REVIEW OF LITERATURE

L-Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) is not especially remarkable as an enzyme. What is unusual about asparaginase, and constitutes the feature responsible for the considerable attention it has attracted, is its antineoplastic activity. This enzyme is a valuable chemotherapeutic agent in the treatment of certain leukemias and a variety of other malignancies in man (Tallal et al., 1970; Crowther, 1971; Jaffe et al., 1971; Ertel et al., 1979; Advani et al., 1983; Abuchowski et al., 1984; MacEwen et al., 1987; Ridgway et al., 1989). But, all L-asparaginases do not possess antineoplastic activities and also are not clinically useful.

DISCOVERY :

The discovery that L-asparaginase has a useful place in the treatment of some forms of malignant disease in man was promoted by Kidd (1953; 1953a), who observed striking regressions after the injection of normal guinea pig serum in mice and rats bearing certain transplanted lymphomas. Sera of other species, such as, horse, rabbit, and human, were not effective. Not long alter Kidd's discovery McCoy et al. (1959) reported the results of a nutritional study of animal cancer cells grown in laboratory culture. Broome (1961), convinced himself that Kidd's results could not be attributed to immunological rejection. Searching for nonimmunological differences between guinea pig serum and the serum of other animals, Broome found that in 1922 Clementi investigated the distribution of amino-acid degrading enzymes in the tissues of different animal species, and observed that L-asparaginase was present in the liver and kidney of many species, but in the blood of only one of the animals that is the studies - guinea pig. Broome (1963, 1963a) then demonstrated that the enzyme of guinea pig serum, called asparaginase is the antileukemia 'factor' responsible for the Kidd's phenomenon. Sera lacking L-asparaginase have no antihymphoma properties. The tumor inhibitory agent and L-asparaginase seemed to be identical in several physical and chemical properties examined, indicating thereby they are one and the same. The antilymphoma activity of L-asparaginase has been confirmed by further works from several laboratories (Mashburn and Wriston, 1963; Broome, 1963; Old et al., 1963; Yellin and Wriston, 1966; 1966a), and is now generally accepted.

MODE OF ACTION :

The simplest exaplanation for the oncolytic activity of asparaginase is that it deprives the tumor of asparagine, whose, only known vital function in mammalian cells is in protein synthesis (Cooney and Handschumacher, 1970; Broome, 1967, 1968; Capizzi et al., 1970; Wriston, 1971; Crowther, 1971). L-Asparaginase exerts its antitumor activity by hydrolysis of asparagine to aspartic acid and ammonia. The depletion of asparagine selectively affects those leukemia cells which possess low levels of asparagine synthetase (Ohnuma et al., 1971). In the absence of asparagine, a decrease of protein and glycoprotein synthesis occurs which ultimately leads to leukemic cell death (Bos mann and Kessel, 1970; Kessel and Bos mann, 1970; Chong and Chang, 1977). During therapy, L-asparagine in the plasma has been reported to fall to undetectable levels (Miller et al., 1969); moreover, in some cases the depression of plasmatic L-asparagine has been protracted (Capizzi et al., 1969). Sobin and Kidd (1965) and Kidd and Sobin (1966, 1966a) showed the effect of guinea pig serum on nucleic acid synthesis appears to be secondary and probably results from the curtailed synthesis of proteins. Bos mann and Kessel (1970) suggested that the rapid turnover rate of membrane glycoproteins explains the rapid cell lysis caused by asparaginase. Broome (1968) proposed that the synthesis and utilization of asparagine were linked, although not necessarily anatomically. Such a link could be an asparaginyl - tRNA ligase with a low K_m, which would permit continued high turnover rates of asparagine at low steady-state levels of the amino acid.

In addition to its effects on protein and nucleic acid synthesis, asparaginase has been shown to cause a number of other alterations in tumor cells which have been suggested as factors contributing to its oncolytic action; examples are changes in the levels of free amino acids (Broome, 1968) and increases in ribonuclease activity (Mashburn and Gordon, 1968; Mashburn and Wriston, 1966; Mashburn and Landin,1968).Several reports have appeared suggesting that E.coli asparaginase acts on cell-surfaces or other macromolecular substrates. The electrophoretic mobility of human peripheral blood lymphocytes was reduced after treatment with the enzyme in vitro (Lajolo et al., 1970). Dods et al. (1972) reported that L-asparaginase (1.5 µg/ml) caused rapid lysis of ghost plasma membranes isolated from cells of Leukemia L5178Y. The study did not include ghosts of other cell lines (e.g. asparagine-resistant tumors) or evidence linking the action of the enzyme preparation to its L-asparaginase activity. Since, rapid lysis is observed when washed L5178Y

cells are put into an asparaginase-free medium (Summers and Handschumacher, 1971), it is not necessary to invoke a direct action of the enzyme on some surface structure to explain its oncolytic activity. Recent re-examination of asparaginase's ability to inhibit mitogenesis, Kefkewitz and Bendich (1984) utilized the asparaginase produced by the bacterium V.succinogenes. They have taken advantage of the recently developed technology of covalent modification of enzymes with methoxy-polyethelene glycol (PEG). PEG-asparaginases are non-immunogenic and non-antigenic also (Abuchowski et al, 1977; Bendich et al., 1982).

