COW BRAIN GLUTAMINASE: PURIFICATION, CHARACTERIZATION, AND MECHANISM OF ACTION

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Biochemistry

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PREFACE

Intent of The Study

Glutamine lies at the center of cellular nitrogen metabolism; glutaminase is the major enzyme facilitating use of the amide nitrogen for a wide variety of biochemical substances. This work was devoted to the characterization of glutaminase.

There is no up-to-date review on glutaminase available. The REVIEW OF LITERATURE is an attempt to accomplish that purpose. This chapter not only summarizes previous works on glutaminase, but also indicates the controversies among workers. The sections are arranged in such a way that the reader can easily compare the experimental results of this work with those of previous works.

All of the critical experiments are described in detail, including the methods for setting them up, in order to enable the reader to judge the validity of the experiments as well as to repeat them. To minimize distraction in reading and to provide a compact picture of this work, results and discussion are presented simultaneously.

To solve problems directly is the principle of this work. For example, a new, quick, and sensitive radiometric assay for glutaminase activity was established to eliminate the uncertainty inherent in most of the existing assays; a new purification procedure with high yield of glutaminase was established to solve the low yield problem. The author realizes that there is no single procedure or set of procedures by which any and every protein can be isolated. Nevertheless, the author attempts to indicate the underlying nature of each purification step.
Acknowledgements

This work was under the direction of Dr. Elizabeth A. Boeker. The author would like to express his sincere appreciation to Dr. Boeker for her advice, guidance, and professional example. The author also would like to thank Dr. T. F. Emery, Dr. T. M. Farley, Dr. R. K. Olsen, and Dr. C. A. Ernststrom for their advice and conscientious help in the preparation of this dissertation.

The unfailing confidence of his parents toward their son has been an unlimited source of courage to the author. Had the courage not been available, it would not have been possible for the author to finish this work. Finally, the author wants to express his deep appreciation to his wife, who patiently endured the many weekends and evenings that were devoted to experimenting and who gave help when it was needed, as always.

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ABSTRACT

Cow Brain Glutaminase: Purification, Characterization, and Mechanism of Action

by

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Utah State University, 1979

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Department: Chemistry and Biochemistry

A simple, quick, and sensitive radiometric assay for glutaminase has been established. This assay uses L-(U-14C)-glutamine as the substrate and measures the 14CO2 produced when the reaction is coupled to glutamate decarboxylase. An efficient purification procedure for cow brain glutaminase has also been developed; the yield is 12%. Steps include acetone extraction, ammonium sulfate fractionation, calcium phosphate gel treatment, and differential gel filtration on Sepharose 4B. The final preparation has a specific activity of 385 μmole/min/mg and shows a single protein band on polyacrylamide gel electrophoresis in sodium dodecyl sulfate; this band corresponds to a subunit molecular weight of 82,000. Polyacrylamide gel electrophoresis studies did not reveal any impurities; the enzyme activity coincided with the protein staining. This enzyme is stable between pH 7.5 and 9.2 and has maximal activity around 8.8. The activity of glutaminase appears to be independent of the nature of the buffer with which it was equilibrated before it was assayed. The enzyme absolutely requires phosphate for activity; the dependence is sigmoidal and has a Hill coefficient of
2.2. The phosphate concentration that gives half maximal velocity is 50 mM. At 0.2 M potassium phosphate (pH 8.8), the dependence of activity on glutamine is hyperbolic; the observed $K_{\text{gln}}$ is 17 mM. Neither ammonium ion (0.1 M) nor citrate, succinate, or glutarate (57 mM) inhibit the enzyme activity. Glutamate inhibits competitively with respect to glutamine. Phosphate affects both $K_{\text{gln}}$ and $K_{\text{glu}}$ the same way. A radioactivity exchange study was not able to detect incorporation of $^{14}$C-glutamate into $^{14}$C-glutamine. The results are consistent with a model in which ammonia is released irreversibly from the enzyme-substrate complex and is the first product to be released.
INTRODUCTION

Glutaminase (E.C.3.5.1.2., L-glutamine amidohydrolase) catalyzes the hydrolytic cleavage of glutamine into glutamate and ammonia. The metabolic pathways in Figure 1 show the importance of this enzyme. Glutaminase and glutamine synthetase interconvert glutamine and glutamate; if unregulated, this pair of enzymes will form a futile cycle that leads only to the net hydrolysis of ATP. Decarboxylation of glutamate is an irreversible step, catalyzed by glutamate decarboxylase. Both glutamate and γ-aminobutyrate, the substrate and the product of this decarboxylase, are important neurotransmitters in mammalian brains (1-11). α-Ketoglutarate, one of the metabolites of the tricarboxylic acid cycle, is formed from glutamate through a reversible reaction catalyzed by glutamate dehydrogenase.

When $^{14}$C-α-ketoglutarate was incubated with a preparation of brain mitochondria, very little $^{14}$C-glutamate was found (12), suggesting that neither glutamate dehydrogenase nor any transaminase catalyzes the major pathway for glutamate synthesis. On the other hand, when $^{14}$C-glutamine was used as a tracer, a large amount of $^{14}$C-glutamate accumulated in the brain mitochondria preparation (12), indicating that glutaminase is the major enzyme responsible for the synthesis of glutamate (13-21, 1). The mechanism of glutamate formation and its regulation are not completely understood. In order to study the control of glutamate formation, it is necessary to purify glutaminase, the key synthetic enzyme.
Figure 1. Pathways to glutamine, glutamate, and γ-aminobutyrate.
Presently, only one purification method is available for brain glutaminase (22). The yield for the enzyme from pig brain was less than 0.4% (specific activity = 106 μmole/min/mg) or less than 3% (specific activity = 358 μmole/min/mg) when the final preparation was dissolved in a Tris buffer or a borate-phosphate buffer, respectively. This purification method does not produce large amounts of enzyme.

To date, including enzymes from all sources, the molecular weight of glutaminase is still ambiguous (22-26), and no amino acid composition has been published. There are at least two factors contributing to the difficulty of glutaminase study. First, glutaminase is a mitochondrial membrane associated enzyme (27-39, 22, 12, 13, 24). It is very difficult to solublize. Second, all the assays used for glutaminase activity determination have limitations, as well be discussed later, which impose restrictions upon the purification procedure.

In order to overcome previous difficulties in enzyme purification and to compile useful and reliable data for glutaminase, this work attempts to (a) establish a new and effective radiometric assay for glutaminase activity, (b) develop a simple and efficient purification procedure for cow brain glutaminase, (c) elucidate the mechanism of action of the enzyme, and (d) define some of the characteristics of the enzyme. As a consequence of this work, further study of brain glutaminase has been greatly simplified.
REVIEW OF LITERATURE

General Background

Inorganic ammonia is formed during the catabolism of amino acids and is strongly toxic to living cells. In higher animals, ammonia is incorporated into glutamine as an important nontoxic intermediate. Glutamine lies at the center of cellular nitrogen metabolism; the amide nitrogen serves as the immediate source of nitrogen atoms for a wide range of biochemical substances (Figure 2). In 1904, S. Long reported that glutamine is deamidated by a great variety of animal tissues (40).

The presence of glutaminase in animal tissues was first reported by H. A. Krebs in 1935 (41). He distinguished between two types of this enzyme, the brain type and the liver type. The brain type was called glutaminase I, and required phosphate for activity (42-43, 45-50). The liver type was called glutaminase II, and was not activated by phosphate but did require an α-keto acid for activity (42, 43, 45, 50-53). This enzyme has been identified as a glutamine-α-keto acid transaminase (42). A liver enzyme with a normal glutaminase activity has been reported by W. E. Knox and his colleagues (54-58); it requires phosphate for activity.

Katunuma et al. first reported in 1966 that two glutaminase isozymes are found in rat kidney; they have subsequently found them in many other animal tissues (59, 60). One isozyme requires phosphate for activity (phosphate-dependent glutaminase) while the other
Figure 2. Metabolic fates of the amide nitrogen of L-glutamine.
does not require phosphate but is activated by maleate (phosphate-independent glutaminase). The phosphate-independent glutaminase has been shown to a γ-transpeptidase; maleate promotes glutaminase activity while blocking transpeptidation (61-63). A mechanism of action of transpeptidation as well as a functional role for maleate has been proposed recently (64).

Kidney phosphate-dependent glutaminase appears to be immunologically (65) and kinetically (66-70) very much like the brain phosphate-dependent glutaminase. The strongest support for this comes from a recent report by Clark and Curthoys (71); they found that the kidney glutaminase, immunoprecipitated from Triton X-100-solubilized mitochondria, is composed primarily of a subunit which has molecular weight almost identical with that of the cow brain glutaminase reported by Boeker and Chiu (72). Phosphate-dependent glutaminase from kidney also showed an immunoprecipitation curve identical with that of the phosphate-dependent glutaminase from small intestine (37). However, antisera prepared against the rat kidney enzyme did not cross-react with glutaminase solubilized from rat liver mitochondria (65). Since these two enzymes also exhibit distinct kinetic properties (54, 65), the liver enzyme has been recognized as a distinct isozyme (65).

Escherichia coli contains also two types of glutaminases, A and B (73-75). Glutaminase A possesses maximal activity at about pH 5 and is present in stationary phase cells. Glutaminase B occurs in log phase cells; like the phosphate-dependent glutaminases of animal tissues, this enzyme is active only when phosphate is present and the pH is greater than 7.
Role of Glutaminase

In brain

Abundant evidence now exists which suggests that glutamate is one of the principle synaptic transmitters in the vertebrate central nervous system (1). It strongly excites almost all neurons examined, and has been considered to be a main excitatory transmitter. The product of glutamate decarboxylation, \( \gamma \)-aminobutyrate, has the opposite effect; it strongly inhibits depolarization of the nerve cell membrane (1-9).

Glutamine has been shown to be the major precursor of transmitters in nerve endings (1, 13). Mammalian cortical synaptosomes incubated in the presence of 0.5 mM glutamine and 2.5 mM glucose showed a 30% increase in transmitter amino acid content over the control without glutamine. Isotope labelling experiments showed that in the presence of glucose, the contribution of glutamine to the total glutamate, aspartate, and \( \gamma \)-aminobutyrate in the tissue relative to glucose was 50-70%; to glutamate alone, it contributed 80%. However, in the absence of glucose, glutamine alone is not an adequate substrate at concentrations up to 0.75 mM; its contribution to energy metabolism seems to be minimal.

