L-ASPARAGINASE AND L-ASPARAGINE METABOLISM[1]

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L-Asparagine, one of the first amino acids to be detected [Delaville (1)] and isolated in crystalline form [Vauquelin & Robiquet (2)], was a logical object for study by early enzymologists. Ferments which released volatile base (NH_3) from L-asparagine were reported as early as 1904 [Lang (3)]. Over the next several decades stereospecific asparaginases were detected in the extracts of many gram-negative bacteria, [Aubel (4), Altenbern & Housewright (5), Ulzino & Imaizumi (6), Rowley & Wriston (7)], including *Escherichia coli* [Tsuji (8)] and *Erwinia carotovora* [Wade et al. (9)], mycobacteria [Saitoo (10), Kirchheimer & Whittaker (11)], yeast and molds [Dox (12)], plants [Grover & Chibnall (13)] and vertebrates [Furth & Freidmann (14), Ohnuma et al. (15)]. In 1922 Clementi, a comparative biochemist, suggested that hepatic L-asparaginase was present only in warm blooded vertebrata (16). He advocated the view that L-asparaginase was an adaptation on the part of these animals to a diet containing L-asparagine. However, Steensholt later demonstrated L-asparaginase in the livers of several cold blooded species [Steensholt (17)]. Clementi, however, did make the germinal observation that the blood of guinea pigs contained an active L-asparaginase (16). Apart from these early taxonomic studies and sporadic reports in the botanic literature, general interest in L-asparaginase was minimal. In 1953 Kidd documented the therapeutic effects of guinea pig serum against the Gardner lymphosarcoma (6C3HED lymphoma) in C3H mice (18, 19). Since this chemotherapeutic activity was still present in serum which had been heated at 56° for 30 minutes, it was presumed not to be mediated by complement. Broome, in a series of definitive papers, convincingly related the antitumor properties of guinea pig serum to its L-asparaginase activity (20–22). Later Mashburn & Wriston demonstrated that L-asparaginase from *Escherichia coli* also was effective against the 6C3HED lymphoma (23, 24). An impressive literature has since developed, documenting these findings and extending them to the therapy of cancer in man.

[1] Abbreviations used in this review: Asp (aspartic acid); Asn (asparagine); I.U. (International Units).

Several reviews have appeared on L-asparaginase. The one by Adamson & Fabro (25) contains valuable experimental therapeutic data in tabular form. Others are by Boiron et al. (26), Marquardt (27), Gallmeir & Schmidt (28), Schmidt & Gallmeir (29), Grundmann (30), and Bauer (31). A companion review of therapeutic studies in man with L-asparaginase in Annual Review of Medicine, 1970, should also be consulted [Capizzi et al. (32)].

Isolation and Properties of L-Asparaginase

The need for large amounts of L-asparaginase for clinical studies has promoted a broad search for suitable sources. Although therapeutic effects have been noted with sera from several members of the superfamily Cavioidea [Old et al. (33), Holmquist (34)] and a purification of the enzyme from guinea pig serum has been reported [Meister (35), Yellin & Wriston (36)], these rodents have not been considered to be a practical source of L-asparaginase. The demonstration by Mashburn & Wriston (24), that one of two L-asparaginases present in *E. coli* was at least as active against experimental tumors as the partially purified preparation from guinea pig serum, greatly increased the scope of the search. Subsequently, several isolation procedures were developed [Roberts et al. (37, 38), Campbell et al. (39), Ho & Milikin (40), Bauer (31), Whelan & Wriston (41)] and some of these were adapted to large scale preparation of the enzyme. In essence these involve harvesting mass cultures, rupturing the cells and fractionation with $(NH_4)_2SO_4$, organic solvents, and chromatography. Two of these procedures give a crystalline product [Ho & Milikin (40), Bauer (31)] which is homogeneous [Whelan & Wriston (41), Bauer (31), Ho & Milikin (40)]. Enzyme preparations from *Serratia marcescens* [Rowley & Wriston (7)], *Erwinia carotovora* [Wade et al. (9)] and guinea pig liver [Suld & Herbut (42)] have also shown antitumor activity; but partially purified L-asparaginases from chicken liver [Ohnuma et al. (15)], yeast [Broome (43)], and *Bacillus coagulans* [Mashburn & Wriston (23, 24)] were either inactive or less effective.

Since most clinical studies employ the enzyme from *E. coli,* its properties will be reviewed in some detail. The specific activity of the best preparations has varied between 300 and 625 I.U./mg of protein [Roberts et al. (38), Whelan & Wriston (41), Ho & Milikin (40)]. In part these differences may relate to the protein standard used in the determination and to the conditions of assay. Several preparations have a molecular weight of approximately 130,000 daltons based on sedimentation studies and Sephadex filtration [Whelan & Wriston (41), Frank & Veros (44), Kirschbaum et al. (45)]. It is presumed that the molecule is composed of subunits, since acid conditions, urea, and guanidine can cause dissociation into forms sedimenting more rapidly in the ultracentrifuge. The isoelectric points of various preparations are reported as 4.35 [Roberts et al. (46)] and 4.85 [Whelan & Wriston (41)]; under certain experimental conditions [Mashburn (47)] values as high as 5.61 have been obtained. It may be that these differences

reflect degrees of aggregation or conformational distortion of a basic 130,000 dalton structure.

Amino acid analysis of L-asparaginase after acid hydrolysis indicates the presence of a large number of L-aspartic acid and L-asparagine residues (15 percent of the residues determined) with very low concentrations of L-cystine, L-tryptophan, and L-histidine [Whelan & Wriston (41)]. Since these latter amino acids are present as simple multiples of six, a hexameric structure has been suggested; however, the exact subunit composition of the coliform enzyme is not yet clear.

