might already have been present after 3–4 min. The period of time required for the first appearance of secreted labelled albumin is only a little shorter than that which we calculate to be required for complete replacement of the intracellular albumin. In our experiments three-quarters of the newly synthesized intracellular albumin was bound to subcellular particles, but the true proportion was probably greater, inasmuch as any disruption or failure to sediment the particles would increase the amount of albumin apparently unbound.

These facts taken together suggest that the process of secretion involves rapid synthesis of albumin molecules, which remain attached to subcellular structures for a relatively much longer period, before being rapidly released from the cell. The process must resemble travel along a pipe line, since the time taken for the first labelled albumin molecules to be secreted in the presence of labelled amino acids is little less than the time taken for secretion of an amount of albumin equal to the whole of the intracellular pool. Furthermore, in an active liver, the time required for synthesis of albumin molecules and their passage to the surface of the cell cannot vary very widely from cell to cell or even within each cell.

SUMMARY

1. An estimate of the amount of intracellular albumin in rat livers has been obtained by an isotope-dilution method, in rats which had received rat [^{131}I]albumin and been kept until equilibrium had been reached between the blood and the liver.

2. Albumin was recovered, by precipitation with antibody and dissociation in a trichloroacetic acid–ethanol mixture, from the livers after homogenization and treatment with deoxycholate, and the specific radioactivity was measured and compared with that of plasma albumin.

3. The estimates of the intracellular albumin pool were 0·3–1·19 mg./g. wet wt. In four of seven experiments the value was close to 0·4 mg./g.

4. The distribution was examined of intracellular albumin between mitochondrial and microsomal fractions and the supernatant. At least three-quarters of the intracellular albumin was associated with the particulate fractions, and was evenly divided between the two.

5. The bearing of these findings on the process of albumin synthesis and secretion are discussed.

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REFERENCES

Askonas, B. A. (1958). *Rec. Trav. chim. Pays-Bas*, **77**, 611.
Cohen, S. M. & Gordon, A. H. (1958). *Biochem. J.* **70**, 544.
Delaville, M., Delaville, G. & Delaville, J. (1953). *Ann. pharm. franç.* **12**, 109.
Freeman, T., Gordon, A. H. & Humphrey, J. H. (1958). *Brit. J. exp. Path.* **39**, 459.
Freund, J. & McDermott, K. (1942). *Proc. Soc. exp. Biol., N.Y.*, **49**, 548.
Gordon, A. H. & Humphrey, J. H. (1960). *Biochem. J.* **75**, 240.
Grabar, P. & Williams, C. A. (1953). *Biochim. biophys. Acta*, **10**, 193.
Holmes, B. E. (1956). *Ciba Foundation Symp., Ionizing Radiation and Cell Metabolism*, p. 225.
Humphrey, J. H. & McFarlane, A. S. (1954). *Biochem. J.* **57**, 195.
Keller, E. B., Zamecnik, P. C. & Loftfield, R. B. (1954). *J. Histochem. Cytochem.* **2**, 378.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
McFarlane, A. S. (1958). *Nature, Lond.*, **182**, 53.
Parkes, A. S. (1946). *J. Hyg., Camb.*, **44**, 491.
Penn, N. W. (1960). *Biochim. biophys. Acta*, **37**, 55.
Peters, T. (1957). *J. biol. Chem.* **229**, 659.
Schneider, W. C. & Hogeboom, G. H. (1951). *Cancer Res.* **11**, 1.

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The Purification of Interferon

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Virus interference may be defined as an effect of a live or inactivated virus on host cells which results in that cell being unable subsequently fully to support the growth of a second virus. In 1957, during a study of interference induced by heat-inactivated influenza virus, Isaacs & Lindenmann (1957) observed the formation of a virus-interfering substance, to which they gave the name 'interferon'. Interferon could not be detected until several hours after treatment with inactivated virus, when it appeared first in the cells and was subsequently released into the surrounding buffer

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solution. Although it is different in a number of ways from the virus particle, it showed the interfering ability of the original virus, and it appears to be the mediator of virus interference (Isaacs & Burke, 1959). It has also been shown that interferon, or a very similar substance, is involved in the resistance shown by persistently infected cell cultures to a second virus infection (Ho & Enders, 1959; Henle, Henle, Deinhardt & Bergs, 1959).

Interferons have been produced by the interaction of a number of different viruses with several kinds of cells. The products are functionally similar, but are not identical, as shown by the limited host-cell specificity of the action of interferon (Tyrrell, 1959; Isaacs & Westwood, 1959*a*). In contrast, interferon produced from inactivated influenza virus is capable of inhibiting the multiplication of a number of viruses, although to different degrees. These include such different viruses as vaccinia virus, which contains deoxyribonucleic acid (Isaacs, Lindenmann & Valentine, 1957), and West Nile virus, which contains ribonucleic acid (Isaacs & Westwood, 1959*b*).

It has previously been shown that interferon, prepared by the interaction of ultraviolet-inactivated influenza virus and chick chorio-allantoic membranes, is probably a protein which is stable over the pH range 2–11, but is destroyed by heating to 100° for 5 min. It is non-dialysable and is destroyed by incubation with trypsin and pepsin, but not by incubation with ribonuclease and deoxyribonuclease (Lindenmann, Burke & Isaacs, 1957). The present work is concerned with the purification and characterization of interferon.

A preliminary account of this work has been published (Burke, 1960).

EXPERIMENTAL

Interferon and interference

Buffer. Earle's buffered saline (Parker, 1950) was used throughout, with the addition of benzylpenicillin (100 i.u./ml.) and streptomycin sulphate (50μg./ml.).

