WORK, E. & DEWEY, D. L. (1953). *J. gen. Microbiol.* 9, 394-409.

The Distribution of α,ϵ -Diaminopimelic Acid among various Micro-organisms

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With a Note by R. L. M. SYNGE on the 'Occurrence of Diaminopimelic Acid in some Intestinal Micro-organisms from Farm Animals'

SUMMARY: Paper chromatograms of hydrolysates of 118 micro-organisms were examined in a study of the distribution of α,ϵ -diaminopimelic acid and other amino-acids. A method for the identification of α,ϵ -diaminopimelic acid is described. Diaminopimelic acid was found in nearly all the bacteria examined, except for the Gram-positive cocci, *Streptomyces* spp., and *Actinomyces* spp. It was also found in blue-green algae but in no other algae, nor in fungi, yeasts, plant viruses, or protozoa.

Each species examined showed a different amino acid composition. β -Alanine and α - and γ -aminobutyric acids were sometimes found, often in several species of the same genus. Seven unidentified ninhydrin-reacting spots were recorded; none of them had the wide distribution of diaminopimelic acid.

During an examination of the amino acids of *Corynebacterium diphtheriae* by paper chromatography, acid hydrolysates of ethanol-washed cells were found to contain an unknown amino acid, which was subsequently isolated and identified as α,ϵ -diaminopimelic acid:

for brevity subsequently referred to here as diaminopimelic acid (Work, 1949; 1950*a, b*; 1951). At the same time, an amino acid with identical chromatographic behaviour was found in hydrolysates from antigenic lipopolysaccharides of *Mycobacterium tuberculosis*, from rumen contents of sheep, and also from soil (Asselineau, Choucroun & Lederer, 1950; Klungsøyr & Synge, footnote in Work, 1950*a*; Bremner, 1950). The isolation of diaminopimelic acid from whole *Myco. tuberculosis* (Work, 1951) confirmed the identity of the amino acid found by Asselineau *et al.* The same amino acid was also identified chromatographically in hydrolysates of *Proteus vulgaris* and *Bacterium coli* (Work, 1950*b*; Dewey & Work, 1952), *Vibrio cholerae* and numerous strains of *Mycobacterium* (Blass, Lecomte & Macheboeuf, 1951; Gendre & Lederer, 1952; Pauletta & DeFranceschi, 1952), but it was not found in *Staphylococcus aureus* (Work, 1950*b*; Gendre & Lederer, 1952). None of these workers found diaminopimelic acid on chromatograms of products of non-bacterial origin, such as yeasts (Lindan & Work, 1951), animal fodders or animal proteins, nor has the amino acid been reported in the extensive literature on chromatography of many tissue fluids or protein hydrolysates. A derivative,

α,ϵ -diamino- β -hydroxypimelic acid was found by Woolley, Schaffner & Braun (1952) in the toxin of *Pseudomonas tabaci*.

The present investigation was undertaken to test the validity of the suggestion made by Work (1951) that diaminopimelic acid might be confined to certain bacteria. A survey of the amino acid composition of some representative bacteria, fungi, algae and other micro-organisms was therefore carried out. A preliminary report has already been given (Work & Dewey, 1952). The work has been extended by Dr R. L. M. Synge, whose quantitative examination for diaminopimelic acid in some ruminant intestinal micro-organisms is given in his note.

METHODS

Micro-organisms used. The micro-organisms were grown and harvested in the normal way for each organism, either in this Department, or by other workers to whom we are much indebted. The organisms were usually washed free of medium, but as the media were found to be free from diaminopimelic acid, washing was not essential and was omitted if not convenient. The organisms were dried, either at 100° or by acetone washing.

Examination for diaminopimelic acid. The essentials of the procedure for examining hydrolysates of micro-organisms for diaminopimelic acid have already been described (Work, 1951). Dried organisms (100–500 mg.) were hydrolysed with 10 ml. 6N-HCl under reflux for 24 hr. HCl was removed from the hydrolysate by three evaporations to dryness *in vacuo*, and the residue was redissolved in water to final concentration 6 ml. equivalent to 1 g. dried organism. Two-dimensional chromatograms were run at 26° from 15 μ l. of hydrolysate (equivalent to 2.5 mg. dry cells) applied to Whatman no. 4 paper (54 $\times$ 45 cm.). Phenol/water (NH_3 + HCN atmosphere) was the first solvent, and collidine/lutidine/water the second solvent (Dent, 1948). Six chromatograms were run simultaneously from the same trough (Lindan & Work, 1951). The solvents were removed from the paper by a stream of air at 45°; spots were developed at 100° after spraying with a solution of ninhydrin in butanol (0.1 %, w/v). Since the amount of hydrolysate used for the chromatograms was based on dry weight, it was sometimes necessary, when examining organisms of abnormal protein content, to make further chromatograms using a different amount of hydrolysate in order to obtain good definition.

