Interferons: Purification and Physicochemical Aspects

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ABSTRACT  Antiviral interferon activity in any one species can be exhibited by a variety of substances that differ in their physical and chemical properties, but the nature of these differences is not understood. Conditions that can lead to the formation of diverse types of interferons have been outlined. Reasons have been adduced why, for certain purposes, purification of interferons is desirable or even necessary, and examples have been presented to show how and to what extent this has been achieved. In spite of some very high purification factors, not a single interferon has been obtained as a pure substance. Therefore, all available knowledge of physical and chemical properties has been obtained by indirect means.

The first interferon was discovered by Isaacs and Lindenmann (1) in 1957, and since then many biochemists have attempted to purify many interferons. It is perhaps worthwhile to pause for a moment and consider why it is sometimes necessary, or at least desirable, to work with purified rather than with crude interferons.

WHY PURIFY INTERFERONS?

Experiments with crude interferons have on several occasions led to wrong interpretations, and examples that show how this danger was sometimes overcome by purification or other means are presented. There are at least five situations where the use of crude interferons is inappropriate.

Firstly, crude interferons, but also sera and tissue extracts from apparently normal animals, culture fluids from uninfected cells, urine, allantoic fluid, egg white, agar overlays, etc., frequently contain other often nonvirus- and nonspecies-specific antiviral substances that may simulate certain aspects of interferon action and may thus mislead the casual observer. A number of specific and nonspecific viral antagonists of this nature is shown in Table I which represents the “list of contents” of a recent review article by Wasserman (2). But there are doubtless others, e.g. Buckler and Baron (3) showed that heterologous, acid-stable, antivaccinia virus activity of crude mouse
interferon and of mouse serum could be readily removed from chick cells by mere washing. The same process also eliminated, again from chick cells, a noninterferon antivaccinia factor, present in normal allantoic fluid. Andrews (4) found that crude chick, calf, monkey, and human interferons had pronounced activity against vaccinia virus in rabbit skin, and even the noninfected, heterologous culture fluids themselves were active in this system. Similarly, the size of vaccinia lesions and the degree of necrosis in monkey skin were not reduced only by monkey interferon, but also by crude chick interferon. We, (5, 6) in collaboration with Doctors Andrews and Finter, found that crude allantoic chick interferon also had some activity in human embryonic lung cells and in mouse L cells against rhinoviruses and Sendai viruses, respectively, but that partly purified and concentrated interferon, with much higher potency in chick cells, had lost its heterologous activity. Sutton and Tyrrell (7) and Sellers and Fitzpatrick (8) reported that crude calf interferon inhibited viruses in human and monkey cells, and heterologous activities of other crude interferons have been described by Pollikoff et al. (9) and by Paucker (10). With hindsight, we would now guess that at least some of these activities were caused by agents other than interferons and that they could probably be eliminated by washing the treated cells or by some purification step.

The second class of active contaminants present sometimes in infected or normal culture fluids, or in crude interferon preparations, are materials capable of actually enhancing virus growth. Some of these, such as the "stimulons" of Chany and his colleagues (11, 12) and various other factors described

| TABLE I |
| --- |
| SOME NATURAL, NONINTERFERON VIRAL INHIBITORS (2) |

| I. Specific Inhibition of Virus Infection |
| --- |
| A Antibody to the virus |
| B Antibody to the host cell |

| II. Nonspecific Inhibition of Virus Infection |
| --- |
| A α-(Neuraminic acid) inhibitors of myxoviruses |
| B γ-Inhibitors of myxoviruses |
| C Mucopolysaccharide inhibitor of Theiler's virus |
| D Mucoid inhibitors of mumps virus and pneumonia virus of mice |
| E Mucoid inhibitors of bacteriophages |
| F Lipid inhibitors in normal sera |
| G Nonlipid inhibitors in normal sera |
| H Inhibitors in the overlay plating agar |
| I Nonspecific inhibitor of bacteriophage T2 |
| J Host-induced modification |
| K Mycoplasma contamination of tissue culture cells |
| L Inapparent and latent infections |
| M Miscellaneous factors in the tissue culture system |
| N Virus inactivation following virus-cell interaction |
| O Inhibition of plant viruses |
by Truden et al. (13), by Sheaff and Stewart (14), by Fournier et al. (15), and by Chany and Robbe-Maridor (16), do so by antagonizing the action of interferons. Other potential proviral contaminants have been described by Righthand and Karzon (17, 18) and by Toyoshima and Vogt (19).

Several naturally occurring factors are known to block the synthesis rather than the action of interferons (20, 21) but these are outside the scope of this discussion.

A third group of substances, those sometimes present in crude interferons, has been found to affect the metabolism of uninfected cells and so could possibly, directly or indirectly, also effect virus proliferation. Isaacs et al. (22) thought that crude chick interferon inhibited oxidative phosphorylation and increased glycolysis of chick embryo cells, but Lampson et al. (23) later showed that purified interferon caused no such effects. Levy et al. (24) found that crude chick interferon inhibited the synthesis of “slowly labeled” RNA of chick fibroblasts, but later Levy and Merigan (25) showed that purified interferon did not impede cellular synthesis of DNA, RNA, or protein. Baron et al. (26) clearly demonstrated that crude mouse interferon inhibited cell growth, but not by virtue of its antiviral activity. Sonnabend et al. (27) reported that crude, but not highly or partly purified, chick interferon inhibited the action of Semliki Forest virus RNA-polymerase. Kerr et al. (28) noted cytopathic effects and reduced ribosomal activity, after chick fibroblasts had been exposed to high concentrations of chick interferon of a specific activity of about 10,000 U/mg protein; but these phenomena did not follow the use of materials that were 3-10 times purer.

