L-Asparaginase from \textit{Proteus vulgaris}

TETSUYA TOSA, RYUJIRO SAN, KOZO YAMAMOTO, MASATOSHI NAKAMURA, KATSUKO ANDO, AND ICHIRO CHIBATA

\textit{Department of Applied Biochemistry, Chemical Research Laboratory, Tanabe Seiyaku Co. Ltd.,}
\textit{Kashima-cho, Higashiyodogawa-ku, Osaka, Japan}

Received for publication 15 March 1971

To produce an immunologically and enzymologically new type of L-asparaginase, 108 strains of bacteria were screened for enzyme production. As a result, 13 bacteria belonging to the genera \textit{Alcaligenes}, \textit{Bacterium}, and \textit{Proteus} were found to produce L-asparaginases in high levels. Among these L-asparaginases, partially purified L-asparaginases from \textit{B. cadaveris} and \textit{P. vulgaris} showed antitumor activity. A partially purified L-asparaginase preparation of \textit{P. vulgaris} did not react with the antibody of \textit{Escherichia coli} L-asparaginase on the Ouchterlony agar plate. Culture conditions for the production of L-asparaginase by \textit{P. vulgaris} were investigated in detail. The enzyme was produced in high yields when cells were grown aerobically in a medium containing sodium fumarate and corn steep liquor. The addition of glucose or ammonium ion to the medium, however, resulted in depressed production of L-asparaginase. Under the optimum conditions, 3,700 international units of L-asparaginase was obtained from 1 liter of culture medium.

Since the observation by Mashburn and Wriston (20) that L-asparaginase (L-asparagine amido-hydrolase, EC 3.5.1.1.) from \textit{Escherichia coli} has an antitumor activity similar to that of guinea pig serum (3, 16, 19), a number of papers have been published on the production and the purification of the enzyme from \textit{E. coli} (1, 2, 5, 7, 13, 17, 24, 26, 29, 30, 33, 34). Thus, a large amount of purified enzyme has been supplied and employed in the clinical tests for acute leukemia and other malignant neoplasms in man (11, 12, 21). However, the administration of such an enzyme protein for a long duration, in general, produces the corresponding antibody in the living bodies, and the antibody causes an anaphylactic shock or neutralization of the drug effect (15). Therefore, the discovery of a new L-asparaginase immunologically different from that of \textit{E. coli} has been greatly desired.

In addition to \textit{E. coli}, some reports have been published on the production of tumor inhibitory L-asparaginase by \textit{Asperillus terreus} (8), \textit{Erwinia aroideae} (22), \textit{E. carotovora} (32), \textit{Mycobacterium tuberculosis} (14, 23), and \textit{Serratia marcescens} (9, 10, 27).

We have screened a number of bacteria for the formation of L-asparaginase and found that \textit{Proteus vulgaris} OUT 8226 can produce the enzyme in significant amounts. The present paper describes the screening of bacteria producing L-asparaginase and the production of the enzyme by \textit{P. vulgaris}.

\section*{MATERIALS AND METHODS}

\textbf{Bacteria.} \textit{Alcaligenes faecalis} OUT 8027 and OUT 8029 and \textit{P. vulgaris} OUT 8226 were obtained from the Faculty of Engineering, Osaka University, Osaka. \textit{P. vulgaris} RIDM OX2 was obtained from the Research Institute for Microbial Disease, Osaka University, Osaka. \textit{P. mirabilis} IFO 3849, \textit{P. morgani} IFO 3848, and \textit{P. vulgaris} IFO 3045 were obtained from the Institute for Fermentation, Osaka. \textit{P. mirabilis} AHU 1467 and AHU 1468 and \textit{P. vulgaris} AHU 1144, AHU 1474, and AHU 1475 were obtained from the Faculty of Agriculture, Hokkaido University, Sapporo. \textit{Bacterium cadaveris} NCTC 6578 was obtained from the Central Public Health Laboratory of the National Collection of Type Cultures, London.

\textbf{Media.} To screen for L-asparaginase-producing bacteria, a medium containing 1% sodium fumarate and 5% corn steep liquor was used. For L-asparaginase production by \textit{P. vulgaris}, the media indicated in the respective tables were used. Corn steep liquor was adjusted to pH 7.0 with sodium hydroxide and boiled for 10 min. The precipitate was removed by filtration, and the clear filtrate was used for the preparation of media. Media used were adjusted to pH 7.0 before autoclaving.