Virus strains. These were the Melbourne (MEL) and PR 8 strains of influenza A.

Preparation of ultraviolet-inactivated virus. Ultraviolet irradiation of virus, partially purified by adsorption on to chick red cells and elution into saline, was carried out as described by Isaacs, Burke & Fadeeva (1958), the dose being $9{\cdot}5 \times 10^5$ ergs/sq.cm. The irradiated virus had an infectivity of less than 2 ID_{50} (two 50% infective doses)/ml. as shown by two consecutive egg passages of undiluted fluid. This represents inactivation of the original virus eluate by approximately 10^{-9}. The virus was stored at $-70°$.

Preparation of interferon. Interferon was prepared by the interaction of ultraviolet-inactivated Melbourne virus and the chorio-allantoic membranes of fertile eggs (Lindenmann *et al.* 1957; Burke & Isaacs, 1958). The ultraviolet-inactivated virus (1000–1500 agglutinating doses per membrane) in 50 ml. of Earle's buffer was shaken for 1–3 hr. with ten chorio-allantoic membranes from fertile eggs which were 10 or 11 days old. The membranes were washed and shaken overnight with 50 ml. of fresh buffer. The buffer was then removed, fresh buffer was added, and shaking was continued for a further 24 hr., after which the membranes were discarded. The two harvests were pooled, clarified by centrifuging at 1000 *g* for 20 min., and stored at 2°. The yield of interferon was affected neither by the variation, between the limits stated, of the time of incubation of the ultraviolet-inactivated virus with the membranes, nor by the age of the eggs; 13- or 14-day-old eggs were found to be as suitable. Some of the interferon used for this work was prepared by the Microbiological Research Establishment, Porton, and by Glaxo Laboratories Ltd.; the process used by the latter is described in the Addendum.

Assay of interferon activity. Small portions of the chorio-allantoic membrane were obtained from 10- or 11-day-old eggs as described by Lindenmann *et al.* (1957), six to eight pieces being taken from each egg. The interferon assay was carried out as previously described with six tubes per experimental group. After incubation for 18–24 hr. the fluids were removed and replaced by a 10^{-4} dilution of PR 8 virus in Earle's buffer. The groups were then incubated for a further 48 hr. before titration of their haemagglutinin content. Occasionally, groups were chilled to 2° after 48 hr. incubation, and titrated the next day, without any apparent change in activity.

In earlier experiments samples were dialysed against Earle's buffer before being tested for biological activity, but later it was found more convenient to test the samples without dialysis, that is, by dilution directly with Earle's buffer. Dilutions were so chosen that the final concentration of salt was insufficient to inhibit production of virus in similarly treated controls. The activity of purified samples tested in this way was higher than when they were tested after dialysis, presumably owing to the avoidance of surface losses during dialysis.

Haemagglutinin titrations were set up and read as described by Lindenmann *et al.* (1957).

There was considerable variation in haemagglutinin titres between the six members of any one group, with a consequent high standard deviation, usually between one and two twofold dilutions. This high standard deviation obscured the nature of the relationship between the concentration of interferon and the degree to which virus yield is depressed, but it was clear that the virus yield increased more rapidly upon dilution of the interferon than was found by Isaacs *et al.* (1957). This difference might be due to a change in the source of interferon: interferon prepared from ultraviolet-inactivated virus contains much less protein than did that prepared from heat-inactivated virus. A number of attempts were made to decrease the standard deviation of the mean haemagglutinin titration, but none were successful. These included the use of different media (Hanks or Geys buffered salt solutions), the additions of 0·1% crystalline bovine plasma albumin (Armour and Co. Ltd., Hampden Park, Eastbourne, Sussex), or 1% gelatin during the first 24 hr. of incubation in an attempt to prevent possible denaturation of interferon, variations in the size and age of the pieces of chorio-allantoic membrane, the use of membrane still attached to the cell as described by Fazekas de St Groth & White (1958) in an attempt to prevent adsorption on the chorionic surface of the mem-

brane, shaking instead of rolling the tubes during incubation, and the use of the Melbourne strain of influenza A, instead of PR 8 as a challenge virus. Difficulties, which will be discussed below, were also encountered in estimating the activity of purified samples.

In practice the best way to compare two different samples of interferon was to test both over a series of twofold dilutions (usually three) and to compare the dilutions which gave similar degrees of interference. This gave a percentage activity recovery for any given stage. This figure, coupled with the protein concentrations, gave a purification factor for this particular stage, the purification factor being defined as the activity recovery divided by the protein recovery.

Chromatography on cellulose ion-exchangers

Reagents. Except where otherwise stated, buffers were made from sodium salts. Their concentrations are stated with respect to the anionic component. Analytical-grade reagents and ion-exchange water were used throughout. Cellulose was Solka-Flok, B.W. 200-mesh; 2-chlorotriethylamine hydrochloride was obtained from Eastman Kodak Ltd. and purified by recrystallization from methanol and ethyl acetate, as described by Press, Porter & Cebra (1960); monochloroacetic acid was reagent-grade; sodium chloromethanesulphonate was prepared as described by Porath (1957).

Cellulose ion-exchangers. Diethylaminoethylcellulose (DEAE-cellulose) and carboxymethylcellulose were prepared as described by Peterson & Sober (1956). The exchangers were regenerated after use by washing with N-NaOH to remove adsorbed proteins, and then washing with water. Sulphomethylcellulose was prepared from graded Solka-Flok and sodium chloromethanesulphonate as described by Porath (1957). It had about 0·3 m-equiv. of acidic groups/g. After use, this exchanger was regenerated by washing with 0·1N-NaOH followed by water until the filtrate was neutral.