The fourth reason for the use of purified interferons is a more mundane one. Whenever highly concentrated interferon preparations are to be used with either cultured cells or in animals, some inert material must be eliminated because a crude interferon containing 5% serum would contain, after 100-fold concentration, some 400-500 mg/ml protein, and it would not be an easily injectable fluid but a viscous semisolid glue.

The fifth situation, requiring the use of really pure interferons, concerns certain approaches to work on their chemical composition and their physical properties. Except with preparations of very high purity (a state, as will be shown later, not yet reached), any direct chemical analysis or measurement of physical parameters can only lead to questionable or valueless results. Several such studies have, however, been published but are, in my view at least, best disregarded, e.g. the conclusion, drawn from the absence of a peak at 280 m\textmu in the UV absorption spectrum, that chick interferon lacks aromatic amino acids (29). Other workers (30) have published chemical data on chick interferon and also a molecular weight (now known to be incorrect) calculated from the position of a visible band in a sucrose gradient; still others (23), using much more highly purified chick interferon, published not only a UV
absorption spectrum but also some chemical data, among them the percentage contents of several amino acids. We, in our turn (5), presented the amino acid composition of what we then thought to be pure chick interferon. Results of other chemical analyses were published for calf interferon (31), for a rabbit interferon-like substance (32), and for mouse and monkey interferons (33). Although these particular results (obtained by direct observation of impure interferons) are probably of little value, it does not mean that we know nothing of their chemical and physical properties. Valuable information has been obtained in an indirect manner, not by studying the interferon itself, but from the effects of various treatments on the antiviral behavior of these preparations. Such experiments have meaning, and results obtained in this way will be discussed later on.

METHODS USED TO PURIFY INTERFERONS

Isaacs and his colleagues (1, 34, 35) showed already in their first publications that chick interferon was, or at least contained, protein. It was precipitated by ammonium sulfate, it was substantially destroyed by trypsin but not by nucleases, it was retained by dialysis tubing, and it could not be sedimented by centrifugation at 100,000 g. The observation that interferon was, at least in part, protein has guided subsequent purification work, and most or all the methods known to protein chemists have been employed. Such methods are, for instance: salting out procedures; precipitation of interferon or of impurities with protein-precipitating acids like trichloroacetic, perchloric, thiocyanic, and others; precipitation with lower alcohols or ketones; adsorption onto and release from various metal hydroxides or silicates; chromatography on ion-exchange resins, -celluloses, or -gels; gel filtration; dialysis; electrophoresis in liquid gradients or solid supports; ultracentrifugation; electrofocusing; and electrodialysis

Interferons that have been purified to varying extents by one or more of these methods include those from species as far apart as chick, mouse, rat, rabbit, monkey, and man. All these were virus-induced; only Dima et al. (33) seem to have attempted to purify nonviral animal interferons.

Considerable work has also been done on the purification of some interferon-like substances that occur in normal and virus-infected plants.

With the exception of chick interferon, none has, until recently, been purified more than about 50-100-fold. During the last year, however, Paucker and his colleagues (36, 37) have prepared mouse interferon of high specific radio- and bioactivities, and Dr. Paucker will present his results later on in this Symposium.

PURIFICATION OF CHICK INTERFERON

Most of the work in our laboratory concerned chick interferon, and its purification is therefore presented in somewhat more detail. Early work by Wagner
(29), Burke (30, 38), Zemla and Vilček (39, 40), Kreuz and Levy (41), and others did not lead to high purification. The first highly purified chick interferon was obtained by Lampson et al. (23). With allantoic interferon as their starting material, they precipitated residual virus and some inert protein with dilute perchloric acid, concentrated the active material by adsorption onto zinc hydroxide, dissolved the adsorbate in dilute acid, and removed zinc ions by dialysis; then they twice chromatographed the interferon on CM-cellulose and finally subjected the peak fractions to alkaline electrophoresis in Pevikon. In this way 4500-fold purification was achieved. Merigan et al. (42), using similar starting material, somewhat modified Lampson’s methods by substituting CM-Sephadex for CM-cellulose and by eluting the activity from the ion-exchange gel by a rising pH gradient instead of stepwise. This led to 6500-fold purification. Our own early purification work (43, 44) also started with allantoic fluid interferon, but we used somewhat different methods after we had discovered some perhaps unexpected properties. We found, for example, that chick interferon was, like serum albumin, soluble in acid aqueous methanol, ethanol, and acetone, but unlike albumin it was also soluble in acid thiocyanate or iodide solutions. After screening a number of potential adsorbents, we found that the synthetic, micronized Na-Al-silicates “Alusil” and “Doucil” (J. Crosfield and Sons, Warrington, England) adsorbed interferon at an acid pH and released it again, somewhat purified and concentrated, on suspension in smaller volumes of solutions of higher pH and ionic concentration. By making use of these findings and by combining them with some other purification steps, allantoic interferon could be purified about 20,000-fold, to a specific activity of $1.6 \times 10^8$ U/mg protein. (Activity was assessed by a CPE protection method, in which chick fibroblasts were challenged with Semliki Forest virus.) In later work (6) using tissue culture interferon, a more potent and much cleaner starting material, the same purification sequences were found applicable. The most highly purified interferon obtained again had a specific activity of $1.6 \times 10^8$ U/mg protein, representing for this type of starting fluid a 3400-fold purification. The process is summarized in Table II.

