Asparaginase and Glutaminase Activities of Bacteria

By H. E. WADE, H. K. ROBINSON AND B. W. PHILLIPS

Microbiological Research Establishment, Porton, Salisbury, Wiltshire

(Accepted for publication 7 September 1971)

SUMMARY

A test-tube method is described for the determinations of L-asparaginase and L-glutaminase in suspensions and extracts of bacteria. An automated modification of the method was used to determine the asparaginase activities of about 200 strains from 78 species and the glutaminase activities of 46 strains from 13 species. Both enzymes were widely distributed and, at pH 8.5, asparaginase was generally the more active. Asparaginase was exceptionally active in some species of *Erwinia*.

INTRODUCTION

Considerable attention has been paid to the enzyme L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) since Broome (1961) showed that it was responsible for the anti-tumour activity of guinea-pig serum (Kidd, 1953); unlike most normal tissue cells some neoplastic cells are unable to survive in the absence of L-asparagine. Mashburn & Wriston (1964) showed that an asparaginase from *Escherichia coli* was equally effective against experimental tumours and its clinical evaluation subsequently revealed its special value in the treatment of acute leukaemia. A serologically distinct asparaginase, which produced similar therapeutic effects, was obtained in much higher yield from the plant pathogen *Erwinia carotovora* (Wade, Elsworth, Herbert, Keppie & Sargeant, 1968; Wade & Rutter, 1970) and came to light as a result of the survey described in this report.

A parallel interest in L-glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2) has arisen from demonstrations that microbial glutaminases also exhibit antitumour activity (El-Asmar & Greenberg, 1966; Roberts, Holcenberg & Dolowy, 1971*a, b*; Broome, 1971) and from a report that the complete regression (caused by L-asparaginase) of a mouse leukaemia can be obtained only under conditions in which the circulating L-glutamine is depleted (Riley, 1970).

The antitumour effects of both amidases are the subject of recent reviews by Capizzi, Bertino & Handschumacher (1970), Cooney & Handschumacher (1970) and Wriston (1971) and of a report on a symposium held in Paris in 1970 (see Broome, 1971). The present report describes the results of screening bacteria for these enzymes using a test-tube method and an automated Technicon method. The results were briefly described in an earlier report (Wade, Robinson & Phillips, 1971).

METHODS

Organisms. Bacteria were generally obtained from national collections: ATCC, American Type Culture Collection, Rockville, U.S.A.; FD, Boots Pure Drug Co. Ltd, Research Department, Antibiotic and Fermentation Division, Nottingham; ICPB, International Collection of Phytopathogenic Bacteria, Davis, California, U.S.A.; MRE, Microbiological Research

Establishment, Salisbury, Wiltshire; NCDO, National Collection of Dairy Organisms, Shinfield, Berkshire; NCIB, National Collection of Industrial Bacteria, Torry Research Station, Aberdeen; NCMB, National Collection of Marine Bacteria, Torry Research Station, Aberdeen; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, Hertfordshire; RES, Rothamstead Experimental Station, Harpenden, Hertfordshire.

Strains of *Serratia marcescens*, identified by the prefix JA, were gifts from Dr A. Alden (University of Sydney).

Media. Plate Count Agar (CM183), Tomato Juice Agar (CM113) and Malt Extract Agar (CM59) were obtained from Oxoid Ltd, London. Lister Peptoné Agar contained: 1 % (w/v) peptone (Evans Medical Supply Ltd, Runcorn, Cheshire), 1 % (w/v) mannitol, 0.5 % NaCl and 1.2 % (w/v) agar. Yeatex Agar contained 3.5 % (w/v) Yeatex (light grade yeast extract from the Trent Yeast Extract Co. Ltd, Burton-on-Trent, Staffordshire) and 1.2 % (w/v) agar. Peptone Agar contained 2 % (w/v) peptone (Evans), 0.5 % NaCl and 1.2 % (w/v) agar. Nutrient Agar contained 2.5 % (w/v) Nutrient Broth no. 2 (Oxoid, CM 67), 0.4 % Yeast Extract (Oxoid L21) and 1.2 % (w/v) agar. Potato Agar contained the filtrate from 200 g. of boiled potatoes in 1 l. 2.4 % (w/v) agar at pH 7.6.

Culture conditions. The examination of different nutrient agars (Table 1) showed that Plate Count Agar induced high levels of L-asparaginase and L-glutaminase. Bacteria generally exhibited the highest activity of L-asparaginase towards the end of a period of maximum growth rate (Roberts, Burson & Hill, 1968; Heinemann & Howard, 1969; Robison & Berk, 1969). For screening, therefore, bacteria were grown on this medium at 30° for 2 days.

