L-Asparagine Amidohydrolase (E. C. 3.5.1.1.) in a Commercial Preparation of *Escherichia coli* ATCC 11246. Some Properties of the Enzyme and Its Possible Use in the Determination of L-Asparagine

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During the last years, several papers have appeared on the enzymatic determination of L-asparagine as well as on the purification and properties of the enzyme L-asparaginase. The analytical enzyme has been derived from guinea pig serum [1,2] and a strain of *Pseudomonas* which also contains L-glutaminase.[3-5] Before analytical use on body fluids and protein hydrolysates, both enzymes must be purified; the guinea pig serum enzyme, by precipitation with Na_2SO_4 and negative adsorption with calcium phosphate gel, and the *Pseudomonas* enzyme, by treatment with butanol, $BaCl_2$, and $(NH_4)_2SO_4$ followed by chromatography on DEAE-cellulose ion exchanger and retreatment with $(NH_4)_2SO_4$. The latter enzyme could not be separated from L-glutaminase by the treatment mentioned or by fractionation by zone electrophoresis and sucrose gradient centrifugation, but the authors consider these two effects to be activities of two very similar molecules rather than of a single molecule.

Two types of L-asparaginase have also been demonstrated in *Escherichia coli* B. These types, named E.C. 1 and E.C. 2, differ in antitumour activity and in their pH dependence.[6] A highly purified preparation of the type E.C. 2 is now commercially available.

However, the present author noticed that another commercial preparation of *E. coli*, generally used for the enzymic determination of L-glutamic acid through decarboxylation by the manometric procedure,[7] contained a powerful L-asparaginase. Several batches of this preparation were tested during the years 1962–1967; all were found to contain about the same amount of this enzyme. The conversion of L-asparagine to L-aspartic acid was confirmed by identification of the latter by infrared spectrophotometry, paper electrophoresis, and paper chromatography. The discovery of L-asparaginase in this preparation, which above the advantage of being commercially available in an already suitable purified state, also is inexpensive, seemed to open a convenient way to determine L-asparagine alone or together with L-glutamic acid, L-glutamine, and L-aspartic acid. This could be done by use of this enzyme alone or by a combination of the actual preparation and another commercially available enzyme. Several methods can be used, *e.g.* ammonia determination, manometry, or manometry combined with ammonia determination in the usual way. The second enzyme in the combination mentioned is a preparation from *Clostridium welchii* which contains L-glutamic acid decarboxylase, L-glutaminase, and L-aspartic acid decarboxylase but no L-asparaginase. The L-aspartic acid decarboxylase is active only in the presence of traces of α-keto acids and is therefore completely inactivated by ketofixatives such as semicarbazide.

This communication deals with some basic properties of the L-asparaginase found in the *E. coli* preparation which are of importance for the subsequent analytical use of the enzyme, such as the influence of enzyme and substrate concentration, pH, and temperature, on the rate of reaction. The specificity of the enzyme was tested only with D-asparagine and L-glutamine, because the manometric determination of L-asparagine by the combined enzyme method used, is founded on the specificity of L-aspartic acid decarboxylase. The effect of added semicarbazide, α-keto acids and D-asparagine was thus studied under conditions recommended for the manometric assay.[7]

Preparations from *E. coli* ATCC 11246 and *Cl. welchii* NCTC 6784 were purchased from Worthington Biochemical Corporation, Freehold, N.J., USA. Amino acids, chromatographically pure, and α-keto acids of *pro analysi* quality were purchased from Mann Research Laboratories, N.Y., USA, buffer salts and semicarbazide from Merck, Darmstadt. Unless otherwise stated, the standard incubation conditions were 0.1 M sodium acetate buffer pH 5.0 at a temperature of 37°C in a total volume of 2.0 ml. The reactions were started by adding enzyme from a stock suspension to the substrate mixture. Both the suspension and the substrate were brought to 37°C before being mixed.

