Sodium chloride added at the same ionic concentration as phosphate possessed no accelerating effect (Fig. 4).

**Effect of Temperature**—Digests consisting of 1 cc. of 0.1 M glutamine, 2 cc. of veronal buffer at pH 8.0, and 1 cc. of either water or 0.8 M phosphate were incubated at 47.4° and 37.0° for several hours. The initial and final pH of each digest was 8.0 ± 0.05, and the data were corrected for the small blanks in the absence of added phosphate. After 1.5 hours, the ammonia
liberated at the higher temperature in the presence of phosphate was 10.6 μM, and at the lower temperature 4.8 μM. After 3.25 hours, the corresponding values were 21.0 and 9.2 μM. On the basis of these data, the average temperature coefficient amounts to about 2.2 for a difference in temperature of 10.4°, or close to 2 for a difference of 10°.

Conversion of Glutamine to Pyrrolidonecarboxylic Acid—The non-enzymatic desamidation of glutamine is accompanied by ring closure to pyrrolidonecarboxylic acid (7, 8, 11). Phosphate, as well as bicarbonate and certain other salts, accelerates the reaction. We have noted that this acceleration by phosphate is not inhibited by pyrrolidonecarboxylic acid or glutamic acid added in a molar ratio of 400:1 of glutamine. When 1 cc. of ammonium glutamate, 2 cc. of veronal buffer at pH 8.0, and 1 cc. of 0.8 M phosphate at pH 8.0 were incubated for 12 hours at 37°, no appreciable loss of carboxyl nitrogen had occurred and thus no appreciable amount of pyrrolidonecarboxylic acid was formed. Under these conditions, therefore, glutamate is not converted to pyrrolidonecarboxylic acid even in the presence of phosphate.

Study of Chloroacetylglutamine—Mixtures of 1 cc. of neutralized 0.05 M chloroacetylglutamine (13), 2 cc. of 0.014 veronal buffer, and 1 cc. of either water or 0.8 M phosphate buffer at pH 8.0 were prepared and allowed to incubate at 47° for 4 and 8 hours. Under these conditions, and especially

![Graph showing effect of pH on the non-enzymatic desamidation of glutamine.](http://www.jbc.org/)

Fig. 4. Effect of pH on the non-enzymatic desamidation of glutamine. X, 0.2 M final phosphate concentration; O, no added salt; A, sodium chloride added at equal ionic strength to phosphate. The mixtures consisted of 1 cc. of 0.05 M glutamine, 2 cc. of veronal buffer at different pH values, and either 1 cc. of water or 0.8 M phosphate. Incubation period, 12 hours at 37°.
in the presence of phosphate, glutamine yields an appreciable amount of its amide nitrogen as ammonia. With chloroacetylglutamine, however, no significant quantities of ammonia were noted, whether phosphate was or was not present. Chloroacetylation of the α-amino group of glutamine obviously blocks pyrrolidonecarboxylic acid formation.

Increased ammonia production from chloroacetylglutamine is noted when concentrated rat liver extracts treated with pyruvate (13) or phosphate (3) are used, but this may be due to a preliminary enzymatic hydrolysis at the peptide bond yielding free glutamine.

Effect of Various Anions on Enzymatic Desamidation of Glutamine—Phosphate, arsenate, and sulfate at 0.02 to 0.20 M concentration accelerate markedly the desamidation of glutamine in rat liver, kidney, spleen, and brain (3–6). Methyl arsenate and phosphate at concentrations lower than 0.02 M do not accelerate (4), but at higher concentrations do accelerate the enzymatic hydrolysis of the amide to practically the same extent as arsenate or phosphate. Since bicarbonate and borate accelerated the non-enzymatic desamidation of glutamine (see above), the effect of these salts at 0.10 M final concentration on the enzymatic desamidation of glutamine with rat liver extracts was studied. The digests consisted of 1 cc. of fresh aqueous rat liver extract equivalent to 330 mg. of tissue, 2 cc. of veronal buffer with and without the salts, and 1 cc. of either water or 0.014 M glutamine. The pH of the digests was 8.2 and the incubation period 1 hour at 37°. No measurable acceleration of desamidation of the glutamine by either borate or bicarbonate was noted.

Effect of pH and of Concentration on Accelerating Effect by Sulfate on Enzymatic Desamidation of Glutamine—The accelerating effect of sulfate on the enzymatic desamidation of glutamine is so unusual that a brief study was made of the reaction. In Fig. 5 is portrayed the effect of pH on this reaction, and it is noted that the pH of 8.0, when optimum desamidation occurs in the presence of sulfate, is practically the same as that of the optimum desamidation of glutamine in the absence of the added salt. Thus, the effect of sulfate in this respect is similar to that of phosphate or arsenate (3–6).

The effect of increasing amounts of sulfate on the enzymatic desamidation of glutamine is shown in Fig. 6. As in the case of added phosphate (3–6), the desamidation of glutamine in the presence of sulfate reaches a maximum value at a molar ratio of about 8 of salt to amide, and thereafter remains appreciably constant up to a final concentration of sulfate of 0.2 M.

Determinations of carboxyl nitrogen in digests of rat liver extracts with glutamine and sulfate revealed no evidence of appreciable pyrrolidonecarboxylic acid formation, for there was no apparent decrease in the car-
Fig. 5. Effect of pH on the enzymatic desamidation of glutamine by rat liver extracts in the presence and absence of added sodium sulfate. The digests consisted of 1 cc. of fresh aqueous extract equivalent to 330 mg. of tissue, 2 cc. of buffer with and without 0.4 M sodium sulfate, and 1 cc. of water or 0.014 M glutamine. X, with SO₄; 0, without SO₄. Veronal buffers were used below pH 8.5, glycine-NaOH buffers above pH 8.5. Incubation period, 1 hour at 37°.

