Preparation and Studies on the Characterization of Sheep Brain Glutamine Synthetase*

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ABSTRACT: Glutamine synthetase from sheep brain has been isolated by a new procedure, which gives much higher yields of the enzyme than have been obtained previously. The new procedure may be conveniently carried out in the laboratory and may be adapted to a 20-fold scale-up for use in a pilot plant. The amino acid composition of the enzyme has been determined. A single N-terminal amino acid, arginine, was found. Close to 65 peptides were obtained after digestion of the enzyme with trypsin and about 16 peptides were found after treatment of the enzyme with cyanogen bromide. These findings are consistent with the view that the eight subunits of the enzyme are identical. The data indicate that 12–14 of the 15 half-cystine residues/enzyme subunit found on amino acid analysis are cysteine residues. Titration with 5,5′-dithiobis(2-nitrobenzoate) indicates that one sulfhydryl group per subunit reacts readily with this reagent and that the other enzyme sulfhydryl groups react more slowly and are presumably buried. The enzyme is inactivated by treatment with *N*-ethylmaleimide and protected from such inactivation by ATP + Mg^{2+}.

Previous studies in this laboratory have dealt with the mechanism of action of glutamine synthetase of brain and with the relationship of the specificity of this enzyme to substrate conformation at the active site (for reviews, see Meister, 1962, 1968). Studies on the irreversible inhibition of glutamine synthetase by L-methionine sulfoximine have shown that inhibition is associated with the ATP-dependent phosphorylation of methionine sulfoximine and the tight binding of 8 moles of methionine sulfoximine phosphate/mole of enzyme (Ronzio and Meister, 1968; Rowe *et al.*, 1968; Ronzio *et al.*, 1969). Haschemeyer (1965, 1966) has found that sheep brain glutamine synthetase consists of eight subunits arranged in a manner analogous to the corners of a cube. It is evident that further information about the structure of the enzyme is needed, and that larger amounts of the enzyme than have previously been available will be required.

The present communication describes an improved method for the isolation of glutamine synthetase from sheep brain. The enzyme probably represents at least 0.2% of the protein present in sheep brain. The new procedure is less time consuming and the yields are four to five times greater than the method used earlier; furthermore, the present method can be adapted to a pilot plant scale. The amino acid composition of the enzyme has been determined and a single N-terminal amino acid has been found. This observation and the finding that the number of peptides found after tryptic digestion is close to one-eighth of the total of (lysine plus arginine) residues is in accord with the view that the subunits are identical. The present studies also show that most of the half-cystine residues of the enzyme exist as cysteine in the native enzyme. The evidence indicates that about eight of the sulfhydryl groups of the enzyme react more readily with 5,5′-dithiobis-(2-nitrobenzoate) than do the remainder. Although the enzyme loses activity rapidly when treated with *N*-ethylmaleimide, such loss of activity does not occur in the presence of ATP and Mg^{2+}.

Experimental Section

Materials. Adenosine 5′-triphosphate, bovine serum albumin, *p*-mercuribenzoate, *N*-ethylmaleimide, and dimethylaminonaphthalene-5-sulfonyl chloride were obtained from the Sigma Chemical Co. 5,5′-Dithiobis(2-nitrobenzoate) was obtained from Aldrich Chemical Co.; hydroxylapatite from Calbiochem; DEAE-cellulose from Brown Co.; dithiothreitol from Nutritional Biochemicals Corp.; trypsin from Worthington Biochemical Corp.; and L-1-tosylamido-2-phenylethyl chloromethyl ketone from Cyclo Chemical Corp. *N*-Ethylmaleimide-1-^{14}C was obtained from Schwarz BioResearch, Inc.