### Table II

#### Purification of Chick Tissue Culture Interferon (6)

| Sample                          | Total units (X 10⁶) | Specific activity (µg/mg protein) | Purification factor | Over-all recovery (%) |
|---------------------------------|---------------------|----------------------------------|---------------------|-----------------------|
| Crude culture fluid             | 22,500              | 5.40                             | 470                 | 100                   |
| pH 7.5 0.5 M KSCN Doucil eluate | 3000                | 4.80                             | 1980                | 4                     |
| pH 3.5 supernatant fluid        | 3000                | 3.60                             | 5180                | 11                    |
| pH 2.0 supernatant fluid        | 3000                | 2.37                             | 15,500              | 33                    |
| Redissolved methanol precipitate| 225                 | 1.80                             | 19,300              | 41                    |
| DEAE-cellulose eluate fractions  | 960                 | 1.14                             | 132,000             | 281                   |
| CM-Sephadex gradient eluate fractions | 25             | 0.40                             | 1,600,000           | 3400                  |

(Continued on next page)
Yet another method that led to highly purified chick interferon was de-
veloped by Bodo (45). By combining and modifying some of Lampson's and
some of Merigan's steps, and by including final filtration through Biogel P-
2000, he purified allantoic fluid interferon 8000 times.

To test for homogeneity, our most highly purified interferon preparations
were subjected to polyacrylamide gel electrophoresis in alkaline solution.
The process was essentially that of Davis (47), but without sample and spacer
gels. On completion of the electrophoresis, the gel column was cut lengthwise
into two equal portions; one was stained with amido black, the other was cut
into 2-mm long pieces which were individually eluted and assayed for inter-
feron content. The results of this experiment are shown in Fig. 1. The region
of peak activity coincided exactly with a sharp double band of protein, raising
the hope that one of the two bands was the interferon. In order to verify this,
electrophoresis was carried out at an acid pH, following essentially the method
of Reisfeld et al. (48). At first all activity was invariably lost, until it was
realized that persulfate, the material used by both Davis and Reisfeld to
polymerize their gels, destroyed interferon in acid solution. Gels were there-
fore polymerized with riboflavin instead of with persulfate, a modification
that permitted electrophoresis also at an acid pH. The results obtained in this
system are shown in Fig. 2. When the same starting material as had been

Figures 1 and 2. Polyacrylamide gel electrophoresis of highly purified chick inter-
feron at pH 8.9 and at pH 4.3. 1 ml samples of interferon (specific activity 1.6 × 10^6
U/mg protein) in 0.01 M phosphate buffer pH 7.5, each mixed with 1 ml of 40% aqueous
sucrose, were applied to gel columns (40 × 6.5 mm). After electrophoresis (6 mA per
column) the gels were cut lengthwise, one-half of each was stained with amido black,
the other was cut into 20 equal portions which were individually eluted for assay. Arrows
denote that, owing to insufficient dilution, assay end points were not reached, i.e.,
actual interferon contents were higher than those shown.
used for alkaline electrophoresis was employed, two protein zones were again observed, this time well separated, but the peak interferon activity was situated between them and was obviously not related to either. Since the starting material itself already had a specific activity of $1.6 \times 10^6$ U/mg protein, and since most of the protein was present as impurity and not as part of the interferon, pure chick interferon will be found to have an extremely high specific activity, and it must be classed among the biologically most potent substances so far known. Attempts to stain similar gels with dyes that would reveal the presence of neutral or acidic polysaccharides led to negative results.

Bodo (45) too, found that his highly purified chick interferon gave rise to several protein bands after acid or alkaline electrophoresis; none of these bands appeared to be associated with the antiviral activity. Moreover, when non-infected allantoic fluid was processed by the same methods, he observed protein profiles identical with those obtained from the interferon.

During our purification work we had several indications that the active material in chick interferon preparations was itself heterogeneous. For example, the distribution of activity after polyacrylamide gel electrophoresis was never confined to a very narrow zone, and the activity, after elution from CM-Sephadex by a rising pH gradient, was usually spread over several fractions. We then discovered that an early, more acid CM-Sephadex eluate fraction, after readsorption onto fresh CM-Sephadex, again eluted at a lower pH than did readsorbed, more alkaline eluate fractions. A possible explanation could have been partial methylation of the interferon during the acid methanol step of the purification process, leading to artificial heterogeneity; it could, however, be shown that this was not so (6). Further tests (49) proved the charge heterogeneity of the interferon; partly purified interferon was adsorbed onto a CM-Sephadex column and then eluted by means of a steeply rising pH gradient. Elution was not performed under conditions of equilibrium, and thus there is no direct correlation between the isoelectric point of any of the individual fractions and the pH at which it was eluted (50). The results of this part of the experiment are shown in Table III.

