IMPROVED METHODS FOR DETECTING α , ϵ -DIAMINO-PIMELIC ACID IN LUNG LESIONS

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Consdén and Glynn (1955) described a simple test for identifying α , ϵ -diaminopimelic acid in tissue hydrolysates and found this amino-acid was present in several lung lesions, including a "Caplan" nodule, which, by the usual tests, contained no viable or histologically recognizable *M. tuberculosis*. Recently, Nethercott and Strawbridge (1956), using this method, confirmed the presence of diaminopimelic acid in lung lesions from a number of cases of pneumoconiosis, and suggested that it was also present in a lymph node from a case of sarcoidosis. The method employed in these investigations will detect down to about 2 μ g. of diaminopimelic acid in 1 mg. of protein, but it seemed important to be able to detect even smaller proportions because this amino-acid would be derived from about fifty times its weight of bacteria. In searching for methods which would handle the relatively large amounts of tissue which had become available and detect smaller proportions of diaminopimelic acid, the method of Work and Denman (1956) and Hoare and Work (1955), which employs chromatographic separation on a column of ion-exchange resin, seemed suitable. We have modified this procedure to deal with protein hydrolysates, and have found it to be about five times more sensitive than the simpler paper method of Consden and Glynn (1955). We report here the results obtained by this method on a number of lung lesions, and we describe also some improvements in the paper method.

Materials

(a) **Normal Lung.**—Normal lung was removed at necropsy from a male aged about 60 who had died after operation for carcinoma of the stomach. The dusty pleural surface was removed, washed five times with physiological saline, twice with water, and finally dehydrated with acetone. The dry weight was 6.1 g.

(b) **Progressive Massive Fibrosis (P.M.F.).**—A specimen was obtained by lobectomy from a miner,

at Sully Hospital, Glamorgan. The specimen was kept ice-cold during transport. The black tissue (weight 2.7 g.) was washed and dried as described above.

(c) **Caplan Nodule.**—A Caplan nodule was obtained at biopsy from a miner. The case history was reported to us from the Pneumoconiosis Research Unit. The patient had been suffering for some years from rheumatoid arthritis, and his sputum was negative for tubercle bacilli on 12 examinations. The biopsied material was transported in alcohol from Wales. The nodules were dissected out after their arrival at these laboratories and specimens were examined histologically. No acid-fast material was present. The air dry weight of the remainder was 0.5 g.

(d) **Caseous Nodule.**—This was the same tissue as that described by Consden and Glynn (1955).

(e) **Tubercle Bacillus Residue.**—This was the residue described by Colover and Consden (1956) and was used as a control diaminopimelic-acid-containing substance in the modified two-dimensional method described below and in paper chromatography.

Pre-treatments of Tissues

Autoclaving.—Where this was carried out, the tissues were autoclaved with 20 times their dry weight of water at 120° C. for three hours. The aqueous portion was separated and the residue was re-autoclaved with fresh water. The final residue was then dehydrated in acetone. When the extracted material was to be examined, it was recovered from the combined aqueous portions by evaporation *in vacuo*. The residues of normal lung (6.1 g.) and P.M.F. (2.7 g.) after autoclaving weighed 2.8 g. and 1.5 g. respectively. The Caplan nodule was not autoclaved.

Hydrolysis and Bromination.—These procedures were carried out as described by Consden and Glynn (1955). Concentrations were adjusted on the basis of N content for the tests described below. Hydrolysates were freed from humin before analysis.

Meso α , ϵ -Diaminopimelic Acid.—This, obtained from L. Light & Co., gave a single spot on paper chromatograms in various solvents.

Modified Two-dimensional Test

The modifications were designed to overcome the tendency of the original method of Consden and Glynn (1955) to produce elongated spots. This is a disadvantage, since the serine spot can sometimes be so elongated as to occur at or near the position occupied by diaminopimelic acid. This effect is probably brought about by inward flow of liquid when the paper chromatogram is sprayed with buffer solution before the ionophoretic stage. In the modified procedure, the stages were reversed; ionophoresis was now carried out first followed by chromatography. The developing solvent in the chromatographic stage was phenol, which was about half saturated with water. This prevented the paper becoming waterlogged, as occurred with water-saturated solvent, due to buffer salt in the paper. Owing to the slowing of chromatographic development with the undersaturated solvent and because the length of the paper in the chromatographic direction was limited by the dimensions of the ionophoresis chamber, the faster No. 4 paper was substituted for Whatman No. 1. Furthermore, this ensured good separation of serine from glutamic acid, which was important, because in phenolic solvents *meso*-diaminopimelic acid has a rather unpredictable R_F value and sometimes moves just ahead of glutamic acid.