Since (a), glutamine can readily pass the blood-brain barrier (76), (b), glutaminase activity in the nerve endings is high (31, 77, 78), and (c), isolated brain mitochondria do not synthesize a substantial amount of glutamate from \( \alpha \)-ketoglutarate (12), glutaminase is thought to play a major role in replenishing the pool of glutamate in mammalian brain (13, 1, 14-21).
In kidney

In 1943, Van Slyke found that 60% of the ammonia excreted by the kidney was derived from glutamine (79), a fact which has since been confirmed (80, 81). The remaining carbon skeleton of glutamine, α-ketoglutarate, can be used by the kidney for glucose synthesis (82, 83). It has been suggested that phosphate-dependent glutaminase enables kidney mitochondria to respire in the presence of glutamine (32, 21, 84).

The mammalian kidney increases its ammonia production as part of the adaptive response to chronic acidosis (49, 85, 86, 79, 88-92). The ammonia is used primarily to titrate acids which are then excreted by the kidney (23, 85). It has been found that it is the phosphate-dependent glutaminase which responds to acidosis as well as to renal ammoniagenesis (27, 65, 91-95): (a) The phosphate-dependent glutaminase solubilized from normal and acidotic rat kidneys are immunologically identical and exhibit similar kinetic properties (65). (b) The phosphate-dependent glutaminase, but not the phosphate-independent enzyme, responds to metabolic acidosis or alkalosis (89, 90, 93). (c) The amount of phosphate-dependent glutaminase increases 20-fold within the proximal convoluted tubules of the rat nephron (65, 89). (d) Actinomycin D prevents elevated ammonia excretion in the acidotic rat (88).

However, in acute acidosis, phosphate-dependent glutaminase does not make responsive changes although the synthesis of ammonia, as in chronic acidosis, is increased (88, 97). It has been proposed that the decreased glutamate concentration in acute acidosis could
stimulate ammonia production by reducing the inhibition of glutamate on the phosphate-dependent glutaminase (94, 98, 99).

The control mechanism for adaptation to either chronic or acute acidosis has been studied rather extensively. Most of the work focuses on (a) α-ketoglutarate, the regulator of the renal mitochondrial glutaminase transport system (100), (b) glutamate, the glutaminase inhibitor which decreases during acidosis (101-103, 97, 98, 94), and (c) phosphoenolpyruvate carboxykinase, the enzyme which could be the major site of metabolic control of gluconeogenesis and ammoniagenesis when glutamate or α-ketoglutarate is converted to glucose (82, 83, 95, 104, 106, 94, 98, 97). So far, none of the studies is conclusive.

In the small intestine

The most abundant amino acid in mammalian plasma, glutamine (107), is taken up and metabolized by the small intestine of animals (108, 109) and men (110). This metabolism of glutamine accounts for 30% of carbon dioxide produced, relative to glucose (108).

A highly active phosphate-dependent glutaminase was found in the mucosal epithelium of rats, dogs, cats, hamsters, rabbits, and monkeys (37); the net uptake of glutamine by intestine was found to be proportional to the blood glutamine concentration and the glutaminase activity. Based on this and experimental data from inhibition studies using glutamine analogs, it has been suggested that phosphate-dependent glutaminase is the major glutamine utilizing enzyme in small intestine (37).

In HeLa cells

When HeLa cells were grown exponentially on glutamine and sugar,
a dramatic phenomenon was observed: the cells used glutamine, not sugar, as the major source for energy (111). Less than 2% of the glutamine was incorporated directly into protein, 35% of the carbon appeared as carbon dioxide, 13% was incorporated into lactate, and about 25% into macromolecules. This result occurred regardless of the sugar used, i.e., fructose, galactose, or glucose, excepting that a large amount of glucose and a small amount of glutamine were used as the growth medium. This experiment also indicated that little sugar carbon was oxidized in the tricarboxylic acid cycle, glycolytic activity was low, and almost all of the carbon arising from fructose was metabolized through the pentose phosphate pathway and was not a source of energy.

Follow-up studies to this recent report on glutamine metabolism should be very interesting. There seems to be little doubt but what glutaminase is responsible for the primary energy producing pathway.

In tumors

Phosphate-dependent glutaminase activity has been found to increase in proportion to the growth and the degree of histological undifferentiation of various tumors (58, 54-56). A series of experiments on hepatic and nonhepatic rat tumors showed that only glutaminase resembling the phosphate-dependent enzyme from kidney or brain was increased (58, 55, 56). Even in a series of hepatomas, neoplasms originating from liver tissue, only the kidney enzyme, and not the liver enzyme, increased. Although the adult liver contains only the liver form, the kidney form occurs before birth (54). Thus, liver has the potential for both forms of the enzyme. It has been suggested
that glutamine hydrolysis may be needed for neoplastic growth, since
the kidney enzyme, which has a much lower Km than the liver enzyme,
can play a more efficient role. In addition, since the liver enzyme
is activated in an allosteric manner by glutamine and inhibited by
adenosine diphosphate (58, 54), enzyme activity, and therefore the
growth of tumor cells, will be regulated by both the substrate concen­
tration and the energy charge.

The parenteral administration of enzymes which degrade amino
acids required only for growth of neoplasms offers a potential cancer
therapy with marked specificity for the tumor (112-114). It has been
shown that asparaginase-resistant Ehrlich carcinomas regressed per­
manently when tumor-bearing mice were injected with bacterial gluta­
minase or glutaminase-asparaginase preparations (115, 116). Therefore,
glutaminase has been studied as an antineoplastic agent (117).
However, all animal glutaminases known so far have a very high Km
(>> 0.1 mM) and also are strongly inhibited by their product; they are
probably not suitable for antitumor treatment.

Subcellular location of glutaminase

Brains of rat (29), pig (22), rabbit (12), chick (31), and toad
(13), kidneys of rat (27, 28, 30, 32, 39, 33) and pig (24, 34), liver
of pig (35, 36), skeletal muscle of rat (38), and mammalian small
intestines (37) have all been found to contain at least one phosphate­
dependent glutaminase. All these enzyme are mitochondrial. Pig
kidney phosphate-dependent glutaminase was found to be located exclu­sively in the mitochondria by Klingman and Handler in 1958 (34); they
used detergent solublization and sonication. Similar techniques were
used later to show that the rat kidney enzyme is bound to the inner membrane of mitochondria (33, 28).

Glutaminase Assay

Glutaminase activity has been measured by a variety of methods, which include ammonia evolution, ammonia sensitive electrodes, paper chromatography, ion exchange column chromatography, and coupling to the glutamate dehydrogenase reaction. Every one of these methods has significant drawbacks. It seems likely that at least some of the confusing and conflicting results for glutaminase are due to the deficiencies of the assays used. In all cases, the assay used must be considered when data are interpreted.

The ammonia formed by glutaminase has been determined by direct or modified Nesslerization (34, 59, 119, 54, 120-122). Since Nessler's reagent is subject to interference by amines, amino acids, proteins, inorganic ions, and organic solvents (123), and since precipitation occurs frequently after the addition of Nessler's reagent (124), the ammonia must be distilled or diffused into an acid before measurement. During distillation or diffusion, glutamine can be hydrolyzed by the strongly basic solution, producing spurious ammonia (125). The adapted ammonia diffusion-titration assay described by Mattenheimer and Debruin appears to have high reproducibility but low accuracy (124). Since the procedure is also tedious and requires skillful operation, this assay is not often used.

Ammonia at concentration higher than 0.1 mM has been measured directly with an ammonia electrode (126, 127). This method requires at least 3 ml of solution and is subject to interference by potassium
ions and volatile amines (125, 128-130). It should be noted that, in general, assays measuring ammonia require that ammonia be removed from the substrate solution before assay.

In chromatographic assays, glutamate is separated from glutamine by paper chromatography, located with ninhydrin, eluted, and measured colorimetrically (131-133, 74). This method is inherently imprecise and requires an overnight separation on paper. A newer assay separates radioactive glutamate by thin-layer chromatography, locates it with ninhydrin and then quantifies the glutamate by radioactivity. This method is very sensitive and requires only 35 minutes for the chromatogram to develop (134). However, when labelled glutamate is heated with ninhydrin, radioactive carbon dioxide is formed and escapes into the air, making the assay unreliable.

Dowex-1 (Cl) has been used to separate radioactive glutamate from glutamine (135, 75). When the pH is higher than 9 both glutamate and glutamine are bound to the resin; below pH 6 both compounds are eluted. At pH 7, 100% of the glutamate is bound while 90-95% of the glutamine is recovered. Since (a) the substrate must be purified before use, (b) pyrroldidonecarboxylate cannot be separated from glutamate at neutral pH, and (c) EDTA, phosphate, chloride, glutamate, and imidazole all interfere with the chromatography, this method apparently is unsuitable for detection of the phosphate-dependent glutaminase.

The most commonly used assays for glutaminase employ coupling to glutamate dehydrogenase to measure either the glutamate or the ammonia formed. Detection is based on the absorbance of NAD(P)H at 340 nm. Assays in which ammonia is measured (133, 24, 74, 133, 136) are sensitive to contaminating ammonia (24, 131) and ammonia from nonenzymatic
hydrolysis of glutamine (137, 125, 124). It is known that α-ketoglu­
tarate undergoes dimerization in solution (125). It is known also
that the presence of α-ketoglutarate, an activator of glutaminase
under some conditions, interferes with the reaction (24).

In the coupled assay where glutamate is determined (89, 13), the
dehydrogenase is not added until after the glutaminase reaction has
been stopped. This method is direct and is not subject to interfer­
ence by most compounds, but it requires that the dehydrogenase re­
action proceed in the unfavorable direction. Besides, several small
volumes transferred by pipets, from a fraction of one microliter to
a few micro liters, are required; the procedure is also tedious.

All assays coupled to glutamate dehydrogenase share the following
drawbacks: (a) Bovine liver glutamate dehydrogenase is inhibited by
a variety of compounds (138); ATP and GTP are powerful inhibitors (139,
140), while ADP is an activator, in the direction of glutamate syn­
thesis (141). (b) Since the phosphate-dependent glutaminase has a pH
optimum around 8.8, the assays must be run at a suboptimal pH to
accommodate the glutamate dehydrogenase action. (c) The assays are
limited by the absorbance of NAD(P)H.

Recently Chiu and Boeker have reported a new radiometric assay
for phosphate-dependent glutaminase (66). The assay is stopped by
reducing the pH to 4.5, where glutaminase is completely inactive.
E. coli glutamate decarboxylase is then added to release all of the
α-carboxyl group from radioactive glutamate. The $^{14}$CO$_2$ is then
trapped and counted. Regardless of the conditions used, the only
requirement for an accurate assay is that enough glutamate decar­
boxylase be added to convert all the glutamate to carbon dioxide.
As will be discussed in detail later, this assay is simple, quick, inexpensive, and practical for handling a large number of samples. The principle limitation of this assay is that it does not measure enzyme activity continuously.