Enzyme determination. Amidase activity was determined in 0.05 M-borate buffer pH 8.5 (0.05 M-Na⁺) at 37° in the presence of 10 mM-substrate. Two methods were used: one a test tube method using direct Nesslerization and the other an automated Technicon method (Wade & Phillips, 1971). Both took advantage of the observation (Wade *et al.* 1971) that the incidence of turbidity which is often encountered in the Nessler reaction, is lower in the presence of EDTA and when alkali is added immediately prior to Nesslerization.

In the test tube assay, bacteria were grown on a solid medium, washed in 10 to 50 vol. (relative to the packed bacteria) of 0.15 M-NaCl containing 10 mM-EDTA (disodium salt) and taken up in about 10 vol. water, while 9.9 ml. of 10 mM-L-asparagine (BDH Chemicals Ltd, Poole, Dorset) or 10 mM-L-glutamine (chromatographically pure, Koch-Light Laboratories Ltd, Colnbrook, Buckinghamshire) in 0.05 M-borate buffer containing 0.1 mM-EDTA were held at 37°. Other preparations of L-glutamine were found unsatisfactory. At zero time, 0.1 ml. of the bacterial suspension, containing about 1 to 2 international units (i.u.) of enzyme/ml., was added and quickly mixed. A sample of 1 ml. was immediately withdrawn and delivered into 2.5 ml. of 0.1 N-trichloroacetic acid at room temperature. Further samples were taken at 10 min. intervals up to 60 min.

Each sample was individually mixed with 1 ml. of 1.0 N-NaOH and after 2 min. 0.5 ml. of Nessler reagent was added (Koch & McMeekin, 1924). Automatic dispensers ensured that the treatment of the whole series of samples with each reagent was carried out as quickly as possible.

After 5 min. from the addition of Nessler reagent to the first sample, the optical densities of the subsequent samples were read against it, in optical cells with 1 or 2 cm. path length and an optical filter with peak transmission at about 400 nm. A plot of optical density against time normally followed a straight line passing through the origin. Standards were prepared by treating 1 ml. of 0.25, 0.5 and 1 mM-NH₄⁺ (ammonium sulphate) with trichloroacetic acid, NaOH and Nessler reagent in the way described above. The linearity of the plot of optical densities is illustrated in Fig. 1 to 3.

The automated assay is described in detail elsewhere (Wade & Phillips, 1971). Suspensions in 0.05 M-borate buffer pH 8.5 (0.05 M-Na⁺) were taken from a Technicon sampler at a rate of 20 samples/h. and incubated with 10 mM-L-asparagine (or L-glutamine) and 1 mM-EDTA for 20 min. at 37°. The ammonia released passed through the membrane of a Technicon dialyser into 0.025 M-Na₂SO₄ and was treated with alkali and Nessler reagent. Standards of 0.1 to 0.8 mM-NH₄⁺ were used, corresponding to enzyme activities of 0.005 to 0.04 i.u./ml.

The enzyme activities in most species are of such a magnitude that the assay could be carried out in tandem with an automated assay for protein. A loopful of bacteria, taken from a fully-grown culture on the surface of a nutrient agar slope, was suspended in borate buffer (10⁷ to 10⁸ bacteria/ml.). The two determinations were synchronized so that each sample produced two peaks on a Technicon two-pen recorder: the height of one indicated the amidase activity and the height of the other the protein concentration. From these measurements the specific activities (i.u./mg. protein) could be calculated.

Protein determination. An automated Lowry method was used which operated over the range 4 to 40 µg./ml. (Wade & Phillips, 1971). Standards were prepared from bovine serum albumin (Fraction V, Armour Pharmaceutical Co. Ltd, Eastbourne, Sussex).

Disruption of bacteria. A suspension (1 to 5 % (v/v)) in borate buffer pH 8.5 was shaken with 15 % (v/v) Ballotini beads grade no. 12 (Jenkins Scientific Apparatus Ltd, Hemel Hempstead) in a disintegrator (Mickle, 1948) in the presence of 0.01 % (v/v) octan-2-ol, at 4° for 20 min.

Fig. 1. The liberation of ammonia from 10 mM-L-asparagine by *Serratia marcescens* MRE UK8 grown on Plate Count Agar at 30° for 2 days and incubated with asparagine at 37° in 0.05 M-borate buffer pH 8.5 (—○—○—) or in 0.05 M-acetate buffer pH 5.5 (—●—●—) or grown in Lister Peptone Agar and incubated in 0.05 M-borate buffer pH 8.5 (—□—□). The asparaginase activities were respectively 0.25, 0.10 and 0.06 i.u./mg. protein.