Numerous methods have been devised for the assay of L-asparaginase activity. Although various units have been used, all references below will be to the standard expression of International Units, micromoles of L-asparagine hydrolyzed per minute at 37°. Although the therapeutically effective enzyme from *E. coli* has a broad pH optimum between pH 5 and 8.5 [Campbell et al. (39)] activity is customarily measured near neutrality. The most common assay procedure is based on the determination of NH_3 by the Nessler reaction [Meister (35)]. This method is suitable for most biological samples at concentrations of enzyme as low as 0.1 International Units/ml. Although good reproducibility can be achieved, the method requires careful attention to reaction conditions and disparate results have been reported from different laboratories on the same sample. To assure the stability of highly purified enzyme during prolonged incubation a stabilizing protein such as albumin must be present. The report of enhancement of coliform L-asparaginase activity by serum [Lee & Bridges (48)] has been attributed by Ho & Jones (49), to stabilization of the enzyme; their studies, carried out at pH 5, might exaggerate this effect. Sensitive alternative methods for the determination of NH_3 have been reported [Mardashev & Lestrovaya (50), Ramadan & Greenberg (51)]. Great sensitivity can be achieved in the assay for L-asparaginase by using ^{14}C-L-asparagine and determining liberated ^{14}C-L-aspartic acid by paper electrophoresis [Broome (52)]. In the authors' laboratories, two other assays are employed. The first, which can detect 0.001 International Units per ml, is based on a spectrophotometric determination at 340_{nm} of the rate of NADH oxidation in a coupled enzyme assay employing excess glutamic-oxaloacetate transaminase and malic dehydrogenase [Cooney & Handschumacher (53)]. A more direct procedure involves the spectrophotometric assay at 274_{nm} of the decomposition of 5-diazo-4-oxo-L-norvaline, an alternative substrate, which gives sensitivity of 0.01 I.U./ml [Handschumacher et al. (54)].

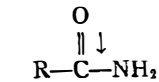
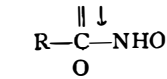
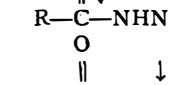
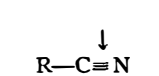
The substrate-specificity of L-asparaginase appears to be restricted to four and five carbon L- or D- α-amino acids with an ω-nitrogen function. Preparations from *E. coli* have shown L-glutaminase activity that is about 2 percent of the L-asparaginase activity [Campbell et al. (39)]. Based on the purification and pH optimum studies, it would appear that the hydrolysis of L-glutamine is an intrinsic property of the enzyme and is not catalyzed by an enzyme contaminant [Campbell et al. (39)]. Several laboratories have

observed L-glutaminase activity in crystalline preparations of L-asparaginase from *E. coli* and we have shown that titration of the active center with 5-diazo-4-oxo-L-norvaline, a specific analogue of L-asparagine, eliminates both enzymatic activities. L-Asparaginase from guinea pig serum, however, does not have detectable L-glutaminase activity [Tower et al. (55)]. D-Asparagine is a poor substrate but a moderately effective inhibitor of the enzymes from *Pseudomonas* and *E. coli* [deGroot & Lichtenstein (56), Handschumacher (57)]. Other substrates, of related structure but low activity, include the β-hydrazide and the β-hydroxamate of L-asparagine [deGroot & Lichtenstein (56)]. It has been reported that preparations from guinea pig serum exhibit minor amounts of amidase activity toward L-leucinamide, L-phenylalaninamide, and L-tyrosinamide [Tower et al. (55)]. Guinea pig serum has also been reported to hydrolyze L-β-cyanoalanine to L-aspartic acid presumably by a nitrilase-type reaction [Giza et al. (59)]. Recent work in this laboratory indicates that L-β-cyanoalanine is converted to NH_3 and L-aspartic acid by a purified L-asparaginase from *E. coli* at 3.5 percent the rate with L-asparagine as substrate [Jackson et al. (58)]. Another unusual catalytic activity of the *E. coli* enzyme is the decomposition of 5-diazo-4-oxo-L-norvaline to N_2 and 5-hydroxy-4-oxo-L-norvaline [Handschumacher (57)]. This reaction is the alternative fate of the enzyme-inhibitor complex discussed below which can also proceed to inactivation of the enzyme by covalent binding to the active site. The enzymic rupture of these C-N bonds can be diagrammed as shown in Table I.

The majority of common enzyme inhibitors have not been reported to inhibit L-asparaginase from *E. coli*. Some of the inhibitors of the guinea pig

TABLE I

Types of C—N Bonds Hydrolyzed by L-Asparaginase (EC-II)

1. Amide	
2. Hydroxamate	
3. Hydrazide	
4. Diazoketone	
5. Nitrile	

enzyme include p-chloromercuriphenyl-sulfonate, $HgCl_2$ zinc ions, D-aspartic acid [Tower et al. (55), α-methyl-DL-aspartic acid, DL-isoasparagine [Lerman & Verevkina (60)] and cysteine sulfonamide [Heymann et al. (61)]. Irreversible inactivation of the substrate site of both the guinea pig and *E. coli* enzymes can be accomplished by exposure to the L-asparagine analogue mentioned above, 5-diazo-4-oxo-L-norvaline [Handschumacher et al. (62), Jackson et al. (58)]. The equivalent analogue of L-glutamine, 6-diazo-5-oxo-L-norleucine, does not cause significant inhibition of L-asparaginase. Titrations of L-asparaginase with its diazoketone analogue suggest that there are four substrate sites on the *E. coli* enzyme and support the concept of a subunit structure. To achieve this site-directed labelling it was necessary to suppress the catalytic decomposition of the diazo-ketone by organic solvents.