\textbf{Culture method.} Unless otherwise noted, the media were distributed in 100-ml amounts to 500-ml shaking flasks, sterilized, and inoculated with a loopful of bacteria. Culture was carried out for 20 hr at 30°C on a reciprocating shaker [140 rev/min; 8-cm stroke; oxygen absorption rate (OAR), 1.0 nmol of O$_2$ absorbed per liter of culture medium per min].

---

1 A part of this investigation was presented at the Annual Meeting of the Agricultural Chemistry of Japan, Fukuoka, Japan, 4 April 1970.
Preparation of cell-free extract. The cells from 100 ml of culture broth were harvested by centrifugation, washed with 0.9% saline solution, and suspended in 50 ml of distilled water. The cell suspension was subjected to sonic treatment at 5 C (10 kc, 20 min), and the centrifuged cell-free extract was used as a l-asparaginase preparation for screening.

Partial purification of L-asparaginase. For antitumor and immunochemical tests, l-asparaginas from A. faecalis, B. cadaveris, and P. vulgaris were partially purified. Bacteria were cultivated in the medium containing 1% sodium fumarate and 5% corn steep liquor for 20 hr, and cell-free extracts were obtained by the method described above. The cell-free extracts were partially purified by ammonium sulfate fractionation. In the case of A. faecalis, the cell-free extract was brought to 40% saturation with ammonium sulfate, and the precipitate was collected by centrifugation. The precipitate was dissolved in a small volume of water and dialyzed against distilled water overnight at 5 C. In soluble material was removed by centrifugation, and the supernatant solution was lyophilized. A partially purified preparation having a specific activity of 3.9 international units (IU) per mg was obtained. In the cases of B. cadaveris and P. vulgaris, the precipitates which were formed between 40 and 80% saturation with ammonium sulfate were collected and treated as described for A. faecalis. The specific activities of the partially purified preparations of B. cadaveris and P. vulgaris were 2.1 and 3.1 IU per mg, respectively.

Assay of l-asparaginase. Reaction mixture containing 0.5 ml of 0.08 M l-asparagine, 1.0 ml of 0.05 M borate buffer (pH 8.4), and 0.5 ml of enzyme solution was incubated for 10 min at 37 C. The reaction was stopped by the addition of 0.5 ml of 15% trichloroacetic acid solution. The precipitated protein was removed by centrifugation, and the liberated ammonia was determined by the indophenol method (28). One l-asparaginase unit (IU) is defined as that amount of enzyme which liberates 1 µmole of ammonia per min at 37 C.

Determination of protein. Protein was determined by the method of Lowry et al. (18).

Antitumor test. Four groups of nine C3H mice were intraperitoneally implanted with 10⁶ G3HED-OG tumor cells. After 24 hr, 6.7 IU of partially purified l-asparaginase preparations of A. faecalis OUT 8027, B. cadaveris NCTC 6578, and P. vulgaris OUT 8226 were dissolved in an aqueous solution of sodium bicarbonate and administered intraperitoneally to the tumor-implanted mice. The survival time of the mice was compared with that of control mice which received only an aqueous solution of sodium bicarbonate.

Preparation of antibody of E. coli l-asparaginase. Three rabbits (female, 2.7 to 2.9 kg) were used for preparing antibody of E. coli l-asparaginase (anti-EC). Crystalline E. coli l-asparaginase dissolved in 0.5 ml of 0.9% saline solution was injected with 0.5 ml of Freund's complete adjuvant into the femoral muscle every 7 days (increasing doses as follows: 1, 1, 3, and 5 mg). After the formation of anti-EC was confirmed, the animals were bled from the carotid artery. The serum was separated by centrifugation and γ-G-immunoglobulin fraction was obtained by ammonium sulfate fractionation. The fraction which precipitated between 0 and 50% saturation was collected and dissolved in a small volume of 0.9% saline solution and dialyzed against 0.9% saline solution overnight at 5 C. The insoluble matter was removed by centrifugation, and the supernatant solution was used as an anti-EC preparation.

