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THE CHROMATOGRAPHIC SEPARATION OF PHAGOCYTIN

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SUMMARY

A chromatographic method of purification has shown that the antibacterial activity of the crude material "phagocytin" is confined to the fraction having a molecular weight range 13,000–14,500 approximately. However, this active material is not sufficiently soluble at physiological pH values to have a clinical application as an antibacterial substance.

INTRODUCTION

The work described in this publication was carried out as part of a programme of investigation involving antibacterial substances of animal origin, and their toxicity towards cultured human cells. The substance here investigated, phagocytin, was first isolated by HIRSCH[1–3]. He showed that rabbit polymorphonuclear leucocytes, obtained as a peritoneal exudate, contained a substance which was soluble in dilute organic acids, and which appeared to be particularly lethal to certain Gram-negative bacteria. He termed the substance "phagocytin". He was able to extract it also from leucocytes from the guinea-pig, the horse and human, but most of his work concerns the rabbit material. He investigated its nature as well as its antibacterial activity and on the basis of its behaviour in strong ammonium sulphate solutions, its inability to pass through dialysis membranes and the partial loss of activity caused by proteolytic enzymes, he suggested that it was a globulin. Further work by HIRSCH and his co-workers[4–9] pointed to its location within the lysosomes, or typical granules of the polymorphonuclear neutrophil and their results suggested that it was released, along with various degradative enzymes, into the vacuole within which the ingested bacterium was contained.

It was of interest, therefore, to examine this substance more closely. In the course of the investigations, it became obvious that the insolubility of all the extract above a pH value of 3.5 prevented a satisfactory examination of its toxicity towards mammalian cells, and therefore this aspect of the study of phagocytin was not pursued. Instead a greater measure of purification was attempted than that originally achieved by HIRSCH to see if an active, soluble substance could be obtained.

MATERIALS AND METHODS

In the preliminary experiments, phagocytin was extracted from rabbit peritoneal exudate cells by the method of HIRSCH[3]. Since this method, in our experience, yielded a small amount of cells in return for the effort expended, an alternative source of polymorphonuclear leucocytes was sought. Whole bovine and ovine blood were tried and rejected on account of their poor erythrocyte sedimentation rate which made separation difficult. Whole porcine blood proved suitable, both in that the erythrocytes separated readily, and in that it was readily available in large volumes from an abattoir. Therefore the erythrocytes from whole, fresh, citrated, pig blood were separated by sedimentation with a 3 % solution of Dextran (average molecular weight 200,000–270,000; B.D.H. Ltd., Poole, England) by the method of SKOOG AND BECK[10]. The remaining suspension of leucocytes was centrifuged for 15 min at 10^3 *g* and the supernatant fluid was discarded. No attempt was made to separate the neutrophils from the other leucocytes since the method of extraction was known to extract no antibacterial substance from other leucocytes[3]. The packed leucocytes were homogenised using a Potter-Elvehjem homogeniser and extracted with 0.34 *M* sucrose and then 0.01 *M* citric acid as described by HIRSCH[3] for rabbit neutrophils. The phagocytin was contained in the citric acid extract.

Separation of the active constituents of the extract was initially attempted by means of salt precipitation with ammonium sulphate and subsequent concentration dialysis through Visking tubing. This method was abandoned for several reasons, the most important of which were the penetration of polyethylene glycol 6000, which was used as a concentrating solution, into the dialysis sac and the difficulties in precipitating, centrifuging and redissolving very small amounts of solid from the solution. The more elegant and more quantitative method of gel filtration was favoured and proved satisfactory.

A column of Sephadex G75 (AB Pharmacia, Uppsala, Sweden) measuring 50 cm × 2.5 cm diameter was prepared in the standard manner by soaking the granules in buffer and pouring the slurry into the glass tube whose end had been sealed with a bed of glass wool and a layer of coarse grain Sephadex G25. The buffer of choice was 0.1 *M* ammonium acetate–acetic acid at pH 3. A capillary outlet tube from the Sephadex column tube was connected to a Uvicord recording spectrophotometer (LKB Produkter AB, Stockholm, Sweden). The absorption of light, at 254 mμ, by the eluate could thus be measured and continuously recorded as it was being collected in 10 ml fractions. The chromatography was carried out at room temperature. The column was calibrated as described by ANDREWS[11] using the following proteins: polymyxin (Burroughs Wellcome Ltd., London, Great Britain), insulin (Allen & Hanbury Ltd., London, Great Britain), cytochrome C (B.D.H. Ltd., Poole, Great Britain), ovalbumin (Sigma Ltd., London, Great Britain), pepsin and bovine plasma albumin (The Armour Pharmaceutical Co., Eastbourne, Great Britain).