Portions of the original eluate fractions were then readsorbed at pH 5.9 onto fresh CM-Sephadex columns and then eluted carefully, this time stepwise and under equilibrium conditions, with buffers of rising pH. In order to eliminate errors possibly caused by adsorption to a solid matrix, some of the original eluate fractions were also subjected to electrofocusing in a liquid pH gradient (51). The pH and interferon content of every fraction was determined, and the results obtained from these experiments are shown in Table IV. The results indicate that the interferon consisted of five, six, or even more components of different charges, but they do not establish whether there is only one interferon adsorbed to or complexed with several differently charged substances, or whether there are several different interferons involved.
### Table III

**Elution from a Carboxymethyl-Sephadex Column of Partly Purified, Concentrated Chick Interferon by Means of a Rising pH Gradient in 0.1 M Phosphate Buffer**

| Fraction code | Volume | pH  | Interferon Recovery |
|---------------|--------|-----|---------------------|
|               | ml     | U/ml| %                   |
| **Column input** |        |    |                     |
| A             | 100    | 5.9 | 25,600              | 100 |
| **0.1 M Phosphate wash pH 6** |      |    |                     |
| B             | 500    | 6.0 | <20                 |     |
| C             | 400    | 6.0 | <20                 |     |
| D             | 100    | 6.0 | <20                 |     |
| **0.1 M Phosphate rising pH eluate fractions** |      |    |                     |
| 1             | 10     | 6.0 | <20                 |     |
| 2             | 10     | 6.0 | <20                 |     |
| 3             | 10     | 6.01| <20                 |     |
| 4             | 10     | 6.02| <20                 |     |
| 5             | 10     | 6.10| 640                 | 0.3 |
| 6             | 10     | 6.29| 3200                | 1.3 |
| 7             | 10     | 6.53| 6400                | 2.5 |
| 8             | 10     | 6.66| 6400                | 2.5 |
| 9             | 10     | 6.79| 12,800              | 5   |
| 10            | 10     | 6.88| 12,800              | 5   |
| 11            | 10     | 6.96| 25,600              | 10  |
| 12            | 10     | 7.02| 12,800              | 5   |
| 13            | 10     | 7.11| 12,800              | 5   |
| 14            | 10     | 7.20| 12,800              | 5   |
| 15            | 10     | 7.28| 12,800              | 5   |
| 16            | 10     | 7.35| 6400                | 2.5 |
| 17            | 10     | 7.50| 4800                | 1.9 |
| 18            | 10     | 7.62| 3200                | 1.3 |
| 19            | 10     | 7.81| 3200                | 1.3 |
| 20            | 10     | 8.11| 6400                | 2.5 |
| 21            | 10     | 8.49| 5400                | 2.5 |
| 22            | 10     | 9.78| 3200                | 1.3 |
| 23            | 10     | 10.27| 640              | 0.3 |
| 24            | 10     | 10.38| 160              | 0.1 |
| 25            | 10     | 10.59| <80              |     |
| 26            | 10     | 10.80| <20              |     |

In view of the recent findings by Schonne et al. (107) that charge microheterogeneity of rabbit interferon was probably caused by varying sialic acid content, it is likely that the same will also be found to apply to chick interferon. On the other hand, very many enzymes and other biologically active proteins exhibit polymorphism, and one should therefore perhaps not exclude the possibility that interferons too may consist of groups of closely related polymorphic units.
### TABLE IV
RECHROMATOGRAPHY ON CARBOXYMETHYL-SEPHADEX COLUMN AND ELECTROFOCUSING OF INTERFERON SOLUTIONS OBTAINED FROM FIRST CARBOXYMETHYL-SEPHADEX FRACTIONATION

| Original eluate fraction | pH of buffer | Eluting peak activity | pH of peak ampholyte fraction |
|--------------------------|--------------|-----------------------|------------------------------|
| 6                        | 6.1          |                       |                              |
| 7                        | 6.4          | 6.40                  |                              |
| 8                        | 6.4          | 6.39                  |                              |
| 9                        | 6.4          |                       |                              |
| 10                       | 6.4          |                       |                              |
| 11                       | 6.4          | 6.58                  |                              |
| 12                       | 6.6          |                       |                              |
| 13                       | 6.6          | 6.69                  |                              |
| 14                       | 6.8          |                       |                              |
| 15                       | 6.8          | 6.86                  |                              |
| 16                       | 6.8          |                       |                              |
| 17                       | 6.9          | 7.23                  |                              |
| 18                       | 6.9          |                       |                              |
| 19                       | 6.9          |                       |                              |
| 20                       | 6.9          |                       |                              |
| 21                       | 7.2          | 7.34                  |                              |
| 22                       | 7.2          |                       |                              |