Details of the modified two-dimensional procedure are briefly as follows. The ionophoresis apparatus was the closed "perspex" box which accommodated paper 24 cm. wide (Consden and Powell, 1955) and up to 40 cm. in length. A sheet of Whatman No. 4 paper of these dimensions was wetted with M/30 phosphate buffer, pH 6.8, and placed in position in the apparatus. Hydrolysate (5–10 μ l.) containing the equivalent of about $\frac{1}{2}$ mg. protein was placed about halfway across the width of the sheet and 8.5 cm. from one edge. Near that edge and in line with the hydrolysate was placed a "marker" spot of orange G. Ionophoresis was carried out for one and a quarter to one and a half hours in M/30 phosphate buffer at a potential across the electrodes of 314 V (potential across the paper, 11 V/cm.) and current, 0.4–0.5 mA/cm. width. After ionophoresis the sheet was dried and the marker dye spots removed by cutting away the edge of the paper containing them. Chromatography was then carried out in phenol containing 14% (w/w) water for 17 hours in an atmosphere of ammonia produced from a solution of 0.3% aqueous ammonia on the floor of the tank. The lower edge of the paper was serrated to facilitate running off of solvent and usually a marker mixture of amino-acids was placed on a suitable part of the sheet as a control. After chromatography, the sheet was dried and treated with ninhydrin in the usual way.

Column Separation

Zeokarb 225 resin (100–200 mesh; 8% cross linked) was cycled with alkali and acid according to Work and Denman (1953) and transferred to a column of

diameter 0.9 cm. The length of the resin in the column was 18 cm. The resin was equilibrated with 1.5 N HCl and washed with 1.5 N HCl until about 50 ml. of eluate gave only a slight deposit on concentration to less than 1 ml. (This procedure was necessary in order to remove substances washed out from the resin which caused distortions in subsequent paper chromatograms.) Amino-acid solution or brominated hydrolysate (0.5–1 ml.) containing the equivalent of up to 200 mg. protein was transferred to the column and eluted with 1.5 N HCl at room temperature. For our purposes, it was found unnecessary to change the eluting solution to 2.5 N HCl (Work and Denman, 1953). Eluates were freed from excess HCl (see below) and concentrated to suitable volume for examination by paper chromatography on Whatman No. 4 paper, in water-saturated-phenol-NH₃. The same column could be prepared for use again by washing for 17 hours with 4 N HCl followed by equilibration with 1.5 N HCl (Work and Denman, 1953).

Results

Colour Reaction of Diaminopimelic Acid with Ninhydrin.—On one-dimensional chromatograms developed in phenolic solvents or on the two-dimensional paper method the initial purple colour of the diaminopimelic acid spot gradually became grey, and finally, after two or three days, yellow, which did not fade in the absence of light. This distinctive colour change with ninhydrin was a useful confirmation in identification. Rhuland, Work, Denman, and Hoare (1955) reported characteristic colour changes on chromatograms which had been developed with an aqueous pyridine-methanol-hydrochloric acid mixture, but this system was not found to be satisfactory for testing column eluates, as it was too insensitive for the small quantities of diaminopimelic acid encountered and because the position of accompanying basic amino-acids overlapped that of diaminopimelic acid.

Separation of Diaminopimelic Acid in a Protein Hydrolysate.—A brominated hydrolysate of normal lung residue containing 23 mg. N (i.e., 144 mg. protein, employing 6.25 as the factor) and 0.5 mg. added diaminopimelic acid in 1 ml. was eluted on the column with 1.5 N hydrochloric acid. Fractions (5 ml.) were collected on a fraction collector (Locarte & Co.) and each fraction was concentrated to small bulk and stood *in vacuo* over potassium hydroxide to remove acid. To each residue was added 100 μ l. water and 5 or 10 μ l. portions of the solutions were used for paper chromatography. Control solutions containing diaminopimelic acid were developed alongside. Cysteic acid was eluted first, followed by the di-