L-Asparagine Metabolism

Full appreciation of the action of L-asparaginase requires a consideration of the metabolism of its principal substrate, L-asparagine. The early literature on this subject is primarily limited to a characterization of the intermediary metabolism of L-asparagine in plants [Vickery & Pucher (63); McRary (64); Yamamoto (65); Panalaks et al. (66); Ressler et al. (67)]. Studies in mammalian systems were undoubtedly hampered by the lack of convenient and sufficiently sensitive methods for assay of the amino acid. A number of analytical techniques have been reported for L-asparagine which are fully adequate for their intended use. Release of NH_3 by a purified, specific L-asparaginase, and measurement by the Nessler reagent [Tower et al. (55)], was successfully used to follow the elevated plasma levels of L-asparagine after oral or intravenous administration of large amounts of this amino acid. Greater sensitivity in the detection of the released NH_3 can be achieved by use of the alkaline phenol method [Ramadan & Greenberg (51)]. Another more sensitive method [Broome (68)] utilized the formation of trinitrophenol derivatives of all reactive compounds in deproteinized tissue or plasma extracts, followed by paper chromatography to resolve the L-asparagine derivative, which was quantitated by colorimetry. This method is sensitive but requires careful technique and does not permit large numbers of samples to be processed. Although automated ion exchange chromatography [Oreskes et al. (69)] must be considered the standard against which other methods are compared, adequate resolution requires critical attention to column conditions. Most commonly used elution systems have provided less than totally satisfactory resolution of L-asparagine from either L-glutamine, L-glutamic acid, or L-serine when physiological fluids are being analyzed. The lithium citrate buffer systems, however, provide the best resolution reported to date [Benson et al. (70)]. This laboratory has used a coupled enzymatic assay for the determination of both L-aspartic acid and L-asparagine in various tissue extracts and body fluids [Cooney & Handschumacher (53)]. L-Aspartic acid in the sample is converted to oxaloacetate

by L-glutamic-oxaloacetate transaminase and quantitated by the oxidation of NADH by malic dehydrogenase with measurements at 340_{nm}. Purified L-asparaginase is then added to convert L-asparagine to L-aspartic acid and the additional oxidation of NADH quantitated in a similar manner. This method can detect 5×10^{-9} moles in 1 ml of tissue or plasma extract and permits many simultaneous assays. However, a more simple, sensitive, and rapid assay system for L-asparagine is needed; particularly one that provides instant inactivation of any L-asparaginase that may be present in the biological sample.

Although the biosynthesis of L-asparagine by partially purified enzyme preparations from Novikoff hepatoma [Patterson & Orr (71)] proceeds by a reaction similar to that reported for the synthesis of L-glutamine, there are several significant differences. L-Glutamine is the preferred amido-donor in the biosynthesis of L-asparagine, whereas NH_3 is ordinarily utilized for the synthesis of L-glutamine. In the synthesis of L-asparagine, ATP undergoes pyrophosphorolytic cleavage to AMP and pyrophosphate, but in the synthesis of L-glutamine, a phosphorolytic cleavage of ATP to ADP and phosphate occurs [Meister (72)]. L-Asparagine biosynthesis in *Lactobacillus arabinosus* [Ravel et al. (73)] and in *Streptococcus bovis* [Burchall et al. (74)] follow the general reaction mechanism seen in mammalian cells:

$$\begin{array}{l} \text{L-aspartic acid} \\ + \\ \text{ATP} \\ + \\ \text{L-glutamine or } NH_3 \end{array} \xrightarrow[\text{cation}]{\text{divalent}} \begin{array}{l} \text{L-asparagine} \\ + \\ \text{AMP} + \text{Pyrophosphate} \\ + \\ \text{L-glutamic acid or } H_2O \end{array}$$

The bacterial systems exhibit a cation specificity for Mn^{++} and the mammalian systems for Mg^{++}; nevertheless, the synthetic reactions appear to be similar in most other respects. A report of L-asparagine biosynthesis in embryonic chick liver suggested that the mitochondrial fraction was an obligatory component of the reaction mix [Arfin (75)]; but recent results from this laboratory indicate that the 100,000xg supernatant fraction is also active in the synthesis of L-asparagine when a proper concentration of ATP is supplied. Rat liver microsomes have also been reported to have L-asparagine synthetase activity [Prager & Bachynsky (76)].

Numerous reports have appeared correlating the therapeutic effects of L-asparaginase against experimental neoplasms with low, or unmeasurable levels of L-asparagine synthetase in whole cells or in cell-free extracts [Patterson & Orr (77); Broome & Schwartz (78); Horowitz et al. (79)]. Low or intermediate levels of enzyme activity are found in extracts of normal tissues, with particularly high levels in testes and brain [Horowitz et al. (79)]. In general, resistant tumors, whether naturally resistant or selected for resistance by treatment with L-asparaginase, possess much higher levels of the biosynthetic enzyme than most normal cells. L-Asparagine is a potent feedback inhibitor of the enzyme from two resistant tumor lines [Patterson

& Orr (71); Prager & Bachynsky (76); Broome (80)] and recent unpublished work by Chou, indicates a competitive relationship to the L-glutamine or NH_3 site. Interestingly, L-asparagine synthetase from guinea pig liver is reported to be insensitive to feedback inhibition by L-asparagine [Holcenberg (81)]. The amount of synthetase activity in certain tissues increases in response to metabolic stress, such as treatment with L-asparaginase [Prager & Bachynsky (76)], feeding a diet deficient in L-asparagine, or the presence of a rapidly growing tumor [Patterson & Orr (82)]. These adaptive changes should not be confused with the high constitutive levels of activity in most resistant experimental tumors.