### PURIFICATION OF HUMAN AND OTHER INTERFERONS

We have also done some work on the purification of human interferon (52, 53). The starting material was Sendai virus-induced leukocyte interferon prepared by the methods of Strander and Cantell (54, 55) and kindly donated by Dr. Cantell. Early preparations contained 10% human or calf serum, later samples only 2% human serum. Many, though not all, of the methods used to purify chick interferon could also be applied to human interferon. But purification, after every stage, was much lower than had been noted with chick interferon. Two of the factors mainly responsible for this are probably the presence of serum and the low isoelectric point of the human interferon (52, 53). High titers of human leukocyte interferon, in contrast to those of chick tissue culture interferon, can seemingly only be obtained in the presence of serum (56), and some serum components, physicochemically similar to the interferon, appear to follow it during the purification processes. The low isoelectric point of the human interferon (see below) makes chromatography on cation exchangers, a key step in the purification of chick interferon, a much less effective procedure. By choosing a suitable combination of methods it has, however, proved possible to concentrate and purify interferon that initially contained 2% serum some 40- and 80-fold, respec-
tively. Such material is being used in various experiments in monkeys and is also intended for human trials in the near future (57).

Several other interferons have been purified in various laboratories by methods already described or by similar techniques, e.g. Merigan et al. (42), Lampson (58), and Falcoff et al. (59) purified various human interferons. Merigan et al. (42), Paucker and Boxaca (60), Falcoff et al. (61), Golgher and Paucker (36), and Stancek and Paucker (37), and others purified mouse interferons of diverse origin; rat interferon was purified by Denys (62), Cocito et al. (63), and Schonne (64); many authors, including Ho (65), Smith and Wagner (66), Ke and Ho (67), and De Clercq et al. (68) have worked on the purification of rabbit interferons; Wagner and Yanazaki (69) prepared and purified radioactive rabbit interferon; and Nagano et al. (32) did extensive work on the purification of interferon-like materials obtained from vaccinia virus-infected rabbit skin. Several workers concentrated calf interferon by nonspecific methods, e.g. Sutton and Tyrrell (7) and Sellers and Fitzpatrick (8); Davies (31, 70) subjected it to a number of purification steps. Russell et al. (71) and Dima et al. (33) did some work on the purification of monkey interferon.

The only publication dealing with the purification of nonvirus-induced interferons is that of Dima et al. (33) who showed that mouse and monkey interferons, induced by Brucella suis, could be fractionated by the methods used by others to purify virus-induced interferons.

Most workers have used well-known separation methods, but there are a few exceptions. Davies (31) adsorbed calf interferon onto small glass beads and eluted it with a mixture of organic solvents; Dima et al. (33), following up some work by Soru and Ionescu-Stoian (72), used a mixed CM-Sephadex/Sephadex G100 column in preference to the single gels for the fractionation of mouse and monkey interferons; De Clercq et al. (68) precipitated various rabbit and human interferons by complexing them with yeast-RNA in acid solution, a method previously used by Charney (73) for the concentration of poliomyelitis viruses; and Bocci et al. (74) indicated a biological procedure. Interferon in rabbit urine, obtained after intravenous inoculation of Sindbis virus, was about 140 times purer than the corresponding serum interferon, and interferonuria was suggested as an excellent condition for obtaining highly purified rabbit interferon.

Although work with several interferons from other animal species has been described in the literature (e.g. experiments carried out with interferons from guinea pig, dog, pig, hamster, bat, and even tortoise and fish), no work concerning their purification has been reported.

**MULTIPICLITY OF INTERFERONS**

Although the charge microheterogeneity of chick (49) and rabbit (107) interferon has only recently been recognized, it has been known for several years
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that cells from one species are capable of producing more than one type of interferon. At one time it was thought that viral stimuli gave rise to neutral or slightly acidic interferons of molecular weights of about 30,000, and that nonviral stimuli produced much larger, more basic, and usually less stable interferons. This view has proved to be an oversimplification, since the kind of interferon that is formed may depend on the following other factors: 

(a) the nature of the inducing stimulus (75),
(b) whether induced in vivo or in cultured cells (76),
(c) the type of cultured cell (77, 78, 94),
(d) the nature of the stimulated organ in the intact animal (79),
(e) the time interval between stimulation and harvest (80),
(f) the presence of stimulus enhancers (81),
(g) the degree of stimulation (82), and
(h) unexplained changes with time of the interferon producing animal strain (83).

Investigations under these varied conditions have revealed many new and unexpected interferons with different molecular weights or charges, or with different heat and acid stabilities. Often more than one interferon is present in one serum or in one culture fluid specimen, a situation that, of course, increases purification difficulties and that is apt, as already mentioned, to confuse interpretations. In spite of this great physicochemical heterogeneity, interferons belonging to one animal species have certain important features in common. Their mode of biological action appears to be the same, they are essentially species specific (though there are exceptions [84]), and they probably have common chemical features, since Boxaca and Paucker (85) showed that mouse interferons, whether large or small, whether induced in vivo or in tissue culture by viral or nonviral agents, could all be neutralized by an antiserum prepared against a virus-induced tissue culture interferon.

The interrelationships between different interferon components of any one species are not understood, except perhaps for the charge microheterogeneity of rabbit interferon (107). For example, it is not known whether large molecular interferons are aggregates of monomers or complexes of monomers with inert substances, or whether they are completely unrelated entities. Attempts to elucidate this complex problem have so far only led to few and tentative results.