Methods. Glutamine synthetase activity was determined by following the formation of γ-glutamylhydroxamate essentially as previously described (Wellner and Meister, 1966). The enzyme solution (0.1 ml) was added to 0.9 ml of a solution (at 37°) containing imidazole-HCl buffer (50 μmoles), $MgCl_2$ (20 μmoles), 2-mercaptoethanol (25 μmoles), sodium L-glutamate (50 μmoles), salt-free hydroxylamine (100 μmoles), and sodium ATP (10 μmoles). After incubation at 37° for 15 min, 1.5 ml of ferric chloride reagent (0.37 M $FeCl_3$, 0.67 N HCl, and 0.20 M trichloroacetic acid) was added. The precipitated protein was removed by centrifugation and the absorbance at 535 mμ was read against a reagent blank. Controls in which enzyme, ATP, and glutamate were separately omitted were carried out; the values of these (usually less than 0.01 absorbance unit) were subtracted from the experimental values. Under these conditions, 1 μmole of γ-glutamylhydroxamate gave an absorbance of 0.340. The rate of the enzyme-catalyzed reaction was linear with enzyme concentration over the range 0.2–1.2 μmole of hydroxamate formed. Protein was

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determined by the procedure of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard; the absorbance of the standard (1 mg/ml) at 278 mμ was 0.660. A unit of enzyme activity is defined as that amount which catalyzes the synthesis of 1 μmole of γ-glutamylhydroxamate under the conditions given above.

Titrations with *p*-mercuribenzoate were carried out as described by Boyer (1954) and Benesch and Benesch (1962); titrations with 5,5′-dithiobis(2-nitrobenzoate) were carried out according to the procedure of Ellman (1959).

Amino acid analyses were carried out on samples of the enzyme that were hydrolyzed in 6 N HCl at 110° for 24–72 hr. The hydrolysates were analyzed with a Beckman amino acid analyzer Model 120 C by the method of Spackman *et al.* (1958).[1] Half-cystine residues were determined after performic acid oxidation of the enzyme according to the procedure of Moore (1963). Tryptophan was determined by the spectrophotometric method of Edelhoch (1967).

Peptide mapping after tryptic digestion of the enzyme was carried out as follows. The enzyme (1 ml; 5 mg) was dialyzed against 1 M NH_4HCO_3 buffer (pH 8.0) for 1 hr at 5°, and then mixed with 0.06 ml of the same buffer containing 0.6 mg of L-1-tosylamido-2-phenylethyl chloromethyl ketone treated trypsin. This solution was shaken at 26°; after 3 and 6 hr an additional 0.6 mg of the trypsin preparation was added. Aliquots (20 μl) were removed at intervals and subjected to descending paper chromatography in a solvent consisting of 1-butanol–acetic acid–water (4:1:5, v/v). On the basis of the appearance of these after spraying with ninhydrin solution, it was concluded that tryptic digestion was complete after 9 hr. After 12-hr incubation with trypsin, the solution was brought to dryness in a vacuum desiccator and the residue was dissolved completely in 0.05 ml of water. Aliquots of this solution (10 μl, containing about 750 μg of peptides) were spotted on Whatman No. 3MM paper and peptide mapping was carried out according to the method of Katz *et al.* (1959).

Treatment of the enzyme with cyanogen bromide was performed as described by Gross and Witkop (1962); peptide mapping was carried out as cited above.

N-Terminal amino acid determination was carried out according to the Woods and Wang (1967) procedure in which dansylamino acids are separated by polyamide thin-layer chromatography, and rendered visible by the use of ultraviolet light.

Polyacrylamide gel electrophoresis was performed as described by Davis (1964) in 0.05 M potassium phosphate buffer (pH 7.5) in 6% gels.

Isolation of the Enzyme. The procedure described here differs from the earlier method (Pamiljans *et al.*, 1962) in that the step involving selective heat denaturation has been omitted. After precipitation of the enzyme at pH 4.3, chromatography is carried out on hydroxylapatite and on diethylaminoethylcellulose. These chromatographic procedures are used in place of the steps used previously involving adsorption on and elution from calcium phosphate gel, fractionation with ammonium sulfate, and free electrophoresis.

STEP 1. EXTRACTION OF ACETONE POWDER. The acetone powder, prepared as described previously (Pamiljans *et al.*, 1962), was suspended in solution A (300 g of powder; 2.25 l of solution A). Solution A consists of 0.15 M potassium chloride, 5 mM 2-mercaptoethanol, and 1 mM EDTA, adjusted to pH 7.2 by addition of sodium hydroxide. The acetone powder suspension was stirred mechanically for 30 min at 26° and was then centrifuged at 27,000*g* at 0° for 20 min. The precipitate was discarded and the supernatant solution (acetone powder extract) was used in the following step.