Although the enzymatic reaction given above is the only established mechanism for the biosynthesis of L-asparagine *de novo* in mammalian systems, several alternate possibilities can be suggested. The demonstration that L-glutaminyl transfer RNA from *E. coli* is capable of accepting L-glutamic acid and of amidating the γ-carboxyl, *in situ* [Wilcox & Nirenberg (83)], suggests the possibility of a corresponding reaction in the case of L-asparaginyl tRNA. It is interesting that L-asparagine itself functions as an efficient amido-donor in the amidation of L-glutamic acid affixed to L-glutaminyl tRNA. Another possible minor source of L-asparagine could be dietary L-β-cyanoalanine, derived from plant sources [Ressler et al. (67)]. Although its nitrile group is hydrolyzed to L-aspartic acid by both guinea pig [Giza et al. (59)] and coliform [Jackson et al. (58)] L-asparaginase, hydrolysis only to the carboxamide is biochemically possible, inasmuch as plants apparently employ this reaction in their biosynthesis of L-asparagine [Ressler et al. (67)]. The transamination of α-ketosuccinamic acid could also yield L-asparagine [Meister et al. (84, 85)], but a source of the keto acid, other than from transamination or α-oxidation of L-asparagine [Meister & Wellner (86)], is not known.

The most important macromolecular fate of L-asparagine is its incorporation into protein. The generally accepted code words for L-asparagine in messenger RNA are AAU and AAC [Nirenberg et al. (87); Söll et al. (88)]. The existence of a separate tRNA synthetase for L-asparagine in extracts of *L. arabinosus* has been shown [Hedgcoth et al. (89)]. The profile of tRNAs charged with radioactive L-asparagine shows multiple peaks in extracts of Novikoff hepatoma but only a single major peak in normal liver [Baliga et al. (90)]. These facts, combined with more physiological studies with ^{15}N-asparagine using goat udders [Sansom & Barry (91)] indicate that intact L-asparagine is incorporated into proteins and that the amidation of L-aspartate after peptide bond formation is not a significant source of L-asparaginyl residues in proteins. Evidence also exists that shows that L-asparagine is not in rapid equilibrium with L-aspartic acid after oral or parenteral administration [Breuer et al. (92)].

As a component of the primary peptide structure of most proteins [Damodaran (93)], L-asparagine can have several roles. One well established function of the β-amide is participation in the linkage of oligosaccharides to

glycoproteins [Neuberger et al. (94)] through a 1-L-β-aspartamido-2-acetamido-1,2-dideoxy-β-D-glucose linkage. Although the reaction by which the glucosyl residue is attached to the peptide chain through the β-amide linkage has not been fully elucidated, an enzyme capable of cleavage of this structure has been isolated from lysosomes [Mahadevan & Tappel (95)]. It is of interest that a human genetic defect exists in which this enzyme is presumably deficient, resulting in the urinary excretion of large amounts of β-aspartyl-glucosamine derivatives [Pollitt & Jenner (96)]. An N-substituted L-asparaginyl residue could play a role in the secondary structure of proteins by analogy to the cross-linkage between adjacent peptide chains in thrombin through an ε-amino of lysine to a γ-carboxyl of L-glutamic acid [Waelsch (97)]; however, evidence for this type of bond, which could be formed by a transamidation reaction, has not been seen by the reviewers. Recent work of Woods & Dixon (98) suggests that in addition to its role in protein synthesis, the carbon skeleton of L-asparagine may serve as a direct precursor for some portion of the DNA isolated from lymphosarcoma cells. The amido-N of L-asparagine functions as the preferential amido donor in the synthesis of 5-phosphoribosylamine by preparations from wheat germ [Kapoor & Waygood (99)]; it has also been reported to serve as a precursor in the biosynthesis of carbamyl phosphate by pea seedlings [Kleczkowski (100)].

The prevention by orally administered L-asparagine of the fatty infiltration of the liver caused by ethanol [Lansford et al. (101)] as well as by ethionine and carbon tetrachloride [Alexander et al. (102)], has not yet been explained on a biochemical basis.

Biological Effects of L-Asparagine Deficiency and L-Asparaginase

L-Asparagine is generally not considered to be an essential amino acid; however, it has been possible to demonstrate that the maximal rate of growth of weanling rats is temporarily retarded if L-asparagine is absent from the diet [Breuer et al. (92)]. Although there is only indirect evidence from the toxicologic studies discussed elsewhere, certain normal cell-types responsible for the synthesis and excretion of proteins appear to be at least partially dependent on exogenous L-asparagine, since decreased levels of plasma albumin and clotting factors have been observed during the initial period of therapy with L-asparaginase [Haskell et al. (103); Capizzi et al. (104)]. It is of interest that there appears to be rigorous homeostatic control of the plasma concentration of L-asparagine in a wide variety of disease states in man [Cooney & Handschumacher (53)]. The existence of native L-asparaginase activity in normal liver, kidneys, and the intestinal tract [Broome (52)] may contribute to regulation of the plasma level of L-asparagine. Nevertheless, large oral doses of L-asparagine are absorbed virtually intact from the gastrointestinal tract, creating elevated plasma levels that quickly return to normal [Meister et al. (105); Tower et al. (55)]. Little can be said about the normal source of plasma L-asparagine. In adult animals, a

diet deficient in L-asparagine does not significantly depress circulating levels of this amino acid [Cooney & Handschumacher (53)] despite the comparatively low levels of L-asparagine synthetase (even after dietary induction) in the organs studied to date. Similarly, the locus and mechanism of homeostatic control of L-asparagine remain to be established.