Davies (70) thought he had evidence that his calf interferon was linked to serum albumin; Nagano et al. (32) believe that their high molecular weight rabbit interferon is a complex of a small active oligo- or polysaccharide with inert protein; Merigan and Kleinenschmidt (76) failed to disaggregate high molecular weight, statolon-induced mouse interferon by exposure to high pH and high ionic concentration; Schonne and his colleagues (86)² treated a high-molecular weight, NDV-induced rabbit tissue culture interferon with 4 M urea and found that it was converted, in very low yield, to a smaller unit,

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¹ Stewart, E., and S. E. Sulkin. 1969. Personal communication.
² Schonne, E., A. Billiaux, E. De Clercq, and P. De Somer. 1969. Personal communication.
similar to one already present in greater abundance in the same culture fluid. Kadri and Kohlhage (87), on the other hand, were unable to interconvert high and low molecular weight components of virus-induced rabbit serum interferon. Coppey and Markovits3 observed that graded UV-light irradiation of virus-infected monkey kidney cells reduced the production of low and high molecular interferons to a similar extent, from which they concluded that the latter was a polymer of the former. Fantes (52, 53) presented some tentative evidence that a high molecular weight component in virus induced human leukocyte interferon could be an aggregation artifact.

The multiplicity of interferons is reminiscent of isoenzymes, a field in which again several different molecules appear to perform one and the same biological function. The position there was summarized by Kaplan (88) who wrote: “An ever increasing number of enzymes have been reported as existing in more than one molecular form. In fact, it appears that the enzyme existing in only one form is an exception.” This could perhaps be said with equal justification of interferons. As an example of this heterogeneity, the properties of a number of rabbit interferons, as described by various authors, are presented in Table V.

| Origin         | Inducer      | IEP  | Mol wt     | Reference |
|----------------|--------------|------|------------|-----------|
| Primary spleen | NDV          | 7.4  | 33,000     | 50        |
| Serum          | NDV          |      | 46,000>100,000 |          |
| Serum          | Endotoxin    |      | 54,000>100,000 | 67, 89   |
| Urine          | NDV          |      | 46,000>100,000 |          |
| Urine          | Endotoxin    |      | 35,000>100,000 |          |
| Serum          | NDV          |      | 51,000>134,000 |          |
| Macrophages    | NDV          | 37,000| 45,000>134,000 | 66       |
| Primary kidney | NDV          |      | 45,000>134,000 |          |
| Serum          | RNA from *P. funiculorum* | 6.9-7.1 | 61,000 130,000 | 90       |
| Serum          | Poly I:C     | 6.8-6.9| 49,000 52,000 | 91       |
| Urine          | Sindbis      | <8.8 | ---        | 92       |
| RK13           | NDV          |      | 40,000>100,000 | *        |
| Serum          | NDV or PR8   | 41,000| 55,000>100,000 | 87       |
| RK13           | NDV          | 5.07-6.69 | ---      | 107      |

* Schonne, E., A. Billiau, E. De Clercq, and P. De Somer. 1969. Personal communication.

3 Coppey, J., and P. Markovits. 1969. Personal communication.
PHYSICOCHEMICAL PROPERTIES

No interferon has so far been prepared in a pure state, and therefore all the facts known have been obtained by indirect means, i.e. by following the fate of the biological activity under various conditions, and not from direct chemical analyses or direct physical observations. However, in view of the already discussed multiplicity of interferons, results obtained from work with crude or partly purified interferons should be carefully scrutinized, since they could easily represent composite values of mixtures of active components.

Molecular weights of interferons have been obtained mainly from gel filtration studies and, to a lesser extent, also from gradient centrifugations. Polson and Vargosco used the rate of diffusion through stretched cellophane to calculate the molecular weight of chick interferon. In general, virus-induced interferons have molecular weights in the region 25,000–35,000, but several minor components of higher molecular weights have also been reported. Interferons obtained after nonviral stimulation often, but not always, have higher molecular weights, but the dividing line between the two groups of interferons is by no means clear-cut. For example, interferons induced in human leukocytes by two nonviral stimuli phytohemagglutinin and the double-stranded RNA complex, formed from polyinosinic and polycytidylic acids, had molecular weights of 18,000 and 25,000, while virus-induced human amnion interferon had a molecular weight of about 160,000 (59).

Isoelectric points have been determined from the pH at which interferons could be eluted from various natural and synthetic ion-exchangers, in particular from CM-Sephadex, by electrophoresis in solid supports or liquid gradients, and, more recently also by electrofocusing (51) and electrodecantation (95). It was first thought that all virus induced interferons were neutral or slightly acidic substances, while nonviral interferons were considered to be more basic (93). Again, this generalization is only partly correct; e.g. Falcoff et al. (61) reported an isoelectric point of pH 8.0, determined by CM-Sephadex chromatography, for virus induced mouse brain interferon, and Stancek and Paucker (108) observed by electrofocusing a component of virus-induced mouse tissue culture interferon isoelectric at pH 10. Fantes (52, 53) found, by CM-Sephadex chromatography, by electrofocusing, and by electrodecantation, that virus-induced human leukocyte interferon was isoelectric near pH 5.2 (with minor peaks at pH 5.7 and pH 6.1). Merigan (96) also reported a low (pH 5.0–5.5) isoelectric point for virus induced human foreskin interferon. In view of the already demonstrated charge microheterogeneities of chick and rabbit interferons (49, 107), one should not ignore the possibility

4 Polson, A., and A. J. Vargosco. 1965. Personal communication.
that the reported isoelectric points of some other interferons are also those of the major components of polymorphic or other mixtures.