L-Asparaginase of E.coli and some other sources, which have limited therapeutic effectiveness against solid tumors in man, is an effective agent for the treatment of acute lymphocytic leukemia (Clarkson et al., 1970). The result has led to the use of other amino acid degrading enzymes for cancer treatment, since tumor cells may lack those enzymes which catalyse the production of non-essential amino acids (Holcenberg, 1983). In tissue culture, glutaminase-asparaginase, which has been purified from Acinetobacter glutaminasificans selectively kills human leukemic leukocytes at approximately one-hundredth the effective concentration of E.coli asparaginase (Schrek et al., 1972) and dictate their selective cytotoxic effects. However, succinylated glutaminase-asparaginase of the Acinetobacter causes considerable neurotoxicity in adult patients (Warrell et al., 1980).

The low levels of asparaginase present in guinea pig serum made it necessary to seek a more practical source of the antineoplastic enzyme. In 1964, Mashburn and Wriston indicated that asparaginase derived from E.coli was an effective anti-tumor agent as that derived from guinea pig serum, thus facilitating the production of larger supplies of the enzyme for further study. The intense oncolytic effect of the enzyme against a spontaneously occuring canine lymphosarcoma drew attention to the asparaginase as a possibly useful drug in clinical medicine (Old et al., 1967). Subsequent clinical trials showed significant activity against acute lymphoblastic leukemia with both the partially purified enzyme from guinea pig serum and E.coli (Dolowy et al., 1966; Hill et al., 1969; Oettgen et al., 1967).

DISTRIBUTION :

Asparaginases are found in diverse sources in nature, including bacteria, mycobacteria, yeasts, molds, microalgaes, plants and vertebrates (Wriston and Yellin, 1973; 1981; Imada et al., 1973; Bilimoria, 1969; Wade et al., 1971; Paul and Cooksey, 1979; 1981; Reddy et al., 1969; Singh & Sukumaron, 1986; Yurek et al., 1983). However, micro-organisms are considered as a major source for practical clinical use. And, among the micro-organisms, the appreciable enzyme activities occured in most members of the family Enterobacteriaceae (Imada et al., 1973). Arima et al. (1972) noticed that many bacteria (mainly Pseudomonas sp.), fungi (mainly Penicillium sp. and Aspergillus sp.), and yeasts produced extracellular L-asparaginase. However, antilymphosarcoma activity was only noticed in the enzymes from Enterobacteriaceae, and some of these enzymes are now used for clinical experiments. Moreover, the content and characteristics of the enzyme may differ even among various strains of the same micro-organism. Recombinants of E.coli have been isolated whose content of asparaginase is greater than E.coli A-1 (Barnes et al., 1977; 1978). The need for large quantities of enzyme for therapeutic work has promoted a search for L-asparaginase in various micro-organisms, as they lend themselves to large-scale production.

L-Asparaginase activity was found in varying quantities in different organisms, and appears to be more common among Gram-negative bacteria. Anti-tumor activity was shown by the enzymes from E.coli, Serratia marcescens (Rowley and Wriston, 1967), Erwinia carotovora (Wade et al., 1968), Erwinia aroideae (Peterson and Ciegler, 1969), Proteus vulgaris (Tosa et al., 1971; 1972), Mycobacterium tuberculosis (Jayaram et al., 1968; Reddy et al., 1969), Vibrio succinogenes (Distasio et al., 1976) recently renamed as Wolinella succinogenes, by Tanner et al. (1981); Ammon et al. (1985).

Scheetz et al. (1971) purified an L-asparaginase from mycelia of Fusarium tricinctum and found the enzyme to be inactive against 6C3HED lymphosarcoma. In 1972, Imada et al. purified the enzyme partially from Tilachlidium humicola, which did not suppress the growth of the lymphosarcoma in mice. De-Angeli et al. (1970) reported that L-asparaginase from mycelia of Aspergillus terreus suppressed Walker 256 ascites carcinoma in rats. Two partial purifications of a Saccharomyces asparaginase have been reported (Broome, 1965; Abdumalikov and Nikolaev, 1967) but this enzyme proved not to have antilymphoma activity. Moreover,

asparginase preparations from culture filtrates of Candida utilis, Hansenula jadini and Rhodotorula rubra did not suppress the growth of tumor (Imada et al., 1972).

A total of 45 species of animals have been examined, but serum asparaginase is found in more than trace amounts only in the guinea pig and other members of the superfamily Cavioidea, and in New World Monkey (Yurek et al., 1983). A partial purification from asparaginase from agouti serum, which contain several times as many units/ml as the guinea pig, has been reported but this enzyme has resisted further purification attempts due to its apparent instability (Calich and Guimaraes, 1976; Wriston, 1981; 1973; 1985).