The absolute nutritional requirement of certain neoplastic cells for L-asparagine has been determined in cell cultures of the Walker 256 carcinosarcoma [Neuman & McCoy (106); McCoy et al. (107)], the L5178Y lymphoblastic leukemia [Haley et al. (108)], and 6C3HED lymphoma [Broome (22)]. The former two cells lines require approximately 1×10^{-5}M L-asparagine for optimal growth. Many other sensitive tumor lines must have similar requirements because of their deficiency in L-asparagine synthetase. Still other neoplastic cell lines may exhibit a relative dependency upon external sources of the amino acid. In a study by Eagle et al. (109) the requirement for L-asparagine of an MDAB-hepatoma cell line and the Jensen sarcoma was dependent upon the concentration of cells in the medium. Although many cells may be able to concentrate L-asparagine by an active transport process to concentrations which are five to ten times that in the medium [Broome (80)], considerable efflux occurs if cells are placed in medium free of L-asparagine. Only if the population is sufficiently dense is it possible for the cells to retain sufficient intracellular amino acid for their normal growth and metabolism.

In addition to its antitumor properties, L-asparaginase produces a number of biologic effects on normal cells *in vivo* and on explanted cells *in vitro*. The first wave of mitosis which follows the partial hepatectomy of rats is inhibited by L-asparaginase [Becker & Broome (110)]; and the blastic transformation of peripheral lymphocytes by phytohemagglutinin is retarded by the enzyme [Astaldi et al. (111); Ohno (112); McElwain & Hayward (113)]. L-Asparaginase also appears to cause greater toxicity to cultured lymphocytes of leukemic patients than to those of normal subjects [Schrek et al. (114)]. These inhibitory effects of coliform L-asparaginase on cultured HeLa and other cell types, however, may reflect its coexistent L-glutaminase activity [Kim et al. (115)]. L-Asparaginase is embryotoxic in rabbits [Adamson & Fabro (116)] and interferes with the development of sea urchin eggs from the gastrula stage [Lallier (117)]. Guinea pig serum retards the normal rate of weight gain of young mice [Yellin & Wriston (118)]; this observation may relate to the effect of an L-asparagine-free diet in weanling rats [Breuer et al. (92)]. In summary, it would appear that L-asparaginase antagonizes growth or repair in systems undergoing rapid protein synthesis.

Antineoplastic Activity of L-Asparaginase

L-Asparaginase has been shown to be effective against over fifty neoplasms of the mouse, three of the rat, and canine lymphosarcoma [Kidd (18, 19); Jameson et al. (119); Herbut & Kramer (120); Kwak et al. (121);

Boyse et al. (122, 123); Mashburn & Wriston (23); Old et al. 124, 125); Horowitz et al. (79)]. The majority of susceptible tumors have been lymphoid in type; however, rat sarcoma ACMC42 and the Walker carcinosarcoma are both responsive to the enzyme. Virus-induced murine leukemia in general is not susceptible to guinea pig serum [Boyse et al. (123)] but Rauscher leukemia is responsive to coliform L-asparaginase [Campbell & Levine (126)].

A wide variety of human neoplasms have been treated with L-asparaginase from *E. coli,* but at this time acute lymphocytic leukemia appears to be almost uniquely sensitive. Complete remissions in various series are achieved in 60 to 80 percent of the patients treated [Hill et al. (134); Oettgen et al. (129)]. Although the duration of unmaintained remissions is relatively short, L-asparaginase is considered to be a useful agent for induction of remission, to be followed by maintenance therapy with other agents. Acute myelocytic leukemia has been less responsive ($<$ 20 percent remission rates) and, in general, requires larger doses of L-asparaginase, with an attendant increase in toxicity. Frequently, a transient decrease in circulating leukemic cells is seen after treatment with the enzyme but apparently an adaptive response terminates this action. No consistent response has been obtained in any solid tumors. For a more detailed review of the clinical trials, the reader is referred to the chapter by Capizzi et al. (32).

There has been an intensive search for possible predictive tests to indicate sensitivity of human neoplasms to L-asparaginase. The cytopathology of cells in short-term culture with and without L-asparagine or L-asparaginase has been explored as a measure of potential sensitivity [Dolowy et al. (127); Schrek et al. (114)]. Macromolecular uptake of radioactive amino acids and nucleosides has also been studied in the presence and absence of both L-asparagine and L-asparaginase [Borella et al. (128); Oettgen et al. (129); Sobin & Kidd (130)]. Experience with these tests, however, indicates that although excellent correlation exists with experimental tumors, results have not been consistently satisfactory in human disease. For this reason, studies of the metabolism of L-asparagine itself in sensitive versus resistant tumor cells have been undertaken. Explanted intact malignant cells from resistant experimental tumors were shown to synthesize and excrete L-asparagine into the medium in short-term culture [Broome (80); Chou et al. (131)]; sensitive experimental tumors, on the other hand, produced only minor amounts of L-asparagine. Related studies with cell-free extracts of experimental tumors also indicate an inverse correlation between sensitivity to L-asparaginase and levels of L-asparagine synthetase [Patterson & Orr (77); Horowitz et al. (79)]. The preliminary application of this method to extracts from human leukemia cells appeared to be of no value as a predictive test before treatment [Haskell & Canellos (132)]. Although the plasma concentration of L-asparagine has been reported to be low in many cases of acute lymphocytic leukemia [Ohnuma et al. (133); Capizzi et al. (104)], this measurement has not proven to be useful in predicting potential responsiveness of the disease. Similarly, preliminary measurements of the rate

of turnover of plasma L-asparagine, using 4-^{14}C-L-asparagine, have failed to distinguish between patients with sensitive or resistant tumors when correction is made for the plasma concentration of L-asparagine [Capizzi et al. (104)]. Nevertheless, the presence of an L-asparagine dependent tumor must perturb the metabolism of L-asparagine in the host in a manner which could be discovered and quantitated.