**Properties of Glutaminase**

**Purification**

Progress in the study of phosphate-dependent glutaminase has been very slow because of the difficulties encountered in extracting and stabilizing this enzyme. The pig kidney enzyme was first solubilized by Otey, Birnbaum, and Greenstein in 1954 (142). Klingman and Handler purified it 200-fold in 1957, by making a mitochondrial extract in borate buffer and chromatographing it on Amberlite XE-97 (34). The same report indicates that numerous attempts at purification, including heat treatment, isoelectric precipitation, protamine precipitation of nucleic acids, fractionation with metals, ammonium sulfate or ethanol fractionation, absorption and elution from calcium phosphate, calcium borate, alumina Cγ gel, Dowex-1 chloride or borate, Dowex-50, Amberlite IRC-4B, IR-400, or XE-64, hydroxyapatite or Celite columns, electrophoresis on potato starch blocks, sodium dodecyl sulfate, digitonin, or deoxycholate treatment, or sonication did not produce satisfactory purification. A year later, Sayre and Roberts obtained the same purification factor by repeated sodium sulfate fractionation of dog kidney homogenate which had been kept frozen for several weeks (119).

The first homogeneous phosphate-dependent glutaminase preparation, isolated from pig kidney, was reported by Kvamme, Tveit, and Svenneby,
in 1970 (24, 132). The main steps of this procedure consisted of sodium sulfate fractionation and alternative solubilization, by dialysis against Tris-HCl buffer, and precipitation with phosphate-borate buffer. This 10,000-fold purification is based on the fact that the enzyme aggregates in phosphate-borate buffer and disaggregates in Tris-HCl buffer. The final preparation, in phosphate-borate buffer, had a specific activity of 320 µmole/min/mg; the yield was 3%. In Tris-HCl buffer, electrophoresis on polyacrylamide gel gave one protein band but no activity could be recovered. Sedimentation velocity studies revealed one symmetrical peak, and the immune diffusion technique did not detect any impurity. In a later report (26), the same enzyme preparation showed two protein bands after polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

The pig brain enzyme was purified 5,000-fold by G. Svenneby in 1970 (131) and then 10,000-fold by Svenneby, Torgner, and Kvamme in 1973 (22). The main steps were acetone extraction, sodium sulfate fractionation, and alternative solubilization and precipitation in Tris-HCl buffer and phosphate-borate buffer. Isoelectric focusing and sedimentation equilibrium studies revealed no impurities. With polyacrylamide gel electrophoresis, one protein band was detected on regular gel, and one major and one minor band in the presence of sodium dodecyl sulfate. The yield of enzyme was 2.8%. The specific activity was 358 µmole/min/mg when the enzyme was dissolved in phosphate-borate buffer.

The rat kidney enzyme was purified by Curthoys, Kuhlenschmidt, and Godfrey in 1976 with 11% recovery (25). The final preparation had a specific activity of 320 µmole/min/mg. The steps were
mitochondrial preparation, digitonin treatment, lyophilization, ammonium sulfate precipitation, and successive gel filtrations. Polyacrylamide gel electrophoresis showed a broad protein band; the enzyme activity recovered from a sliced gel was consistent with the protein band. This is the only report where highly purified glutaminase activity has been recovered from a gel. However, this enzyme would not enter the gel unless 0.1% Triton X-100 was present. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate revealed at least five protein bands (143); further studies suggest that the heterogeneity results from a partial proteolysis which does not inactivate the enzyme (71, 23, 143).

Glutaminase B from E. coli is the only other phosphate-dependent glutaminase which has been purified more than 1,000-fold. Purification steps were streptomycin precipitation, ammonium sulfate fractionation, DEAE chromatographies, Agarose 1.5m filtration, and preparative electrophoresis. The final preparation had a specific activity of 716 μmole/min/mg and the yield was 19%. Estimation by continuous electrophoresis on polyacrylamide gel suggests that the enzyme was 40% pure (75).

It should be noted that almost every report used its own assay method and that the concentrations of phosphate and substrate in the assay solutions were by no means consistent. This may account for some of the apparent variability observed for glutaminase.

**Molecular weight**

Molecular weight estimations for phosphate-dependent glutaminase are very inconsistent so far. There is not one purified enzyme that
has been studied by two independent methods with consistent results. It seems likely either that the enzyme is not completely pure, or that partial proteolysis has occurred (71), or both.

In general, in Tris buffer, glutaminase is in its lowest molecular form, the so-called Tris form. (24, 22, 26, 131, 144, 25, 72). Phosphate induces aggregation, perhaps through dimerization (144). Phosphate and borate together induce further aggregation to a form with molecular weight higher than one million (24). This reversible aggregation has been observed in studies of sedimentation (24, 22, 144), Sephadex G-200 (25), Sepharose 4B (25, 72), and electron microscopy (26, 145, 132). In this section, only the lowest molecular form will be discussed.

Assuming a partial specific volume of 0.732 ml/g for the pig kidney enzyme, the estimated molecular weight by sedimentation equilibrium in 10% sucrose is 170,000 (26). The value estimated from Sephadex G-200 gel filtration and sucrose gradient centrifugation is 140,000-160,000 (24). From electron microscopy, the molecular weight is calculated to be 125,500 if the shape is prolate-ellipsoidal and 187,000 if the shape is cylindrical (26). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate reveals two close bands, corresponding to subunit molecular weight of 53,000 and 61,000 (26). It has been suggested that the molecule may be a trimer, $\alpha_2\beta$ or $\beta_2\alpha$, although this contradicts the electron microscopic observations.

The pig brain enzyme has been studied by sedimentation equilibrium. Earlier work on a partially purified preparation found a molecular weight of 117,000 (146), whereas recent work gave a value of 187,000 for a highly purified preparation (22). By polyacrylamide gel
in sodium dodecyl sulfate, only one protein staining band with a molecular weight of 64,000 was found (22). The idea that pig brain enzyme is a trimer has also been suggested.

Rat kidney glutaminase eluted from a calibrated Sephadex G-200 column with an apparent molecular weight of 190,000, whereas sucrose gradient centrifugation suggests 115,000 (25). In a careful experiment, using techniques similar to these, the molecular weight was redetermined to be 160,000 (144). Upon sodium dodecyl sulfate polyacrylamide gel electrophoresis, the highly purified enzyme exhibited at least five protein staining bands with molecular weights ranging from 57,000 to 75,000 (143). L-2-amino-4-oxo-5-chloropentanoic acid, an analogue of glutamine, has been found to react with each of the protein staining bands (143). Recently, a follow-up report showed that all of these bands are related in structure and that glutaminase, immunoprecipitated from Triton X-100 solubilized mitochondria, is composed primarily of subunits which have molecular weight of 83,000 (71). This value is consistent with the value for cow brain glutaminase reported earlier by Boeker and Chiu (72). All of the results taken together strongly suggest that the heterogeneity in subunit size results from partial proteolysis and that the molecule is a dimer.

The molecular weight of glutaminase B from E. coli has been estimated to be 90,000 by Sephadex G-200 gel filtration and analytical polyacrylamide gel electrophoresis (75). Since the enzyme was only about 40% purified, further investigation is necessary.

**pH optimum and Km**

The activity of glutaminase as a function of pH has been studied
in many tissues with several assays. The tissues include pig brain (131) and kidney (132, 24), rat brain (48) and kidney (59, 34, 60, 147, 25, 39), dog kidney (119, 124), human kidney (124), and rat intestine (37). Since different assays were used, the results are not entirely comparable. However, two conclusions can be drawn. The pH optimum lies between 8 and 9 and enzyme is inactive below 6 or above 11. In addition, the optimal curve is broader when borate is present.

The value of Km for glutaminase has been reported to decrease with increasing phosphate concentration (149, 119, 132), increase with increasing pH (68), and increase with increasing borate concentration (132). The measurement of Km is also sensitive to the purity of enzyme and the assay method. Each determination of Km should be recognized as a result of a particular assay method and set of conditions.

The Km measurements for pig brain glutaminase have been interpreted in a very complicated manner. The value for the enzyme equilibrated in Tris-HCl buffer increases from 5 mM to 20 mM when the pH is increased from 6.5 to 10 (68). Corresponding values for the enzyme in phosphate-borate buffer are 1.8 and 8 mM. The plots of velocity versus glutamine were all sigmoidal, and phosphate concentration up to 300 mM did not cancel the sigmoidal pattern. Decreasing pH lowered the enzyme activity and shifted the pattern from sigmoidal to hyperbolic. It has been suggested that the enzyme is allosteric and that protons may play a role as effectors. In trying to explain the sigmoidal pattern, speculations included the induced fit theory, substrate cooperativity, ligand exclusion theory, and one catalytic site theory.
It was concluded that all theories were possible (68). It should be pointed out that this experiment did not use substrate concentrations higher than 20 mM, the Km was determined with four data points, partially purified enzyme was used, and the enzyme activity was determined by paper chromatography in 300 mM potassium phosphate. A follow-up report found the Km to be in the range of 5 mM to 9 mM (150). The rat brain enzyme has been reported to have a Km of 30-50 mM, using Nesslerization in 0.2 M potassium phosphate at pH 8.6 (60). Another report on rat brain enzyme shows Km values of 16 mM and 11 mM when it was assayed in phosphate concentrations of 5-30 mM and 100 mM, respectively (149). The Km of the pig kidney enzyme has been found to be 4.3 mM and 5.8 mM in Tris-HCl buffer and phosphate buffer, respectively (132). The assay was spectrophotometric, employing coupling to glutamate dehydrogenase to determine the formation of ammonia in 0.1 M sodium phosphate at pH 8. It was found that the Km decreased when the phosphate concentration was increased (67). An earlier report found a Km of 5 mM for the pig kidney enzyme with Nesslerization (34). Dog kidney glutaminase has a Km of about 5 mM when assayed in 0.1 M sodium phosphate; the value decreases with the increase of phosphate concentration from 50 mM to 150 mM (119). The rat kidney enzyme was found to have a Km of 40 mM when assayed in 0.2 M potassium phosphate at pH 8.6 with Nesslerization (60, 59). A Km of 4-10 mM has also been reported for the rat kidney enzyme, regardless of the buffer the enzyme is equilibrated with (89, 25). Here the enzyme activity was detected by using glutamate dehydrogenase to measure the formation of glutamate in 150 mM phosphate at pH 8.6; the Km increased with increasing phosphate concentration. One of two
other reports on rat kidney glutaminase found the \( \text{Km} \) to be 21 mM at 5-30 mM phosphate and 10 mM at 100 mM phosphate (149); the other report gave 5 mM in 0.4 M phosphate (34).

For the enzyme from rat small intestine, \( \text{Km} \) was found to be 2.2 mM (37). The rat liver enzyme is reported to have \( \text{Km} \) values of 28 mM and 80-100 mM (60, 151); the discrepancy apparently arises from the assay method and conditions.