BIOSYNTHESIS :

Here, the in vivo biosynthesis of the enzyme in different bacteria are discussed. L-Asparaginase activity in the micro-organisms was found to vary with growth conditions (Cedar and Schwartz, 1968; Heineman and Howard, 1969; Boeck and Ho, 1973; Liu and Zajic, 1973; 1973a; Netrval, 197,3; Barnes et al., 1977; 1978; Kafkewitz and Goodman, 1974; Albanese and Kafkewitz, 1978; Mikucki and Szarapinska-Kwas, 1979). In E.coli excessive aeration will decrease enzyme synthesis; however, moderate aeration without dissolved oxygen resulted in high enzyme production (Roberts et al., 1968; Barnes et al., 1978).

In Eraroideae (Liu and Zajic, 1973), using yeast extract as a growth-limiting substrate, the relationship between specific growth rate and substrate concentration was determined to fit the Monod equation. The optimum temperature for enzyme production was 24°C, although cell growth was higher at 28°C. In continuous process it was 24°C, and was the same in the batch process. Increasing the temperature from 24 to 28°C resulted a 20% loss of the enzyme yield. Bascomb et al. (1975) reported that highest enzyme yield of the Citrobacter was obtained in corn-steep liquor medium (9.2% w/v) at 37°C. Oxygen limitation was not necessary for high enzyme yield. The L-asparaginase EC-1 isoenzyme of E.coli ATCC 9637 was synthesized in the cells constitutively and its synthesis was not markedly affected by any of the conditions tested (Svobodova and Strbanova-Necinova, 1973).

The synthesis of L-asparaginase II of E.coli is significantly decreased by the addition of glucose to the growth medium, and this effect has been ascribed to

catabolite repression (Cedar and Schwartz, 1968). The role of sugars in decreasing the production of enzyme, L-asparaginase-II was determined in the presence and absence of catabolite-activating protein (CAP), in E.coli (Emmer et al., 1970; De Crombrugghe et al., 1970). Chesney (1983) demonstrated that asparaginase II production was dependent on CAP. The rumen anaerobe V.succinogenes showed best growth when fumarate was provided as the terminal electron acceptor of the formate-oxidizing cytochrome system (Albanese and Kafkewitz, 1978). Yeast extract or enzyme hydrolyzed proteins are effective nutrient sources. The reducing agent added to provide anaerobiosis may be 0.05% sodium thioglycolate 0.05% sodium sulfide, or 0.05% cysteine-hydrochloride (Kafkewitz, 1975).

In Lactobacillus plantarum (Nalepka et al., 1981) the L-asparaginase activity could be significantly increased when the growth medium was supplemented with L-asparagine. Barnes et al. (1978) reported that L-asparaginase synthesis in the recombinants of E.coli A-1 was increased by the addition of oxaloacetate as well as other members of the tricarboxylic acid cycle. In 1971 Tosa et al., found that the enzyme was produced in higher amount when cells of P.vulgaris were grown aerobically in a medium containing sodium fumarate and corn steep liquor and the addition of glucose or ammonium ion to the medium, however, resulted in depressed production of L-asparaginase. Roberts et al. (1968), observed that the good enzyme production of strain E.coli HAP was associated with media containing L-glutamic acid, L-methionine, and lactic acid, and the sodium ion appeared to suppress L-asparaginase production, but the highest yield of enzyme was obtained when cells were grown aerobically.

The L-asparaginase production was also investigated in five strains of S.marcescens in shake-flask experiments and were found to produce the maximum amount of enzyme at 48 h after inoculation (Heinemann and Howard, 1969). Imada et al. (1973) were unable to detect L-asparaginase production by the strains of Staphylococcus sp.. Mikucki and Pluta, (1976) however, showed that all these strains produced L-asparaginase but at a varying levels. Mikucki and Sparapinska-Kwaszewska, (1979), deduced that the staphylococcal L-asparaginase is not induced by the substrate L-asparagine.

PURIFICATION, PROPERTIES, AND COMPARATIVE BIOCHEMISTRY OF L-ASPARAGINASE :

Several L-asparaginases have been so far purified from different micro-organisms to study their antineoplastic activities. Few of them are extensively used and relevant for the treatment of antineoplastic diseases. Here the review has been restricted and centralized only on the aspects of asparaginases of E.coli, guinea pig serum, Er.carotovora, V.succinogenes, S.marcescens, and P.vulgaris.