STEP 2. PRECIPITATION OF THE ENZYME AT pH 4.3. The acetone powder extract was cooled in an ice bath and sufficient 1 N acetic acid (usually 110–125 ml) was added with mechanical stirring over a period of 1 hr to bring the pH to 4.3. The precipitate was collected by centrifugation at 27,000*g* at 0° for 20 min and it was then dissolved in 150 ml of solution A at 0°. The pH of this solution was rapidly adjusted to 7.2 by addition of 1 M ammonium hydroxide. The resulting suspension was centrifuged at 43,000*g* at 0° for 20 min. Both the precipitate and the supernatant solution were saved. The precipitate was suspended in 125 ml of solution A and then centrifuged at 43,000*g* at 0° for 20 min. The two supernatant solutions were combined and centrifuged again in a Spinco Model L centrifuge at about 55,000*g* at 0° to remove turbidity. The clarified supernatant solution was then dialyzed for 18 hr at 4° against 10 l. of 0.01 M potassium phosphate buffer (pH 7.2) containing 0.01 M 2-mercaptoethanol.

The enzyme may be stored (at 0–4°) at this point provided that 0.002 M EDTA is added to the solution used for dialysis. Under these conditions, the enzyme is stable for several weeks at 4°. However, the EDTA must be removed in order to carry out successful chromatography on hydroxylapatite in step 3.

STEP 3. CHROMATOGRAPHY ON HYDROXYLAPATITE. Dry hydroxylapatite (Bio-Rad, 100 g) was suspended in 400 ml of 0.01 M potassium phosphate buffer (pH 7.2) at 4° and allowed to stand at this temperature for at least 4 hr. The sintered glass plate at the bottom of a column (4.5 × 30 cm) was covered with a plastic filter cloth or a piece of filter paper and the column was filled to about two-thirds of its volume by adding buffer. Then the suspension of hydroxylapatite was poured into the column and the hydroxylapatite was allowed to settle. A bed depth of about 10 cm was obtained; this depth is sufficient for the successful chromatography of about 5 g of protein. The column was equilibrated with 200 ml of 0.01 M potassium phosphate buffer (pH 7.2) containing 2 mM dithiothreitol. A flow rate of less than 200 ml/hr was employed.

The dialyzed solution obtained in step 2 was carefully poured into the column, the top of which was protected by a plastic filter cloth or a piece of filter paper, at a rate less than 200 ml/hr. Elution was then carried out with 0.1 M potassium phosphate buffer (pH 7.2) containing 2 mM dithiothreitol. When the absorbance of the effluent solution at 280 mμ decreased to about 0.05, elution was begun with a linear gradient established between 300 ml of 0.15 M potassium phosphate buffer (pH 7.2) and 300 ml of 0.3 M potassium phosphate buffer (pH 7.2); both buffers contained 2 mM dithiothreitol. The enzyme emerged from the column when the phosphate concentration was about 0.225 M. The fractions containing enzyme activity were pooled and dialyzed for 18 hr at 4° against 10 l. of 0.005 M potassium phosphate buffer (pH 7.2) containing 0.01 M 2-mercaptoethanol and 2 mM EDTA.

STEP 4. CHROMATOGRAPHY ON DEAE-CELLULOSE. About 100 ml of a slurry (two parts DEAE-cellulose and one part 0.005 M potassium phosphate buffer (pH 8.0) containing 2

[1] The authors wish to thank Dr. Daniel Wellner and Mr. Michae Davis for the amino acid analyses.

TABLE I: Summary of the Purification of Glutamine Synthetase from Sheep Brain.[a]

Step	Vol (ml)	Protein Total (mg)	Protein Concn (mg/ml)	Activity Total Units	Activity Concn (units/ml)	Activity Sp Act. (units/mg)	Activity Yield (%)
1. Acetone powder extract	1,610	57,600	35.7	49,000	30.4	0.85	[100]
2. Acid precipitation	255	5,150	20.2	29,400	115	5.7	60
3. Hydroxylapatite chromatography	240	421	1.76	19,800	82.4	47.0	40
4. DEAE-cellulose chromatography	65	62.9	0.966	11,700	180	186	24

[a] See the text; from 300 g of sheep brain acetone powder.

mM EDTA) was poured into a column (2.5 × 25 cm). The cellulose was allowed to settle and then 500 ml of 0.005 M potassium phosphate buffer (pH 8.0) containing 2 mM EDTA and 2 mM dithiothreitol was passed through the column. The final bed depth was about 10 cm. The flow rate was no greater than 500 ml/hr.