After the preliminary chromatograms, the remaining hydrolysate was electro dialysed to remove all basic and acidic substances. This was carried out after removal of humin by centrifugation, the hydrolysate and humin washings being diluted suitably and placed in the centre compartment (10 ml. capacity) of an electro dialysis apparatus described by Work (1950*a*), using formalin-treated parchment semipermeable membranes. A d.c. potential was applied until 1 hr. after the initial current (about 100 ma.) had fallen to a minimal value (10 ma.) and the pH value of the centre compartment had reached neutrality. The contents of the neutral centre compartment were concentrated to their original volume and chromatographed as before. If no spot was found in the position of diaminopimelic acid (spot 17, Fig. 1), the chroma-

tograms were repeated using larger volumes (up to 200 μ l.) of electro dialysed hydrolysate; if no spot then appeared the organism was said to contain no diaminopimelic acid. A spot in the correct position on any chromatogram from an electro dialysed hydrolysate was taken as evidence for diaminopimelic acid only if three conditions were fulfilled: (1) Absence of glutamic acid and aspartic acid spots, showing that electro dialysis was complete. When the condition was not fulfilled electro dialysis was repeated, to be sure that ethanolamine-*o*-phosphoric acid was removed. (2) Presence of the spot on chromatograms run from electro dialysed hydrolysate treated with 20 μ l. H_2O_2 (100 vol.) + 2.5 μ l. ammonium molybdate (0.4%, w/v), thus distinguishing it from cystine (Dent, 1948). (3) Exact matching of the position of the spot with that produced by an authentic sample of diaminopimelic acid chromatographed under identical conditions.

Rough assessments of diaminopimelic acid concentration were made by visual comparison of the size and colour strength of spots given by known amounts of the amino-acid, with the size and strength of the spot in chromatograms of the electro dialysed hydrolysed micro-organisms. All other spots on the chromatograms were recorded; any unidentified spots were noted but not usually further investigated.

Preparation of fractions from organisms. Ethanolic extracts were made by extracting the wet organisms three times for 24 hr. each with 10 vol. of 70% (w/v) aqueous ethanol; the combined extract was shaken with 8 vol. chloroform and the aqueous supernatant phase used for chromatography (Lindan & Work, 1951). *Bact. coli* soluble protein fraction was prepared by grinding acetone-dried cells as a paste with 'Filter-cel' (Johns-Manville) and 0.1M-phosphate buffer (pH 6.8) in the ratio cells/Filter-cel/buffer/1 : 2 : 6. The mixture was centrifuged at 25,000*g* for 40 min. and the supernatant solution dialysed against water.

C. diphtheriae protein fractions were prepared by extracting acetone-dried cells (45 g.) for 2 days at +2° with 2200 ml. 2% (w/v) ammonium sulphate solution. The mixture was filtered, the filtrate brought to 0.8% saturation with ammonium sulphate and the resulting precipitate collected by filtration. The filtrate was saturated with ammonium sulphate and the resulting precipitate collected. The material precipitated at 0.8% saturation was dissolved in water and further fractionated with ammonium sulphate, precipitates being collected at 0.5 and 0.8% saturation. The three precipitates were dissolved in water and dialysed. All these procedures were carried out at +2°.

RESULTS

Chromatography of diaminopimelic acid

The behaviour of diaminopimelic acid on paper chromatograms with various solvent systems is shown in Table 1, with figures for neighbouring amino-acids included for reference. (R_f values in other solvents are quoted by Gendreau & Lederer, 1952; and Wright & Cresson, 1953). Figs. 1 and 2 are typical chromatograms of a bacterial hydrolysate before and after electro dialysis. Table 1 and Figs. 1 and 2 show that the R_f value of an amino-acid is by no

means an absolute constant but varies with the pH of the solvent system and with the composition of the mixture being chromatographed (see Landua, Fuerst & Awapara, 1951). The R_f value of the diaminopimelic acid spot is particularly sensitive to pH, varying from 0.18 when the acidic whole hydrolysate is chromatographed, to 0.33 when the neutral electrolysate is examined.

Table 1. *Chromatographic behaviour in various aqueous solvent systems of diaminopimelic acid compared with neighbouring amino acids*

Except where stated, amino acids were applied to chromatograms as hydrochlorides in dilute HCl.

Solvent system ...	Phenol,* NH ₃ atmos.	Phenol,* acetic atmos.	Colli- dine*/ lutidine	Butanol†/ acetic acid	Ethanol‡/ ammonia	Pyridine§/ amyl alcohol	Cresol
Characteristic recorded ...	R_f	R_f	R gly.	R gly.	R_f	R gly.	R_f
Diaminopimelic acid	0.33 ¶ 0.25	0.14	0.22	0.17	0.14	0.37	0.02
Aspartic acid	0.19	0.21	0.5	0.53	0.20	0.38	0.02
Glutamic acid	0.28	0.38	0.62	1.0	0.27	0.5	0.09
Glycine	0.44	0.41	1.0	1.0	0.46	1.0	0.14
Cystine	0.25	0.22	0.3	0.17	0.24	0.46	0.04
Ethanolamine-o- phosphoric acid	0.26	0.39	0.22	0.23	0.36	0.36	0.09

* Dent, 1948.