The ion-exchangers were prepared for use by washing in a sintered-glass filter funnel with 0·1M-buffer of the appropriate pH and then with buffer of correct molarity until the pH of the eluate and buffer were the same. They were used to prepare columns, which were washed with buffer overnight at 2°. Concentration-gradient elution was carried out by running a buffer of higher concentration from a reservoir into a mixing vessel of constant volume containing the buffer of lower concentration. All columns were run at 2°.

Phosphate concentration in the eluate from DEAE-cellulose column was determined by a micromodification of the method of Allen (1940). and chloride concentrations by calculation from the formula given by Press *et al.* (1960).

Starch-gel electrophoresis

The following buffers were used: glycine–NaOH, pH 8·9, contained 50 m-moles of glycine and 8 m-moles of NaOH/l., while the buffer for the bridge solution contained 0·3 mole of H_3BO_3 and 0·06 mole of NaOH/l. (Smithies, 1955); KCl–HCl buffer, pH 2, was that described by Gomori (1955), with a buffer containing 0·2 mole of KCl and 10 m-moles of HCl/l. as a bridge solution.

Starch-gel electrophoresis was carried out as described by Smithies (1955) with hydrolysed starch obtained from Connaught Medical Research Laboratories, Toronto, Canada. The gel was prepared and poured into a Perspex tray, 25 cm. × 4 cm. and 6 mm. deep, and allowed to set for at least 1 hr.

Electrophoresis in the glycine–NaOH buffer was for 6 hr. at 11 mA and 8 v/cm. or 16 hr. at 5 mA and 4 v/cm. In the KCl–HCl buffer the current was 5 mA for 18 hr. at 0·5 v/cm. The gel was cut and stained with Amido-Black as described by Smithies (1955). Attempts to recover protein with biological activity from the unstained section of the gel by freezing, thawing and centrifuging the appropriate section were unsuccessful. Electrophoresis as described by Gordon (1960) was much more satisfactory.

Large-scale starch-gel electrophoresis was carried out in a tray 25 cm. × 15 cm. × 0·9 cm. The protein sample (0·8 ml. of solution containing 30 mg. of protein/ml.) was applied to a 0·9 cm. × 13 cm. strip of Whatman seed test paper and placed in a slit cut near one end of the starch block. After electrophoresis for 18 hr. at 25 mA and 4 v/cm., a thin slice was removed from the top of the block and stained with Amido-Black. Protein was recovered from the unstained part of the block by electrophoresis of the appropriate section.

Analytical methods

Protein. The concentration of protein solutions was determined by measuring their absorption at 280 mμ, or by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine plasma albumin as a standard. Samples were dialysed against 0·9% NaCl soln. before determination by the Lowry method, since benzylpenicillin at a concentration of 100 i.u./ml. gave a considerable blank. Comparison of the results obtained by measurement of the absorption at 280 mμ and by the Lowry method showed that the proteins fractionated on the ion-exchangers had an extinction coefficient at 280 mμ of 0·7–0·9 when measured in a 1 cm. cell at a concentration of 1 mg./ml.

Ribonucleic acid. The method of Webb (1956) was used with ribose as a standard. The purine ribonucleotides were assumed to account for half of the ribose content of the ribonucleic acid (RNA). The quality of the xylene was important, and best results were obtained with xylene which had been shaken with conc. H_2SO_4 and redistilled.

Deoxyribonucleic acid. Burton's (1956) modification of the Dische reaction was used with highly polymerized salmon-sperm deoxyribonucleic acid (DNA) (California Corp. for Biochemical Research, Los Angeles 63, Calif., U.S.A.) as a standard.

Carbohydrate. The method of Dische (1955) was used with glucose as a standard.

Hexosamine. The hexosamine analysis was performed by Dr H. R. Perkins. Hexosamine as 'glucosamine' was estimated after separation from amino acids on a cation-exchange resin (Boas, 1953) by the method of Rondle & Morgan (1955).

Ultracentrifugal analysis. The work with the ultracentrifuge was done by Dr P. A. Charlwood using a Spinco Model E ultracentrifuge. Runs were done in 0·15M-KCl + 21·5 mM-NaH_2PO_4 + 9·5 mM-Na_2HPO_4 buffer, pH 6·4, close to 20°, at 52 640 rev./min. in the 12 mm. cell. Photographs were taken at about 16 min. intervals. Molecular-weight determinations were by the method of Archibald (1947) (as used by Charlwood (1957)) at a speed of 17 550 rev./min., photographs being taken at 8 min. intervals between about 30 and 60 min. after reaching speed. The partial specific volume was assumed to be 0·74.

RESULTS

Initial 100-fold concentration of interferon

The crude interferon preparation contained 150–200μg. of protein/ml., 4–8μg. of RNA/ml. and 6–12μg. of DNA acid/ml. and was faintly stained with haemoglobin. At a concentration of 100μg. of protein/ml. it depressed virus growth in the test system used to below 1% of that obtained in controls. The purification procedure for 5 l. of interferon is summarized in Table 1.

Protein fractionation was not attempted with the original solution because of the small amount of protein present, and attention was therefore first turned to concentration. A 10- to 30-fold concentration without loss of activity could be achieved by precipitation with ammonium sulphate. Solid ammonium sulphate was added to 73% saturation, and the solution left overnight at 2°. The precipitate was removed by centrifuging for 1 hr. at 2° and 1800 **g**, and was dissolved in a small volume of Earle's buffered saline.