The dialyzed solution obtained in step 3 was brought to pH 8.0 at 0° by cautious addition of 1 N ammonium hydroxide. This solution was poured into the column at a flow rate no greater than 250 ml/hr. The enzyme was washed into the column with 0.005 M potassium phosphate buffer (pH 8.0) containing 2 mM EDTA and 2 mM dithiothreitol. When the absorbance of the effluent solution at 280 mμ decreased to about 0.05 (after about 150 ml of solution had emerged from the column), the buffer was changed to 0.01 M potassium phosphate (pH 6.4) containing 2 mM EDTA and 2 mM dithiothreitol. The enzyme emerged from the column when the pH of the effluent was 7.3–7.5 or when about 125–150 ml of the pH 6.4 buffer had passed through the column. The fractions containing enzyme activity were combined and dithiothreitol was added to obtain a final concentration of 5 mM. This solution was lyophilized and the dry powder was stored at −20°. (2-Mercaptoethanol, which is removed by lyophilization, cannot be used in place of dithiothreitol.)

A summary of the purification is given in Table I.

The procedure described above yields a preparation of the enzyme which appears to be identical with that obtained by the earlier procedure. This conclusion is based on (a) determinations of specific activity, (b) relative activity toward L-glutamate, D-glutamate, α-methyl L-glutamate, *threo*-β-methyl D-glutamate, and *threo*-γ-methyl L-glutamate (Kagan *et al.*, 1965; Kagan and Meister, 1966a,b), and (c) sedimentation coefficient (15 S), molecular weight (about 500,000), and electron microscopic appearance (Haschemeyer, 1965, 1966).[2] The enzyme is homogeneous in the analytical ultracentrifuge and on electrophoresis on polyacrylamide gel.

Amino Acid Composition and Related Studies. The amino acid composition of the enzyme is given in Table II; the values obtained in analyses on two different preparations of the enzyme are in close agreement. The enzyme contains about 120 alf-cystine residues (or about 15/subunit). The only N-terminal amino acid found was arginine.[3] The enzyme was digested with trypsin as described under Methods; peptide mapping was carried out on five separate digests. In one map between 53 and 58 peptides were distinguished; in the four other maps between 59 and 75 peptides were counted (average estimate, about 65). One-eighth of the total number of arginine and lysine residues is 546/8 or 68, which is not far from the average number of peptides found on tryptic digestion. It is of interest that tryptic digestion of a sample of enzyme that had been inhibited by incubation with ATP, Mg^{2+}, and L-methionine sulfoximine (Ronzio *et al.*, 1969) was far from complete, even after 24-hr digestion. This finding is probably related to the observation that the methionine sulfoximine inhibited enzyme undergoes extensive aggregation (Wilk *et al.*, 1968). Peptide mapping was also carried out after treating the enzyme with cyanogen bromide as described under Methods; 16 ninhydrin-positive spots were found plus 5 very much fainter spots. The number of peptides found after cyanogen bromide treatment therefore appears to be in the range 16–21; one-eighth of the total number of methionine residues is 119/8 or 15.

Effect of Sulfhydryl Reagents. Determination of the sulfhydryl groups of the enzyme by titration with *p*-mercurbenzoate gave a value of 106 ± 10 moles per mole of enzyme. This result is not far from the values obtained for cysteic acid on amino acid analysis.

When the enzyme was treated with 5,5′-dithiobis(2-nitrobenzoate), the formation of 2-nitro-5-thiobenzoate followed a biphasic curve (Figure 1). The initial rate, after correction for the rate of the slower reaction, was extrapolated to zero time; a value of 8.1 moles of sulfhydryl/mole of enzyme was thus obtained. Determinations of enzyme activity were carried out after 1-, 12-, and 30-min incubation with the reagent: 88–95, 85, and 73%, respectively, of the initial activity was found. Further incubation of the enzyme with the reagent was accompanied by continued reaction. After 18 hr, about 63 moles of sulfhydryl was titrated per mole of enzyme and the formation of product was still increasing; however, some of the protein had precipitated.