The citric acid extract of each of three batches of pig leucocytes was added in ten volumes of 2 ml each to the top of the Sephadex column and washed through with the acetate buffer from a reservoir which maintained a constant hydrostatic pressure. The collected 10 ml fractions were stored at —20° until all the original 20 ml of each extract had been passed through the column, whereupon the corresponding fractions from each of these ten separations were pooled, lyophilised, redissolved in 1 ml 0.01 *M*

citric acid solution, and assayed for antibacterial activity. The lyophilised batches of product from each of the three samples of pig blood were, however, kept separate, so that, in effect results would be obtained in triplicate *de novo*. In view of the assay results for antibacterial activity (Table I), those fractions containing solutes of lower molecular weight (*i.e.* eluted from the column after 120 ml, as shown in Fig. 1) were pooled for further separation, lyophilised and redissolved in 10 ml distilled water. This concentrate was passed through a Sephadex G25 column prepared in the same manner as the G75 column. Since the chemical nature of the substances present was unknown, the G25 column was not calibrated and this was subsequently found to be unnecessary (see Results).

The eluate from the G25 column was divided into groups of three or four 5 ml fractions which were then pooled with the same groups from subsequent passages of the low molecular weight concentrate through the same column, lyophilised, redissolved in 0.01 *M* citric acid and assayed for antibacterial activity. In addition, the groups were assayed for citric acid using the assay advocated in the British Pharmacopoeia[12].

The necessity to test large numbers of fractions for antibacterial activity dictated that the method used should be simple, sensitive and quantitative. An agar diffusion test was preferred to serial dilution methods as being less laborious. A range of seven commercially available agar gels was tested and Oxoid Sensitivity Test Agar (S.T. Agar, Oxoid Ltd., London, Great Britain) was found to be suitable since of all the types tested, only in S.T. agar did the citric acid extract cause zones of inhibition of growth of test organism. The organism used in the test was *E. coli* N.C.T.C. 414, maintained in Oxoid Nutrient Broth.

Since nutrient agar preparations are usually opalescent, accurate measurement of zones of growth inhibition is made less difficult by the incorporation of an indicator in the gel. Triphenyltetrazolium chloride (TTC) has been frequently used as an indicator of metabolism and growth and it has found particular use in bacteriology[13–16], where, *inter alia*, it has been used to determine zones of inhibition by flooding penicillin assay plates with a solution of TTC prior to measurement[17]. The metabolic reduction reactions of the organisms reduce the colourless TTC to a red formazan.

The medium for our experiments was prepared as follows. Volumes of 10 ml of S.T. agar 4 % in distilled water were sterilised by autoclaving. Similar volumes of 0.1 *M* citrate buffer (double strength) for pH 6 were also prepared and autoclaved. Separate autoclaving was necessary, otherwise hydrolysis of the agar by the citric acid would occur. When required, the agar was melted, cooled to 50° and mixed with buffer also at 50°. To this mixture were added 0.2 ml 10 % TTC solution, sterilised by filtration, and 0.2 ml of a saline suspension of an overnight culture of *E. coli*. After gentle shaking, the mixture was poured into 100 mm petri dishes and allowed to set. Wells were cut in the agar using a 7 mm sterile cork-borer and into these wells aliquots of the sample to be tested were pipetted. Areas of growth were shown by red, opaque areas and inhibition, where it occurred, was observed as colourless areas.

RESULTS

The optical density of the eluate from the G75 column recorded as it emerged from the column is shown in Fig. 1. The results of the antibacterial tests carried out

on the fractions are given in Table I. The optical density of the eluate from the G25 column is shown in Fig. 2 and the results of the antibacterial tests and citrate estimations which were carried out on the fractions from the G25 column are given in Table II.

The antibacterial effect shown by the fractions containing low molecular weight solutes can be seen to be associated with the fractions containing the citric acid originally used in the extracting solution (Table II). The concentration procedures

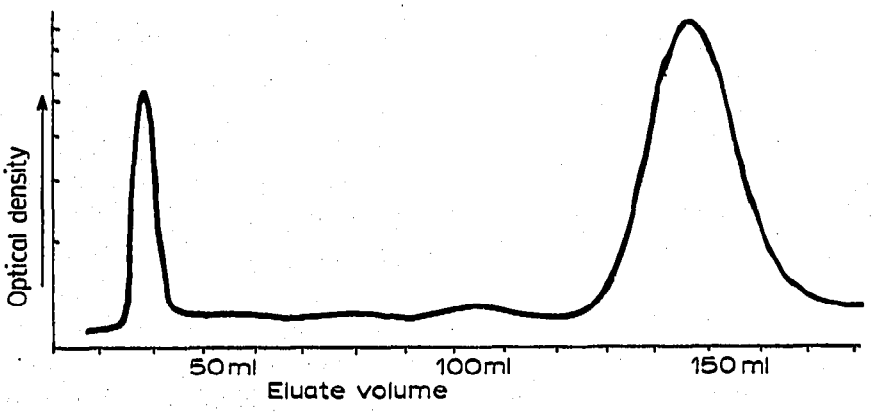

Fig. 1. Chromatogram of 0.01 *M* citric acid extract of leucocytes eluting from a Sephadex G75 column, measured at 254 mμ.