Immunodiffusion. Experiments were conducted by the Ouchterlony double-diffusion method (31). For the test of immunological difference between l-asparaginase from E. coli and l-asparaginas from other sources, the following antigen and antibody systems were employed. The center well received undiluted anti-EC (28 mg of protein per ml), and lateral wells received l-asparaginase preparations dissolved in 0.9% saline solution at the concentration of 150 IU per ml. The loaded gels were developed in a humidifier at 25 C for 40 hr. To clarify cross-reactivity of P. vulgaris l-asparaginase preparation with anti-EC, three levels of the P. vulgaris preparation (20, 100, and 300 IU per ml) and two levels of anti-EC (2.8 and 14 mg of protein per ml) were prepared. These antigen and antibody solutions were loaded on agar plates and developed in a humidifier at 25 C for 24 to 72 hr.

RESULTS AND DISCUSSION

Screening of bacteria for l-asparaginase production. For l-asparaginase production, 108 strains of bacteria were tested. The enzyme was found to be formed by many bacteria of which A. faecalis, B. cadaveris, P. mirabilis, P. morganii, and P. vulgaris produced it in high concentrations (Table 1).

Antitumor activity of l-asparaginas. Two kinds of l-asparaginase have been known to be produced by microorganisms; however, only one

| TABLE 1. Production of l-asparaginase by various bacteriaa | l-Asparaginase activity (IU per ml of culture medium) |
|-----------------------------------------------------------|-------------------------------------------------|
| Bacteria                                                   |                                                |
| Alicaligenes faecalis OUT 8027                            | 0.98                                            |
| A. faecalis OUT 8029                                       | 0.80                                            |
| Bacterium cadaveris NCTC 6578                             | 0.27                                            |
| Proteus mirabilis AHU 1467                                 | 1.00                                            |
| P. mirabilis AHU 1468                                      | 0.33                                            |
| P. mirabilis IFO 3849                                      | 0.51                                            |
| P. morganii IFO 3848                                      | 0.47                                            |
| P. vulgaris AHU 1144                                      | 1.15                                            |
| P. vulgaris AHU 1474                                      | 2.83                                            |
| P. vulgaris AHU 1475                                      | 1.23                                            |
| P. vulgaris IFO 3045                                      | 1.58                                            |
| P. vulgaris RIMD OX2                                      | 2.32                                            |
| P. vulgaris OUT 8226                                      | 2.93                                            |

a Culture of the bacteria was carried out for 20 hr.
of these possesses antitumor activity. This activity has been known to vary with strains or culture conditions of microorganisms. For example, it was reported that L-asparaginase from Bacillus coagulans (20) or yeast (4) showed no antitumor activity. M. tuberculosis (14, 23), on the other hand, produced two kinds of L-asparaginases in the cells, but only one of them was effective against animal tumors. Similarly, E. coli L-asparaginase I and L-asparaginase II were produced simultaneously in the cells (5, 6, 7, 29), but only the latter showed antitumor activity.

Figure 1 shows the results of antitumor test of partially purified L-asparaginase preparations from A. faecalis, B. cadaveris, and P. vulgaris. The animals which had been treated with L-asparaginase preparations from B. cadaveris and P. vulgaris survived for a much longer period than did the control animals: nine mice in the control group died within 11 to 13 days after implantation of 6C3HED-OG tumor cells, whereas eight of nine mice treated with L-asparaginase (6.7 IU) from B. cadaveris and seven of nine mice treated with L-asparaginase from P. vulgaris survived for

**Table 2. Effect of media on the production of L-asparaginase by P. vulgaris**

| Constituents of media | L-Asparaginase activity (IU) |
|-----------------------|-----------------------------|
|                       | Per ml of culture medium | Per mg of protein |
| Basal constituents    | Additions                  |                |
| Meat extract (1%)     | None                       | 1.2            | 0.64 |
| Peptone (1%)          | Glucose (1%)               | 0.6            | 0.38 |
| Yeast extract (1.25%) | Sodium citrate (1%)        | 1.1            | 0.58 |
| NaCl (0.5%)           | Sodium fumarate (1%)       | 1.1            | 0.54 |
| Corn steep liquor (5%)| None                       | 1.6            | 0.98 |
|                       | Glucose (0.5%)             | 0.1            | 0.08 |
|                       | Glucose (1%)               | 0.1            | 0.13 |
|                       | Glycerin (1%)              | 0.1            | 0.14 |
|                       | Sodium citrate (1%)        | 2.0            | 0.67 |
|                       | Sodium fumarate (0.5%)     | 2.3            | 0.98 |
|                       | Sodium fumarate (1%)       | 2.9            | 1.20 |
|                       | Sodium fumarate (1.5%)     | 3.1            | 1.07 |
|                       | Sodium fumarate (2%)       | 3.3            | 0.97 |
|                       | Sodium fumarate (3%)       | 2.7            | 0.85 |
|                       | Ammonium fumarate (1%)     | 2.3            | 1.02 |
|                       | Glucose (0.5%)             | 0.1            | 0.08 |
|                       | Sodium fumarate (1%)       | 0.9            | 0.60 |
TABLE 3. Effect of ammonium salts on the production of L-asparaginase by *P. vulgaris*