† Campbell, Work & Mellanby, 1951.

‡ Ethanol 77: 2N ammonia 33.

§ Pyridine 35: amyl alcohol 35: water 30. Edman, 1945.

|| Ratio: $\frac{\text{distance run by amino acid}}{\text{distance run by glycine}}$.

¶ Free amino acid.

The minimum detectable concentration of diaminopimelic acid on a two-dimensional phenol/collidine chromatogram was 2 $\mu\text{g.}$; on one-dimensional chromatograms in phenol it was as low as 0.5 $\mu\text{g.}$ During electrodialysis, varying proportions (up to 50 %) of diaminopimelic acid passed to the cathode with the other neutral amino-acids. Therefore, when no diaminopimelic acid spot was apparent on chromatograms from 200 $\mu\text{l.}$ of electrolysed hydrolysate (= 33 mg. dry cells), the diaminopimelic acid content of the cells must have been less than *c.* 0.02 %.

Absence of diaminopimelic acid from growth media

Hydrolysates of peptone, casein, yeast extract, blood, Lab Lemco, gelatin and agar-agar were all found to be free from diaminopimelic acid. It is thus evident that the usual growth media do not contain diaminopimelic acid.

Chromatography of whole micro-organisms

Table 2 shows the results obtained from the examination of chromatograms of hydrolysed micro-organisms. The approximate amount of diaminopimelic acid found in each organism is given; in certain cases (marked by §) the findings were checked by the use of an enzyme, found in the coli-aerogenes group of bacteria, which specifically decarboxylates diaminopimelic acid

Table 2. *Diaminopimelic acid (DAP) and other amino acids not common to protein hydrolysates, observed in chromatograms of hydrolysed micro-organisms examined both before and after electro dialysis*

Only one strain of each organism was examined, unless otherwise indicated by a number in brackets after the specific name.

Family	Organism	DAP content*	Other spots†	Source of cultures examined‡
Gram-negative EUBACTERIALES				
Nitrobacteriaceae	<i>Thiobacillus denitrificans</i>	+	G	23
Pseudomonadaceae	<i>Pseudomonas aeruginosa</i>	+	—	27
	<i>Acetobacter xylinum</i>	+	13	27
	<i>A. mobile</i>	tr.	—	27
	<i>Vibrio cholerae</i> , Ogawa	++	16, D	1
	<i>V. comma</i> , El Tor	+	16	1
	<i>Desulphovibrio desulphuricans</i>	+	—	18
Azotobacteriaceae	<i>Azotobacter chroococcum</i>	+	—	23
Rhizobiaceae	<i>Rhizobium</i> sp.	+	—	29
Neisseriaceae	<i>Neisseria catarrhalis</i>	+	—	30
Enterobacteriaceae	<i>Bacterium coli</i> , type 1 (7)	+	—	1, 26, 30
	<i>Aerobacter aerogenes</i>	+	—	27
	<i>Klebsiella pneumoniae</i> (2)	+	D (both)	28, 30
	<i>Proteus vulgaris</i> (2)	+, tr.	—	25, 27
	<i>Salmonella typhi</i> (4)	+	16 (all)	1
			D (3 str.)	
	<i>Salmonella typhimurium</i>	++§	—	1
	<i>Shigella dysenteriae</i> (2, rough and smooth)	+	—	13
	<i>S. paradyenteriae</i>	+	14	1
Parvobacteriaceae	<i>Pasteurella pestis</i> , virulent (3)	++ , tr.	16 (all)	13
	<i>P. pestis</i> , avirulent (3)	+, tr.	16 (all)	13
			14 (1 str.)	
	<i>Brucella abortus</i> , virulent	++§	16	13
	<i>B. abortus</i> , avirulent	+	—	11
	<i>Haemophilus bronchisepticus</i>	tr.	—	20
	<i>H. pertussis</i>	tr.	—	20
Gram-positive EUBACTERIALES				
Micrococcaceae	<i>Staphylococcus aureus</i> (<i>Micrococcus pyogenes</i> var. <i>aureus</i>)	0	—	1, 25
	<i>Micrococcus lysodeikticus</i>	0	—	27
	<i>Sarcina lutea</i>	0	—	27
Lactobacteriaceae	<i>Streptococcus pneumoniae</i>	0	—	20
	<i>Strep. pyogenes</i>	0	—	1
	<i>Strep. faecalis</i> (2)	0	—	27
	<i>Leuconostoc mesenteroides</i>	0	—	27
	<i>Lactobacillus plantarum</i> (arabinosus)	++	13	27
	<i>Propionibacterium rubrum</i>	++	B	9
	<i>P. thoenii</i>	++	—	9
	<i>P. zeae</i>	++	—	9
	<i>P. peterssonii</i>	++	13	9
	<i>P. jensenii</i>	++	13, 19	9
	<i>P. arabinosum</i>	++	13, B	9
	<i>P. pentosaceum</i>	++	13, B, 19	9

Table 2 (cont.)