Only little is known about the chemical make-up of interferons, and this again has been established by indirect means. From the fact that every interferon so far tested was destroyed by proteolytic, but not by other enzymes, it is generally agreed that interferons are or at least contain protein. Only Nagano et al. (32) do not agree with this view. They believe that, at least the antiviral substance isolated by them from vaccinia virus-infected rabbit skin, is a protein-free active oligo- or polysaccharide that can be loosely bound to a variety of inactive proteins. However, both the free saccharide and the saccharide-containing glycoprotein have a number of properties that can only be reconciled with difficulty with those generally accepted for interferons. The views of the Japanese workers were summarized by Nagano (97) and by Okazaki (98), and differences of the factors from other interferons were discussed by Fantes (99, 100).

Carbohydrate has also been found in hydrolysates of some other interferons, e.g., by Burke (30, 38), Lampson et al. (23), Davies (31), and Fantes and Furminger (46). These results were obtained from materials now known to have been impure and thus do not prove that carbohydrate is an integral part of the interferon molecule. The activities of some of these and of some other (e.g. Schonne [64] and Ke and Ho [101]) interferons were also shown to be sensitive to periodate. This again suggests but does not prove the presence of essential carbohydrates, because several carbohydrate-free, biologically active proteins have been destroyed by very mild periodate treatment (46, 102–104). Gresser and Thomas on the other hand, found that the addition of several periodate-sensitive amino acids did not protect mouse brain interferon from destruction by periodate, a result favoring the hypothesis that a sugar is an essential part of this interferon. The presence of the carbohydrate sialic acid in rabbit interferon does not appear to be needed for antiviral activity (107).

Apart from periodate, several other chemicals have been used in attempts to throw some light onto the chemical composition of interferons. For example, high molarities of urea are believed to have no effect on chick interferon (35, 42), but mouse (42) and rat (64) interferons are destroyed by this reagent. Kadri and Kohlhage (87) found that the high molecular but not the low molecular components of virus induced rabbit serum interferon were urea sensitive. These results show that hydrogen bonding is apparently essential in some but not in all interferons.

The fact that neither nucleases nor ether inactivate interferons suggests the absence of nucleic acids and lipids as essential parts of interferons.

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5 Gresser, I., and M. T. Thomas. 1968. Personal communication.
Purified chick interferon has been exposed to a number of chemicals that are known to react, under defined conditions, with specific amino acids or with specific bonds in protein molecules. It was found that antiviral activity was destroyed by all reagents that oxidatively or reductively attack disulfide bonds, by reagents that alter amino groups, and by cyanogen bromide, while several other reagents did not influence the activity. From these results, Fantes and O’Neill (105) concluded that chick interferon must have at least one disulfide bond, at least one free amino group, and at least one methionine residue, but that hydroxyl or sulfhydryl groups, if present, were nonessential parts of the interferon molecule. The results of this work are summarized in Tables VI and VII. Intact disulfide bonds are also needed for the activities of mouse (42), rat (64), and possible rabbit (101) interferons; the latter also contains essential methionine (101).

Some other chemical or physical treatments have an effect on the stability

### Table VI

| Reagent                      | Concentration | pH | Duration of reaction | Temp. of reaction | Residual activity | Protein groups primarily attacked |
|------------------------------|---------------|----|----------------------|-------------------|------------------|----------------------------------|
| Acetic anhydride             | 5.5           | 7.5| 2                    | 0-4              | <5, <10          | -NH₂(a), -OH                     |
|                              | 55.0          | 7.5| 2                    | 0-4              | <5, <10          | -NH₂(a and e), -OH               |
| Benzoylchloride              | 25.0          | 7.5| 2                    | 0-4              | <5, <10          | -NH₂                             |
| Benzylchloride               | 25.0          | 7.5| 2                    | 0-4              | 100, 100         | -NH₂ (at elevated temp. only)    |
| Fluoro-dinitro-benzene       | 8.1           | 8.0| 2                    | 0-4              | 65, 35           | -NH₃, -OH*, -SH                  |
|                             | 81.4          | 8.0| 2                    | 0-4              | 35, 25           | -NH₃, -OH*, -SH                  |
| Nitrous acid                 | 1000          | 4.0| 0.5-0.75             | 0-4              | 15               | -NH₃, -SH, -OH*                  |
|                              | 1000          | 4.0| 1.5                  | 0-4              | 10               | -NH₃, -SH, -OH*                  |
|                              | 1000          | 4.0| 3                    | 0-4              | 5                | -NH₃, -SH, -OH*                  |
| p-Cl-mercuribenzoate         | 0.0735        | 6.8| 0.33                 | R†               | 100, 100, 100, 100| -SH                              |
| Phenyl-isocyanate            | 9.3           | 8.1| 0.25                 | 0-4              | 10, <5           | -NH₃, -OH*                      |
|                             | 9.3           | 5.8| 0.25                 | 0-4              | 100, 50, 100, 50, 100| -SH                              |