After dialysis against several changes of Earle's buffered saline the solution was dialysed overnight against an 0·1M-potassium chloride–hydrochloric acid buffer (pH 2·0) and then finally against Earle's buffered saline. Dialysis against buffer, pH 2·0, served as a convenient sterilizing step and also caused the precipitation of some heavily pigmented material. It also inactivated traces of virus which might complicate the assay of interferon. The precipitate was removed by low-speed centrifuging, and the supernatant further concentrated to about 0·5% of the original volume by ultrafiltration through 18/32 Visking (Visking Corp., Chicago, Ill., U.S.A.) dialysis tubing supported by a sleeve of silk net under 60 cm. Hg pressure against Earle's buffered saline. This procedure, which was carried out at 2°, was primarily a concentration step, but some purification was also achieved without loss of activity.

Some attempted methods of purification

A number of procedures were investigated in attempts to fractionate either the 30-fold or the 200-fold concentrated solutions prepared as described above. They were only partially successful and are summarized briefly below.

Electrophoresis of interferon, either on paper or on columns of ethanol-treated cellulose (Porath, 1956), was not pursued, owing to poor recoveries of biological activity. Starch-block electrophoresis (Kunkel & Slater, 1952) effected very little purification.

Stepwise addition of ammonium sulphate (at pH 7·0) or of ethanol at 0–5° (Askonas, 1951) to interferon gave a series of precipitates all containing biological activity. Because of its lack of promise this approach was not pursued any further.

No significant purification was obtained by chromatography on a column of hydroxylapatite (Tiselius, Hjerten & Levin, 1956), or on the carboxylic cation-exchange resin Amberlite IRC-50 (Hirs, 1955).

Purification of interferon by chromatography on cellulose ion-exchangers

Choice of conditions. In preliminary experiments, interferon was chromatographed on DEAE-cellulose columns at pH values of 5·5, 6·0 and 7·0 without any loss of biological activity, but the behaviour on carboxymethylcellulose columns was anomalous. On chromatography on carboxymethylcellulose at pH 5·5 in 0·01M-acetate, interferon was not retained by the column, but at pH 5·0 it was adsorbed irreversibly and could not be eluted by 2M-acetate, pH 5·0, or by 2M-acetate, pH 6·0. Further study showed that at pH 5·2 interferon was irreversibly adsorbed, whereas at pH 5·3 it was not retained by the column. This anomalous behaviour may be due to the fact that the carboxyl

Table 1. *Purification of interferon*

	Stage	Average purification factor	Average activity recovery
Procedure 1	1. Ammonium sulphate precipitation followed by pressure dialysis	2·7	100
	2. Chromatography on sulphomethylcellulose at pH 2·0	2·1	60
	3. Concentration, adjustment to pH 6·6 and chromatography on DEAE-cellulose at pH 6·6	1·6	100
	4. Concentration, adjustment to pH 5·8, followed by chromatography on DEAE-cellulose at pH 5·8 (twice)	2·0	12
	Overall (procedure 1)	18	7
Procedure 2	1, 2 and 3 as procedure 1		
	4. Adjustment to pH 4·5 and chromatography on DEAE-cellulose at pH 4·5	2·4	100
	5. Adjustment to pH 5·8 and chromatography on DEAE-cellulose at pH 5·8	0·8	25
	Overall (procedure 2)	17	15

groups of the ion-exchanger are not fully ionized at these pH values. Sulphomethylcellulose (Porath, 1957) was therefore prepared as the more strongly acidic groups are completely ionized at this pH. Anomalous behaviour was again observed at pH 5·0, the percentage recovery varying with the buffer used and also with the batch of ion-exchanger. The use of pH values lower than 5·0 was therefore investigated, and chromatography of interferon on sulphomethylcellulose at pH 2·0 was found to be satisfactory. Almost complete recovery of biological activity could be obtained, but on both sulphomethylcellulose columns at pH 2·0 and DEAE-cellulose columns at pH 5·8 and 6·6 there was always considerable trailing of the activity. It was therefore difficult to obtain complete resolution of interferon from other components, and this decreased the purification obtained at any given stage. As a result, it was advisable to use four modified cellulose ion-exchange columns in sequence in order to obtain a homogeneous product. Two procedures for purification were worked out (Table 1), of which the second is the better. They were carried out as follows.

Chromatography on sulphomethylcellulose. The 200-fold concentrated solution (about 25 ml.) was dialysed for 24 hr. against two changes of 0·1M-potassium chloride–hydrochloric acid buffer, pH 2·0. The precipitate was removed by centrifuging for 20 min. at 1000 *g* and the supernatant run slowly on a pre-equilibrated sulphomethylcellulose column (25 cm. × 1·5 cm.²). Under these conditions, the proteins were adsorbed as a dark-brown band at the top of the column. The column was then washed with 0·1M-potassium chloride–hydrochloric acid buffer, pH 2·0, until the extinction at 280 mμ was falling sharply. A concentration gradient was then applied by running 2M-potassium chloride, adjusted to pH 2·0 with hydrochloric acid, into a 250 ml. mixing vessel containing 0·1M-potassium chloride–hydrochloric acid buffer. Preliminary experiments had shown that, although interferon was stable for at least 3 days at 2° in 0·1M-potassium chloride–hydrochloric acid buffer, pH 2·0, activity dropped to about 50% in one day at 2° in M-potassium chloride adjusted with hydrochloric acid to pH 2·0. The fractions (10 ml.) were therefore neutralized with 0·1N-NaOH immediately after collection and before the extinction at 280 mμ was read. As shown in Fig. 1, a single broad peak of protein was obtained with the biological activity concentrated in the later fractions. Appropriate fractions were then pooled, the fractions containing the protein peak but little biological activity being rejected. An average of 60% of the activity was recovered with a purification factor of 2·1.