Family	Organism	DAP content*	Other spots†	Source of cultures examined‡
Gram-positive EUBACTERIALES (cont.)				
Corynebacteriaceae	<i>Corynebacterium diphtheriae</i> (2)	++§	—	14
Bacillaceae	<i>Bacillus subtilis</i>	+	—	27
	<i>B. brevis</i>	tr.	—	17
	<i>B. pumilus</i>	+	F, 13	8
	<i>Clostridium novyi</i> (oedematiens)	+	—	20
	<i>Cl. perfringens</i> (welchii) (2)	++	13 (1 str.)	14
	<i>Cl. tetani</i>	0	13	14
RHODOBACTERIINEAE				
Athiorhodaceae	<i>Rhodopseudomonas palustris</i> (2)	++§	—	21, 23
	<i>R. spheroides</i> (2)	++	{ 13 (both) 14 (1 str.)	23
	<i>Rhodopseudomonas</i> sp.	++	—	24
	<i>Rhodospirillum rubrum</i>	+	—	23
ACTINOMYCETALES				
Mycobacteriaceae	<i>Mycobacterium tuberculosis</i> var. <i>hominis</i>	++	—	11
	<i>Myco. tuberculosis</i> var. <i>bovis</i>	++	19	11
	<i>Myco. tuberculosis</i> var. <i>bovis</i> (BCG)	++	19	11
	<i>Myco. avium</i>	++	19	11
	<i>Actinomyces</i> spp.	0	A	Local
Actinomycetaceae	<i>Streptomyces</i> spp. (3)	0§	A (all)	6
MYXOBACTERIALES				
Cytophagaceae	<i>Cytophaga globulosa</i>	+	—	23
	<i>Cytophaga</i> sp. (marine)	+	—	23
ALGAE				
Myxophyceae	<i>Anabaena cylindrica</i>	+	D	10
	<i>Oscillatoria</i> sp.	+	—	10
	<i>Mastigocladus laminosus</i>	+	—	10
Xanthophyceae	<i>Tribonema aequale</i>	0	—	10
Chlorophyceae	<i>Chlorella pyrenoidosa</i>	0	—	10
	<i>Acetabularia mediterranea</i>	0	—	5
Bacillariophyceae	<i>Navicula pelliculosa</i>	0	—	10
Euglenineae	<i>Euglena gracilis</i>	0	—	10
Phaeophyceae	<i>Fucus serratus</i>	0	—	3
Rhodophyceae	<i>Rhodymenia palmata</i>	0	—	3
Unclassified	'Acid algae' (Allen, 1952)	0	—	23
YEASTS				
	Brewer's yeast¶	0	—	Commer.
	Baker's yeast¶	0	—	Commer.
	<i>Saccharomyces fragilis</i>	0	—	7
FUNGI				
Ascomycetes	<i>Neurospora crassa</i>	0	19	15
Fungi Imperfecti	<i>Aspergillus flavus-oryzae</i>	0	19	4
	<i>A. glaucus</i>	0	19	4
	<i>A. niger</i>	0	—	4

Table 2 (cont.)

Family	Organism	DAP content*	Other spots†	Source of cultures examined‡
FUNGI (cont.)				
Fungi Imperfecti	<i>A. oryzae</i>	0	19	4
	<i>A. ruber</i>	0	19	4
	<i>Penicillium cyclopium</i>	0	13	Local
	<i>P. notatum</i>	0	—	4
	<i>P. spinulosum</i>	0	13	Local
	<i>Scopulariopsis brevicaulis</i>	0	—	4
	<i>Cephalosporium acremonium</i>	0	—	6
	<i>Microsporum audouini</i>	0	—	12
	<i>M. canis</i>	0	—	12
	<i>M. gypseum</i>	0	14, 19	2
	<i>Trichophyton rubrum</i>	0	—	12
Basidiomycetes	<i>Schizophyllum commune</i>	0	—	4
PROTOZOA				
	<i>Strigomonas oncopelti</i>	0	—	19
	<i>Tetrahymena pyriformis</i>	0	15	19
PLANT VIRUS				
	Tobacco mosaic	0	—	16
	Turnip yellow mosaic	0	E	16

* 0 = less than 0.02 % (dry wt.); tr. = up to about 0.1 %; + = 0.1–0.8 %; ++ = more than 0.8 %.

† All spots not normally found in chromatograms of protein hydrolysates. See black spots, Fig. 3 for key to location.

‡ See list of acknowledgements.

§ Checked by specific decarboxylase (Dewey, 1952).

|| Work (1951).

¶ Lindan & Work (1951).

(Dewey & Work, 1952; Dewey, 1952). Any unidentified spots found on the chromatograms are also recorded, a key to their position being given in Fig. 3. Also included in Fig. 3 and Table 2 are α - and γ -aminobutyric acids and β -alanine. These amino-acids are not normally found in protein hydrolysates but are known to be present in non-protein fractions of some micro-organisms (Work, 1949; Lindan & Work, 1951; Fowden, 1951; Blass *et al.* 1951; Pauletta & Defranceschi, 1952).