**Activation and inhibition**

Glutaminase requires phosphate or a phosphate-containing compound for activity. Reports stating that glutaminase shows minor activity in the absence of phosphate are probably in error. This is probably due to contamination of the preparation by phosphate-independent glutaminase or by phosphate itself.

The pig brain enzyme has a \( K_{0.5} \) for phosphate of 13.5 mM and 69 mM when equilibrated with Tris-HCl buffer or phosphate-borate buffer, respectively (68). The Hill coefficient is 1.8 regardless of the buffer (68). However, these \( K_{0.5} \) for phosphate values may not be very accurate; the enzyme was not highly purified and showed some activity at zero phosphate concentration. The rat brain enzyme has been reported to have a \( K_{0.5} \) for phosphate of 100 mM; the assay mixture contained only 5 mM glutamine, a very low concentration (44). Another report shows a \( K_{0.5} \) for phosphate of 600 mM when the rat brain enzyme was assayed in 20 mM glutamine (60).

The \( K_{0.5} \) for phosphate for the pig kidney enzyme has been shown to be 25 mM (87) and 50 mM (132), with Hill coefficients of 1.78 and 1.04, when equilibrated in Tris-HCl and phosphate buffer, respectively...
An earlier report showed a $K_{0.5}$ for phosphate of 50 mM regardless of the buffer (34).

The rat kidney enzyme has a $K_{0.5}$ for phosphate of 35 mM (89). The effects of phosphate on sedimentation coefficient and activity are consistent and can be used to calculate a $K_{0.5}$ for phosphate of 40-50 mM (144).

The small intestine enzyme from the rat has a $K_{0.5}$ for phosphate of 22 mM. Unlike all enzymes previously described in this section, the plot of activity versus phosphate concentration for this enzyme is hyperbolic rather than sigmoidal (37).

Electron microscopic study of the pig kidney enzyme suggests that phosphate causes dimerization of the lowest molecular weight form, or Tris form (26). Sucrose gradient sedimentation and glutaminase activity studies of the rat kidney enzyme demonstrated that increasing phosphate concentration increases both the sedimentation coefficient and the activity of the Tris form the same way, suggesting that there is a correlation between dimer formation and activation (144). Z. Kovacevic suggested that the binding site of glutaminase for phosphate is embedded in the mitochondrial membrane and the binding site for the substrate is exposed toward to matrix space (96). The influx of phosphate across the mitochondrial membrane may bring about the association of inactive Tris form and, therefore, activate the enzyme.

Reports on the effects of ammonia and carboxylic acids on phosphate-dependent glutaminase are contradictory. This probably originates in the assays used. In the presence of effectors the assay situation is very complex, since the effector may affect both
glutaminase and the coupling enzyme, as well as the sensitivity of measurement.

Some reports show that ammonia is competitive inhibitor of the dog (119) and pig (34) kidney enzymes whereas other reports show that ammonia activates the pig kidney (24, 132) and pig brain (68) enzymes.

Physiologically important carboxylic acids such as pyruvate, succinate, malate, malonate, oxaloacetate, α-ketoglutarate, acetate, and citrate have been found both to activate (39, 132, 150, 149, 104, 68, 25, 59, 37) and inhibit (90) glutaminase. It has been suggested that, in the absence of phosphate, there is a correlation between activation and the number of carboxyl groups per molecule (149, 68).

L-2-amino-4-oxo-chloropentanoic acid, a chloroketone, and 6-diazo-5-oxo-L-norleucine (DON) have been shown to specifically and irreversibly inhibit the glutamine dependent enzyme activity by covalent binding at the glutamine (105, 37, 148, 69, 152) or glutamate (10, 143) binding sites. These analogues have been used to identify the glutamine binding peptides in sodium dodecyl sulfate polyacrylamide gels (143) and to study the binding of glutamine and glutamate to phosphate-dependent glutaminase (23, 143). It has been suggested that DON and the chloroketone act on rat kidney enzyme at the binding sites for glutamine and glutamate, respectively (23). This suggestion is based on the fact that incubation of the enzyme with DON prevents binding of the chloroketone only slightly. However, it should be noted that the enzyme preparation is heterogeneous, the Tris in the enzyme buffer may prevent the binding of DON to the enzyme, and that DON does protect up to 30% of the enzyme. A study on the rat small intestine enzyme shows that glutamine protects against inactivation
by both DON and chloroketone in vivo as well as in vitro (37). This study indicates that both DON and chloroketone are acting upon the same site, presumably the glutamine binding site.

In the absence of phosphate, glutaminase can be activated by phosphate containing compounds such as acetyl-CoA (70), fatty acyl-CoA derivatives (70), riboflavin-5-P (44), and tri- and diphosphate nucleotides (44, 149). Acetyl-CoA was reported to have a $K_a$ of 0.2 mM (70); its activation is accompanied by an increase in the sedimentation coefficient. Fatty acyl-CoA derivatives are more effective activators when the fatty acyl group is unsaturated; they are more effective inhibitors when the fatty acyl chain is elongated (70). It has been speculated that acetyl-CoA and fatty acyl-CoA derivatives might regulate the glutaminase activity in vivo (70). Tri- and diphosphate nucleotides were reported to activate the rat brain enzyme more strongly than phosphate itself on the basis of phosphate equivalents (44). On the contrary, the rat kidney enzyme was no more activated by tri- or diphosphate nucleotides than by phosphate itself (25). Studies on the rat kidney and brain enzymes suggest that GTP-cGMP or ATP-cAMP might regulate glutaminase; GTP (or ATP) activates the enzyme and cGMP (or cAMP) inhibits it (149). Glutaminase B from *E. coli* shows the opposite regulation; ATP and ADP inhibit the enzyme whereas AMP activates it (153).

In 1935 H. A. Krebs reported that the brain type glutaminase was strongly inhibited by glutamate (41). It was then reported in 1958 that glutamate inhibited both the pig kidney (34) and dog kidney (119) enzymes noncompetitively. Since then inhibition by glutamate has been reported for the pig kidney (87, 67, 132), pig brain (68), and rat
kidney (60, 23, 144) enzymes; i.e., almost all phosphate-dependent glutaminases studied. Studies on the pig kidney enzyme showed that changing the phosphate concentration in the assay mixture from 50 mM to 150 mM could shift the inhibition pattern from noncompetitive to competitive (67). Generally, it appears that the enzyme is more sensitive to glutamate inhibition when it is equilibrated with Tris buffer than it is in phosphate-borate buffer (87, 68). In both the dog kidney (119) and pig kidney (87) enzymes glutamate was found to compete with phosphate; however, the same study group also observed noncompetitive inhibition (67, 132).

Glutaminase B from *E. coli* behaves uniquely toward glutamate. Incubation of the enzyme with 20 mM glutamate at 4°C causes a 2-fold activation, while addition of 20 mM glutamate to the assay mixture results in a 10% inhibition of the enzyme activity (104). It has been suggested that glutaminase B may have two binding sites for glutamate, and that activation by glutamate may occur slowly at an allosteric site.

**Mechanism of action**

Klingman and Handler proposed a mechanism of action for pig kidney glutaminase (34) in which ammonia is released first, reversibly, and glutamate is released second, irreversibly. This mechanism is based on the following findings: (a) Ammonia acts as a competitive inhibitor. (b) Glutamate acts as a noncompetitive inhibitor. (c) The enzyme did not catalyze the exchange of $^{14}$C-glutamate into $^{14}$C-glutamine but did cause incorporation of $^{15}$NH$_3$ into glutamine.
Based on a series of kinetic studies, Tveit, Svenneby, and Kvanme proposed dead-end complex models for the mechanism of action of kidney glutaminase (67, 132). Both models assume that the reaction sequence is ordered and that glutamate is the first product to be released. One of the models proposes that glutamate binds with free enzyme, thus forming a dead-end complex. The other model proposes that glutamate binds the enzyme only when phosphate is also bound, and that phosphate binds only the free enzyme. They conclude that the dead-end complex models can not be considered to be fully established but that other, more complicated mechanisms, may be possible (67).
EXPERIMENTAL PROCEDURES

Materials

Fresh cow brains were obtained from the E. A. Miller & Son Packing Co., Hyrum, Utah. Sepharose 4B was purchased from Pharmacia. Ultrafiltration cells and XM300 membrane were products of Amicon Co. L-(U-14C)-glutamine and L-(U-14C)-glutamic acid were obtained from New England Nuclear. 2,5-Diphenyloxazole, scintillation grade, was from Amersham/Searle. Glutamate decarboxylase, a crude acetone powder from Escherichia coli, type I, was obtained from Sigma Chemical Co..
Calcium phosphate gel was prepared according to the method of Keilin and Hartree (154). The gel was washed at least three times with ten volumes of distilled water before use. Arginine decarboxylase was a generous gift from Dr. E. A. Boeker. The other reagents were analytical grade.

Glutamate Decarboxylase

One gram of acetone powder from E. coli was suspended in 10 ml of 0.1 M potassium phosphate, 1 mM dithiothreitol, and 1 mM pyridoxal phosphate (pH 4.2), homogenized in a Potter-Elvehjem homogenizer with a teflon pestle, and then centrifuged at 10,000g for 10 minutes at 0°C. The supernatant was collected and used directly for most assays. For kinetic study and/or for precise measurements, the above supernatant was subjected to further purification. Two grams of ammonium sulfate were added to the supernatant; the precipitate obtained by
centrifugation at 10,000g for 10 minutes at 0°C was discarded. One gram of ammonium sulfate was added to the resulting supernatant and another centrifugation was performed, at 30,000g for 30 minutes at 0°C. The precipitate was dissolved in 5 ml of 0.1 M potassium phosphate, 1 mM dithiothreitol, and 1 mM pyridoxal phosphate (pH 4.2). The final preparation had a glutamate decarboxylase activity of 50 μmole/min/mg and showed no detectable glutaminase activity for up to two hours under standard assay conditions.

For glutamate decarboxylase assay, 200 μl of substrate solution containing 20 mM L-(U-14C)-glutamate (2.7 x 10^5 CPM) in 0.2 M pyridine-HCl (pH 4.5) was added with 100 μl of enzyme solution. After 10 minutes incubation at 38°C, the reaction was stopped by adding 25 μl of 100% trichloroacetate. The mixture was incubated for an additional 20 minutes, in order to transfer all the 14CO₂ evolved to the trapping solution. The technique of 14CO₂ trapping and counting is identical with that of glutaminase assay, which will be described in detail in the next section. Unless otherwise indicated, the enzyme had a specific activity of 4 μmole/min/mg; 37 μg of enzyme was used per assay, and the effective specific activity of the carboxyl group of the substrate was 5830 CPM/μmole, taking into account the assay efficiency.