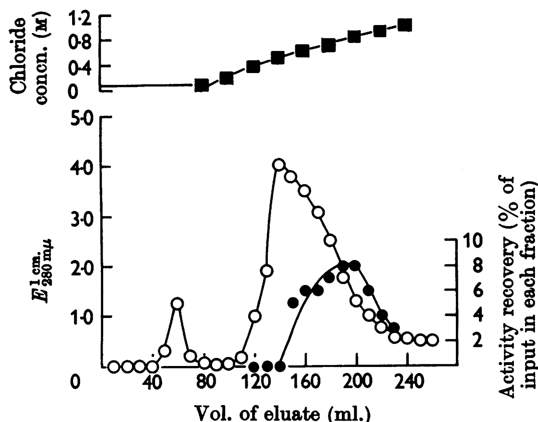

Fig. 1. Chromatography of interferon (200-fold concentrated) on a sulphomethylcellulose column 25 cm. × 1·5 cm.² by gradient elution with potassium chloride–hydrochloric acid buffer, 0·1–2M, pH 2·0. Volume of mixing chamber was 250 ml. ○, Extinction coefficient; ●, percentage activity recovery; ■, chloride concentration in eluate.

Chromatography on diethylaminoethylcellulose. Preliminary experiments showed that there was a considerable loss of activity when the sulphomethylcellulose eluate was concentrated by pressure dialysis against 0·01M-phosphate, pH 5·8, and the precipitate removed before the run on a DEAE-cellulose column. The loss was probably due to adsorption on to the precipitate. However, the eluate could be pressure-dialysed against 0·01M-phosphate, pH 6·6, and the precipitate removed without any loss of activity. The supernatant (2–3 ml.) was run slowly on a previously equilibrated DEAE-cellulose column (10 cm. × 0·3 cm.²).

Small columns were used in this and subsequent fractionations since preliminary experiments had shown that more activity could be recovered by using small columns and steep eluting gradients, i.e. when protein concentrations were kept high. The column was well washed with 0·01M-phosphate, pH 6·6, and then a phosphate gradient started by running M-potassium phosphate buffer, pH 6·6, into a 50 ml. mixing vessel containing 0·01M-phosphate, pH 6·6. A change in pH, similar to that described by Ryle & Porter (1959), was observed when the phosphate concentration of the eluate began to rise, but it was much smaller than when sodium chloride in 0·01M-phosphate, pH 6·6, was used as the eluting buffer. Fractions (2·5 ml.) were collected and the resulting chromatogram is shown in Fig. 2. A single peak was obtained with 100% activity recovery and a purification factor of 1·5-fold. The peak was shown to contain four components stained by Amido-Black by starch-gel electrophoresis at pH 8·9 (Fig. 3). When the components were individually recovered by electrophoresis and tested for their ability to inhibit the multiplication

of influenza virus, only one, fraction A, was found to be active. Preparative starch-gel electrophoresis of 30 mg. of protein from the DEAE-cellulose column in a large block of starch gel gave only about 20% recovery of biological activity. This approach to large-scale purification was therefore not pursued further.

Interferon could be separated from the other components, B, C and D (Fig. 3), by repeated chromatography at pH 5·8 on a DEAE-cellulose column with an increasing phosphate gradient (Table 1, procedure 1), or, better, by running on a DEAE-cellulose column at pH 4·5 (Table 1, procedure 2).

In the first procedure, the DEAE-cellulose eluate obtained at pH 6·6 was concentrated to 2–3 ml. by pressure-dialysis and then dialysed against several changes of 0·01M-phosphate, pH 5·8. A small amount of precipitate was formed which carried down some of the biological activity. The supernatant had little biological activity and chromatography on DEAE-cellulose at pH 5·8 gave a product consisting of components B and C. Therefore the precipitate was not removed after dialysis against 0·01M-phosphate, pH 5·8, and all the material was adsorbed on a previously equilibrated DEAE-cellulose column (10 cm. × 0·3 cm.²). After thorough washing a phosphate gradient was started by running 0·5M-potassium phosphate, pH 5·8, into a 50 ml. mixing vessel containing 0·01M-phosphate, pH 5·8. Two partially resolved peaks were obtained, as shown in Fig. 4, the first of which contained the biological activity. Starch-gel electrophoresis showed that the first peak consisted mainly of band A (Fig. 3), whereas the second peak consisted of bands B and C. The fractions constituting the first peak were pooled, concentrated, dialysed against 0·01M-phosphate buffer, pH 5·8, and run again on DEAE-cellulose at pH 5·8, as above. Two partially resolved peaks were again obtained, the first of which was shown to give a single band on starch-gel electrophoresis at both pH 8·9 and pH 2·0. However, examination in the analytical ultracentrifuge by Dr P. A. Charlwood demonstrated the presence of about 20% of a lighter component.