Diaminopimelic acid was found in chromatograms from all the bacteria examined with the exception of *Actinomyces* spp., *Streptomyces* spp., the Gram-positive cocci, and the one strain of *Clostridium tetani* examined. The amino acid was also found in the Myxophyceae (blue-green algae), but not in any other algae, nor in fungi, yeasts, protozoa, and plant viruses. Concentrations of diaminopimelic acid varied from about 2 % (dry weight) to trace amounts of about 0.02 % which were only demonstrable by grossly over-loading the chromatogram with the other neutral amino-acids.

Seven unidentified spots were found. Some (such as E, F or G) occurred in chromatograms of only one type of organism, others (e.g. A, D) were distributed among certain genera. The substances responsible for spots A, E, F and G were neutral, the others moved from the centre compartment during electrodialysis but their direction of movement was not investigated. Spot F

appears to correspond in position to taurine, while spot E resembles diiodo-tyrosine in position but not in colour (Dent, 1948).

β -Alanine (characterized by a vivid blue colour with ninhydrin and migration from the neutral compartment on electrodialysis) was observed only in certain genera of Gram-negative Eubacteriales.

α -Aminobutyric acid was found in only three organisms, all unrelated. γ -Aminobutyric acid was more widely distributed, except among the Gram-negative Eubacteriales.

The chromatograms all showed the presence of most of the amino-acids usually found in proteins; the technique used did not however separate phenylalanine from the leucine isomers, nor lysine from hydroxylysine and ornithine; histidine was seldom observed. Hydroxyproline was found once only, in a high concentration on chromatograms from the ciliate *Tetrahymena piriformis*. The general pattern of the spots showed that there were significant variations in concentrations of individual amino acids from different organisms.

Diaminopimelic acid in certain cell fractions

Table 3 represents the results of examination of hydrolysates of various types of fractions obtained from micro-organisms. No diaminopimelic acid was found in the soluble amino acid fraction from two Gram-negative bacteria and a blue green alga, although such fractions from some other bacteria are known to contain it (Work, 1949; Blass *et al.* 1951). Various soluble protein fractions from *Bact. coli* and *C. diphtheriae* contained diaminopimelic acid, as did the crude fraction remaining after separation of the insoluble cell walls. The purest preparation of *Sh. dysenteriae* endotoxin was free from diaminopimelic acid, showing that the trace found in the less pure preparation was a contaminant.

Table 3. *Distribution of diaminopimelic acid (DAP) in some cell fractions*

Organism	Fraction	Source of fraction	DAP content
<i>Bact. coli</i>	Ethanolic extract	Local	0
	Whole cytoplasmic contents	M. R. J. Salton	tr.
	Extractable protein	Local	tr.
<i>Sh. shigae</i>	Endotoxin	W. T. J. Morgan	tr.
	Endotoxin, further purified	W. T. J. Morgan	0
<i>Anabaena cylindrica</i>	Ethanolic extract	Local	0
<i>Br. abortus</i>	Ethanolic extract	Local	0
<i>C. diphtheriae</i>	Phenol-soluble fraction	E. S. Holdsworth	+
	Protein {	ppt. 0.5 sat. Am. SO ₄ Local	+
		ppt. 0.8 sat. Am. SO ₄ Local	+
		ppt. 1.0 sat. Am. SO ₄ Local	tr.

DISCUSSION

Identification of diaminopimelic acid

The chromatographic identification of diaminopimelic acid in acid hydrolysates of proteins is complicated by several factors. First, on chromatograms diaminopimelic acid lies near to both aspartic acid and glutamic acid; in fact, with the distortion in this region due to mineral acid ions, overlapping often

occurs, especially when large amounts of hydrolysate are used in attempts to detect low concentrations of diaminopimelic acid. Cystine and ethanolamine phosphoric acid can be confused with diaminopimelic acid. Cystine is found in hydrolysates of nearly all proteins. Its behaviour on chromatograms is unpredictable; sometimes it occupies the same position as diaminopimelic acid, but usually part of the cystine is oxidized on the paper to cysteic acid (spot 18), and the remainder either gives no spot, or spots in positions 18A or 18B. Treatment with peroxide results in complete conversion of all these forms to cysteic acid, and thus avoids any danger of confusion with diaminopimelic acid. Ethanolamine phosphoric acid is a common constituent of animal tissue extracts and has also been found in yeast (Lindan & Work, 1951, Campbell & Work, 1952; Miettinen, 1951). Although its R_f value in butanol/acetic acid is slightly higher than that of diaminopimelic acid, the difference is not sufficiently great for purposes of identification. However, the acidic nature of ethanolamine phosphoric acid enables it to be separated by electrodialysis from the neutral diaminopimelic acid. The presence in micro-organisms, other than yeast, of ethanolamine phosphoric acid has not been reported, but in chromatograms of some cocci we found a spot in the expected position which was not subsequently apparent in the neutral fraction; no further examination was carried out.