Although resistance to therapy with L-asparaginase in general appears to correlate with the level of L-asparagine synthetase, other mechanisms might be considered. Accelerated clearance of L-asparaginase by immunological phenomena has been reported; this compromises the course of therapy and may be considered a form of host resistance [Hill et al. (134); Capizzi et al. (104)]. Another mechanism of resistance might involve the development, by malignant cells, of more efficient means of extracting L-asparagine, either from the plasma, erythrocytes, or other adjacent cells which have been shown not to be totally depleted by treatment with L-asparaginase [Broome (80); Cooney & Handschumacher (53)].

Biochemical and Biological Effects of Therapy with L-Asparaginase

The most prompt biochemical effect of L-asparaginase is the hydrolysis of the L-asparagine of plasma to L-aspartic acid which tends to accumulate in abnormal amounts [Broome (80); Miller et al. (135)]. In contrast to plasma, the L-asparagine content of canine erythrocytes is slowly depleted by L-asparaginase [Handschumacher (57)]. In humans, a profound depletion of erythrocytic L-asparagine has been reported [Miller et al. (135)]. In biopsies from a dog with lymphosarcoma [Handschumacher (57)] and in necropsies of mice bearing the 6C3HED lymphoma [Broome (80)], L-asparagine in tumor tissues was depressed but never eliminated by L-asparaginase; similarly the hepatic L-asparagine of mice is decreased but not eliminated by treatment with L-asparaginase [Broome (80)]. Because of the L-glutaminase activity of the coliform enzyme [Campbell et al. (39)], elevated levels of L-glutamic acid circulate after injection of the enzyme [Miller et al. (135)]. Ammonia, the other product of this amidohydrolysis, has also been found to accumulate [Hill et al. (134)]. Paradoxically, following therapy of a murine lymphoma with L-asparaginase, Broome has reported a rise in the concentration of L-glutamine in the tumor cells [Broome (80)].

Protein synthesis is the first cellular function to be depressed by L-asparaginase; later the syntheses of DNA and RNA are inhibited [Sobin & Kidd (130); Mashburn & Gordon (136); Ellem et al. (137)]. Associated changes after L-asparaginase treatment are an increase in ribonuclease activity in extracts of lymphosarcoma [Mashburn & Wriston (138); Mashburn & Landen (139)], and a depression of the endogenous RNA polymerase activity of regenerating liver [Becker & Broome (140)]. When therapy with L-asparaginase is terminated, pretreatment concentrations of L-asparagine in plasma are regained very slowly. Even when no enzyme is detectable in the

plasma by a variety of sensitive methods, the amino acid is often depressed [Broome & Schwartz (78); Miller et al. (135); Capizzi et al. (104)]. This delay has been attributed to on-going hydrolysis by sequestered enzyme [Broome (52)]. L-Glutamine, on the other hand, rapidly regains its pretreatment concentration.

Only certain L-asparaginases are oncolytic; of the two coliform enzymes, EC-I, eluted from DEAE cellulose with sodium phosphate buffer, was chemotherapeutically inactive, whereas EC-II, eluted with potassium phosphate, exhibited full antitumor potency [Campbell et al. (39)]. Several groups have studied the biological basis for differences in the therapeutic effects of various preparations of L-asparaginase. Potency has been related to the rate of clearance of the two coliform enzymes; the non-oncolytic EC-I was removed from the plasma so rapidly after intraperitoneal injection that no true clearance could be measured [Boyse et al. (123)]. Similarly rapid clearance of the yeast enzyme has been reported [Broome (43)].

Clearance, however, is not the sole determinant of chemotherapeutic potency. Schwartz et al. (143) have stressed the fact that the affinity of the enzyme for L-asparagine is an important factor in its antitumor activity. Boyse et al. (123) had demonstrated that following treatment of the 6C3HED lymphoma with equal unitage of EC-II or of guinea pig L-asparaginase, the coliform enzyme could temporarily eradicate subcutaneous tumor nodules whereas the enzyme from guinea pig serum effected only a moderate reduction in tumor mass despite approximately equal rates of clearance ($T_{½} = \sim 24$ hr). Broome (144) has suggested that differences in the affinity of L-asparaginase from different sources can account for these observations.

At least one group of agents is able significantly to accelerate the clearance of L-asparaginase. Estrogen treatment of tumor-bearing mice, before therapy with L-asparaginase, reduces by about 35 percent the levels of enzyme attained [Broome (43)], a result that may explain the observation that estrogens antagonize the antitumor effects of guinea pig serum [Kim (145)]. Little is known of the fate of L-asparaginase after its clearance from plasma; Broome (52) has shown that one day after injection the concentration of enzyme activity in the liver is greater than in the plasma. Studies with thoracic duct lymph have shown that L-asparaginase given intravenously appears in lymph at a concentration up to 20 percent of that in plasma [Ho & Frei (146)]. Intramuscular injection gives lower but more sustained blood levels; no enzyme could be detected in the plasma of subjects given oral L-asparaginase. Intravenously administered L-asparaginase is ordinarily undetectable in the urine [Tomao et al. (147)], but minimal levels can be measured radiometrically both in the urine and spinal fluid of some patients given large doses [Ho & Frei (146)]. While it is likely that the enzyme alone, or an enzyme-antibody complex, is phagocytized by the reticuloendothelial system, to date this phenomenon has not been directly demonstrated.