The reaction was followed by the formation of L-aspartic acid, which was determined by separation from L-asparagine, proteins, and polysaccharides by paper electrophoresis followed by ninhydrin reaction and photometry.[8]

The relationship between reaction rate and enzyme concentration proved linear when 10 μmoles of L-asparagine were incubated with 0.2, 1.0, 2.0, 5.0, or 10.0 mg, respectively, of *E. coli* preparation.

K_m was found to be 2.3×10^{-3} M at pH 5.0 when 2 mg of *E. coli* preparation was incubated with L-asparagine in increasing concentration.

The effect of pH was studied on 40 μmoles of L-asparagine and 2 mg of *E. coli* preparation in 0.1 M buffers of sodium acetate (pH 4.0 and 5.0), sodium phosphate (pH 6.0, 6.5, 7.0, 7.2, 7.5) and tris(hydroxymethyl)-aminomethane-hydrochloric acid (pH 8.5 and 9.0). This gave a smooth curve with its maximum between pH 6.7–6.9, a value lower than that reported for the L-asparaginase from guinea pig and *Pseudomonas*. The result indicates a resemblance between this enzyme and the L-asparaginase type E.C. 2.[6]

The enzyme seems to be stable at temperatures up to 45°C at which the reaction rates are constant for at least 1 h. At 60°C, however, the activity begins to decrease after a few minutes of incubation. An activation energy based upon data obtained between 25° and 45°C was calculated as 5.1 kJ/mole.

At pH 7 the rate of reaction was increased 57 % by oxaloacetic acid and 38 % by α-ketoglutaric above that at pH 5.0 with no additions, but only very little by pyruvic acid (2.9 μmoles of each acid added to the reaction mixture). At pH 5 the acids produced no increase in the rate of reaction but 10 μmoles of D-asparagine lowered it 2 %. Both D-asparagine and L-glutamine were unaffected by the enzyme.

Table 1 shows that it is possible to determine L-asparagine manometrically by using a combination of two commercially available enzyme preparations. D-Asparagine and L-glutamine were not attacked by the combination of enzymes.

From the analytical point of view this preparation is very useful for determination of L-asparagine. The choice of method for following the activity of the enzyme and thus for determining L-asparagine must depend upon the circumstances under which the determination is to be made, the degree of sensitivity and accuracy desired, etc. It can be done, as shown by paper electrophoresis and manometrically, but other methods are of course ammonia determination in the usual way or the very sensitive method of Ramadan and Greenberg.[3] Before ammonia determinations can be done the specificity test must, however, be extended to check that no compounds other than L-asparagine can be sources of ammonia, under standardized conditions.

Table 1. Manometric determination of L-asparagine.

The incubation mixtures contained 5.0 μmoles of L-asparagine. The enzyme preparations from *E. coli* and *Cl. welchii* were pipetted separately to the side arms of the Warburg vessels; both were tipped in at the same time. Before use the *Cl. welchii* preparation was dialysed against sodium acetate buffer in order to remove ninhydrinpositive compounds which had been detected by paper electrophoresis. (Mean value of 6 determinations, standard deviation 0.8 μl at 120 and 135 min).

Incubation time, min	0	15	30	45	90	120	135
μl CO_2 produced	0	20	45	72	109	110	110

Value found 110 μl. Calculated value 112 μl. Yield 98 %.

The method described above could, somewhat modified, also be used for the measurement of the activity of the enzyme L-asparaginase.

In the manometric method L-glutamic acid, L-glutamine, and L-aspartic acid interfere with the determination. If one or more of these compounds are present, they can, however, be decarboxylated by the enzymes in the *Cl. welchii* preparation alone before addition of the *E. coli* preparation. Theoretically, the proper combination of the enzymes in these two preparations, α-keto acid, and semicarbazide, should enable individual determinations — and not only differential determinations — of L-glutamic and L-aspartic acids and their amides by using two Warburg vessels.