In this survey the routine method employed for the identification of diaminopimelic acid consisted of two-dimensional chromatography of the peroxide-treated neutral fraction of the electrodialysed hydrolysate. Removal of all acidic ions avoided both confusion with ethanolamine phosphoric acid and interference by aspartic and glutamic acids. This enabled large amounts of material to be chromatographed, and thus revealed diaminopimelic acid in trace amounts. Two-dimensional chromatography was necessary to check completeness of electrodialysis, and to ensure identification of diaminopimelic acid. The justification for this step lies in the discovery in chromatograms of certain Actinomycetales of a spot (A) with an R_f value in collidine similar to that of diaminopimelic acid, but with a slightly higher R_f value in phenol. Had a one-dimensional chromatogram been used, this spot (A) might have been wrongly identified as diaminopimelic acid. The disadvantage of the routine use of two-dimensional chromatograms lies in their unsuitability for quantitative work on large numbers of samples; consequently we made no attempt to carry out exact estimations of diaminopimelic acid.

Provided the identity of the spot under investigation is certain, one-dimensional chromatography can be used for quantitative purposes. For the chromatographic estimation of diaminopimelic acid, Synge (see Note) used collidine as the solvent for the electrodialysed neutral amino-acid fraction, while Gendre & Lederer (1952) used butanol/formic acid after preliminary adsorption on acid alumina. We had hoped to develop the use of diaminopimelic acid decarboxylase as a quantitative method, but the enzyme proved to be inhibited by constituents of the crude hydrolysate (Dewey, 1952), and while it could be used on the electrodialysed hydrolysate, the losses involved in electrodialysis were too variable for quantitative work.

Diaminopimelic acid in bacterial cells

The function of diaminopimelic acid in the cell appears to be multiple. Present evidence suggests that it exists largely in the bound form, since it is still present in cell residues after extraction of soluble nitrogenous components (Work, 1950*a*; Blass *et al.* 1951; Pauletta & Defranceschi, 1952). Its presence in cellular proteins has been demonstrated by Gendre & Lederer (1952) and by our findings (Table 3), but it is not present in purified *Sh. shigae* endotoxin, purified diphtheria toxin or the PPD of tuberculin (Work, 1951). High concentrations of diaminopimelic acid were found by Powell & Strange (1953) in a peptide excreted by germinating spores of *B. subtilis*, and it was also present in insoluble cell wall proteins of *C. diphtheriae* and in separated cell walls of some Gram-negative organisms such as *Bact. coli* (Holdsworth, 1952; Salton, 1953). It is possible that in tough rigid cell walls, diaminopimelic acid might be playing the part of an insolubilizing cross-linking agent, in a manner similar to that played by cystine in the keratinous groups of proteins.

In some micro-organisms, diaminopimelic acid was found in the soluble amino-acid fraction which probably represents part of the cellular metabolic pool (see Work, 1949; Blass *et al.* 1951; Pauletta & Defranceschi, 1952). It is possible that the age of the culture, its treatment after harvesting or the activity of the cellular diaminopimelic acid decarboxylase (Dewey & Work, 1952) may determine whether or not diaminopimelic acid will be found among the free amino-acids. The diaminopimelic acid of the metabolic pool may not only be used for incorporation into proteins or other molecules, it may also act as a precursor of lysine; this is suggested from enzymic studies and from an examination of the nutritional requirements of various mutants of *Bact. coli* (Dewey & Work, 1952; Davis, 1952). A mechanism exists in *Bact. coli* for synthesis of large amounts of diaminopimelic acid, as is shown by accumulation of the amino-acid (250 mg./l.) in the culture fluid of a lysine-requiring mutant (Davis, 1952; Work & Denman, 1953; Wright & Cresson, 1953).

Diaminopimelic acid distribution and the classification of micro-organisms

Our survey of the distribution of diaminopimelic acid among micro-organisms, although incomplete, showed that this amino-acid was widely distributed among bacteria, but did not occur in any organisms unrelated to bacteria. No obvious relationship was found between gross diaminopimelic acid contents of bacteria and various properties which have been used in the numerous systems of bacterial classification, such as nutritional requirements, biochemical activities, Gram reaction, acid-fastness, immunological properties and pathogenicity. The absence of diaminopimelic acid from the Gram-positive cocci is one of the most striking facts which emerges from the survey; this absence contrasts with the results obtained from the other Lactobacteriaceae and the one Gram-negative coccus examined. In the Actinomycetales, there is a sharp differentiation between the mycobacteria, which contain diaminopimelic acid, and the actinomyces and streptomyces, where

it is absent and another diaminodicarboxylic acid is found (Work, 1953). The presence of diaminopimelic acid in the three blue-green algae examined is of interest in view of the divergence of opinion as to the existence of a morphological similarity between the Myxophyceae and bacteria, the biochemical similarities being unquestioned (Stanier & Van Niel, 1941; Pringsheim, 1949). Our findings lend additional support to the suggestion of Stanier & Van Niel that the Myxophyceae and the Schizomycetes should be grouped into one kingdom, the Monera.

Diaminopimelic acid appears to be a cell constituent which differentiates certain bacteria not only from other bacterial species but also from all other micro-organisms. We suggest that the occurrence of diaminopimelic acid might qualify as a feature to be considered in bacterial classification. It is a physiological character directly concerned with biosynthetic aspects of cellular metabolism, and might have greater significance than many of the catabolic properties of organisms which are now used as differentiating characteristics.