RESULTS

Choice of medium

Serratia marcescens produced a higher concentration of L-asparaginase when grown on Plate Count Agar than when grown on Lister Peptone Agar (Fig. 1). Another comparison, using *Erwinia carotovora* and the automated method of enzyme determination, confirmed the high production of asparaginase on Plate Count Agar and indicated a broad correlation between the concentration of enzyme produced and the concentration of assimilable carbo-

hydrate (Table 1). The inability of this species to hydrolyse starch probably accounts for the high production of enzyme on Potato Agar.

The production of L-glutaminase appears to follow the same trend (Table 1).

Fig. 2. The liberation of ammonia by *Erwinia carotovora* NCPPB 1066 grown on Plate Count Agar at 30° for 2 days, when incubated at 37° in 0.05 M-borate buffer pH 8.5 with 10 mM-L-asparagine (—○—○—) or 10 mM-L-glutamine (—□—□—). The concentrations of bacterial protein were 5 and 40 µg./ml. respectively.

Table 1. Influence of growth medium upon the production of asparaginase and glutaminase by *Erwinia carotovora*

Erwinia carotovora NCPPB 1066 was trained on each medium by more than three passages. Automated enzyme assays were carried out after 2 days growth at 30°. The two determinations were carried out on different preparations of bacteria.

Agar medium	Principal constituents (% w/v)			Enzyme activity (i.u./mg.)	
	Carbohydrate	Yeast or beef extract	Tryptone or peptone	L-Asparaginase	L-Glutaminase
Plate count	0.1 (glucose)	0.25	0.5	5.3	0.23
Yeatex	—	3.0	—	4.6	0.26
Potato	<0.04 (polysaccharide)	—	—	4.4	0.25
Peptone	—	—	0.5	3.4	0.26
Nutrient	—	0.4	2.5	2.7	0.22
Malt extract	3.0 (malt extract)	—	0.5	0.70	0.10
Tomato juice	2.0 (tomato extract)	—	2.0	0.69	0.08

Reliability of amidase determinations

Ammonia was released at a uniform rate during the degradation of L-asparagine and L-glutamine by *Erwinia carotovora* (Fig. 2) and of L-asparagine by *Serratia marcescens* (Fig. 1) and *Escherichia coli* (Fig. 3). Other buffers such as formate at pH 3.5, acetate at pH 4.5, phosphate at pH 6.5 to 7.5 (prepared with 0.05 M-NaOH) and tris at pH 8 (prepared with 0.05 M-HCl) could also be used in the test tube method without interference with the Nessler reactions. The values obtained were within 10 % of those obtained from the automated method when comparisons were made with suspensions of bacteria or preparations of purified L-asparaginase.

Table 2. *Reliability of the asparaginase activities of whole bacteria as a measure of the total enzyme present*

Bacteria were grown at 30° for 2 days on Plate Count Agar in two Petri dishes, washed in 5 ml. of borate buffer pH 8.5 and made up to 9 ml. in buffer. One half of each suspension was disrupted in a Mickle (1948) disintegrator. A supernatant fraction was obtained by centrifuging at 1000g for 20 min. Enzyme activity was determined by the automated method.

Bacterium	Enzyme activity of whole organisms (i.u./mg. protein)	Enzyme activity of disrupted organisms	
		Unfractionated (i.u./mg. protein)	Supernatant fraction (% of unfractionated activity)
<i>Pseudomonas fluorescens</i> NCTC 8248	0.15	0.12	91
<i>Alcaligenes viscolactis</i> ATCC 9036	0.19	0.24	70
<i>Escherichia coli</i> NCIB 9001	0.11	0.11	100
<i>Erwinia carotovora</i> NCPFB 1066	4.5	4.7	100
<i>Serratia marcescens</i> MRE UK8	0.18	0.18	94
<i>Proteus vulgaris</i> MRE 402	0.31	0.13	86
<i>Micrococcus lysodeikticus</i> ATCC 4698	0.12	0.08	93
<i>Bacillus subtilis</i> MRE strain	0.5	0.47	80
<i>B. megaterium</i> NCTC 2607	0.31	0.19	84

Some species were examined more closely to determine to what extent the values obtained were a reliable measure of the total enzyme present in the bacteria. The results (Table 2) suggest that in fact slightly higher values are obtained from whole organisms than from disrupted preparations. Of the nine species examined, only *Alcaligenes viscolactis* exhibited a higher level of activity when disrupted.