E.coli asparaginase :

L-Asparaginase content varies widely in different E.coli strains, some having no activity at all. Two asparaginases with properties unmistakeably different from each other are present in at least two E.coli strains. Among these, two asparaginase designated EC-1 and EC-2 have been found in E.coli B (Campbell et al., 1967; Mashburn and Wriston, 1964), and Schwartz et al. (1966). The EC-1 enzyme from E.coli B, and asparaginase I from K-12 do not have antilymphoma activity, but the others would be taken to mean the enzyme with antilymphoa activity. The two enzymes of E.coli B differ with respect to their solubility and chromatographic behaviour as well as their activity as a function of pH. The amount of each enzyme present in a mixture can be determined by a comparison of the total L-asparaginase activity at pH 5.0 to that at pH 8.4. The procedure described by Whelan and Wriston, (1969) for the purification of L-asparaginase from E.coli B involved 5 steps from the crude extract, four of them chromatographic or electrophoretic, and gave homogeneous enzyme with a 15% recovery. Arens et al. (1970) described a simpler procedure suitable for large-scale purification (applied to E.coli ATCC 9637 cells). Their procedure involved heat-treatment, 2 solvent fractionations, and a gel filtration step, and gave homogeneous enzyme with an overall yield of 4.5%. Ho et al. (1970) have also described a 5 step procedure involving 2 chromatographic steps that yields crystalline enzyme with a 16% yield. The specific activity of all these preparations is about the same (approx. 280 IU/mg protein). In all these cases the enzyme is a tetramer with identical sub-units. Amino acid compositions of several different E.coli asparaginases have been established (Kristiansen et al., 1970; Nakamura et al., 1971) and one enzyme, from E.coli A-1-3, was completely sequenced (Maita and Matsuda, 1980).

E.coli asparaginase hydrolyzes L-glutamine and D-asparagine at 4 to 5% respectively, of the rate at which it hydrolyzes L-asparagine, but is not inhibited by L or D-aspartic acids or by L or D-glutamic acids (Petz et al., 1979). There are sequence homologies between E.coli asparaginase and glutaminase-asparaginase enzymes from Acinetobacter and Pseudomenas 7A, but the site labelled by [^{14}C] diazooxonorleucine (DON) in the mixed amidases is different from that labelled by [^{14}C] DONV (which is an inhibitor or an alternate substrate, depending on reaction conditions) in E.coli asparaginase (Holcenberg et al., 1975; 1979; Steckel et al., 1983). An apparent K_m of 1.25×10^{-5} M has been reported by Broome (1968). At 0.1% enzyme concentration the MW, as determined by sedimentation equilibrium, is approx. 125,000. Numerous modifications and coupling studies have been carried out with asparaginase for a variety of purposes, such as, to study factors influencing clearance from the circulation, to modify antigenicity, and to provide clinically more useful forms (Allison et al., 1972; Aplin and Wriston, 1981; Nickel et al., 1982; Ashihara et al., 1978; Matsushima et al., 1980).

Guinea pig serum asparaginase :

Yellin and Wriston, (1966) purified the asparaginase from guinea pig serum. It was purified by 4 steps of the purification procedure : i) Fractionation with Na_2SO_4, ii) Sephadex G-200 gel filtration, iii) DEAE-cellulose column chromatography, and iv) Calcium hydroxylapatite column chromatography. The specific activity of the purified enzyme was 46.5 units/mg protein and was an overall 9.4% recovery. The enzyme is stable for at least 6 months at -20°C; to repeated freezing and thawing; and to heating at 55°C for 10 min (Tower et al. 1963; Yellin and Wriston, 1966). The enzyme has a MW of approx. 138,000. The $S_{20,w}$ is 6.55_S. The optimal pH range in 0.1 M sodium borate buffer is from 7.5 to 8.5 (Yellin and Wriston, 1966a),although Tower et al. (1963) reported a pH optimum of 9.6 for 100-fold purified material in buffers of lower ionic strength than those used by Meister (T/2 = 0.2 instead of 0.4). The K_m value has been reported as 2.2×1.0^{-3} M (Tower et al., 1963). The enzyme is inhibited by pCMB and $HgCl_2$ (40 and 83% inhibition, respectively at 0.1 mM) but not by L-glutamic and or L-glutamine, 10 mM, of a large number of amides examined, only L-asparagine (100%), D-asparagine (3%), L-leucine amide (5.3%), L-phenylalanine amide (8.2%), L-tyrosine amide (8.5%) were hydrolyzed (Tower et al., 1963; Meister et al., 1955). L-Glutamine was not hydrolyzed. Guinea pig serum asparaginase also catalyzes hydrolysis of L- B -aspartyl

hydroxylamine, and synthesis of the hydroxamate from asparagine and hydroxylamine, but all these reactions proceed much more slowly than the hydrolysis of L-asparagine (Meister et al., 1955).

The enzyme is responsible for the antilymphoma activity of guinea pig serum (Broome, 1963; 1963a; Mashburn and Wriston, 1963), but because of the difficulty of obtaining sufficient quantities of purified material, most of the clinical trials designed to explore the chemotherapeutic potential of asparaginase have been carried out with the E.coli enzyme (Dolowy et al., 1966; Oettgen et al., 1967). The most important feature related to guinea pig serum asparaginase is that the enzyme is free of L-glutaminase activity as contamination and the enzyme showed no immunosuppression during the treatment of neoplasia (Capizzi and Cheng, 1981).