Treatment of the enzyme with *N*-ethylmaleimide led to

[2] We are indebted to Dr. Rudy H. Haschemeyer for advice and assistance in connection with the ultracentrifugal determinations and for the electron microscope studies.

[3] The N-terminal amino acid of the enzyme isolated by the earlier procedure (Pamiljans *et al.*, 1962) was found to be arginine as determined by the DNP method (Sanger, 1945) by Mr. Stuart Flashman in Dr. Rudy H. Haschemeyer's laboratory (1965).

TABLE II: Amino Acid Analyses of Sheep Brain Glutamine Synthetase.

Amino Acid	Moles of Amino Acid/500,000 g of Enzyme[a]	
	Prepn 1	Prepn 2
Lysine	275	265
Histidine	125	118
Arginine	276	276
Aspartic acid	451	471
Threonine	222[b]	222[b]
Serine	273[b]	265[b]
Glutamic acid	485	488
Proline	279	260
Glycine	456	470
Alanine	327	317
Half cystine[c]	121	119
Valine	202[d]	190[e]
Methionine	119	119
Isoleucine	235	231
Leucine	246	247
Tyrosine	176	177
Phenylalanine	230	221
Tryptophan[f]	76	76

[a] The values were averaged (24 and 48 hr, preparation 1; 24, 48, and 72 hr, preparation 2), except as noted. [b] Extrapolated. [c] 24-hr hydrolysis; as cysteic acid, procedure of Moore (1963). [d] 48-hr hydrolysis. [e] 72-hr hydrolysis. [f] Determined spectrophotometrically by the procedure of Edelhoch (1967).

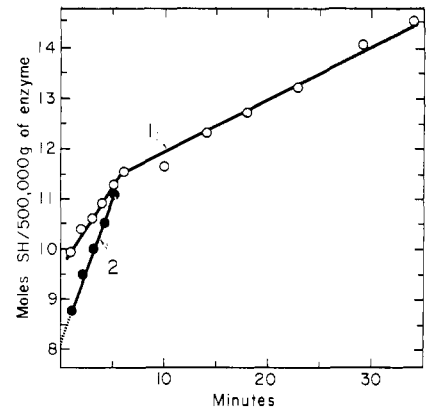

FIGURE 1: Titration of the enzyme with 5,5′-dithiobis(2-nitrobenzoate). The enzyme (0.37 mg in 0.70 ml of 0.01 M potassium phosphate buffer, pH 7.2) was treated at 26° with 0.025 ml of 0.5 M potassium phosphate buffer (pH 7.2), 0.025 ml of 0.5 M EDTA, and 0.02 ml of 0.01 M 5,5′-dithiobis(2-nitrobenzoate); the absorbance at 412 mμ was recorded. The ordinate gives the calculated moles of sulfhydryl groups per mole of enzyme. Curve 1: observed; curve 2: initial rate corrected for rate of slower reaction.

rapid loss of catalytic activity and concomitant precipitation of the enzyme. However, treatment of the enzyme with *N*-ethylmaleimide in the presence of ATP and Mg^{2+} was not accompanied by loss of activity or precipitation. When [^{14}C]*N*-ethylmaleimide was used and the enzyme was then separated by gel filtration, substantial amounts of radioactivity remained associated with the enzyme. In a representative experiment, the enzyme (0.6 mg) was incubated at 37° for 45 min in 0.7 ml of 0.1 M potassium phosphate buffer (pH 7.2) containing 0.02 M $MgCl_2$ and 0.01 M ATP; then, 0.15 ml containing [^{14}C]*N*-ethylmaleimide (0.033 M; 1 μCi/μmole) was added. After 10 min the mixture was added to the top of a column of Sephadex G-50 (0.7 × 24 cm); elution was carried out with 0.05 M potassium phosphate buffer (pH 7.2) and the fractions containing protein were assayed for protein and ^{14}C; 10.0 moles of ^{14}C was found per mole of enzyme.