Determinations carried out on disrupted preparations may be subject to error through the assimilation of ammonia or through the instability of the enzymes. When the release of ammonia from L-asparagine was followed in a suspension of *Escherichia coli* and in a cell-free extract a lag was observed in the latter which could not be avoided by incubating the extract for 20 min. (Fig. 3).

Varying degrees of instability of L-asparaginase were observed in preparations of disrupted bacteria (Table 2). Instability was very pronounced in *Bacillus megaterium*; after

storage for 2 days at 2 to 4° the preparation had lost about 80 % of its activity. A similar preparation from *Escherichia coli* lost 30 % of its activity. When *E. coli* ATCC 11303 was disrupted in a Hughes Press (Hughes, 1951) and fractionated into a cell-wall fraction, a ribosome fraction and a soluble fraction (Wade, 1961), L-asparaginase could be recovered without loss from the soluble fraction where it was unstable particularly when exposed to air. Under aerobic conditions, glutathione and cysteine offered some protection but EDTA was more effective (Table 3) probably by inhibiting metal-dependent proteinases.

Fig. 3. The liberation of ammonia from 10 mM-L-asparagine when *Escherichia coli* ATCC 11303 which was grown on Plate Count Agar at 30° for 2 days, was incubated with asparagine at 37° in 0.05 M-borate buffer pH 8.5 (—○—○—) and the lag produced when a disrupted (Hughes, 1951) preparation of the same organisms was incubated under these conditions (—□—□—). The lag was not avoided by incubating the disrupted bacteria in the buffer for 20 min. before adding asparagine (—●—●—).

L-Asparaginase activities of bacteria

The results of these preliminary studies showed that the degradation of L-asparagine and L-glutamine by suspensions of whole bacteria occurs at a uniform rate and provides in most cases a reliable measure of the total complement of amidase in the cell. The screening procedure was therefore applied to fresh preparations of bacteria which had been grown to maturity on Plate Count Agar.

The results are summarized in Table 4. Where more than one value is given against a strain, the values were obtained from different cultures of the bacterium and determined on different occasions. Particular attention was paid to strains of *Escherichia coli*, *Serratia marcescens* and *Erwinia carotovora*, the asparaginases from which have been used clinically.

Table 3. *Protective effect of anaerobiosis and EDTA upon the stability of asparaginase in a soluble extract from Escherichia coli*

A soluble fraction (about 5 mg. protein/ml.) from *E. coli* ATCC 11303 was stored at 2 to 4° for 17 days in the absence or presence of 5 mM-reducing agent and 10 mM-EDTA. Anaerobic conditions were obtained by storing the solutions in Thunberg tubes which were then evacuated to remove dissolved gases and sealed. Aerobic conditions were maintained for solutions in similar tubes by allowing access to air. Enzyme activity was determined by the test tube method.

Reducing reagent	Enzyme activity (i.u./mg. protein)		
	Anaerobic without EDTA	Aerobic	
		without EDTA	with EDTA
None	0	0	1.1
Ascorbic acid	1.0	0	1.2
Thioglycolic acid	1.2	0	1.1
Glutathione	1.2	0.31	1.1
Cysteine	0.68	0.09	1.1

Of the species examined those from the genus *Erwinia* displayed the highest activity. Bacteria with very low activities appeared irregularly throughout the different groups of bacteria.

L-Glutaminase activities of bacteria

Specific glutaminases from *Escherichia coli*, *Proteus morganii* and *Clostridium welchii* have optimal activities at about pH 5 (see Hughes & Williamson, 1952). Such enzymes however would be unsuitable for clinical use where they would be required to operate at pH 7.5. The clinically effective glutaminases described by Roberts *et al.* (1971*b*) and Broome (1971) have optima above neutrality. Screening for glutaminase was therefore carried out at pH 8.5 under similar conditions to those used for the assay of L-asparaginase. At this pH, bacteria appear to be generally less active towards L-glutamine than towards L-asparagine and none of the species examined displayed high levels of glutaminase activity (Table 5).

All the bacterial asparaginases which have been characterized have significant levels of glutaminase activity.

During the clinical evaluation of asparaginases from *Escherichia coli* and *Erwinia carotovora*, a low order of activity towards L-glutamine has at different times been thought responsible for toxic side-effects and has been credited with therapeutic activity. It was of interest therefore to obtain a series of asparaginases with different degrees of activity towards L-glutamine. Several strains of *Erwinia carotovora* were examined for the purpose of selecting those which were likely to provide such a series. Determinations of both L-asparaginase and L-glutaminase on common suspensions of these strains revealed wide differences among them (Table 6).