Although in many cases the gross distribution of diaminopimelic acid in whole cells cannot be correlated with any obvious characteristic of the organism, it is possible that when definite fractions containing the amino-acid have been separated and studied they may be found to be directly connected with certain cellular functions. In the case of *Myco. tuberculosis*, an antigenic lipopolysaccharide fraction from human strains was found by Asselineau & Lederer (1950) to contain diaminopimelic acid only when the strain was a virulent one, although whole cells from virulent or avirulent strains contained the amino acid (Gendre & Lederer, 1952). The presence of high concentrations of diaminopimelic acid in the cell wall fractions of *Bact. coli* and *C. diphtheriae* does not imply that these fractions always represent the bulk of the amino-acid, since organisms without rigid cell walls such as myxobacteria and Myxophyceae also contain substantial amounts of it.

Amino acids other than diaminopimelic acid

The survey of two-dimensional chromatograms of the large number of micro-organisms examined enables some generalizations about the amino acid patterns to be suggested. It appears that each species has a characteristic overall amino acid composition. The recording of unidentified ninhydrin-reacting substances, and amino acids found usually only in the free state, has yielded some interesting results. For example, every strain examined in certain genera of Gram-negative Eubacteriales contained relatively high concentrations of β -alanine, but rarely showed the presence of γ -aminobutyric acid, which was found fairly frequently in other organisms. It should be emphasized that examination of crude or electro-dialysed hydrolysates of whole cells would not reveal trace amounts of amino acids confined to certain cellular fractions, so that failure to record them in this survey does not imply their absence from the cell. For example, in *C. diphtheriae*, hydrolysates of whole cells did not reveal α - or γ -aminobutyric acids or β -alanine, all of which were known to occur in the soluble amino acid fraction; while hydroxyproline

was found only in the insoluble fraction after removal of the soluble amino-acids (Work, 1949). Therefore, when whole cell hydrolysates are found by this technique to contain a particular amino acid which does not normally occur in proteins, the organism can be considered to contain unusually large amounts of that amino acid.

The number of unidentified spots observed seems small considering the number of organisms examined. However, the primary object of the investigation was to examine the distribution of diaminopimelic acid, not to search for other new compounds. It is noteworthy that, although some of the unidentified spots were found in chromatograms from more than one type of micro-organism, none of them had the wide distribution of diaminopimelic acid.

We are most grateful to the undermentioned for growing organisms for us (numbers are used for reference in Table 2): 1, Lt-Col. H. J. Bensted; 2, Mrs M. Bentley; 3, Dr W. A. P. Black; 4, Prof. F. Challenger; 5, Dr H. Chantrenne; 6, Dr G. C. Codner; 7, Dr R. Davis; 8, Prof. F. Egami; 9, Dr S. R. Elsdon; 10, Dr G. E. Fogg; 11, Dr H. H. Green; 12, Dr P. J. Hare; 13, Dr D. W. Henderson; 14, Prof. B. C. J. G. Knight; 15, Prof. H. A. Krebs; 16, Dr R. Markham; 17, Dr G. F. Newton; 18, Dr J. R. Postgate; 19, Dr Muriel Robertson; 20, Mr A. F. B. Standfast; 21, Dr J. Tosic; 22, Dr W. E. van Heyningen; 23, Dr C. B. van Niel; 24, Dr J. M. Wiame; 25, Dr T. S. Work.

We also acknowledge cultures from: 26, Dr B. Davis; 27, Dr E. F. Gale; 28, Mr H. Proom; 29, Dr H. G. Thornton; 30, Prof. Wilson Smith.

Technical assistance was given by R. Broadman, R. F. Denman and Miss B. C. Knight.

One of us, D. L. D., was in receipt of a grant from the Rockefeller Research Fund of this Medical School.

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**Note on the Occurrence of Diaminopimelic Acid in some Intestinal
Micro-organisms from Farm Animals**

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Micro-organisms were analysed for diaminopimelic acid by a procedure similar to that described above by Work & Dewey. Freeze-dried preparations of the micro-organisms (which had been washed on the centrifuge with distilled water) were hydrolysed for 24 hr. in 6N-HCl at 105°. After evaporation of excess HCl *in vacuo* the residue was dissolved in water and subjected to ionophoresis in a 4-compartment diaphragm cell (Synge, 1951), maintaining the pH at 6. The contents of the specimen compartment were concentrated *in vacuo* to suitable volume; a measured amount of this solution, to which had been added 0.1% (w/v) of ammonium vanadate, was pipetted for chromatography on to the starting line of a 35 cm. tall cylinder of Munktell OB filter paper (Grycksbo Pappersbruk AB, Grycksbo, Sweden). After treatment of the spot with hydrogen peroxide (100 vol.; see Dent, 1948) the chromatogram was developed one-dimensionally (upwards) with water-commercial collidine mixture (Yorkshire Tar Distillers, Ltd., Cleckheaton). Graded known amounts (0.2–0.7 µg. N) of α,ϵ -diaminopimelic acid (isolated from *Corynebacterium diphtheriae* by Dr Elizabeth Work) were pipetted on to the paper (as hydrochloride in aqueous solution) and chromatographed in parallel without H₂O₂ treatment. After spraying with ninhydrin solution in the usual way, visual comparison was made of the unknown and control diaminopimelic acid spots, which had the lowest *R_f* values. A control analysis of an acid hydrolysate of 50 mg. casein gave no visible spot in the diaminopimelic acid position. On repeating with addition of 0.1 mg. diaminopimelic acid an apparent recovery of 83% was obtained. The loss was mainly into the cathode compartment of the diaphragm cell. The same recovery was assumed in calculating all the data given below. An amount of diaminopimelic acid corresponding to 0.1% or more of the N of the micro-organisms could be detected by this procedure.