| Ammonium salts | L-Asparaginase activity (IU) | Per ml of culture medium | Per mg of protein |
|----------------|-----------------------------|--------------------------|-------------------|
| None           | 2.9                         | 1.20                     |
| NH₄Cl (0.5%)   | 1.6                         | 0.79                     |
| NH₄Cl (1.0%)   | 1.4                         | 0.69                     |
| NH₄Cl (1.5%)   | 0.9                         | 0.55                     |
| NH₄NO₃ (2.0%)  | 0.9                         | 0.54                     |
| NH₄NO₃ (1.0%)  | 1.4                         | 0.65                     |
| (NH₄)₂SO₄ (1.0%) | 1.8                     | 0.92                     |

*P. vulgaris* was cultured in the medium containing 1% sodium fumarate, 5% corn steep liquor, and the desired weight of ammonium salts. Culture was carried out for 20 hr.

over 6 months. Further, the autopsy findings of dead animals treated with both L-asparaginase preparations revealed that the death was not caused by tumor but was due to other causes. These results show that partially purified L-asparaginases from *B. cadaveris* and *P. vulgaris* have strong antitumor activity. All of the animals treated with L-asparaginase preparation from *A. faecalis* OUT 8027 died earlier than the controls. The death was probably caused by an endotoxin in the partially purified preparation.

**Immunodiffusion test.** To determine the immunological difference between L-asparaginase from *E. coli* and L-asparaginases from three other bacteria, an immunodiffusion experiment was carried out by using anti-EC. Although a faint precipitin line was observed between anti-EC and *B. cadaveris* L-asparaginase preparation, no line was detected between the other two preparations (Fig. 2). L-Asparaginase preparations were used by dissolving them in 0.9% saline solution at a concentration of 150 IU per ml. To clarify cross-reactivity of *P. vulgaris* L-asparaginase preparation with anti-EC, three different levels of antigen-antibody systems were tested, but no cross-reaction was detected in any instance, indicating that L-asparaginase produced by *P. vulgaris* is immunologically different from that of *E. coli*. Therefore, *P. vulgaris* L-asparaginase could possibly become a new useful antitumor drug.

**Effect of culture conditions on L-asparaginase production by *P. vulgaris*.** On the basis of antitumor and immunodiffusion properties, *P. vulgaris* OUT 8226 was chosen as a preferred L-asparaginase-producing bacterium, and the culture conditions for production of the enzyme were studied in detail.

The culture was grown in different media for 20 hr, and L-asparaginase activity produced in the cells was measured (Table 2). The largest quantity of L-asparaginase was produced when *P. vulgaris* was grown in the medium containing 2% sodium fumarate and 5% corn steep liquor, but its production was decreased by the addition of glucose (Table 2) or ammonium salts (Table 3). A similar depression by glucose was also observed by Cedar and Schwartz (7) and Roberts et al. (24) in *E. coli*.

The addition of L-aspartic acid or L-glutamic acid to the medium containing sodium fumarate and corn steep liquor slightly enhanced enzyme production (Table 4), but these amino acids may not be considered specific inducers for enzyme formation.

The effect of aeration on L-asparaginase production is shown in Table 5. Although Cedar and Schwartz (7) reported that *E. coli* K-12 L-asparaginase (II) was produced under anaero-

| Constituents of media | L-Asparaginase activity (IU) | Per ml of culture medium | Per mg of protein |
|----------------------|-----------------------------|--------------------------|-------------------|
| Sodium fumarate (1%) | None                        | 2.9                      | 1.20              |
| Corn steep liquor (5%)| L-Asparagine (1%)           | 2.2                      | 0.97              |
|                      | L-Aspartic acid (1%)        | 3.2                      | 1.12              |
|                      | L-Glutamic acid (1%)        | 3.6                      | 1.11              |
| Sodium fumarate (2%) | None                        | 3.3                      | 0.97              |
| Corn steep liquor (5%)| L-Asparagine (1%)           | 3.2                      | 0.95              |
|                      | L-Aspartic acid (1%)        | 3.6                      | 1.12              |
|                      | L-Glutamic acid (1%)        | 3.7                      | 1.18              |