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On the Crystal Structure of Copper Wolframate

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Investigations have been started at this Institute on phases occurring in the Cu—Mo—O and Cu—W—O systems in order to elucidate their crystal structures and electric and magnetic properties. Some results of studies on the copper-wolfram-oxygen system obtained so far are reported below.

Samples were prepared by mixing CuO, Cu_2O, WO_3, and WO_2 in various proportions and heating them in evacuated, sealed platinum tubes at about 800°C for a period of several days. Powder patterns taken of the preparations indicated the formation of two intermediate phases, Cu_3WO_6 and a second which we will here designate $CuWO_{4-x}$ (see below). The former is a previously unknown phase with a cubic structure which will be reported elsewhere.[1] The latter appears to be identical with the phase reported as $Cu_{0.77}WO_3$ by Conroy and Sienko [2] and later as $Cu_xWO_{3+\delta}$ by Sienko and Weller.[3]

Preparations made with starting compositions around $CuWO_4$ gave powder patterns of the type listed in Table 1. The bulk of these samples consisted of dark yellow brown, polyhedric crystals which were more or less transparent but which all seemed to be twinned. With the aid of precession and Weissenberg photographs the powder pattern could be indexed on the basis of the triclinic unit cell parameters given in Table 2. By the transformation (0,1,0/−1,0,−1/0,0,1) an alternative cell is obtained which is in close agreement with that reported for "$Cu_{0.77}WO_3$".[2]

Table 1. X-Ray powder diffraction data for $CuWO_{4-x}$, $CuK\alpha_1$ radiation ($\lambda = 1.54051$ Å) $\Delta = (\sin^2\theta_{obs} - \sin^2\theta_{calc})$.

I	d_{obs} Å	$\sin^2\theta_{obs} \times 10^5$	$h\,k\,l$	$\Delta \times 10^5$
m	5.799	1764	0 1 0	− 5
s	4.662	2730	1 0 0	0
w	3.876	3949	1 1 0	− 4
mw	3.771	4171	0 $\bar{1}$ 1	4
m	3.684	4371	0 1 $\bar{1}$	2
ms	3.433	5033	1 $\bar{1}$ 0	−12
			1 0 $\bar{1}$	12
ft	3.304	5435	1 0 1	− 1
s	3.113	6124	1 1 $\bar{1}$	−19
m	2.962	6760	1 1 1	− 1
w	2.895	7081	0 2 0	5
m	2.824	7438	1 $\bar{1}$ $\bar{1}$	1
m	2.7848	7650	1 $\bar{1}$ 1	0
w	2.6094	8713	1 2 0	− 1
mw	2.5159	9373	0 $\bar{2}$ 1	1
m	2.4632	9778	0 2 $\bar{1}$	1
s	2.4359	9999	0 0 2	3
m	2.3311	10918	2 0 0	− 2
w	2.2593	11623	1 2 1	0
mw	2.1954	12309	1 0 $\bar{2}$	− 2
ft	2.1373	12988	2 0 $\bar{1}$	−15
w	2.1239	13152	1 0 2	11
mw	2.1100	13326	1 1 $\bar{2}$	− 5
mw	2.1036	13407	1 $\bar{2}$ 1	6
			1 $\bar{2}$ $\bar{1}$	16
w	2.0903	13578	2 1 $\bar{1}$	− 2
w	2.0743	13789	2 $\bar{1}$ 0	9
mw	2.0176	14575	1 1 2	8
mw	2.0148	14615	2 1 1	2
w	1.9997	14837	1 $\bar{1}$ $\bar{2}$	9
m	1.9731	15239	1 $\bar{1}$ 2	−14

Weissenberg data were collected from a twinned crystal using Mo$K\alpha$ radiation. Positions for the metal atoms were derived from a three-dimensional Patterson synthesis assuming space group $P\bar{1}$. Probable oxygen positions were located in a difference synthesis. The subsequent least-squares refinement gave an R-value of 0.20 which was considered reasonable in view of the inherently poor data obtainable from this twinned crystal. The resulting B-values and interatomic distances indicate

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