TABLE I

ASSAY RESULTS FOR ANTIBACTERIAL ACTIVITY OF VARIOUS FRACTIONS OF SEPHADEX G75 ELUATE

Fraction (ml)	*0–60*	*60–70*	*70–80*	*80–90*	*90–100*	*100–130*	*130–170*
Diameter* of zone of inhibition (mm)	0	5	12	10	8.5	0	17

* The figures for diameter are the mean of ten separate measurements performed on two separate occasions using the same batch of pig blood. The results have been confirmed using the two other batches of pig blood.

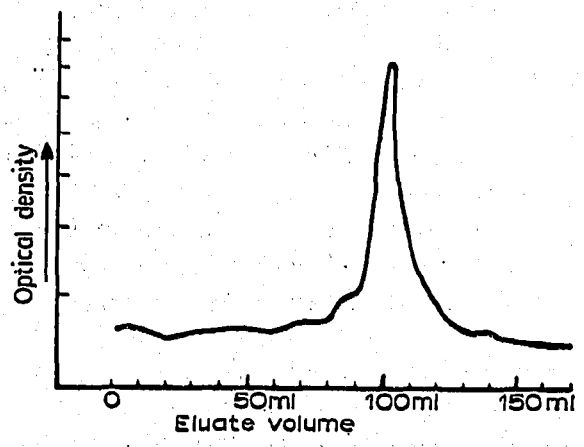

Fig. 2. Chromatogram of 120–170 ml fractions of a G75 column eluate (Fig. 1) concentrated and added to a G25 column, measured at 254 mμ.

TABLE II

ASSAY RESULTS FOR ANTIBACTERIAL ACTIVITY OF VARIOUS FRACTIONS OF SEPHADEX G25 ELUATE

Fraction (ml)	*20–60*	*60–80*	*80–95*	*95–135*	*135–155*
Diameter* of zone of inhibition (mm)	0	11	10.5	0	0
Volume* of NaOH solution equivalent to citric acid content (ml)	0	0.47	0.61	0.23	0

* The values are the mean of three measurements whose scatter was not more than 0.5 mm or 0.05 ml respectively.

would raise the concentration of citric acid above the bacteriostatic level and it is presumed that this is the explanation for the observed bacterial inhibition in the fractions of volumes beyond 120 ml of Fig. 1. This is the reason for calibration of the G25 column becoming superfluous.

The conversion of the block diagram of the antibacterial activity of the fractions of the G75 eluate into the best fit parabola was performed using the formula $y = a + bx + cx^2$, and the individual results from the average of which the block diagram had been constructed. The result of this conversion is depicted in Fig. 3. According to this method, the peak of antibacterial activity occurred after 80 ml had eluted from the G75 column. The calibration graph for the G75 column is shown in Fig. 4.

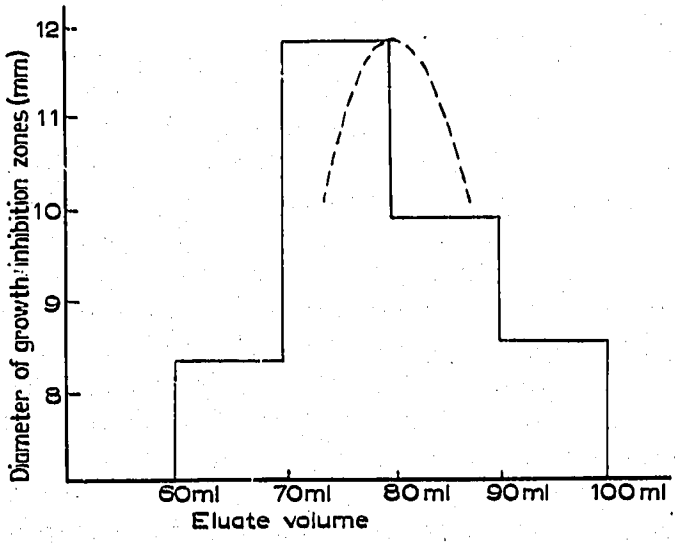

Fig. 3. Block diagram of antibacterial activity of fractions with best fit parabola superimposed.