Glutaminase Assays

200 μl of a solution containing 0.2 M L-(U-14C)-glutamine (170,000 CPM) in 0.2 M potassium phosphate buffer (pH 8.8) was added to a stoppered 13 x 85 mm test tube and equilibrated at 38°C for five minutes. The reaction was begun by adding glutaminase in 100 μl of 0.2 M potassium phosphate (pH 8.8). After 10 minutes, the reaction
was stopped by adding enough 0.4 M pyridine-HCl (pH 1.2) to change the pH of the final mixture to pH 4.5 ± 0.2. A 10 x 35 mm piece of pleated filter paper was soaked with 25 μl of a solution of 2-aminoethanol : 2-methoxylethanol = 1 : 2 and inserted in the upper part of the tube. 100 μl of a glutamate decarboxylase preparation containing an activity of at least 20 μmole/min/ml was added to the mixture to release all the α-carboxyl groups of glutamate as carbon dioxide. The test tube was immediately recapped with a rubber stopper. After 30 minutes, the filter paper was transferred to a vial containing 10 ml of scintillant (6 grams 2,5-diphenyloxazole, 514 ml Triton X-100, 212 ml absolute ethanol, 74 ml 2-methoxyethanol, 1200 ml xylene, and 200 ml distilled water) and counted in a Beckman CPM 100 scintillation counter. The assay was standardized by adding sufficient enzyme, first glutaminase and then glutamate decarboxylase, to exhaust the substrate. The effective specific activity for glutamine was calculated from the exhaustion value and the amount of glutamine used; it normally was about 70%. When less than one-third of the substrate was reacted, the assay was linear with time up to 5 hours and with protein concentration up to 50 mg/ml. For kinetic studies, less than 5% of the substrate was reacted in most cases; the detected velocities should, therefore, approach initial velocities.

Most samples were either assayed directly or diluted with the sample buffer if needed; unless otherwise specified, no effort has been made to standardize the phosphate concentration and the pH of samples before they were assayed. Control assays were done either by replacing the enzyme solution with buffer or by stopping the reaction with pyridine-HCl solution before the enzyme was added; either
way, the results were the same. The experimental data were the
detected values minus the control.

Protein Determinations

Protein concentrations were determined according to the Coomassie
blue dye-binding method of Bradford (155) with some modifications.
A solution of 200 mg Coomassie brilliant blue G, 100 ml of 95% ethanol,
and 200 ml of 85% (w/v) phosphoric acid was stored in a refrigerator
at 5°C as a stock solution. For an assay, one volume of stock solu­
tion was diluted with seven volumes of distilled water, then 2.5 ml
were pipetted into a 12 x 100 mm test tube. Two minutes after the
addition of 100 ul sample solution, the absorbance at 595 nm was
measured with a Bausch & Lomb spectrophotometer. Bovine serum albumin
was used to make a standard curve; the standard curve was linear from
2 ug to 20 ug.

Paper Chromatography

Samples were applied to a 2 x 45 cm strip of Whatman #1 paper,
and chromatographed downward for four hours in water saturated phenol
containing 0.04% 8-hydroxyquinoline (156). After air drying, the
strips were either stained with ninhydrin in 95% ethanol or were cut
into 1/4 inch sections and transferred to 10 ml scintillant and counted.
In one experiment, for the study of reversibility of the glutaminase
reaction, 0.3 ml of a solution containing 27 mM L-(U-14C)-glutamic
acid and 67 mM ammonium ion in 0.2 M phosphate buffer (pH 8.8) was
incubated at 38°C either in the absence or presence of glutaminase
at a concentration of 0.56 umole/min/ml. After 10 hours, 0.4 ml of
0.4 M pyridine-HCl (pH 1.2) and 0.1 ml of 0.2 M glutamine were added, and 50 μl aliquots containing 18,000 CPM were chromatographed and counted as described above. In the other experiment, 0.4 ml of a solution containing 20 mM L-(U-14C)-glutamic acid, 50 mM ammonium ion and 50 mM glutamine in 0.2 M phosphate buffer (pH 8.8) was incubated overnight at 38 C either in the absence or presence of glutaminase at a concentration of 0.42 μmole/min/ml; 50 μl aliquots containing 33,000 CPM were chromatographed and counted as before.

Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed according to Davis et al. (157). Samples containing 20% sucrose were run on 7.5% polyacrylamide gels at 3 mA/gel. For subunit molecular weight estimation, glutaminase was preheated at 70 C for 20 minutes in the presence of 1% sodium dodecyl sulfate, 1% mercaptoethanol, 10% glycerol, and 10 mM sodium phosphate (pH 7.2), and then run on 7.5% polyacrylamide gel in 0.1% sodium dodecyl sulfate at 8 mA/gel. In all cases, 0.002% bromophenol blue was used as a marker; gels were stained in a solution of 0.25% Coomassie blue in methanol : acetic acid : water = 5 : 1 : 5 for one hour and destained in a solution of 7.5% acetic acid and 5% methanol.

Glutaminase Purification Procedure

Acetone extractions

Fresh cow brains were chilled in ice. The cerebellum, outer membrane, and as much white matter as possible were removed, and the brains were stored at -20 C overnight or longer. The frozen brains
were cut into small pieces and homogenized five minutes in five volumes of -80 C acetone in a Sorvall Omni-mixer at speed five. This and all succeeding steps were carried out in a well-ventilated cold room at 4 C. The homogenized suspension was filtered on a Buchner funnel using Whatman #1 filter paper. The filter cake was rehomogenized in acetone and then filtered again. A wet weight of 100 grams brain produced about 12 grams of light pink acetone powder. The acetone powder has been stored at -20 C for more than a year without loss of activity.

Ammonium sulfate fractionation

Two hundreds grams of acetone powder was suspended in 4 liters of 20 mM sodium borate and 2 mM EDTA (pH 8.6), homogenized at 240 rpm in a Potter-Elvehjem homogenizer with a teflon pestle, and centrifuged at 16,000g for 20 minutes at 0 C. Unless otherwise indicated, all centrifugation conditions were the same as these. Solid MgCl$_2$·6H$_2$O was added with stirring to make the solution 20 mM in Mg$^{++}$; then 0.5 gram protamine sulfate was added. One hour later, 265 grams solid ammonium sulfate was added slowly with stirring. The suspension was centrifuged.

Another 400 grams solid ammonium sulfate was added to the resulting supernatant and a centrifugation performed. The precipitate was suspended in 94 ml of 20 mM sodium borate and 2 mM EDTA (pH 8.6); the resulting suspension had a specific acivity of about 1.5 n mole/min/mg.

Calcium phosphate gel treatment

One hundred grams of calcium phosphate gel (wet weight) were added to the above suspension which was then centrifuged at 5,000g for 10
minutes at 0 C. The supernatant was discarded and the gel was washed with 100 ml of 20 mM sodium borate and 2 mM EDTA (pH 8.6); the supernatant was again discarded. The glutaminase was washed off the gel using 260 ml of 100 mM potassium phosphate, 100 mM sodium pyrophosphate, and 10 mM sodium borate (pH 8.8). The supernatant was then concentrated to 25 ml in an Amicon ultrafiltration cell using a Diaflo XM300 membrane.

**Sepharose 4B columns**

The concentrated solution was dialyzed in four steps against four liters of 100 mM potassium phosphate, 100 mM sodium pyrophosphate, and 10 mM sodium borate (pH 8.8), four hours for each step. After insoluble material was removed by centrifugation, the sample was loaded on the top of a 4.2 x 60 cm Sepharose 4B column equilibrated with 100 mM potassium phosphate, 100 mM sodium pyrophosphate, and 10 mM sodium borate (pH 8.8). The column was eluted with the same buffer. Glutaminase activity appeared slightly after the void volume. Fractions with specific activity higher than 4 μmole/min/mg were pooled and concentrated to 13 ml in an Amicon ultrafiltration cell as before. After dialysis in four steps against four liters of 10 mM Tris-HCl, 100 mM sodium pyrophosphate, and 0.2 mM dithiothreitol (pH 8.8), the insoluble material was removed by centrifugation. The sample was loaded on the top of a 2.6 x 100 cm Sepharose 4B column equilibrated with 10 mM Tris-HCl, 100 mM sodium pyrophosphate, and 0.2 mM dithiothreitol (pH 8.8). The column was eluted with the same buffer. Active fractions eluted in about two and one half times the void volume. Appropriate fractions were pooled.
For the pH-activity profile, glutaminase was prepared by dialyzing against 0.1 M potassium phosphate (pH 8.8), or 10 mM Tris-HCl and 0.2 mM EDTA (pH 8.8), or 10 mM sodium borate, 0.1 M potassium phosphate, and 0.1 M sodium pyrophosphate (pH 8.8) before assay. The substrate solutions were made from different combinations of two solutions, one containing 0.2 M $K_2PO_4$ and 0.2 M glutamine and the other containing 0.2 M $KH_2PO_4$ and 0.2 M glutamine. All assays were done according to the standard method.

The stability experiment was completed with the standard assay except that glutaminase was incubated at the indicated pH for 5 hours at 38°C before the assay. To do this, glutaminase in 100 µl of 0.2 M potassium phosphate buffers (pH 8.8) was added with different volumes of either 0.2 M $K_2PO_4$ or 0.2 M $KH_2PO_4$ to shift the pH. At the end of the incubation, the pH was brought back to 8.8 with appropriate volumes of the alternate potassium phosphate solution, all to a final volume of 491 µl. 100 µl of each final enzyme solution was then used for the assay.
RESULTS AND DISCUSSION

Glutaminase Assay

Figure 3 shows that the assay for glutamate decarboxylase activity was linear with time and protein concentration when less than one half of the substrate was reacted. Figure 4 shows that neither glutamine, aspartate, nor γ-aminobutyrate interfere with the decarboxylation assay up to concentrations of 20 mM, 4 mM, and 20 mM, respectively. α-Keto-glutarate inhibits glutamate decarboxylase competitively; this inhibition can be released by increasing the glutamate (data not shown).