Fig. 6. Effect of concentration of sulfate on the enzymatic desamidation of glutamine. The digests consisted of 1 cc. of fresh aqueous rat liver extract equivalent to 330 mg. of tissue, 2 cc. of veronal acetate buffer at pH 8.0 with and without added sulfate, and 1 cc. of water or 0.014 M glutamine. Incubation period, 1 hour at 37°.
DESAMIDATION OF GLUTAMINE

Boxyl nitrogen titer after most of the substrate had been desamidated. Pyrrolidonecarboxylic acid itself was not attacked by rat liver, kidney, or brain extracts, whether added phosphate was present or not.

DISCUSSION

A summary of the effect of various anions on the respective enzymatic and non-enzymatic desamidation of glutamine is given in Table I. Phosphate, phosphite, arsenate, and methyl arsenate accelerate both the enzym-

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<th>Anion*</th>
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<tr>
<td></td>
<td>Non-enzymatic†</td>
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<tr>
<td>Phosphate</td>
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<tr>
<td>Phosphite</td>
<td>1.5 ‚M</td>
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<td>Pyruvate</td>
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* All sodium salts.
† The data were taken from Fig. 2. The experimental conditions and inactive anions are stated in the text.
‡ The data were obtained from (3–6, 14), Fig. 5, and the text of the present paper. Aqueous rat liver extracts were used. In all cases but pyruvate, the enzymatic desamidation refers to the activity of the widely distributed glutaminase I in rat tissues. Pyruvate is concerned only with the hepatic system, glutaminase II. The data on this system were obtained after 4 hours of incubation at pH 7.5.

The enzymatic and non-enzymatic desamidation of glutamine thus differ in at least three particulars: namely (1), the former yields glutamic acid and ammonia, whereas the latter reaction yields pyrrolidonecarboxylic
acid and ammonia; (b) the former is accelerated by sulfate and pyruvate
but not by borate or bicarbonate, whereas the latter reaction is acceler-
ated by borate and bicarbonate but not by sulfate or pyruvate; and (c)
the enzymatic desamidation possesses an optimum at pH 8.0 in rat liver
and kidney extracts, at pH 8.8 in rat brain extracts (3-6), and at pH 7.5
with dog kidney (2), whether added active anion is or is not present, where-
as the non-enzymatic desamidation of glutamine possesses no optimum but
simply increases progressively at either acid or alkaline reactions (Figs.
4 and 5). Moreover, the accelerating effect of phosphate on the non-
enzymatic reaction increases with increasing pH and decreases with de-
creasing pH.

The complete enzymatic desamidation of glutamine is accomplished with-
out detectable pyrrolidonecarboxylic acid formation. No enzyme is pres-
ent, in any of the tissues studied, which is capable of hydrolyzing pyr-
rrolidonecarboxylic acid to glutamic acid; and hence the primary action of
tissue glutaminase is undoubtedly a simple hydrolysis at the amide bond to
yield glutamic acid and ammonia. Since ammonium glutamate under the
non-enzymatic conditions employed does not yield appreciable amounts of
pyrrolidonecarboxylic acid, the non-enzymatic desamidation of glutamine
is in effect the result of a simultaneous ring closure of the glutamine and not
of a primary hydrolysis of the amide bond.

Bicarbonate, which accelerates the non-enzymatic desamidation of glu-
tamine to an extent not much less than that of phosphate, has no influence
on the enzymatic reaction under conditions whereby phosphate accelerates
the reaction nearly to completion. It is true that the enzymatic reaction is
very much faster than the non-enzymatic reaction, and, if bicarbonate had
a very weak effect on the enzymatic desamidation, it might not have been
noted in the relatively short period of incubation used (1 hour at 37°C). On
the other hand, sulfate accelerates the enzymatic desamidation of glutamine
to a degree not much less than that of phosphate, but possesses no demon-
strable effect on the non-enzymatic desamidation of glutamine under condi-
tions whereby phosphate (and bicarbonate) considerably accelerates the
reaction.

The data presented appear to indicate that the effect of the active anions
on the enzymatic and on the non-enzymatic desamidation of glutamine may
be fundamentally different. It is quite possible that the acceleration of
non-enzymatic reaction by the active ions may be due to the formation
of a highly labile intermediate complex of glutamine and anion in minute
amounts. The presence of such a possible complex cannot be excluded in
the course of the enzymatic reaction, but the enzyme itself, together with
other tissue components in the crude extract, may produce conditions
involving new specificities.
Summary

1. The effect of various anions on the desamidation of glutamine in veronal-buffered solution has been studied and compared with the effect of the same ions on the enzymatic desamidation of this substrate in rat tissue aqueous extracts.

2. The non-enzymatic reaction which leads to the formation of pyrrolidonecarboxylic acid and ammonia is accelerated by phosphate, phosphite, arsenate, methyl arsenate, borate, and bicarbonate, and is not accelerated by sulfate, nitrate, acetate, chloride, or pyruvate at equivalent concentrations. Chloroacetylglutamine is not affected, whether phosphate is or is not present. The enzymatic reaction which leads to the formation of glutamic acid and ammonia is accelerated by phosphate, phosphite, arsenate, methyl arsenate, sulfate, and pyruvate, and is not accelerated by borate, bicarbonate, nitrate, acetate, or chloride at equivalent concentrations.

3. The desamidation of glutamine in tissue extracts occurs at an optimum at the same pH, whether accelerating salt has been added or not. The accelerating effect of phosphate on the non-enzymatic desamidation of glutamine increases with increasing pH, and decreases with decreasing pH.

4. The several differences between the phenomena of enzymatic and non-enzymatic desamidation reactions suggest that they occur by different mechanisms.

Bibliography

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