It is likely that the differences observed between these strains of *Erwinia carotovora* reflect differences in the specificities of a single enzyme for the amides rather than differences in the relative quantities of a specific glutaminase and an asparaginase, since the ratio

Table 4. *Asparaginase activities of bacteria*

Bacteria were grown on Plate Count Agar at 30° for 2 days and asparaginase activity determined by the automated method.

Species	Strain	L-Asparaginase activity (i.u./mg. protein)
<i>Rhodopseudomonas spheroides</i>	NCIB 8253	< 0·03
<i>Thiobacillus denitrificans</i>	MRE 1106	< 0·03
<i>Pseudomonas aeruginosa</i>	ACTC 7700, NCTC 1999, NCIB 8626	0·17–0·23
<i>P. angulata</i>	FD 496	0·21
<i>P. chlororaphis</i>	NCIB 9402	0·13
<i>P. cichorii</i>	NCPPB 907	0·15, 0·1
<i>P. fluorescens</i>	KB 1	0·15, 0·12
<i>P. fluorescens</i>	NCTC 8248, 8729	0·15–0·17
<i>P. iodinum</i>	NCDO 754	0·08, 0·19
<i>P. lemonnieri</i>	NCIB 8917	0·13
<i>P. milden</i>	NCDO 754	0·06, 0·05
<i>P. maculicola</i>	NCPPB 952	< 0·02
<i>P. morsprunorum</i>	FD 644	0·02, < 0·03
<i>P. phaseolicola</i>	NCPPB 52	0·10
<i>P. primulae</i>	NCPPB 133	0·11
<i>P. putida</i>	C 1 B	0·24
<i>P. syncyanea</i>	NCDO 795	0·025
<i>P. syringae</i>	FD 495	0·19
<i>Xanthomonas begoniae</i>	NCPPB 241	0·06
<i>X. campestris</i>	NCPPB 528	0·066
<i>X. juglandis</i>	ICPB XJ 107	0·1, 0·04, 0·03
<i>X. hederæ</i>	ICPB XH 1	0·03
<i>X. pelargonii</i>	ICPB XP 121	0·10
<i>Aeromonas hydrophila</i>	NCMB 72	< 0·04
<i>A. liquefaciens</i>	NCMB 87	0·10
<i>Azotomonas insolita</i>	NCIB 8627	0·10, 0·08
<i>Mycoplana bullata</i>	ATCC 4278	0·06
<i>M. dimorpha</i>	NCIB 9439	< 0·02
<i>Alcaligenes bookeri</i>	ATCC 15099	0·11
<i>A. faecalis</i>	ATCC 8748	0·05
<i>A. viscolactis</i>	ATCC 9036	0·13, 0·19, 0·10
<i>A. metalcaligenes</i>	NCIB 9018	< 0·03, 0·05
<i>Escherichia coli</i>	ATCC 11303	} 0·08–0·11
	MRE 161, 165	
	NCIB 8269, 9001, 9270	
	NCTC 1639	
<i>E. coli</i>	MRE 162/6, 164	} 0·12–0·18
	NCTC 8164, 8196	
<i>Citrobacter freundii</i>	NCTC 6071	0·31, 0·27
<i>C. freundii</i>	NCTC 8165	0·55, 0·51
<i>C. intermedia Type I</i>	NCIB 3735	0·38
<i>C. intermedia Type II</i>	NCIB 6071	0·74
<i>Aerobacter aerogenes</i>	MRE 0013	< 0·03
<i>Erwinia amylovora</i>	NCPPB 595	0·43
<i>E. aroideae</i>	NCPPB 495, 497, 612, 1275, 1276, 1639	} 0·21–0·5
<i>E. aroideae</i>	NCPPB 340, 370, 371, 499, 514, 547, 929, 956, 1230, 1231	
<i>E. aroideae</i>	NCPPB 66, 355, 493, 494, 498, 546, 550, 552, 1236	} 1·6–3·8
<i>E. atroseptica</i>	NCPPB 274, 276, 309, 334, 433, 435, 1277	
<i>E. atroseptica</i>	NCPPB 138, 313, 437, 979, 1640, 2043	} 0·59–0·88
<i>E. atroseptica</i>	NCPPB 1590	
<i>E. carnegieana</i>	NCPPB 671	0·02, 0·11
<i>E. carnegieana</i>	NCPPB 672	0·10

Table 4 (cont.)