Glutamate decarboxylase was used to release the α-carboxyl group of glutamate, the product of glutaminase action on glutamine. The specificity of this enzyme has been studied extensively (158-165, 118). However, there is no glutamate decarboxylase free from glutaminase activity commercially available so far. A simple partial purification of glutamate decarboxylase was developed for this dissertation, starting with commercially available acetone powder of E. coli. This contains both glutaminase A and glutaminase B (73-75). Since glutaminase A is active at pH 4.5, it will interfere with the glutaminase activity measurement. Therefore, the effect of glutaminase A on the radiometric assay was carefully investigated. The results in Table 1 show that (a) glutamate decarboxylase does not use glutamine as a substrate, (b) glutamate decarboxylase is contaminated with a glutaminase which is active at pH 4.5, and (c) partial purification removed some of the contamination.
Figure 3. Glutamate decarboxylase activity at varying time or protein concentration. The assay method was described under "EXPERIMENTAL PROCEDURES," excepting that the assay time or the protein concentration was that as indicated.
Figure 4. The effects of free amino acids on glutamate decarboxylase activity. Standard method for glutamate decarboxylase assay described under "EXPERIMENTAL PROCEDURES" was used, excepting that the substrate solution contains free amino acid as indicated, glutamine (●); γ-aminobutyrate (★); asparatate (★); α-ketoglutarate (★). Each point represents the average of three tests. 21 μg of glutamate decarboxylase was used for each assay.
Table 1. Effects of glutamate decarboxylase on glutaminase assay

<table>
<thead>
<tr>
<th>Test Condition&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Glutamate Decarboxylase &lt;sup&gt;b&lt;/sup&gt; Used in Assay</th>
<th>Incubation Time&lt;sup&gt;c&lt;/sup&gt;, Minute</th>
<th>Radioactivity Detected, CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. No glutaminase added</td>
<td>a. buffer only</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>b. unpurified</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>c. partially purified</td>
<td>300</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>b. unpurified</td>
<td>30</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>c. partially purified</td>
<td>300</td>
<td>650</td>
</tr>
<tr>
<td></td>
<td>b. unpurified</td>
<td>30</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>c. partially purified</td>
<td>300</td>
<td>150</td>
</tr>
<tr>
<td>2. Glutamine exhausted with glutaminase</td>
<td>a. buffer only</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>b. unpurified</td>
<td>300</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>b. unpurified</td>
<td>30</td>
<td>17,500</td>
</tr>
<tr>
<td></td>
<td>c. partially purified</td>
<td>300</td>
<td>18,500</td>
</tr>
<tr>
<td></td>
<td>b. unpurified</td>
<td>30</td>
<td>17,000</td>
</tr>
<tr>
<td></td>
<td>c. partially purified</td>
<td>300</td>
<td>18,450</td>
</tr>
<tr>
<td>3. Glutamate substituted for glutamine</td>
<td>a. buffer only</td>
<td>30</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>b. unpurified</td>
<td>300</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>b. unpurified</td>
<td>30</td>
<td>18,400</td>
</tr>
<tr>
<td></td>
<td>c. partially purified</td>
<td>300</td>
<td>18,450</td>
</tr>
<tr>
<td></td>
<td>b. unpurified</td>
<td>30</td>
<td>18,550</td>
</tr>
<tr>
<td>4. Glutamate substituted for 1/8 of the glutamine</td>
<td>a. buffer only</td>
<td>30</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>b. unpurified</td>
<td>300</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>b. unpurified</td>
<td>30</td>
<td>2,400</td>
</tr>
<tr>
<td></td>
<td>c. partially purified</td>
<td>300</td>
<td>3,000</td>
</tr>
<tr>
<td></td>
<td>b. unpurified</td>
<td>30</td>
<td>2,350</td>
</tr>
<tr>
<td></td>
<td>c. partially purified</td>
<td>300</td>
<td>2,500</td>
</tr>
</tbody>
</table>

<sup>a</sup>The assay was described in "EXPERIMENTAL PROCEDURES." Substrate exhaustion gave 18,500 CPM.

<sup>b</sup>Partial purification of glutamate decarboxylase was described in "EXPERIMENTAL PROCEDURES." Both unpurified and partially purified glutamate decarboxylase had an activity of 15 umole/min/ml.

<sup>c</sup>Incubation time was the time between the addition of glutamate decarboxylase and the removal of filter paper.
Since less than 5% of the glutamine present was allowed to react in most kinetic assays and since it was impossible to obtain glutamate decayboxylase absolutely free from glutaminase activity, each batch of partially purified glutamate decarboxylase was checked. Test condition 1c for 30 minutes and test condition 4c for 300 minutes (Table 1) were used to see whether a glutamate decarboxylase preparation was suitable for kinetic studies and to determine how much enzyme should be used at a certain incubation time while keeping the control relatively low.

Figure 5 shows that at least the first one-third of the substrate is consumed linearly with time in the glutaminase assay. The assay is sensitive enough to detect 50 umoles of substrate with confidence. The precision is restricted by pipetting error rather than the assay itself. Since the entire assay is run in a single test tube, no solution transfer is necessary. Pipetting error is restricted to two possibilities: pipetting of the substrate and the sample solutions. One assay requires 10 minutes for the reaction and 30 minutes for CO₂ release and trapping; one extra minute is needed per additional assay, up to nine assays.

Since glutamate decarboxylase is added after the glutaminase reaction has been stopped, the coupling system does not affect the assay. There is, therefore, no reason for assays to be done at sub-optimal pH. The glutaminase reaction can be stopped and the conditions for glutamate decarboxylase activity can be achieved in a single step by changing the pH from 8.8 to 4.5 with pyridine-HCl, since the phosphate-dependent glutaminase has a pH optimum around 8.8 and is irreversibly damaged below pH 6.5, while glutamate decarboxylase has a pH optimum at 4.5 and is completely inactive above pH 6.
Figure 5. Activity profile of glutaminase assay. The enzyme has a specific activity of 2.5 µmole/min/mg. 100 µl of 0.2 M potassium phosphate buffer (pH 8.8) containing 0.22 mg protein was assayed according to the standard method described under "EXPERIMENTAL PROCEDURES." The specificity of the substrate is 610 CPM/µmole glutamine. Each point represents the mean of two determinations.
Controls show that ammonia, up to 100 mM, and citrate, succinate, and glutarate up to 57 mM do not interfere with the assay. Nonenzymatic hydrolysis of glutamine is frequently a problem in glutaminase assays. However, it produces pyrrolidinocarboxylic acid (166, 137) which is not a substrate for glutamate decarboxylase. Hence, substrate pretreatment is not necessary. It is still advisable to prepare fresh substrate solution just before use.

Control experiments also show that the trapping solution is able to form carbamate with at least 40 μmoles of carbon dioxide (167). Also, there is no indication that diffused 2-aminoethanol (167) inhibits decarboxylation.

**Glutaminase Purification (Table 2)**

Acetone extraction removed most of the acetone soluble materials from the brain extract, disrupted the mitochondrial membranes, and rendered the glutaminase soluble. The possibility of protease action on glutaminase apparently was also eliminated by this treatment, since the enzyme was stable throughout the entire subsequent purification and showed a single band after sodium dodecyl sulfate gel electrophoresis.

A solution of 20 mM sodium borate and 2 mM EDTA (pH 8.6) was used to suspend the acetone powder. This buffer was used to aggregate and stabilize glutaminase. It is necessary to use borate concentrations of 20 mM or more; if the borate concentration used was 10 mM, for instance, the pH of the enzyme solution after the ammonium sulfate fractionation would be around 6.5. Glutaminase is not stable at this pH. If a buffer of 0.2 M potassium phosphate (pH 8.8) replaces 20 mM sodium
Table 2. Purification of cow brain glutaminase

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Protein (mg)</th>
<th>Activity (μmole/min)</th>
<th>Specific Activity (μmole/min/mg)</th>
<th>Activity Recovery (%)</th>
<th>280/260&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>11,100</td>
<td>-</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>Acetone Powder</td>
<td>58,200</td>
<td>8,880</td>
<td>0.15</td>
<td>80</td>
<td>0.79</td>
</tr>
<tr>
<td>Ammonium Sulfate</td>
<td>3,528</td>
<td>5,770</td>
<td>1.46</td>
<td>52</td>
<td>0.5</td>
</tr>
<tr>
<td>Calcium Phosphate</td>
<td>2,346</td>
<td>5,866</td>
<td>2.5</td>
<td>53</td>
<td>0.78</td>
</tr>
<tr>
<td>Sepharose 4B Column I</td>
<td>174</td>
<td>2,628</td>
<td>15.1</td>
<td>29.6</td>
<td>0.86</td>
</tr>
<tr>
<td>Sepharose 4B Column II</td>
<td>3.4</td>
<td>1,310</td>
<td>385</td>
<td>12</td>
<td>1.48&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>UV absorbance
<sup>b</sup>The purification started with 1,670 g of cow brain after the removal of membrane.
<sup>c</sup>Enzyme was dialyzed against 10 mM Tris-HCl, 100 mM potassium chloride, and 1 mM dithiothreitol (pH 8.8) before measurement.
borate and 2 mM EDTA (pH 8.6) in suspending the acetone powder, the
recovery after ammonium sulfate fractionation is 20% higher, but the
viscosity of the sample is so high that further purification is practi-
cally impossible.

After the initial extraction, magnesium chloride and protamine
sulfate were used to activate nucleotidases and to precipitate nucleic
acids, respectively. Since the direct addition of nucleotidases to
the sample did not decrease the 280/260 ratio and since addition of
0.1 g streptomycin per 100 ml sample eliminated the glutaminase acti-
vity completely, neither of these alternative procedure was adapted.

Ammonium sulfate fractionation between 7% and 15% was very effec-
tive in removing impurities, as reflected by the increase of specific
activity and the sharp decrease of total protein. Since the radio-
metric assay was used, neither ammonium ions nor sulfate ions inter-
fered with the activity measurements. The 280/260 ratio decreased to
0.5 at this stage, indicating that protein impurities had been removed
more effectively than nucleic acids.

Calcium phosphate gel treatment removed some nucleic acids, as
judged by the increased 280/260 ratio and the decreased viscosity.
Glutaminase was bound to the calcium phosphate gel in the absence of
phosphate, and released by 0.2 M phosphate. Without this treatment
the sample sticks to the top of chromatographic columns, making sepa-
rations impossible.

Ultrafiltration served three purposes: (a) it concentrated the
sample solution without changing the sample buffer, (b) it removed some
impurities with molecular weights lower than 300,000, and (c) it
decreased the viscosity of the sample since some viscous materials stuck to the membrane.

Glutaminase aggregates in 0.1 M potassium phosphate, 0.1 M sodium pyrophosphate, and 10 mM sodium borate (pH 8.8) and dissociates in 10 mM Tris-HCl, 0.1 M sodium pyrophosphate, and 0.2 mM dithiothreitol (pH 8.8). Most of the impurities do not show this behavior. Taking advantage of this, Sepharose 4B columns were used to differentiate glutaminase from impurities by eluting first with associating buffer and then with dissociating buffer. In the first column, glutaminase was highly aggregated and appeared slightly after the void volume (Figure 6-a). In the second column, the glutaminase activity coincided with a minor protein peak that appeared well after the void volume (Figure 6-b). This method is reproducible if the sample size is approximately the same.