Trials of coliform L-asparaginase against murine neoplasms showed that therapy on the day of inoculation was considerably inferior to therapy of an established tumor [Boyse et al. (123)]. It was demonstrated that a reduced rate of clearance of the enzyme from the plasma caused by the LDH virus, present in the tumor inoculum, was responsible for this puzzling chemotherapeutic effect [Old et al. (141); Riley (142)]. If mice carried the LDH agent prior to inoculation of tumor, early therapy with L-asparaginase was equally as effective as delayed therapy. The half-life of coliform L-asparaginase in mice infected with the LDH virus is from 19–24 hours compared to 2–3 hours in uninfected mice [Boyse et al. (123); Broome (52)]. In monkeys the $T_{1/2}$ is about 12 hours, in dogs about 16 hours [Schein et al. (148)], and in man between 8 and 26 hours, depending on the preparation [Tomao et al. (147)].

The route of parenteral administration (intraperitoneal, subcutaneous, or intravenous) of L-asparaginase has not been found to alter significantly the oncolytic potency [Boyse et al. (123)].

It must be assumed that the primary action of L-asparaginase is the elimination of circulating pools of L-asparagine. Although the enzymatic activity achieved in the plasma is ordinarily far in excess of that required to eliminate L-asparagine from the circulation, improved effects are sometimes seen at higher doses; these might reflect a more effective reduction of L-asparagine pools in the interstitial spaces, or even within certain target cells with limited ability to synthesize this amino acid. For example, it is interesting that treatment of intracerebral 6C3HED lymphoma in mice, by intraperitoneal administration of *E. coli* or guinea pig serum L-asparaginase has caused prolonged regression of established tumors [Dolowy et al. (149)], although there is minimal entry of L-asparaginase into the cerebrospinal fluid. The absence of the amino acid in the plasma apparently leads to its eventual depletion in the CSF and the consequent therapeutic effect. The alternative of another metabolite in mammals, aside from L-asparagine and L-glutamine, which might serve as a substrate for the enzyme, cannot however be eliminated at this time. If the affinity of the enzyme for this conjectural substrate is low, higher concentrations of enzyme will be needed to hydrolyze it and a dose-response pattern will emerge.

The therapy of experimental and human neoplasms with L-asparaginase has evoked a significant number of allergic reactions, but their incidence has not been as great as anticipated. Of particular interest is the absence of allergy in some patients maintained for several months on L-asparaginase after the induction of a complete remission. The generally low incidence of allergic reactions may reflect a form of immune paralysis created by the large amounts of bacterial protein (20–250 mg/day) given for 20 or more days. Alternatively, the biochemical action of the enzyme may comprise the ability of the host to exhibit a normal response. Despite these considerations urticarial reactions in humans have appeared with moderate frequency [Hill et al. (134); Oettgen et al. (129); Haskell et al. (103)]; and precipitating

antibodies have been detected in mice [Roberts et al. (37); Vadlamudi et al. (150]; rabbits [Schein et al. (148)]; and a significant percentage of human subjects [Oettgen et al. (129); Khan (151); Capizzi et al. (104; Peterson et al. (152)]. Moreover, in the course of continued therapy, an abnormally rapid disappearance of L-asparaginase from the plasma has sometimes developed in certain patients whose initial clearance of the enzyme had proceeded at the normal rate. Precipitating antibodies have sometimes accompanied this accelerated clearance. The appearance of precipitin has also been accompanied by diminished therapeutic efficacy of the enzyme in mice [Roberts et al. (37)] and man [Peterson et al. (152)]. Although the antigen-antibody complex is likely to be cleared rapidly by the reticuloendothelial system, it is not necessarily inert: evidence of catalytically active enzyme-antibody complexes has in fact been obtained in several patients [Peterson et al. (152)].

The occurrence of anaphylactic reactions in man apparently is somewhat dependent upon the preparation of enzyme used, but preliminary reports suggest that it is a general phenomenon attributable to the proteinaceous nature of the agent [Haskell et al. (103); Capizzi et al. (104)]. Although cross-reaction has been noted between the enzyme from *E. coli* and from *S. marcescens* [Boyd (153); Khan & Hill (154)], preparations of L-asparaginase from *E. carotovora* do not significantly cross-react with the enzyme from *E. coli* [Wade et al. (9); Peterson et al. (152)]. This has permitted the successful continued treatment with enzyme from *E. carotovora* of an L-asparagine-dependent neoplasm after the occurrence of an allergic reaction to enzyme from *E. coli* [Alexander (155)].

Toxicity

The most general toxic effect of coliform L-asparaginase is inanition [Schein et al. (148)]. Mice, monkeys, dogs, and human beings lose appetite and weight during treatment with large doses of enzyme [Haskell et al. (103); Schein et al. (148); Oettgen et al. (129); Hill et al. (134)]. Although fatty metamorphosis of the liver has been reported in monkeys and man treated with EC-II [Schein et al. (148); Gross et al. (156); Haskell et al. (103); Hill et al. (134)] pure preparations do not cause this change in mice [Gross et al. (156)]. Moreover, in a limited toxicologic trial, L-asparaginase from guinea pig serum did not cause fatty livers in monkeys [Dolowy et al. (158)], so that L-asparagine deprivation may not be the sole causal factor of hepatic steatosis. Although pre- or post-treatment with oral L-glutamine or L-asparagine failed to prevent or correct the hepatic fatty changes in monkeys given 1000 I. U. of EC-II kg/day × 5, it should be stressed that these amino acids had to traverse a plasma compartment whose amidohydrolytic capacity was tremendous.