Species	Strain	L-Asparaginase activity (i.u./mg. protein)
<i>E. carotovora</i>	NCPBP 1281, 1625, 1743, 1746, } 1747, 1791, 1847, 2042	0.2-0.6
<i>E. carotovora</i>	NCPBP 491, 516, 1280, 1744, } 1748, 1848, 1860	0.7-0.95
<i>E. carotovora</i>	NCPBP 312, 392, 395, 438, 468, } 898, 1740, 1741, 1742	1.1-1.6
<i>E. carotovora</i>	NCPBP 551, 708, 1065, 1066, } 1120, 1121, 1125	2.9-5.3
<i>E. chrysanthemi</i>	NCPBP 394, 402, 426, 430, } 533, 910, 911	0.11-0.39
<i>E. chrysanthemi</i>	NCPBP 395, 429, 511, 517, 518, } 568, 1515, 2027	0.4-0.66
<i>E. chrysanthemi</i>	NCPBP 913	1.6
<i>E. cypripedii</i>	NCPBP 741	0.17
<i>E. cypripedii</i>	NCPBP 750	0.39
<i>E. cypripedii</i>	NCPBP 752	0.08
<i>E. cytolytica</i>	NCPBP 1385	0.29
<i>E. maydis</i>	NCPBP 1851	3.3
<i>E. nigritflavens</i>	NCPBP 564	0.15
<i>E. rhapontica</i>	NCPBP 139	0.19
<i>E. rhapontica</i>	NCPBP 1578, 1739	0.06-0.08
<i>E. uredovora</i>	NCPBP 1416	0.27
<i>Serratia kiliense</i>	NCTC 4619	0.14, 0.12
<i>S. marcescens</i>	JA 130, 131, 150, 190, 280, } 440, MRE UK8, NCIB 4612, } 8266, 8889	0.18-0.30
<i>S. marcescens</i>	JA 110, 140, 200, 210, 230, } 260, 311, 321, 430, NCIB } 9155	0.31-0.47
<i>Proteus vulgaris</i>	MRE 402, NCTC 1635, 8065, } 8066	0.15-0.46
<i>P. vulgaris</i>	ATCC 12454, 13315	0.61-0.63
<i>Micrococcus conglomeratus</i>	NCIB 2677	0.10
<i>M. freudenreichii</i>	NCIB 2699	< 0.05
<i>M. lysodeikticus</i>	ATCC 4698, NCTC 1377	0.12-0.17
<i>M. radiodurans</i>	ATCC 13939	0.10
<i>Sarcina aurantiaca</i>	ATCC 146	0.25
<i>S. flava</i>	MRE strain	0.12
<i>S. lutea</i>	MRE 304, NCTC 7564	0.05-0.08
<i>S. species</i>	NCTC 7582	0.013
<i>Streptococcus faecalis</i>	MRE 297	0.14, 0.17
<i>S. faecalis</i>	NCIB 6782	< 0.02
<i>Lactobacillus casei</i>	NCIB 4113	< 0.02
<i>Corynebacterium fascians</i>	NCPBP 1488	0.34
<i>C. viscosum</i>	NCTC 2416	0.16
<i>C. xerosis</i>	ATCC 9755	0.07
<i>Microbacterium lacticum</i>	NCIB 8540	0.05
<i>Arthrobacter aurescens</i>	NCIB 8912	0.04
<i>A. citreus</i>	NCIB 8915	0.11
<i>Bacillus cereus</i>	NCTC 7464	0.15
<i>B. circulans</i>	NCTC 7578	0.03, < 0.02
<i>B. coagulans</i>	NCTC 3991	0.03
<i>B. lentis</i>	NCTC 4842	0.10
<i>B. megaterium</i>	NCTC 2607	0.31, 0.12
<i>B. pumilus</i>	NCTC 7576	0.80
<i>B. sphaericus</i>	NCTC 7582	0.04
<i>B. subtilis</i>	MRE strain	0.50
<i>Nocardia coelica</i>	NCIB 8939	1.1
<i>N. erythropolis</i>	NCIB 9151	1.5
<i>Thermopolyspora glauca</i>	RES A 66	< 0.05

Table 5. *L-Glutaminase activities of bacteria*

Bacteria were grown on Plate Count Agar at 30° for 2 days and L-glutaminase activities determined by the automated method at pH 8·5.