E.carotovora asparaginase :

Laboureur et al., 1971 stated that Erwinia, Proteus, Serratia showed only a single asparaginase but two isoenzymes may exist simultaneously in Pseudomonas. However, not all asparaginases are clinically useful. The enzymes derived from E.coli and Er.carotovora have adequate enzymatic activity and circulation half-lives to be of clinical value (Crowther, 1971; Cooney and Handschumacher, 1970). Although the bacterial L-asparaginases from E.coli and Erwinia have similar molecular weights (approx. 140,000), the difference in isoelectric points (4.5 for E.coli asparaginase II and 8.5 to 9.0 for Erwinia) would suggest that these two proteins are not homologous (Wade et al., 1968; North et al., 1969). E.carotovora L-asparaginase has a K_m of $\sim 10^{-5}$ and V_{max} of 0.9 μmole of ammonia released/min/unit of enzyme for L-asparaginase and 1.1×10^{-3} M for L-glutaminase.

The covalent attachment of poly-DL-alanine peptides to lysyl residue on the surface of E.carotovora L-asparaginase has produced a modified enzyme which is much less immunogenic in mice and demonstrates 100-fold longer plasma half-life in the Rhesus monkey (Uren et al., 1982). A direct comparison between the asparaginases from E.coli and Er.carotovora has been made by Cammack et al. (1972), who found that the Erwinia enzyme is more stable than the E.coli enzyme in the alkaline pH region. In contrast with the E.coli asparaginase (Lilly), Erwinia asparaginase dissociates only to the extent of about 50% on 48 h of exposure to

8 M urea in 0.2 M phosphate buffer, pH 7.4. Full dissociation of the Erwinia enzymes can be achieved only at pH values well away from the isoelectric region (8.5 to 9.0).

V.succinogenes asparaginase :

L-Asparaginase of this organism is associated with the cytoplasm (Krautheim et al., 1982). Homogeneous L-asparaginase with anti-lymphoma activity was prepared from V.succinogenes (now renamed as W.succinogenes), an anaerobic bacterium from the bovine rumen. An overall yield of pure asparaginase of 40 to 45% and a specific activity of 200 ± 2 IU/mg protein were obtained (Distasio et al., 1976). The pure enzyme can be stored at $-20^{o}C$ for atleast 3 months with no loss of activity. The isoelectric point of the asparaginase is 8.74. No carbohydrate, phosphorus, tryptophan, disulfide or sulfhydryl groups were detected. The enzyme has a MW of 146,000 and a subunit MW of approx. 37,000. The K_m of the enzyme for L-asparagine is 4.78×10^{-5} M and the pH optimum of the asparaginase reaction is 7.3. D-Asparagine was hydrolyzed at significant rates; the activity of the enzyme for L-glutamine was 130 to 600 fold less than that of other therapeutically effective L-asparaginases of bacterial origin (e.g., E.coli). The L-asparaginase from V.succinogenes is immunologically distinct from the L-asparaginase (EC-2) of E.coli.

Normal dogs were able to tolerate large doses of polyethylene glycol conjugates with V.succinogenes L-asparaginase (PEG-asparaginase) without any significant toxic reactions. MacEwen et al. (1987) reported that PEG-asparaginase has anti-tumor activity in canine malignant lymphoma when used alone or in combination with other chemotherapeutic agents. Both PEG-asparaginases (or E.coli and V. succinogenes) have circulating half-lives ($t_{\frac{1}{2}}$) of about 4 days. The native E.coli enzyme has $t_{\frac{1}{2}}$ of less than 6 h; the native V.succinogenes enzyme has a $t_{\frac{1}{2}}$ too short to be determined accurately in the studied conditions. Both PEG-asparaginases were found to be effective anti-tumor agents with comparable activity (Abuchowski and Davis, 1981). Durden and Distasio (1980; 1981) have presented data comparing the immunosuppressive effects of asparaginase EC and asparaginase VS. They concluded that glutaminase free asparaginase VS is non-immunosuppressive, whereas asparaginase EC causes immunosuppression.

S.marcescens asparaginase :

Boyd and Phillips, (1971) purified L-asparaginase from S.marcescens ATCC 60. The specific activity of the homogeneous purified enzyme was 255.0 IU/mg protein, and 12% recovery was found. An apparent K_m of 1.0×10^{-4} M was obtained at two different enzyme concentrations. Investigators reported that the enzyme from E.coli and S.marcescens had the same sedimentation constant of 7.6S, indicating similar apparent molecular weights. The inhibition of the 6C3HED lymphoma in C3H mice was tested. Complete regression of this tumor was obtained with a smaller dose of the enzyme from S.marcescens than with enzyme from E.coli.