* -OH from tyrosine.
† R = room temperature.
TABLE VII
EFFECT OF REDUCING AND OTHER REAGENTS ON ANTIVIRAL ACTIVITY OF CHICK INTERFERON

| Reagent                     | Concentration | pH | Duration | Temp. of reaction | Residual activity | Protein groups primarily attacked |
|-----------------------------|---------------|----|----------|-------------------|-------------------|----------------------------------|
|                             | m M           |    | hr       | °C                | %                |                                  |
| Benzyl mercaptan            | 100           | 5.0| 8        | R*                | <5, <10          | -S--S--                          |
| Benzyl mercaptan            | 100           | 5.0| 72       | R                 | <5, <10          | -S--S--                          |
| Sodium thioglycollate       | 1000          | 5.0| 1        | R                 | 10, 10           | -S--S--                          |
| Sodium thioglycollate       | 1000          | 5.0| 18       | R                 | 5, <5            | -S--S--                          |
| 2-Mercaptoethanol           | 200           | 7.2| 24       | 4                 | 25, 50           | -S--S--                          |
| 2-Mercaptoethanol           | 200           | 7.2| 24       | 4                 | 20, 35           | -S--S-- then -SH                  |
| followed by iodoacetate     | 10            | 7.2| 24       | 4                 | -SH              |                                  |
| Iodoacetate                 | 10            | 7.2| 24       | 4                 | 65, 100          | -SH                              |
| Bromoacetate                | 5             | 5.0| 3        | 37                | 100, 65          | Imidazole (histidine)            |
| Bromoacetate                | 5             | 8.2| 3        | 37                | 100, 100         | -SH                              |
| Cysteine                    | 25            | 5.0| 3        | 37                | 50, 50           | -S--S--                          |
| Cysteine                    | 25            | 8.2| 3        | 37                | 50, 50           | -S--S--                          |
| Bromoacetate + cysteine     | 5+25          | 5.0| 3        | 37                | 70, 50           | Imidazole histidine/             |
|                             |               |    |          |                   |                  | -S--S--                          |
| Bromoacetate + cysteine     | 5+25          | 8.2| 3        | 37                | 40, 50           | -SH/-S--S--                      |
| K-Cyanate                   | 0.5           | 6.0| 24       | R                 | 100, 100         | -SH                              |
| K-Cyanate                   | 5             | 6.0| 24       | R                 | 100, 100         | -SH                              |
| K-Cyanate                   | 50            | 6.0| 24       | R                 | 100, 100         | -SH                              |
| Cyanogen-bromide            | 16.5          | 2.0| 24       | R                 | <5, 10           | γ-S-methyl of methionine         |

* R = room temperature

TABLE VIII
LOSS OF ACTIVITY OF CRUDE HUMAN LEUKOCYTE INTERFERON DUE TO SURFACE DENATURATION AND PROTECTIVE EFFECT OF ANTIFOAM P2000

| Gas            | 0 hr | 8 hr | 24 hr | 48 hr | 0 hr | 8 hr | 24 hr | 48 hr |
|----------------|------|------|-------|-------|------|------|-------|-------|
| None           | 10,240 | 5120 | 10,240 | 10,240 | 10,240 | 10,240 | 10,240 | 10,240 |
| Air            | 640  | 160  | 80    | 5120  | 5120  | 10,240 | 10,240 | 10,240 |
| N₂             | 1280 | <320 | <320  | 10,240 | 20,480 | 10,240 | 10,240 | 10,240 |
| CO₂            | 1280 | <160 | <160  | 10,240 | 10,240 | 10,240 | 10,240 | 10,240 |

of interferons, without however helping to elucidate their structure or composition. Most interferons are remarkably stable to acid and heat. We found that beta-propiolactone rapidly destroyed chick (43) and human (52, 53) interferons (a view not shared by Lo Grippo and Hayashi [106]), and we also observed that γ-ray irradiation with a total dose of 2.69 Mrad destroyed 90% of the activity of crude human leukocyte interferon (52, 53). This interferon has been reputed to be unstable. To ascertain whether instability could be
attributed to atmospheric oxidation, air, nitrogen, and carbon dioxide were bubbled through samples of the interferon (52, 53). In fact all three treatments led to inactivation, which could not therefore have been caused by oxidation. The considerable frothing noted in each of the tests raised the suspicion that surface inactivation could have taken place. The experiments were therefore repeated in the presence of an antifoaming agent (Shell P2000, a polypropylene glycol). The antifoam suppressed both frothing and inactivation of the interferon. The results of the experiment are summarized in Table VIII.

SUMMARY AND CONCLUSIONS

In spite of extensive work, not a single interferon has so far been obtained in a pure state. Therefore, all available knowledge of physicochemical properties of interferons had to be derived by indirect means.

Chick interferon, the most studied of all, was still heavily contaminated with inert proteins after 20,000-fold purification. If other interferons are essentially similar to chick interferon (and there are at present no indications that they are not) then it will take a long time before the first pure interferon will become available.

For most biological work, existing purified interferons are adequate. New approaches should become possible with the advent of interferons of high radioactive even if not of chemical purity.

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