Discussion

The method described here for the isolation of glutamine synthetase from sheep brain has been used repeatedly by a number of investigators in this laboratory during the last 2 years. The yields have varied from 15 to 30% and the lyophilized enzyme has been found to be stable when stored at −20° for at least 7 months. The procedure has been successfully scaled up by factors of 10–20-fold; we have carried out the preparation of the acetone powder[4] and the first 2 steps at the New England Enzyme Center.[5] In the large-scale procedure the material obtained before dialysis in step 2 is brought to 2 mM with respect to dithiothreitol and then lyophilized. The lyophilized material is stored at −20°; it is suspended in buffer in the laboratory and then dialyzed as described under step 2 prior to chromatography on large columns.

The studies described here are consistent with the conclusion that the subunits of the enzyme are identical. The findings indicate that all or almost all of the half-cystine residues of the isolated enzyme are in the cysteine form. Titration with 5,5′-dithiobis(2-nitrobenzoate) shows that about 1 mole of sulfhydryl/enzyme subunit reacts relatively rapidly with this reagent; presumably, the remainder, which react more slowly, are buried in the molecule. It is of interest that inhibition of the enzyme by *N*-ethylmaleimide is prevented by ATP plus Mg^{2+}; however, the enzyme thus protected binds close to 1 mole of *N*-ethylmaleimide per subunit. The nature of this binding requires additional study.

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[4] About 80 kg of powder are usually obtained from 500 kg of fresh sheep brain.

[5] We thank Dr. Stanley Charm, Director of the New England Enzyme Center, Tufts University School of Medicine, for his valuable contribution to this work.

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Inhibition of Glutamine Synthetase by Methionine Sulfoximine. Studies on Methionine Sulfoximine Phosphate*

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ABSTRACT: The irreversible inhibition of glutamine synthetase by methionine sulfoximine is associated with the tight binding to the enzyme of adenosine diphosphate and methionine sulfoximine phosphate; the latter compound can be cleaved nonenzymatically and also by phosphatases to equimolar amounts of methionine sulfoximine and inorganic phosphate. Methionine sulfoximine phosphate is oxidized by L-amino acid oxidase to yield a phosphate-containing product. The chemical synthesis of methionine sulfoximine phosphate was achieved by treating methionine sulfoximine methyl ester with cyanoethyl phosphate and dicyclohexylcarbodiimide; the product was isolated from the reaction mixture and crystallized. The enzyme- and chemically synthesized methionine sulfoximine phosphate products are identical as judged by paper chromatography in five solvent systems, paper electrophoresis at pH 2.8, 5.5, 7.5, and 9.0, relative stability at various values of pH, and the rates of cleavage in 1 N HCl at 100° and by phosphatases. Treatment of methionine sulfoximine phosphate with hydrosulfite gave a product exhibiting the properties of methionine sulfimine phosphate, indicating that the phosphoryl moiety of methionine sulfoximine phosphate is attached to the sulfoximine nitrogen atom. L-Methionine sulfoximine phosphate inhibits glutamine synthetase in the absence of added nucleotide and metal ions; however, L-methionine sulfoximine phosphate inhibits the enzyme much more effectively in the presence of Mg^{2+} and adenosine diphosphate (and to a lesser extent adenosine triphosphate).

Previous studies in this laboratory (Ronzio *et al.*, 1969a) have shown that the irreversible inhibition of glutamine synthetase from sheep brain by L-methionine sulfoximine in the presence of ATP and Mg^{2+} (or Mn^{2+}) is associated with the tight binding to the enzyme of close to 8 moles each of methionine sulfoximine phosphate and ADP. It was established that the methionine sulfoximine phosphate derivative obtained from the enzyme (by brief heating at 100° or by treatment with perchloric acid) could be converted by treatment with acid or with several phosphatases into equimolar amounts of methionine sulfoximine and P_i. The present work, which was undertaken in order to further characterize the enzyme-bound methionine sulfoximine derivative, indicates that the phosphoryl moiety of this compound is attached to the sulfoximine nitrogen atom. The chemical synthesis of L-methionine sulfoximine phosphate has been achieved, and studies on the inhibition of glutamine synthetase by this compound have been carried out.

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