P.vulgaris asparaginase :

L-Asparaginase from P.vulgaris was purified by the following steps : cell lysis by lysozyme and toluene treatment, pH treatment, ammonium sulfate fractionation, Sephadex G-100 gel filtration, DEAE-Sephadex chromatography, and crystalization by the addition of ammonium sulfate. The procedure yields the crystalline enzyme with 1 30% recovery of the activity in crude extracts (Tosa et al., 1972). The crystalline enzyme appears to be homogeneous, as judged by ultracentrifugation, disc electrophoresis, and isoelectric focussing with carrier ampholytes. Isoelectric point is 5.08. Specific activity of this crystalline enzyme is 300 IU/mg. The crystalline enzyme hydrolyzes L-asparagine, D-asparagine, L-glutamine, and some analogs of L-asparagine. Michaelis constants for L-asparagine, D-asparagine, and L-glutamine are 2.6×10^{-5} M, 4.3×10^{-4} M, and 5×10^{-3} M, respectively. The pH optimum for L-asparagine hydrolysis is between pH 7 and 8. Although the enzyme is inactivated by heat, organic solvents, and chymotrypsin treatments, the presence of L-asparagine or its analogs protects the enzyme from the inactivation caused by these treatment. The enzyme activity is cleared from mouse and rabbit plasma with half-life values of only 110 and 130 min, respectively.

A.glutaminasificans glutaminase-asparaginase (AGA) :

Roberts et al. (1972) have purified a glutaminase-asparaginase from the Acinetobacter to essential homogeneity in high yield. This enzyme, with an activity ratio toward L-glutamine and L-asparagine of 1.2 to 1.0 has four subunits and the

same MW (130,000) of E.coli asparaginase. The isoelectric point unusually (pI 8.43), and the enzyme contains no disulfide or sulfhydryl groups and no carbohydrate. The values for L-glutamine and L-asparagine are both low and very similar, (i.e., 4.8×10^{-6} and 5.8×10^{-6}), respectively, and the enzyme catalyzes the hydrolysis of the D-isomers as well, at about one third of the rate for L-isomers.

Antitumor activity of AGA and other purified glutaminase-asparaginase (i.e., Pseudomonas 7A glutaminase - asparaginase) is also related to their plasma half-lives (Clarkson et al., 1970). Succinylation and glycolysation of AGA have been shown to increase its half-life about 10 fold (Holcenberg et al., 1975). In tissue culture experiments, the AGA selectively kills human leukemic leukocytes at approximately one-hundredth the effective concentration of E.coli asparaginase (Schrek, 1967; 1971). In 1980, Warrell et al. reported that succinylated AGA (SAGA) causes considerable neurotoxicity in adult patients. Based on the kinetic data it has been claimed that, the SAGA which has a 10-fold increase of plasma half-life in animals and humans and, thus, has benefit as a cancer chemotherapeutic agent, retained its catalytic activity and maintained K_m and V_{max} values similar to the native enzyme (Steckel et al., 1983).

ANTINEOPLASTIC ACTIVITY, BIOLOGICAL APPLICATION AND COMBINATION CHEMOTHERAPY :

L-Asparaginase is effective against more than 50 neoplasms of the mouse, 3 of the rat, and spontaneous canine lymphosarcomas. In general, virus induced murine leukemias, except that induced by the Rauscher leukemia virus, are not affected by asparaginase (Jackson and Handschumacher, 1970; Capizzi and Cheng, 1981). Clinical trials of asparaginase have, to date, been conducted mostly in acute leukemia. The most responsive disease to date is acute lymphoblastic leukemia (AA) (Pratt & Johnson, 1971; Capizzi et al., 1970; Advani et al., 1983; Homans et al., 1987; Ridgway et al., 1989). Initial clinical trials were conducted in patients who were refractory to conventional chemotherapy; complete remission rates ranging from 30% to 60% were reported with perhaps the higher response rate being achieved in children in their first relapse after a lengthy remission (Grundmann and Oettgen, 1970; Haskel et al., 1967; Sutow et al., 1971; 1976). A randomized trial in 413 children with advanced ALL by the children's Cancer Study Group has shown that the optimal dose of asparaginase, when used as a single agent, in the range of

6000 IU/M^2 given twice weekly (Ertel et al., 1979). An increase in dose after a failure of response to lower doses is usually not productive of an improved response rate (Capizzi et al., 1971; Clarkson et al., 1970). The clinical response associated with intermittent injections (once, twice or three times a week) has been comparable to that achieved with daily administration (Ohnuma et al., 1971; 1972; Jaffe et al., 1972). However, the frequency of anaphylaxis is increased with asparaginase is given at weekly intervals (Capizzi, 1975). Serious allergic reactions appear to be reduced with i.m. route of administration compared with that which occurs following intravenous administration (Ertel et al., 1979).

Continued therapy beyond a point is usually not worth-while (Ertel, 1979). Once a remission has been achieved, the duration of remission is not prolonged by continued treatment with asparaginase, the medium duration of remissions being 2-4 months. Thus, when used as a single agent, asparaginase falls within the category of an "inducing agent" rather than "maintaining agent". Second complete remissions have been achieved in 50% of patients who responded to a first course of asparaginase and relapsed on other maintenance therapy (Capizzi. et al., 1971; Sutow et al., 1971).