In the second procedure the DEAE-cellulose pH 6·6 eluate was dialysed against 0·01M-acetate, pH 4·5, and run on to a previously equilibrated DEAE-cellulose column (10 cm. × 0·3 cm.²). Interferon was not retained by the column, and the biological activity was recovered quantitatively in the eluate. The other components B, C and D (Fig. 3), which had been shown to be present by starch-gel electrophoresis, were retained by the column. They could, however, be eluted by a gradient running up to 0·4M-acetate, pH 4·5, with a 50 ml. mixer. The

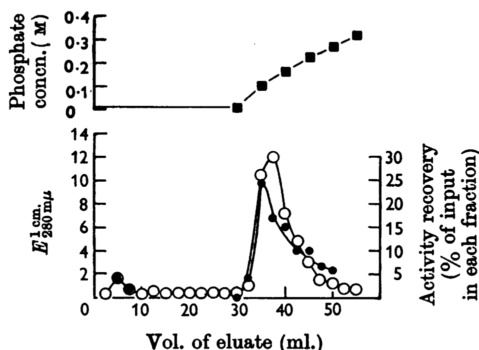

Fig. 2. Chromatography of interferon (sulphomethyl-cellulose eluate) on a DEAE-cellulose column (10 cm. × 0·3 cm.²) by gradient elution with potassium phosphate buffer, 0·01–1M, pH 6·6. Volume of mixing chamber was 50 ml. ○, Extinction coefficient; ●, percentage activity recovery; ■, phosphate concentration in eluate.

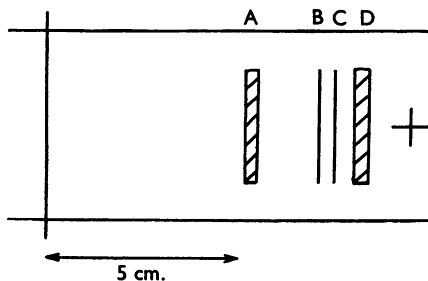

Fig. 3. Starch-gel electrophoresis of pH 6·6 DEAE-cellulose eluate in glycine–NaOH buffer, pH 8·9; 8 v/cm. for 6 hr.

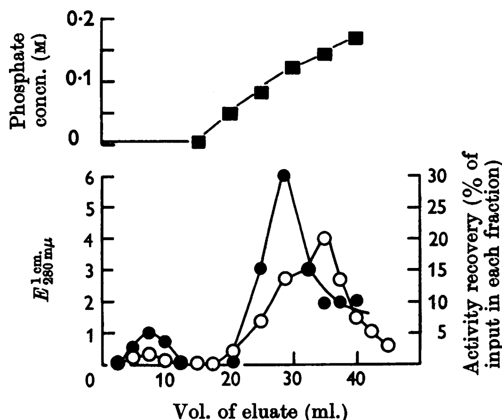

Fig. 4. Chromatography of interferon (pH 6·6, DEAE-cellulose eluate) on a DEAE-cellulose column (10 cm. × 0·3 cm.²) by gradient elution with potassium phosphate, 0·01–0·5M, pH 5·8. Volume of mixing chamber was 50 ml. ○, Extinction coefficient; ●, percentage activity recovery; ■, phosphate concentration in eluate.

biologically active eluate was pressure-dialysed against 0·01M-phosphate, pH 5·8, and chromatographed on a DEAE-cellulose column at this pH under conditions which have been described above. A single symmetrical peak was obtained (Fig. 5), which gave a single band on starch-gel electrophoresis at both pH 8·9 and pH 2·0. Only about 25% of the biological activity was recovered. This poor recovery was observed whenever pure or nearly pure interferon was chromatographed. Good recoveries were obtained when less-pure material was chromatographed on DEAE-cellulose at pH 5·8, and the loss was probably due to surface losses.

Attempts to obtain better activity recoveries by using silicone-treated pipettes in the interferon assay were not noticeably effective.

Ultracentrifugal analysis

Three preparations were examined by Dr P. A. Charlwood in the Spinco analytical ultracentrifuge, the first two prepared by procedure 1 (Table 1) and the third by procedure 2 (Table 1). The first preparation had almost no biological activity and its characteristics are recorded only for the light they throw on the behaviour of the second preparation. It was prepared by chromatography of material of low activity on DEAE-cellulose at pH 5·8 and was shown by starch-gel electrophoresis to consist of components B and C (Fig. 3). Examination in the ultracentrifuge revealed a single component of $S_{20,w}$ 3·34 and of molecular weight 46 000. The two components, which do not differ appreciably in charge, must have very similar molecular weights.

The second preparation had been purified by chromatography on DEAE-cellulose at pH 5·8 as described in procedure 1. Ultracentrifugal analysis revealed two components, the lighter one accounting for about 20% of the material. The $S_{20,w}$ of the heavier component was 4·84s and this agrees well with the value of $S_{20,w}$ found for interferon in the third preparation. The lighter component was probably composed of a mixture of components B and C (Fig. 3) which have already been shown to be separated only with difficulty from interferon by chromatography on DEAE-cellulose at pH 5·8.

The third preparation had been purified by procedure 2 (Table 1). This behaved in the ultracentrifuge as a single component with a molecular weight of 63 000 and a sedimentation constant of 4·77s (at 4% concentration).

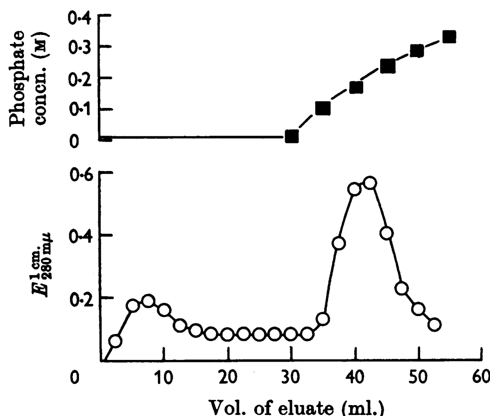

Fig. 5. Chromatography of interferon (pH 4·5, DEAE-cellulose eluate) on a DEAE-cellulose column (10 cm. × 0·3 cm.²) by gradient elution with potassium phosphate, 0·01–0·5M, pH 5·8. Volume of mixing chamber was 50 ml. ○, Extinction coefficient; ■, phosphate concentration in eluate.