* Culture was carried out for 20 hr.
Table 5. Effect of aeration on the production of L-asparaginase by P. vulgaris

| Aeration rate (OAR) | Cultivation time (hr) | L-Asparaginase activity (IU per ml of culture medium) |
|---------------------|-----------------------|------------------------------------------------------|
| Stationary culture  | 20                    | 0.6                                                  |
|                     | 44                    | 1.0                                                  |
| 0.5                 | 20                    | 1.3                                                  |
|                     | 44                    | 3.0                                                  |
| 0.7                 | 20                    | 2.4                                                  |
|                     | 44                    | 3.0                                                  |
| 1.0                 | 20                    | 2.9                                                  |
|                     | 44                    | 3.1                                                  |
| 2.0                 | 20                    | 2.7                                                  |
|                     | 44                    | 2.2                                                  |

a OAR, millimoles of O₂ absorbed per liter of culture medium per minute.

bic conditions, its formation by P. vulgaris as well as E. coli HAPOne greatly increased under aerobic conditions (24).

Studies on the purification and some enzymatic and physicochemical properties of L-asparaginase from P. vulgaris will be published elsewhere.

ACKNOWLEDGMENTS

We are indebted to K. Yamada, School of Medicine, University of Nagoya, for the antitumor test. We are grateful to T. Takayanagi, managing director of this company, and K. Fujii, director of this laboratory, for their helpful advice and encouragement in this study.