The overall yield of this purification was 12%, four times higher than that obtained for the pig brain enzyme (22). Based on weight, only one-tenth as much cow brain as pig brain is required to produce comparable activity. The specific activity of the acetone powder is ten times that of pig brain preparation. The final preparation had a specific activity of 385 μmole/min/mg and showed a single protein band in polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Figure 7). In discontinuous polyacrylamide gel electrophoresis, a broad protein band one-fifth of the way down the gel and a band on the top of the gel were observed (Figure 8). The activity profile for this gel was coincided with the protein staining (Figure 9). This experiment indicates that some of the sample was highly aggregated and was just barely able to get into the gel while the rest migrated for some
Figure 6. Elution profiles of Sepharose 4B columns equilibrated with 0.1 M potassium phosphate, 0.1 M sodium pyrophosphate, and 10 mM sodium borate (pH 8.8) (a) and 10 mM Tris-HCl, 0.1 M sodium pyrophosphate, and 0.2 mM dithiothreitol (pH 8.8) (b). The arrows indicate the elution positions of void volumes. Experimental details were described under "EXPERIMENTAL PROCEDURES."
Figure 7. Polyacrylamide gel electrophoresis of cow brain glutaminase in sodium dodecyl sulfate. 10 μg of the final preparation was used per gel. The method is described under "EXPERIMENTAL PROCEDURES."
Figure 8. Discontinuous polyacrylamide gel electrophoresis of cow brain glutaminase. 20 μg of the final preparation in 0.1 M potassium phosphate, 0.1 M sodium pyrophosphate, and 10 mM sodium borate (pH 8.8) was used. The method is described under "EXPERIMENTAL PROCEDURES."
Figure 9. Glutaminase activity recovery from discontinuous polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis of highly purified glutaminase in 0.1 M potassium phosphate, 0.1 M sodium pyrophosphate, and 10 mM sodium borate (pH 8.8). 10 μg enzyme was electrophoresed at room temperature. For measurement of activity, a parallel gel without protein staining was sliced into 25 slices, 2 mm each, after electrophoresis. Each slice was soaked in 200 μl of 0.1 M potassium phosphate, 0.1 M sodium pyrophosphate, and 10 mM sodium borate (pH 8.8) overnight at room temperature and then assayed. Electrophoresis was performed according to "EXPERIMENTAL PROCEDURES."
distance into the gel. This total recovery from the gel was about 12%. The reason for the relatively low activity in the first slice is unknown. The extraction may be incomplete, or some of the activity may remain in the stacking gel, or both. This is the first report that highly purified glutaminase would migrate into polyacrylamide gel and remain active in the absence of Triton X-100.

A second parallel gel was sliced and soaked in 0.1 M potassium phosphate, 0.1 M sodium pyrophosphate, and 10 mM sodium borate (pH 8.8) as described in Figure 9, and each slice was then subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate. When this was done, a single band similar to the one shown in Figure 7 was observed, coincident with the enzyme activity profile and the protein staining shown in Figure 9.

The 280/260 of the final preparation in 10 mM Tris-HCl, 0.1 M sodium pyrophosphate, and 0.2 mM dithiothreitol (pH 8.8) is 1.48. The ratio was 1.1 when the same preparation was dialyzed against 0.1 M potassium phosphate, 0.1 M sodium pyrophosphate, and 10 mM sodium borate (pH 8.8). These values are consistent with results reported for pig kidney enzyme (132), where 280/260 is about 1.5 and 1.2 in the two buffers.

Subunit Molecular Weight of Glutaminase

Cow brain glutaminase shows a single protein band after polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Figure 7). A single protein band suggests that this enzyme has not been subjected to protease action during purification. The subunit molecular weight estimated from five standards is 82,000 ± 2,000 (Figure 10). This value
Figure 10. Estimation of cow brain glutaminase subunit molecular weight by electrophoresis on polyacrylamide gel in sodium dodecyl sulfate. 10 μg of the final preparation was applied to the gel. The method is described under "EXPERIMENTAL PROCEDURES." The arrow indicates the position of glutaminase. Abbreviations: ADC, arginine decarboxylase; BSA, bovine serum albumin; CAT, catalase; GDH, glutamate dehydrogenase; OA, ovalbumin.
is higher than that found for the pig brain and kidney enzymes (22, 26) but agrees well with that of the rat kidney enzyme (71, 143). If the molecular weight of native cow brain glutaminase is close to that of other glutaminases, the subunit molecular weight would suggest that the native enzyme is a dimer.

**Effect of pH On Activity and Stability**

The activity and stability of glutaminase as a function of pH are shown in Figure 11. The enzyme has highest activity at pH 8.8 regardless of the buffer that it was equilibrated with. In both phosphate and Tris-HCl buffers, the enzyme gave a similar activity curve whereas in phosphate-borate buffer a broader curve was observed. This result is consistent with all reports on phosphate-dependent glutaminase (24, 131, 37, 25, 39, 48, 60, 124, 147, 59, 132, 34, 119, 75).

When incubated in 0.2 M phosphate, glutaminase is stable over a fairly wide pH range; it retains essentially full activity from pH 7.5 to 9.2. This pH stability plateau is a great advantage in the enzyme purification procedure; in this pH range no irreversible enzyme denaturation will occur. This stability curve is the first reported for glutaminase.

**Activators and Inhibitors of Glutaminase**

Figure 12 shows that glutaminase absolutely requires phosphate for activity; the equilibration buffer does not affect the results. The dependence on phosphate is sigmoidal and has a Hill coefficient of 2.2. The phosphate concentration that gives half maximal velocity is 50 mM, consistent with that for the pig brain (68), pig kidney (87, 34),
Figure 11. Activity (——) and stability (---) of glutaminase as a function of pH. Experimental details are given in "EXPERIMENTAL PROCEDURES." The glutaminase used for both experiments had a specific activity of 21 μmole/min/mg. In both cases, parallel experiments without enzyme were conducted for pH measurements. The enzyme was equilibrated in 0.1 M potassium phosphate (○), or 10 mM Tris-HCl and 0.2 mM EDTA (○), or 10 mM sodium borate, 0.1 M potassium phosphate, and 0.1 M sodium pyrophosphate (□ or ●), all at pH 8.8.
Figure 12. Effect of phosphate on glutaminase. Enzyme with a specific activity of 24 μmole/min/mg was dialyzed against 10 mM Tris-HCl and 10 mM EDTA (pH 8.5) (○) or 0.1 M potassium phosphate (pH 8.5) (●); the final protein concentrations were 0.2 and 0.14 mg/ml, respectively. 100 μl of each solution was added to 200 μl of 0.2 M glutamine containing potassium phosphate as appropriate (pH 8.5); assays were run at 38 °C for 15 minutes.
and rat kidney (144) enzymes. In an additional experiment (not shown), glutaminase was equilibrated in 0.1 M potassium phosphate, 0.1 M sodium pyrophosphate, and 10 mM sodium borate (pH 8.5) and assayed in the presence of additional phosphate. If the concentrations of phosphate and pyrophosphate in the assay are summed, the results match those shown very closely. Since a phosphate dependent molecular weight change has been reported either to be correlated with phosphate activation (144) or to occur only at very high protein concentration (87, 144, 71, 150), this complexity has been reduced as much as possible by minimizing the change of enzyme concentration in this experiment.

Figure 13 shows that at high phosphate and low enzyme concentration, $K_m$ (the slope) and $V_{max}$ are independent of the equilibration buffer. This is consistent with the results in Figure 12 and with reports on pig and rat kidney glutaminases (34, 89, 25). It is not, however, consistent with results reported for the pig brain enzyme, which suggest that it is an hysteretic enzyme that responds slowly to changes in ligand concentration (150).

Sucinate, citrate and glutarate do not affect cow brain glutaminase activity at either high (200 mM) or low (57 mM) phosphate. In the experiment shown in Table 3, substrate solutions were 0.2 M glutamine (pH 8.8), with or without 0.2 M potassium phosphate; carboxylic acid solutions were 50 mM (pH 8.8), with or without 0.2 M potassium phosphate. 100 μl of a carboxylic acid solution was added to 200 μl of a substrate solution before the addition of 100 μl of glutaminase in 0.2 M potassium phosphate (pH 8.8). Assays were performed at 38 C for 10 minutes. These results are consistent with those for the rat kidney,
Figure 13. Effect of pretreatment on glutaminase. Highly purified glutaminase was dialyzed against 10 mM Tris-HCl and 0.4 mM EDTA (○), 0.1 M potassium phosphate (□), and 0.1 M potassium phosphate, 0.1 M sodium pyrophosphate, and 10 mM sodium borate (●), all at pH 8.5, to final protein concentrations of 0.48, 0.25, and 0.29 mg/ml, respectively. 100 µl of each solution was then added to 200 µl of 0.2 M potassium phosphate (pH 8.5) containing glutamine as appropriate; assays were carried out at 38 C for 15 minutes.
Table 3. Effect of carboxylic acids on glutaminase activity

<table>
<thead>
<tr>
<th>Additions</th>
<th>Activity, umoles/min/ml, at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>200 mM Pi</td>
</tr>
<tr>
<td>None</td>
<td>10.4</td>
</tr>
<tr>
<td>12.5 mM succinate</td>
<td>10.0</td>
</tr>
<tr>
<td>12.5 mM glutarate</td>
<td>10.5</td>
</tr>
<tr>
<td>12.5 mM citrate</td>
<td>10.2</td>
</tr>
</tbody>
</table>

*aThe enzyme had a specific activity of 21 umole/min/mg; 50 µg was used per assay.*
rat brain (149) and pig brain (68) enzymes, which showed no significant activation due to carboxylic acid in the presence of 7.5 mM or higher phosphate.

Figure 14 shows that ammonium ion concentrations up to 0.1 M do not affect the activity of the enzyme at 0.2 M phosphate. In this experiment, the enzyme was dissolved in 0.2 M potassium phosphate buffer (pH 8.8) and had a specific activity of 1.2 µmole/min/mg. 100 µl of this solution containing 116 µg protein was added to 300 µl of a solution containing appropriate concentrations of glutamine, ammonium ion, and 0.2 M phosphate (pH 8.8); all assays were done at 38°C for 15 minutes.

Figure 14 also shows that decreasing phosphate concentrations decrease the apparent $V_{\text{max}}$ and increase $K_m$ ($K_{\text{glu}}$). It is not surprising that phosphate affects both $K_{\text{glu}}$ and $V_{\text{max}}$. When reviewed separately, phosphate-caused decreases in $K_{\text{glu}}$ have been reported for rat brain (149), dog kidney (119), pig kidney (132), and rat kidney (149) enzymes. All previous studies show that increasing phosphate increases the enzyme activity. The overall phosphate effect in Figure 14 is consistent with the study of pig kidney enzyme (67). This result indicates that the activation role of phosphate cannot be filled by glutamine and that the binding sites for phosphate and glutamine are exclusive.