carboxylic amino-acids and the lower mono-amino-monocarboxylic acids. Valine, leucine, and proline were next eluted. After 100 ml., the next 20 ml. were free from amino-acids. Diaminopimelic acid began to appear at 125 ml. and was eluted in the next 40 ml. The diaminopimelic acid was accompanied by amino-acids, presumed to be basic, having high R_F values in phenol-ammonia, which continued to be eluted after the diaminopimelic acid. Since their presence did not interfere in the detection of diaminopimelic acid, no attempt was made to remove them. The lower limit of detection of diaminopimelic acid was 0.5–1 $\mu\text{g.}$ in 5 $\mu\text{l.}$ Comparison with standard amounts on the paper chromatograms indicated substantial recovery of the added diaminopimelic acid. When a similar amount of normal lung hydrolysate, which contained no added diaminopimelic acid, was fractionated on the column this amino-acid was not found.

Application of Tests to Pathological Tissues

Caseous Tissue.—The modified two-dimensional method showed that diaminopimelic acid was clearly present in the residue after autoclaving, but its presence in the extract was doubtful. However, when fractionated in the column, the extract was found to contain this amino-acid. A brominated hydrolysate of the extract containing 8 mg. N, i.e., about 50 mg. protein, was separated on the column. Successive eluates, 60, 60, 35, 45, and 25 ml., were collected, concentrated to a final volume of 100 $\mu\text{l.}$, and 5 $\mu\text{l.}$ taken for paper chromatography. Diaminopimelic acid was found in the third and fourth eluates (confirmed by mixed chromatograms with added diaminopimelic acid) and, by comparison with known amounts, the total quantity present was estimated to be about 60 $\mu\text{g.}$ The autoclaved residue by the column method contained about five times this concentration.

Progressive Massive Fibrosis.—The two-dimensional method failed to establish with certainty that diaminopimelic acid was present, either in the residue or autoclaved extract. A hydrolysate containing about 30 mg. N was then separated on the column. Tests for diaminopimelic acid in the appropriate effluents were negative or doubtful and it was concluded that diaminopimelic acid if present amounted to not more than 0.01% of the protein.

Caplan Nodule.—A sample, 0.6 ml., of a brominated hydrolysate of the unautoclaved tissue containing 60 mg. protein was fractionated on the column and diaminopimelic acid was found

to be present (chromatogram and mixed chromatogram). The amount was calculated to be 0.04% of the weight of the protein. The concentrated eluate was tested further by the two-dimensional ionophoresis-chromatography method and diaminopimelic acid was shown to be present by the occurrence of the spot in the expected position and by its colour changes.

Discussion

The tests described here have confirmed that most of the diaminopimelic acid is left in the residue after autoclaving. However, if sufficient material is available, autoclaving can be dispensed with for column fractionation, since most of the amino-acids are relatively less strongly adsorbed and easily separated from the diaminopimelic acid. Though less sensitive, the two-dimensional paper method is convenient for tissues containing more than about 2 $\mu\text{g./mg.}$ protein. Conventional two-dimensional paper chromatography has a similar order of sensitivity but is less specific for diaminopimelic acid.

The amount of diaminopimelic acid found in the caseous tissues confirms the suggestion by Consden and Glynn (1955) of a total bacterial infection amounting to about 20% of the lesion, despite the absence of evidence for the presence of living tubercle bacilli. The Caplan tissue contained diaminopimelic acid corresponding to about 2% of bacteria. This figure is to be compared with three or four times that amount found by Consden and Glynn (1955) in another Caplan specimen. In contrast, both P.M.F. tissues so far examined appear to contain little or no diaminopimelic acid. It would, of course, be premature at this stage to suggest that infection was associated with one type of lesion and not with the other, especially in view of Nethercott and Strawbridge's (1956) findings of diaminopimelic acid in a high proportion of P.M.F. specimens. The column method, because of its high specificity and sensitivity for diaminopimelic acid, would appear to be suitable for the examination of a series of lung lesions in order to establish whether or not bacterial infection is implicated.

Summary

A modification of an earlier two-dimensional method for detecting α,ϵ -diaminopimelic acid in tissues is described, and also a procedure for concentrating this amino-acid in hydrolysates in a column, a method suitable for larger quantities of tissue.

These methods were used to re-examine a caseous nodule and to analyse further specimens of "progressive massive fibrosis" and "Caplan" nodules from cases of pneumoconiosis.

It was confirmed that the caseous tissue contained diaminopimelic acid amounting to about 0.4% of the protein. The Caplan nodule contained 0.04%. Diaminopimelic acid was not found in the P.M.F. tissue.

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