LITERATURE CITED

1. Arens, A., E. Rauenbusch, E. Irion, O. Wagner, K. Bauer, and W. Kaufmann. 1970. Isolation and properties of L-asparaginase from Escherichia coli. Hoppe-Seyler's Z. Physiol. Chem. 351:197-212.
2. Bilimoria, M. H. 1969. Conditions for the production of L-asparaginase 2 by coliform bacteria. Appl. Microbiol. 18:1025-1030.
3. Broome, J. D. 1961. Evidence that the L-asparaginase activity of guinea pig serum is responsible for its antilymphoma effects. Nature (London) 191:1114-1115.
4. Broome, J. D. 1965. Antilymphoma activity of L-asparaginase in vivo: clearance rates of enzyme preparation from guinea pig serum and yeast in relation to their effect on tumor growth. J. Nat. Cancer Inst. 35:967-974.
5. Campbell, H. A., L. T. Mashburn, E. A. Boyse, and L. J. Old. 1967. Two L-asparaginases from Escherichia coli B. Their separation, purification, and antitumor activity. Biochemistry 6:721-730.
6. Cedar, H., and J. H. Schwartz. 1967. Localization of the two L-asparaginases in anaerobically grown Escherichia coli B. J. Biol. Chem. 242:3753-3755.
7. Cedar, H., and J. H. Schwartz. 1968. Production of L-asparaginase II by Escherichia coli. J. Bacteriol. 96:2043-2048.
8. DeAngel, L. C., F. Pochiari, A. Tonolo, V. E. Zurita, E. Ciardani, and A. Perin. 1970. Effect of L-asparaginase from Aspergillus terreus on ascites sarcoma in the rat. Nature (London) 225:549-550.
9. Heinemann, B., and A. J. Howard. 1969. Production of tumor-inhibitory L-asparaginase by submerged growth of Serratia marcescens. Appl. Microbiol. 18:550-554.
10. Heinemann, B., A. J. Howard, and H. J. Palozz. 1970. Influence of dissolved oxygen levels on production of L-asparaginase and prodigiosin by Serratia marcescens. Appl. Microbiol. 19:800-804.
11. Hill, J. M., E. Leob, A. MacLellan, A. Khan, J. Roberts, W. F. Schields, and N. O. Hill. 1969. Response to highly purified L-asparaginase during therapy of acute leukemia. Cancer Res. 29:1574-1580.
12. Hill, J. M., J. Roberts, E. Leob, A. Khan, A. Macelilan, and R. W. Hill. 1967. L-asparaginase therapy for leukemia and other malignant neoplasms. J. Amer. Med. As. 292:882-888.
13. Ho, P. P. K., E. B. Milkin, J. J. Bobbitt, E. L. Grinnan, P. J. Burck, B. H. Frank, L. D. Bocek, and R. W. Squires. 1970. Crystalline L-asparaginase from Escherichia coli B 1. Purification and chemical characterization. J. Biol. Chem. 245:3708-3715.
14. Jayaram, H. N., T. Ramakrishnan, and C. S. Vaidyanathan. 1968. L-asparaginase from Mycobacterium tuberculosis strain H₃₇Ra and H₃₇Rₐ. Arch. Biochem. Biophys. 126:165-174.
15. Khan, A., and J. M. Hill. 1969. Neutralizing precipitin in the serum of a patient treated with L-asparaginase. J. Lab. Clin. Med. 73:846-852.
16. Kidd, J. G. 1953. Regression of transplanted lymphomas induced in vivo by means of normal guinea pig serum. I. Course of transplanted cancers of various kinds in mice and rats given guinea pig serum. J. Exp. Med. 98:565-582.
17. Kristiansen, T., M. Einarsen, L. Sundberg, and J. Porath. 1970. Purification of L-asparaginase from E. coli by specific adsorption and desorption. FEBS Lett. 7:294-296.
18. Lowry, O. H., N. J. Rosebrough, A. J. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
19. Mashburn, L. T., and J. C. Wriston, Jr. 1963. Tumor inhibitory effect of L-asparaginase. Biochem. Biophys. Res. Commun. 12:50-55.
20. Mashburn, L. T., and J. C. Wriston, Jr. 1964. Tumor inhibitory effect of L-asparaginase from Escherichia coli. Arch. Biochem. Biophys. 105:450-452.
21. Oetken, H. F., J. L. Old, E. A. Boyse, H. A. Campbell, F. S. Phillips, B. D. Clarkson, L. Tallal, R. D. Leeper, M. K. Schwartz, and J. H. Kim. 1967. Inhibition of leukemias in man by L-asparaginase. Cancer Res. 27:2619-2631.
22. Peterson, R. E., and A. Cleg. 1969. L-Asparaginase production by Erwinia avrdae. Appl. Microbiol. 18:64-67.
23. Reddy, V. V. S., H. N. Jayaram, M. Sirsi, and T. Ramakrishnan. 1969. Inhibitory activity of L-asparaginase from Mycobacterium tuberculosis on Yoshida ascites sarcoma in rats. Arch. Biochem. Biophys. 132:262-267.
24. Roberts, J., G. Burson, and J. M. Hill. 1968. New procedures for purification of L-asparaginase with high yield from Escherichia coli. J. Bacteriol. 95:2117-2123.
25. Roberts, J., M. D. Prager, and N. Bachaknsky. 1966. The antitumor activity of Escherichia coli L-asparaginase. Cancer Res. 26:2213-2217.
26. Robison, R. S., and B. Berk. 1969. L-Asparaginase synthesis by Escherichia coli B. Biotechnol. Bioeng. 11:1211-1225.
27. Rowley, B., and J. C. Wriston, Jr. 1967. Partial purification and antilymphoma activity of Serratia marcescens L-asparaginase. Biochem. Biophys. Res. Commun. 28:160-165.
28. Russell, J. A. 1944. The colorimetric estimation of small amounts of ammonia by the phenol-hypochlorite reaction. J. Biol. Chem. 156:457-461.
29. Schwartz, J. H., J. Y. Reeves, and J. D. Broome. 1966. Two L-asparaginases from E. coli and their action against tumors. Proc. Nat. Acad. Sci. U.S.A. 56:1516–1519.

30. Stollar, D., and L. Levine. 1963. Two-dimensional immunodiffusion, p. 848–854. In S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 6. Academic Press Inc., New York.

31. Staerk, J., H. Haupt, and T. Kranz. 1970. Crystallization and properties of L-asparaginase from Escherichia coli. Experientia 26:131–132.

32. Wade, H. E., R. Elsworth, D. Herbert, J. Keppie, and K. Sargeant. 1968. A new L-asparaginase with antitumor activity. Lancet 2:776–777.

33. Wagner, O., K. Bauer, E. Irion, E. Rauenbusch, W. Kaufmann, and A. Arens. 1969. Polyethylene glycol for fractionation and crystallization of L-asparaginase. Angew. Chem. Int. Ed. Engl. 8:885–886.

34. Whelan, H. A., and J. C. Wriston, Jr. 1969. Purification and properties of asparaginase from Escherichia coli B. Biochemistry 8:2386–2393.