Bromsulfonphthalein retention provides the most consistent index of L-asparaginase hepatotoxicity in monkeys. If therapy is continued, and the diet is enriched in protein and vitamins, the retention of dye disappears [Rakieten et al. (157)]. An analogous acclimatization has been seen in the

clinic: abnormalities of hepatic function, including derangements of the clotting mechanism [Haskell et al. (103); Gralnick & Henry (159); Khan (151)] have been found to return to normal despite continued treatment with L-asparaginase [Capizzi et al. (104)]. The liver apparently adapts to continuous deprivation of L-asparagine by a documented increase in L-asparagine synthetase. In addition to lowered levels of clotting factors, rather consistent hypoalbuminemia and hypocholesterolemia have been noted in patients receiving the enzyme [Canellos et al. (160)]. It is likely that the synthesis of still other proteins will be found to be depressed by L-asparaginase when a thorough search is made.

Preliminary evidence of pancreatic, central nervous system, and renal toxicity has appeared at high and intermediate dosages of enzyme [Haskell et al. (103)]. Although elevated levels of blood ammonia were seen in a number of patients [Hill et al. (134)] the relationship of this finding to clinical symptoms of neurotoxicity is unclear. Rabbits, of the lower species studied to date, are uniquely sensitive to this neurotoxic action of L-asparaginase [Oettgen et al. (129)], but the signs of intoxication—consisting of fasciculations and fatal convulsions—are of such irregular incidence, that adequate study has been difficult.

It should be mentioned lastly that, apart from weight loss, dogs are resistant to virtually all of the toxic effects of the enzyme [Schein et al. (148)].

Combination Chemotherapy

Combination chemotherapy with L-asparaginase and other agents has met with considerable success in certain experimental tumor systems. An additive effect of L-asparaginase and bis-β-chloroethyl nitrosourea against the intracerebrally-inoculated L-asparaginase-susceptible leukemias L5178Y and EARAD1 has been demonstrated [Burchenal & Dollinger (161)]. L-Asparaginase is also additive or synergistic with 6-hydroxylaminopurine riboside and diphenyldiacetyl urea diguanylhydrazone. When the L-glutamine analogues, azaserine or azotomycin were used in conjunction with the enzyme in the therapy of the L5178Y ascites tumor, additive effects were also seen [Jacobs et al. (162)]. Using the P1798 lymphosarcoma a significant increase in survival was observed when L-asparaginase was given before azaserine or vinblastine, and when azaserine was alternated with the enzyme [Mashburn (163)]. However, neither 6-diazo-5-oxo-L-norleucine nor azaserine, could confer sensitivity on leukemia L1210, which is resistant to L-asparaginase alone.

In contrast to these encouraging experimental studies, a preliminary clinical trial of L-asparaginase and oral azaserine [Leventhal (164)] did not afford a significant potentiation in the treatment of lymphoblastic leukemia of childhood.

Alternate Modes of L-Asparagine Deprivation

Theoretically, a state of L-asparagine depletion of dependent cells could be produced by means other than parenteral therapy with L-asparaginase.

Preliminary hemodialysis studies have revealed that the membranes of the clinically-useful dialysis machines will ultrafilter L-asparagine in large amounts over a 6-hour period, but homeostatic mechanisms prevent a decrease in the plasma concentration of free L-asparagine [Handschumacher (57)]. The introduction of L-asparaginase into the outer bath-fluid might augment the rate of removal of substrate, but to date this experiment has not been reported. Dietary deprivation of L-asparagine is a second possible means of lowering L-asparagine in the body fluids, since the amino acid is a constituent of vegetables and fruits, as well as most proteins; however, in mice, an L-asparagine free diet does not significantly lower the concentration of L-asparagine in the plasma [Cooney & Handschumacher (53)]. Undoubtably, enhanced biosynthesis of L-asparagine by many tissues [Prager & Bachynsky (76); Patterson & Orr (82)] compensates for the deprivation.

Analogues of L-asparagine should provide another reasonable means of achieving inhibition of the synthesis of L-asparagine because of the potent feedback control which it exerts on its own synthesis in most mammals. 5-Diazo-4-oxo-L-norvaline, a diazoketo analogue of L-asparagine and alkylator of L-asparaginase (see above), and 5-chloro-4-oxo-L-norvaline are effective inhibitors of L-asparagine synthesis by whole cells and cell free extracts [Chou et al. (131)] but the potential of these agents as synergists of L-asparaginase in the treatment of experimental tumors has not yet been fully explored. While it might be possible to reduce the biosynthesis of L-asparagine with analogues of the precursors of its synthesis—ATP, L-aspartic acid, and L-glutamine or NH_3—these substances are of such general importance that excessive toxicity might be anticipated. It should be noted, though, that inhibition of the biosynthesis of L-asparagine *in vitro* has been reported with the analogues of L-glutamine: azaserine and 6-diazo-5-oxo-L-norleucine [Patterson & Orr (71)]. Lastly, the catalysis of the hydrolysis of L-asparagine by several rare earth metals, particularly lanthanum, might be considered for future exploration [Bamann & Trapmann (165); Kyker (166)].

Much of the work in this field is still in progress and therefore unavailable for citation at this time. Apologies must be made to those whose efforts have not been noted, and for omissions in attribution. Although at present the therapeutic efficacy of L-asparaginase in the treatment of human disease appears to be limited to the prompt induction of remission in both virgin and veteran cases of acute lymphocytic leukemia, this enzyme has engendered a major interest in the potential use of other enzymes in the therapy of human disease. Much also remains to be done to exploit fully the unique nutritional requirement of some cells for L-asparagine.

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