L-Asparaginase could also exerted its inhibitory effects on solid tumors in adults (Clarkson et al., 1970). While asparaginase does not cross blood-brain barrier to an appreciable degree, the systemic use of the drug has been found effective against intracerebrally inoculated L5178Y leukemia in mice (Burchenal et al., 1970; Dolowy et al., 1966) and in humans with central nervous system (CNS) leukemia (Tallal et al., 1970). Systemic L-asparaginase therapy may be a feasible means of treating CNS involvement in Rhesus monkeys and humans with ALL and there is no therapeutic advantage to using intrathecal L-asparaginase (Riccardi et al., 1981). The minimal plasma level of L-asparaginase necessary to deplete CSF asparagine in both species was 0.1 IU/ml.

Long-term survival in acute lymphoblastic leukemia (ALL) patients has been achieved as a result of combination chemotherapy, optimal dose scheduling, CNS prophylaxis, and intensive treatment during remission and vigorous supportive care (Pinkel, 1979). Asparaginase is a logical candidate for use in combination with other drugs (e.g. Vincristine, Vinblastine, Methotrexate, Adriamycin, 6-Thioguanine, Actinomycin-D and VM-26) because it has a mechanism of action quite different

from other antineoplastic agents, there is no cross-resistance with the other drugs. Combinations were more effective when the other drug was given before or simultaneously with the first dose of asparaginase (Burchenal & Karnufsky, 1970). Combination of glutamine antagonists and asparaginase are very effective against murine tumors (Jacobs et al., 1970; Mashburn, 1970). Sutow et al. (1976) reported a 67% complete remission (CR) rate with the combination of prednisone, vincistrine and asparaginase (PVA) in the 'PV-resistant patients' compared with an 87% CR rate in those patients who were apparently not refractory to the drug. The addition of cyclophosphamide or the daunorubicin to PVA or VPA does not effect the efficacy or effect of the complete remission rate produced by VPA or PVA alone (Trueworthy et al., 1978; Chessels and Cornbleet, 1979). The sequential use of asparaginase after a 4 week course of PV results in significantly longer remission durations than does prior treatment with asparaginase or the simultaneous use of three drugs (Jones et al., 1977; Spiers et al., 1977).

Glutaminase-asparaginase from A.glutaminasificans (ATCC 27197) in preclinical testing showed a broader spectrum of antitumor activity than L-asparaginase (Schmid and Roberts, 1974; Holcenberg and Roberts, 1977; Roberts et al., 1979), furthermore, several tumor cell lines resistant to L-asparagine deprivation were killed by depletion of L-glutamine (Roberts et al., 1971; Roberts et al., 1979). Succinylated Acinatobacter glutaminase - asparaginase (SAGA) causes considerable neurotoxicity in adult - patients (Warrellet al., 1980).

HOST-TOXICITY AND RESISTANCE TO ASPARAGINASE :

L-Asparaginase of bacterial origin has been used extensively during the last several years in the treatment of lymphatic leukemia (Oettgen et al., 1967; Ertel et al., 1979). L-Asparaginase is effective in inducing complete remission in upto 60% of human patients with acute lymphoblastic leukemia (Hill et al., 1967; Jaffe et al., 1973). The remission of the tumors is dose dependent (Ertel et al., 1979) and considered to be due primarily to the deprivation of the cells of L-asparagine and to some extent of L-glutamine (Holcenberg et al., 1975). However, the short biological half-life of the enzyme necessitates frequent administration, which often leads to antibody production and hypersensitivity (Killander et al., 1976). The antigenic determinants of the E.coli and Erwinia-derived enzymes, which have

comparable enzymatic and therapeutic properties, are sufficiently different so that they do not cross-react immunologically. Thus patients allergic to the E.coli enzyme have been successfully treated with the Erwinia enzyme without the immediate appearance of an anaphylactic or other allergic reaction (Capizzi et al., 1970; Ohnuma et al., 1972). With continued use, the Erwinia enzyme is just allergic as the E.coli enzyme. Moreover, asparaginase induced immunosuppression has been attributed to the glutaminase action possessed by the E.coli and Erwinia asparaginases now in clinical use (Ashworth & MacLennan, 1974; Hersh, 1971). Kafkewitz and Goodman showed that the anaerobic bacteria V.succinogenes (VS) produces very little glutaminase activity (Distasio et al., 1977) and further studies have shown that PEG-asparaginase of VS origin is non-immunogenic and still possess significant antitumor activity in murine models (Bendich et al., 1982) and effective against canine spontaneously occuring malignant lymphoma (MacEwen et al., 1987).

E.coli asparaginase given in vivo suppresses a wide variety of immunological responses, including production of antibody to SRBC (Ohno & Hersh, 1970) suggesting that the asparaginase damages T-cells more than the B-cells (Berenbaum et al., 1973; Ohno & Hersh, 1970a).

Warrell et al. (1980) investigated the pharmacology and toxicology of succinylated Acinetobacter glutaminase-asparaginase (SAGA) after both single-dose and serial daily dose injections in 20 adult patients. The investigation concluded that SAGA causes considerable neurotoxicity in adults which requires close patient monitoring.