DISCUSSION

The use of gel filtration as a means of molecular weight estimation is now generally accepted in work dealing with large molecules[11,18–20]. The accuracy of the method where the molecules involved are approximately spherical is about 12% (ref. 20). It has advantages over other methods such as simplicity and cheapness of equipment and the simultaneous purification of the sample. Indeed in this particular instance estimation of molecular weight is a by-product of attempts to purify and

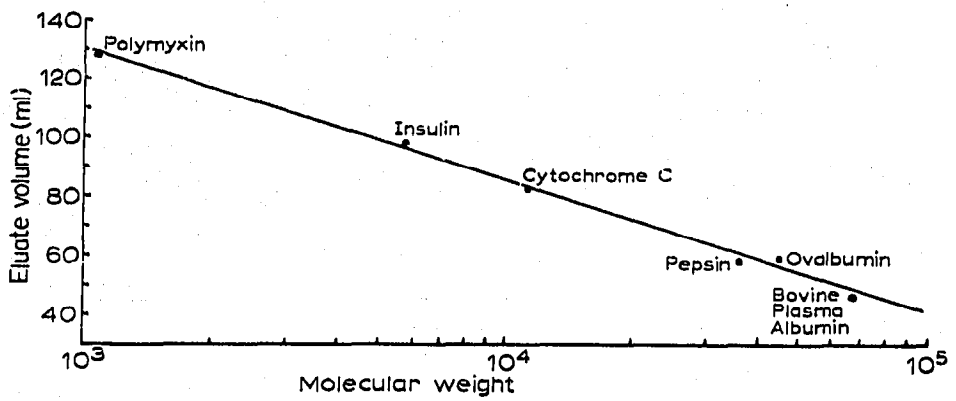

Fig. 4. Calibration plot of a G75 column. Elution volume (V_e) plotted against log molecular weight of proteins which were dissolved in 0.1 *M* acetic acid.

render soluble, in medium of a pH suitable for the maintenance of living cells, an otherwise acid-soluble substance.

The method is not, however, without its drawbacks. The assumption made in this work is that phagocytin is a protein. HIRSCH's original experiments were only suggestive and not in fact confirmatory that this was so. In our work we were not primarily concerned with the true chemical composition of phagocytin since we were seeking a clinically useful material, and the small amounts available demanded equipment for absolute chemical analysis to which we had not access. We confirmed HIRSCH's findings in our preliminary experiments.

If the substance is indeed a protein, it may still be conjugated with a carbohydrate or other prosthetic group. If this is the case, or if the molecule deviates drastically from the globular shape, then its passage through the pores of the dextran gel will be affected. This situation has been examined by ANDREWS[18] who could find no correlation between the migration rates of glycoproteins and their structure, but found that the greater the deviation of an all-protein molecule from the spherical shape, the more was its progress through the dextran gel impeded. Such molecules, *e.g.* fibrinogen, were eluted from columns much later than their molecular weights led one to expect. Other factors which might interfere with progress through the gel matrix such as aromatic adsorption or ionic concentration effects[21] can be discounted. In the former instance the lack of an ultraviolet absorbing group in the molecule (see Fig. 1 and later discussion) discounts the suggestion of the presence of an aromatic group, and in the latter instance, at the low pH used, the molecule was completely soluble and hence presumably fully hydrated. The other proteins used in the calibration were passed through at the same pH. Their elution volumes plotted against molecular weight showed the same straight line (Fig. 4) as found at neutral pH by ANDREWS[11]. The phagocytin molecule is therefore presumed to show no anomalous behaviour because of pH and ionic content of the elution fluid.

In order to obtain a figure for elution volume for a molecular weight estimation, the peak of an elution curve is usually chosen. However, it is unavoidable that the results for identifying these fractions containing antibacterial activity should take the form of a block diagram rather than a continuous curve with a clear peak. This is brought about by the requirement for an irreducible minimum volume for antibacterial testing.

It has been contended that single substances are eluted from columns in essentially symmetrical peaks[21]. This is true for the upper portion of the elution curve and therefore the block diagram of our results may be converted to a theoretical best fit parabola (Fig. 2) with its peak at a value of 80 ml. This gives a K_d value of 0.3 (ref. 19), and a value of 13,000 for the molecular weight. In our experience the whole elution curves of some substances, *e.g.* plasma albumin and insulin, tend to be slightly but noticeably skewed to the left with a steep leading edge and a more gently sloping trailing edge. Such a curve would fit more closely the observed shape of the block diagram and would have a peak about 78 ml. The resultant molecular weight of 14,500 is within the 12 % accuracy figure already quoted[20]. Therefore the best fit parabola need not be discounted.

The optical density elution graph (Fig. 1) shows no peak at the point of maximum antibacterial activity. This does not negate the possibility that phagocytin is a protein since the ultraviolet absorption of proteins is due to their aromatic amino acid content. There are several already known proteins which lack such acids and presumably there are many more.

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