Species	Strain	L-Glutaminase activity (i.u./mg. protein)
<i>Rhodopseudomonas spheroides</i>	NCIB 8253	< 0·03
<i>Pseudomonas fluorescens</i>	NCTC 8729	0·077
<i>Xanthomonas juglandis</i>	ICPB XJ 107	< 0·033
<i>Aeromonas liquifaciens</i>	NCMB 87	0·04
<i>Alcaligenes metalcaligenes</i>	NCIB 9018	< 0·03
<i>Escherichia coli</i>	ATCC 11303, NCTC 8164, 9001	0·02–0·03
<i>E. coli</i>	MRE 162/6, 165	0·046–0·065
<i>Aerobacter aerogenes</i>	MRE 0013	< 0·03
<i>Serratia marcescens</i>	NCIB 8889	0·06
<i>Proteus vulgaris</i>	NCTC 8065	0·025
<i>Micrococcus lysodeikticus</i>	ATCC 4698	0·05
<i>Bacillus megaterium</i>	NCTC 2607	0·026
<i>B. subtilis</i>	MRE strain	0·033

Table 6. *L-Glutaminase activities of NCPPB strains of Erwinia carotovora*

Bacteria were grown on Plate Count Agar at 30° for 2 days and glutaminase and asparaginase activities determined on samples of the same suspension of organisms by the automated method at pH 8·5.

Strain	L-Glutaminase activity (i.u./mg. protein)	L-Glutaminase activity L-Asparaginase activity
		(%)
312	0·03	2·2
392	0·04	2·6
395	0·03	2·5
438	0·036	2·8
468	0·028	2·6
491	0·034	4·9
516	0·089	11·0
551	0·38	8·3
708	0·64	14·0
898	0·14	9·3
1066	0·24	8·2
1120	0·70	19·0
1121	0·33	8·2
1125	0·48	9·2
1280	0·095	10·0
1281	0·06	11·0
1625	0·033	5·7
1740	0·052	3·8
1741	0·046	3·9
1742	0·045	4·1
1743	0·06	33·0
1744	0·036	5·3
1747	0·044	7·3
1746	0·108	26·0
1748	0·057	6·0
1791	0·085	37·0
1847	0·07	31·0
1860	0·05	6·7
1848	0·059	9·6
2042	0·037	6·6

of glutaminase to asparaginase activity in a purified asparaginase from *E. carotovora* NCPPB 1066 and in partially purified preparations of the enzyme from *E. carotovora* NCPPB 312 and 1120 are of the same order as the ratio of these activities in the intact bacteria (Table 6).

DISCUSSION

The data in Table 2 support the general belief that the activities of most microbial asparaginases are fully expressed in the intact organism and suggest that these enzymes are generally located in the periplasm like the antitumour asparaginase from *Escherichia coli* (Cedar & Schwartz, 1967). In applying the assay to whole organisms, however, any cytoplasmic asparaginases that are present will be overlooked. This is of no more than theoretical interest since such asparaginases, like the cytoplasmic asparaginase from *E. coli* (Cedar & Schwartz, 1967), are unlikely to have a high affinity for asparagine (in competition with protein-synthesizing enzymes) and unlikely therefore to be of much therapeutic value (Broome, 1968).

The choice of buffer for the determination of enzyme took account of the fact that those bacterial asparaginases which have been characterized have optima above neutrality and that the presence of borate ions and EDTA would discourage growth and the assimilation of ammonia during the period of incubation. The borate ion is in fact more toxic to the growth of bacteria than nitrite or fluoride ions (Porter, 1946).

The low concentration of glucose (0.1 %) in Plate Count Agar used for the comparison of species in Tables 4 to 6, encouraged in most species a rapid initial rate of growth before the bacteria became dependent upon amino acids and peptides for a source of energy. The presence of this level of glucose appeared to be essential for the production of L-asparaginase from *Escherichia coli* growing in a casein hydrolysate medium and its exhaustion preceded the synthesis of the enzyme (Robison & Berk, 1969).

The depressing effects of higher concentrations of carbohydrates (Table 1) has been observed by others. Roberts, Burson & Hill (1968) showed that the addition of carbohydrates to a medium of corn-steep liquor reduced the production of asparaginase by *Escherichia coli*. A similar observation was made with *Serratia marcescens* (Heinemann & Howard, 1969). It is difficult from batch cultures such as these to establish whether the low yield of asparaginase is the result of an excess of carbohydrate or of a low pH. Continuous cultures of *S. marcescens* and *Erwinia carotovora*, however, have indicated that the concentration of carbohydrate is primarily responsible (D. Herbert, D. S. Callow and C. G. T. Evans, private communication).

The high yields of asparaginase obtained with unsupplemented Plate Count Agar, discouraged studies into methods for inducing the enzyme. The production from *Escherichia coli* was not increased when a complex medium (Roberts *et al.* 1968; Robison & Berk, 1969) or an amino acid free medium (D. Herbert, private communication) were supplemented with L-asparagine. Only among species of *Pseudomonas* has there been any evidence of a marked induction by asparagine or aspartic acid (Zanin, Berezov, Nikolaev & Abdumalikov, 1969).