The use of L-asparaginase during remission induction in children with leukemia is associated with coagulation abnormalities and platelet function which may present at haemorrhage (Homans et al., 1987). Unlike the guinea pig enzyme (Dolowy et al., 1966), clinical use of the microbial asparaginases are associated with pronounced toxicity including liver and pancreas dysfunction and immunosuppression (Ohno and Hersh, 1970; Haskell et al., 1969).

Of particular concern are the reports of CNS thrombosis, haemorrhage, or haemorrhagic infarctions occuring in patients treated with L-asparaginase (Packer et al., 1985). Abnormal laboratory parameters include deficient of qualitatively abnormal clotting factors (Pui et al., 1985; Ramsay et al., 1977), decreased fibrino-

lysis (Vellenga et al., 1984), abnormalities of antithrombin III (AT III) (Andersen et al., 1979; Vellenga et al., 1980; Buchanan and Holtkamp, 1980), and abnormal platelet aggregation (Shapiro et al., 1980; Pui et al., 1983).

Despite the immunosuppressive effects of asparaginase, the enzyme is immunogenic and can elicit the production of IgM, IgG, and IgE anti-asparaginase antibodies (Paterson & Orr, 1969; Killander et al., 1976). The E.coli enzyme inhibits mitogen-induced lymphocyte blastogenesis in vitro (Astaldi et al., 1969; Ohno and Hersh, 1970), immunoglobulin production induced by a variety of antigens (Berenbaum, 1970), as well as cell-mediated immune reactions (Schulten et al., 1969; Ohno and Hersh, 1970a).Nevertheless, immune reactions to asparaginase constitute the primary limitation to its clinical use.

CLINICAL EFFICACY :

Production of antibody to the enzyme can result in loss of effectiveness of the drug due to shortened half-life in the plasma (Prager&Derr,1971; Goldberg et al., 1973). The immunological problem can be circumvented by sequential therapy with serologically unrelated asparaginases (Goldberg et al., 1973; King et al., 1974). Thus, patients allergic to the E.coli enzyme have been successfully treated with the Erwinia enzyme without the immediate appearance of an anaphylactic or other allergic reaction (Capizzi et al., 1970; Ohnuma et al., 1972).

E.coli asparaginase treated with polyethylene glycol 5000 will not react with antiserum made against the native enzyme; however, its enzymatic activity was reduced to 7% (Ashihara et al., 1978). E.coli L-asparaginase has been insolubilized by covalent attachment to partially hydrolyzed nylon tubing via the bifunctional reagent glutaraldehyde (Allison et al., 1972). Uren and Ragin, (1979) reported that the attachment of a poly-DL-alanyl peptide to the E.coli or Erwinia enzyme retained 50-65% of the enzyme activity and did not alter the K_m for asparagine. Its cross-reactivity with antiserum against the parental enzyme was, however, reduced by 300 and 500 fold, respectively. Succinylation of both the E.coli and Erwinia enzymes have been performed. Upto 40% of the lysyl residues have been succinylated without attenuation of enzyme activity (Shifrin et al., 1973). Uren et al. (1982) investigated that the covalent attachment of poly-DL-alanine peptides to lysyl residues on the

surface of Er.carotovora L-asparaginase has produced a modified enzyme which is much less immunogenic in mice and demonstrates 100-fold longer plasma half-life in the Rhesus monkey. L-Asparaginase has been encapsulated in intact erythrocytes and the enzyme removed all detectable asparagine from the plasma in vivo for at least two weeks after the injection of the loaded cells (Alpar and Lewis, 1985). In vitro evidence suggested that the asparagine entered the cell and was metabolized by the loaded enzyme in situ. When the encapsulated asparaginase was tested against the 6C3HED tumor in C3H mice the encapsulated preparation was superior to the free enzyme in treating the tumor and was the only treatment to produce 'cured' mice. When the L-asparaginase micro-particles are embeded in a polyacrylamide gel the duration of action was prolonged upto $\sim$ 25 days after implantation in the rat (Edman and Sjoholm, 1981). The antitumor effect of immobilized L-asparaginase was tested against lymphoid leukemia (6C3HED) in C3H mice (Edman and Sjoholm, 1983).

Durden and Distasio, (1980, 1981) compared that the glutaminase-free asparaginase from V.succinogenes (VS) is non-immunosuppressive, whereas asparaginase of E.coli (EC) causes immunosuppression. Abuchowski and Davis, (1981) also investigated the non-immunosuppressive effects of PEG-asparaginase VS and immunosuppressive effect of PEG-asparaginase EC, since both enzymes have long and similar circulating lives. Both PEG-asparaginases and native asparaginases of VS or EC were found to be an effective antitumor enzyme in a standard animal test system. Measurements of the mitogen induced blastogenic responses of splenocytes, harvested 5 days after in vivo administration of the PEG-enzymes, showed that PEG-asparaginase VS was not immunosuppressive, whereas PEG-asparaginase EC caused immunosuppression (Bendich et al., 1982). But, asparaginase VS was not proved useful in the response of human tumors or lymphatic leukemias.

Several considerations have suggested further research for new asparaginases.