The results obtained with a number of micro-organisms are given in Table 1. In agreement with Dewey & Work negative results were obtained with commercial preparations of *Torula utilis* and a *Saccharomyces* sp.

This work was undertaken after observations by Klungsoyr & Synge (see footnote, Work, 1950) of the occurrence of diaminopimelic acid in hydrolysates of rumen contents and of its absence from hydrolysates of all the usual animal feeding stuffs so far examined. It was hoped that it might serve as a means of measuring the amounts of microbial protein present in the rumen on normal diets in the same way as lysine was used as a marker with a special lysine-deficient diet containing zein by McDonald (1948). However, the differences in diaminopimelic acid content of the different micro-organisms show that changes in the flora could invalidate any estimate of total microbial

protein made on this basis. Nevertheless, study of the diaminopimelic acid content of rumen contents may in future prove useful for checking claims that a particular organism has increased in numbers or become ecologically dominant. The suggestion has been made (Synge, 1952) that, in view of the

Table 1. *Diaminopimelic acid content of micro-organisms*

Organism	Reference	N (micro-Kjeldahl, as % of air-dry preparation)	Diamino-pimelic acid (N as % total N of preparation)
<i>Clostridium butyricum</i> (pig-caecum strain)	Baker, Nasr, Morrice & Bruce (1951)	8.1	0.4
<i>Cl. butyricum</i> (sheep-rumen strain)	Masson (1950)	9.6	0.6
<i>Cl. welchii</i> (sheep-rumen strain)	Isolated by Miss M. J. Masson	12.5	1.6
<i>Ruminococcus flavefaciens</i> (strain S)	Sijpesteijn (1951)	9.3	1.0
<i>Streptococcus faecalis</i> (sheep-rumen strain)	Masson (1950); Moir & Masson (1952, no. 38)	8.4	None
Amylolytic streptococcus (4 sheep-rumen strains)	Macpherson (1953); cf. Macpherson & Oxford (1952)	6.6-9.3	None
Coliform xylose fermenter (culture 14)	Heald (1952 <i>a</i> , <i>b</i>)	10.1	0.8

high content of diaminopimelic acid in *Ruminococcus flavefaciens*, this substance may be required as a growth factor by the organism, which would explain why clostridial extracts were found by Sijpesteijn (1951) to stimulate growth while yeast extracts were less effective. The ruminococcus used in the present work was grown on a liquid medium without clostridial extract.

I am grateful to Dr S. R. Elsdon, Dr P. J. Heald, Miss Margaret Macpherson and Miss Marjorie Masson who provided cultures for this work, and to Mr J. Wood for technical assistance.

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Fig. 1

Fig. 2

Fig. 3

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EXPLANATION OF PLATE

- Fig. 1. Chromatogram of crude hydrolysate of *Rhodopseudomonas spheroides* (1.6 mg. dry wt.). For key to numbers see Fig. 3.
- Fig. 2. Chromatogram of H_2O_2 treated electro dialysed hydrolysate of *Rhodopseudomonas spheroides* (8.2 mg. dry wt.). For key to numbers see Fig. 3.
- Fig. 3. Key to ninhydrin-reacting spots found on chromatograms of hydrolysates of micro-organisms. First solvent phenol (NH_3 atmosphere), second solvent, collidine/lutidine. Clear circles represent amino-acids which are normal protein constituents. 1, aspartic acid; 2, glutamic acid; 3, serine; 4, glycine; 5, threonine; 6, alanine; 7, valine; 7A, methionine sulfoxide; 7B, methionine sulphone; 8, leucines, methionine and phenylalanine; 9, tyrosin; 10, proline; 11, arginine; 12, lysine; 18, cysteic acid; 18A and 18B, some positions occupied by cystine or its oxidation products (spot 17, below, also corresponds to cystine); 19, glucosamine.* Black circles represent additional spots not normally found in bacterial proteins. 13, γ -aminobutyric acid; 14, α -aminobutyric acid; 15, hydroxyproline; 16, β -alanine; 17, ethanolamine phosphoric acid and diaminopimelic acid.* A,* B, C, D, E, F, G, unidentified; A, E, F and G are neutral.

* R_f in phenol variable, according to pH.

(Received 8 May 1953)