The use of a nutrient agar simplified the screening process by avoiding the necessity for washing the bacteria. Bacteria transferred directly from its surface into buffer introduced negligible quantities of ammonia and other interfering substances from the medium and thus avoided the need for a substrate-free control in the enzyme assay. Such a control could not be omitted with suspensions which had been stored as pastes at 2 to 4° or with preparations of disrupted bacteria.

The levels of asparaginase reported here are generally of the same order as those reported from liquid cultures of similar composition by Peterson & Ceigler (1969) and Zanin *et al.* (1969) but are generally lower than those reported by Roberts *et al.* (1968) from corn-steep liquor. The general conclusion of these data and those set out in Tables 4 and 5 is that L-asparaginase and L-glutaminase are very common among bacteria. The results offer only a little help to taxonomy but establish the existence of a relatively few species which are capable of intense asparaginase activity.

Several species of bacteria have received special attention with a view to the therapeutic use of their amidases. One of the first was *Pseudomonas aeruginosa* (Greenberg, Blumenthal & Ramadan, 1964). Its amidase has antitumour activity and showed about equal activity towards L-asparagine and L-glutamine. A similar enzyme has been described in several reports by Mardashev (see Mardashev, Eremenko & Nikolaev, 1970). An amidase from a species of *Xanthomonas* has a greater activity towards L-glutamine; its extremely high affinity for both substrates makes it very effective against tumours (Broome, 1971). A similar nonspecific amidase has been purified from a Gram-negative species of *Diplococcus* by Roberts, Holcenberg & Dolowy (1971*a*). None of the Pseudomonadaceae examined showed very high levels of asparaginase activity (Table 4).

The increase in asparaginase activity observed when suspensions of *Alcaligenes viscolactis* were disrupted (Table 2) suggests either the presence of a significant amount of enzyme in the cytoplasm or the ability of the whole organism to assimilate ammonia under the alkaline conditions of the assay. The generally low values observed in this genus (Table 4) may therefore be underestimates of the enzyme content.

The purification of L-asparaginase from *Escherichia coli* brought to light the existence of two asparaginases in this species, only one of which exhibits antitumour activity (Roberts, Prager & Bachynsky, 1966; Schwartz, Reeves & Broome, 1966; Campbell, Mashburn, Boyse & Old, 1967). Using strain K 12, Cedar & Schwartz (1967) showed that the ineffective one is not released upon forming sphaeroplasts and is therefore probably located in the cytoplasm.

The strains of *Escherichia coli* examined here (Table 4) are not very active against L-asparagine, much higher activities have been reported in other media. Strain ATCC 11303, for example, produced more than ten times the level reported here when grown on corn-steep liquor (Roberts *et al.* 1968). Under the standard growth conditions used for screening, the citric acid assimilating species of *Citrobacter*, *C. freundii* and *C. intermedia*, produce higher levels of asparaginase than *E. coli*. A modification of the conditions of growth would perhaps widen this distinction and provide a quantitative character of taxonomical value.

The measurement of asparaginase activity may be of more immediate benefit for distinguishing species of *Erwinia* from strains of *Escherichia coli*. In contrast to the latter, many strains of *Erwinia* exhibit high levels of asparaginase activity (Table 4). Of these, strains of *Erwinia carotovora*, *E. aroideae* and *E. maydis* produce the highest. The early examination of species of *Erwinia* in the screening programme was prompted by the knowledge that L-asparagine is a common plant storage product and the thought that some plant pathogens would be well equipped to degrade it. In the event, however, there appears to be no simple correlation between the asparaginase activity of a strain and the likely asparagine content of its host plant.

Strains of *Serratia marcescens* display exceptionally uniform levels of asparaginase activity (Table 4). An early demonstration of antitumour activity by Rowley & Wriston (1967) prompted the large-scale production of this enzyme (Heinemann & Howard, 1969).

Of the species of *Bacillus* examined, *Bacillus pumilus* and *B. subtilis* were distinguished by

high asparaginase activities (Table 4). The asparaginase of *B. coagulans* was isolated and shown to have no antitumour activity by Mashburn & Wriston (1964). This and the observations of others led to an appreciation of the special properties which are essential for the expression of this activity (Broome, 1968; Rutter & Wade, 1971).

The *Nocardia* species (Table 4) appear to contrast with species of *Actinomyces* examined by Zanin *et al.* (1969), in having prominent asparaginase activities.

We are indebted to Mr W. J. Brent for technical assistance. The work was supported by a grant from the Department of Health and Social Security.

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