Properties of interferon

The most active preparation of interferon obtained was the eluate from the pH 4·5 DEAE-cellulose column in procedure 2, stage 4. When diluted to a protein concentration of 6μg./ml. it depressed virus growth, in the test system used, to 3% of that obtained in controls. This material had been purified about 20-fold, as shown by the purification factor and also by comparison of the protein concentrations of preparations of crude and purified material having comparable biological activities.

The preparation analysed was that obtained from procedure 2, stage 5, and this exhibited a typical protein ultraviolet spectrum. The results of analyses expressed as % of protein concentration were: RNA, < 0·03; DNA, 0·3; carbohydrate, 1·6; hexosamine, 2·4.

DISCUSSION

Three kinds of evidence for the purity of the final interferon preparation have been obtained. Chromatography on DEAE-cellulose at pH 5·8 with an increasing phosphate gradient gave a single symmetrical peak, and starch-gel electrophoresis at both pH 8·9 and 2 revealed only a single band. Finally, examination in the analytical ultracentrifuge showed a single component of $S_{20,w}$ 4·77 and molecular weight 63 000.

From the Svedberg equation and the measured values of the sedimentation constant and the molecular weight, a value for the diffusion coefficient of $D_{20} = 7{\cdot}07 \times 10^{-7}$cm.²/sec. can be obtained. This is in quite good agreement with the mean value of $7{\cdot}4 \times 10^{-7}$ cm.²/sec. obtained by Porterfield, Burke & Allison (1960) by measurement of the rate of diffusion of interferon through agar gel, and used by them to calculate a maximum molecular weight. The value for the diffusion coefficient obtained by Porterfield *et al.* (1960) depends on the measurement of the rate of diffusion in agar

of the biological activity; and, although this method is not a precise one, the agreement between the two values for the diffusion coefficient indicates that the biologically active molecule is the component observed in the ultracentrifuge.

An estimate of the isoelectric point of interferon can be made from the chromatography data. Interferon is retained by a DEAE-cellulose column at pH 5·0 but not at pH 4·5, and this suggests that the isoelectric point of interferon lies between pH 4·5 and 5·0. Wagner (1960) has suggested that interferon is a basic protein because of its adsorption by bentonite. However, the chromatography data do not support this suggestion.

Analysis of the purified preparations of interferon shows that it is a protein containing small amounts of carbohydrates and no nucleic acid. The nucleic acids have been involved so deeply in the mechanism of virus multiplication that previous work, which on the basis of experiments with enzymes indicated that interferon was a protein rather than a nucleoprotein, was cautiously interpreted, since some nucleoproteins (e.g. elementary virus particles) resistant to nucleases are known. The evidence that interferon is a protein may be summarized as follows: first, the behaviour on treatment with proteolytic enzymes; secondly, the molecular weight of 63 000, which excludes the possibility of its being nucleoprotein; and thirdly, the analysis of the purified material.

SUMMARY

1. Interferon, prepared by the interaction of ultraviolet-inactivated influenza virus and chick chorio-allantoic membranes, has been purified about 20-fold.

2. The purified preparation of interferon could not be resolved by chromatography on modified cellulose ion-exchange columns. It gave a single band on starch-gel electrophoresis at pH 8·9 and 2. Examination in the ultracentrifuge revealed a single component of molecular weight 63 000 ($S_{20,w}$ 4·77).

3. The purified preparation is a protein containing no nucleic acids and only small amounts of carbohydrates.

I wish to express my thanks to a number of my colleagues, particularly to Dr A. Isaacs for advice and for many stimulating discussions, to Dr J. Walker, for his interest in this work, and to Professor R. R. Porter for helpful discussions on ion-exchange chromatography. I am also indebted to Dr P. A. Charlwood for determining the sedimentation constant and molecular weight of interferon, to Dr H. R. Perkins for the hexosamine analysis, and to Dr A. H. Gordon for help with the electrophoretic elution from starch gel. I would like to thank Miss V. Coker and Miss R. Woolgar for able technical assistance. This work was greatly facilitated through supplies of interferon kindly made at M.R.E. Porton and Glaxo Laboratories Ltd.

REFERENCES

Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.

Archibald, W. J. (1947). *J. phys. Chem.* **51**, 1204.

Askonas, B. A. (1951). *Biochem. J.* **48**, 42.

Boas, N. F. (1953). *J. biol. Chem.* **204**, 553.

Burke, D. C. (1960). *Biochem. J.* **76**, 50 P.

Burke, D. C. & Isaacs, A. (1958). *Brit. J. exp. Path.* **39**, 452.

Burton, K. (1956). *Biochem. J.* **62**, 315.

Charlwood, P. A. (1957). *Trans. Faraday Soc.* **53**, 871.

Dische, Z. (1955). *Meth. biochem. Anal.* **2**, 321.

Fazekas de St Groth, G. & White, D. O. (1958). *J. Hyg., Camb.*, **56**, 151.

Gomori, G. (1955). In *Methods in Enzymology*, vol. 1, p. 138. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Gordon, A. H. (1960). *Biochim. biophys. Acta*, **42**, 23.

Henle, W., Henle, G., Deinhardt, F. & Bergs, V. V. (1959). *J. exp. Med.* **110**, 525.