Figures 15 and 16 show that glutamate is a competitive inhibitor for cow brain glutaminase at both high (200 mM) and low (50 mM) phosphate. At 200 mM phosphate, the value for $K_{\text{glu}}$ obtained from the replot is 19 mM (Figure 15). At 50 mM phosphate, $K_{\text{glu}}$ is 25 mM (Figure 16). When identical axes are used, the replots in Figures 15 and 16 are parallel, i.e., phosphate affects both $K_{\text{glu}}$ and $K_{\text{glu}}$ the same way. The
Figure 14. Glutaminase activity at fixed concentrations of phosphate and ammonium ion. Round symbols: activity at 0.2 M phosphate (pH 8.8) with appropriate replacement of potassium by ammonium ion. The final concentrations were 9 to 150 mM glutamine in 50(○), 100(△), or 200(●) mM ammonium ion and 0.2 M phosphate (pH 8.8). Open symbols: activity in the absence of ammonium ion. The final concentrations were 9 to 150 mM glutamine in 50(□), 100(▲), or 200(○) mM phosphate (pH 8.8).
Figure 15. Glutaminase activity at 0.2 M KPi and fixed concentrations of glutamate. The glutaminase in 0.2 M KPi (pH 8.8) had a specific activity of 1.2 μmole/min/mg. 116 μg protein in 100 μl solution was added to 300 μl of a solution containing appropriate concentrations of glutamine, glutamate, and 0.2 M KPi (pH 8.8); all assays were stopped after 15 minutes at 38 C. The final concentrations were 9 to 150 mM glutamine in 0(O), 6.3(©), 12.5(©) or 25 ©) mM glutamate, and 0.2 M KPi (pH 8.8).
Figure 16. Glutaminase activity at 50 mM KPi and fixed concentrations of glutamate. The enzyme in 50 mM KPi (pH 8.8) had a specific activity of 1.2 umole/min/mg. 116 µg protein in 100 µl solution was added to 300 µl of a solution containing appropriate concentrations of glutamine, glutamate, and 50 mM KPi (pH 8.8); all assays were stopped after 15 minutes incubation at 38 C. The final concentrations were 9 to 150 mM glutamine in 0(□), 6.3(■), or 25(▲) mM glutamate and 50 mM KPi (pH 8.8).
competitive inhibition by glutamate is in agreement with the study of the pig kidney enzyme equilibrated with a Tris buffer (132, 67). Although glutamate was shown to be a noncompetitive inhibitor for pig kidney glutaminase when the enzyme was equilibrated in either phosphate or phosphate-borate buffer, it has been observed that increasing the phosphate concentration can shift the noncompetitive inhibition to a competitive one (67). The effect of phosphate on glutaminase can not be interpreted in any simple way without additional experiments.

In an additional experiment shown in Figure 17, $K_{gln}$ was measured on the most highly purified cow brain glutaminase that was obtained. The slope in Figure 17 gives a value of 17 mM for $K_{gln}$. This value is about twice that of the pig brain enzyme (150, 68, 119). Although the enzyme source, enzyme purity, assay method, and assay conditions are not comparable among workers, it nevertheless appears that assays which use potassium phosphate consistently show a $K_{gln}$ higher than those which use sodium phosphate (60, 59, 150, 68, 25, 89, 119).

**Mechanism of Action**

The results in Figure 14 show that ammonium ion up to 0.1 M has no affect to the enzyme activity, suggesting that the release of ammonia may be irreversible. This is supported by the experiments shown in Figure 18.

In the top experiment, ammonium ion was incubated with $^{14}$C-glutamate in the presence of phosphate and glutaminase; cold glutamine was added as a carrier before chromatography. The radioactivity corresponding to glutamine is 530 CPM for both the experiment and the control, indicating that no $^{14}$C-glutamine was formed from $^{14}$C-glutamate. The
Figure 17. Km of cow brain glutaminase. The enzyme has a specific activity of 360 μmole/min/mg. 0.98 μg of enzyme in 100 µl of 0.2 M potassium phosphate buffer (pH 8.8) was assayed with standard method stated in "EXPERIMENTAL PROCEDURES," excepting that the substrate concentration were those as indicated. One μmole glutamine corresponds to 415 CPM. Two sets of data are shown.
Figure 18. Reversibility of glutaminase reaction. Top: \(^{14}\text{C-}
\text{glutamate and ammonium ion in 0.2 M phosphate buffer (pH 8.8) were in-
cubated in the absence (○) or presence (x) of glutaminase and the
mixture was chromatographed and counted as described under "EXPERI-
MENTAL PROCEDURES." The strip shown corresponds to the experiment with
enzyme; the control without enzyme gives an identical pattern. Bottom:
\(^{14}\text{C-glu}
\text{tamate, ammonium ion, and glutamine in 0.2 M phosphate buffer
(pH 8.8) were incubated in the absence (○) or presence (x) of gluta-
minase and the mixture was chromatographed and counted as described
under "EXPERIMENTAL PROCEDURES." The paper strip corresponds to the
experiment without enzyme; the experiment with enzyme present shows a
very faint spot in the position of glutamine. Both experiments had a
radioactivity recovery of about 83\%.
lower experiment differs from the upper one in that cold glutamine was present in incubation. The radioactivity corresponding to glutamine was 1240 CPM for the control and 1280 CPM for the experiment. The ninhydrin stained strip of the experiment (not shown) has a heavier spot corresponding to glutamate, and only a very faint spot corresponding to glutamine. Although the $^{14}$C-glutamate appears to contain a substance that chromatographs in the same position as glutamine, it is clear that glutamine is not being formed from glutamate under these conditions. If 1% of the glutamate present had been converted to glutamine, the increase in radioactivity would have corresponded to 180 and 330 CPM in the upper and lower chromatograms, respectively. This level could have been detected.

This observation that the glutaminase reaction is not significantly reversible is consistent with an irreversible release of $\text{NH}_3$, as suggested by the inhibition data. Since all reports are consistent with a result that glutamate is an inhibitor, it is reasonable to suggest that its release is reversible. There are then two possible mechanisms for glutaminase action. One of these is shown in Figure 19. In the other mechanism, the order of product release is reversed. Neither mechanism makes any effort to assign a role to phosphate; the data available are not adequate for this purpose.

Using a steady state approach, a rate equation for the mechanism shown in Figure 19 can be obtained. The derivation is shown in the Appendix. The result is

$$v = \frac{V_m}{1 + \frac{K_{\text{qln}}}{[\text{GLN}]} + \frac{K_{\text{qln}} \cdot [\text{GLU}]}{K_{\text{glu}}}} \quad \ldots \ldots \quad (1)$$
Figure 19. The mechanism of action of glutaminase.
or \[ v = V_m - K_{gln} \left( 1 + \frac{[GLU]}{K_{glu}} \right) \frac{v}{[GLN]} \]. 

From equation 2 it is clear that glutamate will not affect the intercept (apparent maximum velocity) but will alter the slope (apparent Km for glutamine); i.e., glutamate will appear to inhibit competitively with respect to glutamine. This is consistent with the results in Figures 15 and 16.

Similar methods can be used to obtain a rate equation for the mechanism in which glutamate is the first product released. In this case, the result is:

\[ v = V_m (1 + \frac{[GLU]}{K_{glu}}) - K_{gln} \left( 1 + \frac{K'_l}{K_{gln} K_{glu}} \frac{v}{[GLN]} \right). \]

For this mechanism, then, the presence of glutamate should change both the intercept and the slope.

The data presented here support the mechanism shown in Figure 19. This is consistent with the model of Klingman and Handler (34) but is not consistent with the dead-end complex models proposed by Tveit, Svenneby, and Kvamme (67, 132). Since homogeneous cow brain glutaminase is now available, direct ligand binding studies should provide the evidence to confirm the mechanism of glutaminase.
LITERATURE CITED


APPENDIX

The proposed mechanism is shown in Figure 19, page 68. Using the steady state assumption,

\[ \frac{d[E-Glu]}{dt} = k_3[E-Gln] + k_6[E][Glu] - k_5[E-Glu] = 0 \quad (1) \]

\[ \frac{d[E-Gln]}{dt} = k_1[E][Gln] - (k_2 + k_3)[E-Gln] = 0 \quad (2) \]

From eqn. (1):

\[ [E-Glu] = \frac{(k_3[E-Gln] + k_6[E][Glu])}{k_5} \quad (3) \]

From eqn. (2):

\[ [E-Gln] = \frac{k_1[E][Gln]}{(k_2 + k_3)} \quad (4) \]

Substituting for \([E-Gln]\) in eqn. (3),

\[ [E-Glu] = \frac{(k_3k_1[E][Gln]/(k_2 + k_3) + k_6[E][Glu]/k_5)}{k_5} \quad (5) \]

Now, \([E_T] = [E] + [E-Gln] + [E-Glu] \quad (6)\)

Substituting eqns. (4) and (5) in eqn. (6),

\[ [E_T] = [E](1 + k_1[Gln]/(k_2 + k_3) + (k_3k_1[Gln]/(k_2 + k_3) + k_6[Glu]/k_5) \]

or

\[ [E] = \frac{[E_T]/(1 + k_1[Gln]/(k_2 + k_3) + (k_3k_1[Gln]/(k_2 + k_3) + k_6[Glu]/k_5)}{k_5}) \quad (7) \]

Now, \[v = k_5[E-Glu] - k_6[E][Glu] \quad (8)\]

Substituting eqn. (5) in eqn. (8),

\[ v = k_5[E](k_3k_1[Gln]/(k_2 + k_3) + k_6[Glu]/k_5) - k_6[E][Glu] \]

or
\[ v = [E](k_3 k_1 [\text{Gln}]/(k_2 + k_3)) \]  

Substituting eqn. (7) in eqn. (9) gives

\[ v = [E](k_1 k_3 [\text{Gln}]/(k_2 + k_3) + k_1 [\text{Gln}]/(k_2 + k_3) + k_3 k_1 [\text{Gln}]/(k_2 + k_3) + k_1 k_3 k_5 [\text{Glul}]/k_5 + k_6 [\text{Glul}]/k_5) \]  

Dividing top and bottom by \( k_1 (k_3 + k_5) [\text{Gln}]/k_5 (k_2 + k_3) \) gives

\[ v = [E](k_3 k_5/(k_3 + k_5)/(k_5 (k_2 + k_3)/k_1 (k_3 + k_5) [\text{Gln}]) + 1 \]  

Definitions:

\[ V_m = k_3 k_5 [E_T]/(k_3 + k_5) \]

\[ K_{\text{glu}} = k_5/k_6 \]

\[ K_{\text{gln}} = k_5 (k_2 + k_3)/k_1 (k_3 + k_5) \]

Then

\[ v = \frac{V_m}{1 + \frac{K_{\text{gln}}}{[\text{Gln}]} + \frac{K_{\text{gln}}}{[\text{Gln}]} \cdot \frac{[\text{GLU}]}{K_{\text{glu}}}} \]
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