Hirs, C. H. W. (1955). *Methods in Enzymology*, vol. 1, p. 113. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Ho, M. & Enders, J. F. (1959). *Virology*, **9**, 446.

Isaacs, A. & Burke, D. C. (1959). *Brit. med. Bull.* **15**, no. 3, 185.

Isaacs, A., Burke, D. C. & Fadeeva, L. (1958). *Brit. J. exp. Path.* **39**, 447.

Isaacs, A. & Lindenmann, J. (1957). *Proc. Roy. Soc.* B, **147**, 258.

Isaacs, A., Lindenmann, J. & Valentine, R. C. (1957). *Proc. Roy. Soc.* B, **147**, 268.

Isaacs, A. & Westwood, M. A. (1959*a*). *Lancet*, ii, 324.

Isaacs, A. & Westwood, M. A. (1959*b*). *Nature, Lond.*, **184**, 1232.

Kunkel, H. G. & Slater, R. J. (1952). *J. clin. Invest.* **31**, 677.

Lindenmann, J., Burke, D. C. & Isaacs, A. (1957). *Brit. J. exp. Path.* **38**, 551.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

Parker, R. C. (1950). *Methods of Tissue Culture*, p. 81. London and Toronto: Cassell and Co. Ltd.

Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.

Porath, J. (1956). *Biochim. biophys. Acta*, **22**, 151.

Porath, J. (1957). *Ark. Kemi*, **11**, 97.

Porterfield, J. S., Burke, D. C. & Allison, A. C. (1960). *Virology* (in the Press).

Press, E. M., Porter, R. R. & Cebra, J. (1960). *Biochem. J.* **74**, 501.

Rondle, C. J. M. & Morgan, W. T. J. (1955). *Biochem. J.* **61**, 586.

Ryle, A. P. & Porter, R. R. (1959). *Biochem. J.* **73**, 75.

Smithies, O. (1955). *Biochem. J.* **61**, 629.

Tiselius, A., Hjerten, S. & Levin, O. (1956). *Arch. Biochem. Biophys.* **65**, 132.

Tyrrell, D. A. J. (1959). *Nature, Lond.*, **184**, 452.

Wagner, R. R. (1960). *Bact. Rev.* **24**, 151.

Webb, J. M. (1956). *J. biol. Chem.* **221**, 635.

ADDENDUM

Preparation of Interferon

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The method used was basically that described by Burke & Isaacs (1958) and consisted in exposing chick chorio-allantoic membranes to influenza virus (Melbourne), irradiated by ultraviolet light, and harvesting the culture fluid.

The inactivated seed virus was prepared by irradiating 3 ml. of allantoic fluid containing the Melbourne strain of influenza virus in a 4 in. Petri dish for 1 min., 7 cm. beneath a Phillips T.U.V. tube with a maximum emission at 2537Å. The haemagglutinating capacity of the inactivated-virus preparation was determined by making serial twofold dilutions of this material in 0·9% NaCl soln. in 0·25 ml. volumes and adding an equal volume of 0·5% fowl red-cell suspension to each dilution. After settling at room temperature the sedimentation patterns were observed. One agglutinating unit was taken as the highest dilution giving partial agglutination, and the titres were expressed as the reciprocal of the initial dilutions of inactivated virus at the end point. The seeding of the 5 l. glass bottles containing the membranes was at the rate of 1000 haemagglutinating units per membrane.

Eggs were incubated in large commercial incubators for 10 days, and the fertile eggs, after swabbing with 50% aqueous ethanol, were drilled through the end opposite the air sac. After removal of this part of the shell, the embryo, yolk sac and allantoic fluid were pulled out and discarded. The chorio-allantoic membranes were removed and placed in a beaker of Earle's medium (40 membranes per beaker). The Earle's medium at all stages was reinforced with 500μg. of benzylpenicillin/ml. and 500μg. of streptomycin sulphate/ml.

Each membrane was washed individually in more Earle's medium to clear it of yolk, albumin and blood, and 40 washed membranes were placed in a 5 l. glass bottle containing 200 ml. of medium. These bottles were seeded with ultraviolet-irradiated Melbourne virus and rocked in a 37° incubator for 3 hr.

After incubation for 3 hr. with seed virus, the membranes were removed from the bottles, washed in Earle's medium to remove surplus virus and put into further 5 l. bottles, each containing 200 ml. of Earle's medium. They were then rocked at 37° for 16–18 hr., when the first harvest was taken. The membranes were reincubated as before in Earle's solution in fresh 5 l. bottles for a second harvest.

The culture fluid containing interferon was harvested in 500 ml. blood bottles and clarified by centrifuging at 320 *g* for 20 min. The supernatant interferon solution was removed and tested for sterility. The second harvest received identical treatment.

REFERENCE

Burke, D. C. & Isaacs, A. (1958). *Brit. J. exp. Path.* **39**, 78.

Biochem. J. (1961) **78**, 564

Lipo-amino Acid Complexes from *Bacillus megaterium* and their Possible Role in Protein Synthesis

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In the course of studies of protein synthesis in cell-free systems derived from *Bacillus megaterium*, it was found that some of the labelled amino acids apparently incorporated into protein could be solubilized by methanol, lipase or dilute alkali and obtained in a form no longer precipitable by trichloroacetic acid (Hunter, Brookes, Crathorn & Butler, 1959). When the labelled membrane fraction was extracted with deoxycholate, it was further found that the solubilized material contained protein having a higher specific radioactivity than the protein of the insoluble portion. These results stimulated a study of the possible role of lipids in the amino acid incorporation process, and it has