JOURNAL OF VIROLOGY, Oct. 1967, p. 883-890

Vol. 1, No. 5
Printed in U.S.A.

# Characterization of Virus- and Endotoxin-induced Interferons Obtained from the Serum and Urine of Rabbits

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Received for publication 19 June 1967

Interferons induced in the rabbit by Newcastle disease virus or by endotoxin have been further characterized as to their physicochemical stability and molecular size by Sephadex G-100 gel filtration. Endotoxin-induced interferon obtained from serum was more labile than virus-induced interferon. Both endotoxin and virus induced interferons obtained from serum contained two peaks: a minor high molecular weight (>100,000) peak and a major lower molecular weight peak. The molecular weight of the major peak induced by endotoxin was 54,000, and that induced by Newcastle disease virus was 46,000. The gel filtration pattern of interferon recovered from the urine of animals inoculated with virus reflected faithfully the pattern found in serum except that there was proportionately less of the high molecular weight peak. However, the urine interferon from endotoxin-inoculated animals contained only one broad peak with a molecular weight of 35,000. This was not the peak fraction present in the serum of such animals. It is postulated that this may represent the basic unit of endotoxin-induced interferon, and that the serum components are either polymers or conjugates of this basic unit.

It was apparent when we first studied endotoxin-induced interferon (EII) in the rabbit (5) that this inhibitor differed physicochemically from virus-induced interferon (VII). Interferon induced by endotoxin was inactivated by heating at 56 C for 30 min and at *p*H 2.5 for 5 hr, procedures which left VII largely unaffected. The possibility that there is more than one type of interferon was suggested. Subsequently, many differences between the mechanisms of induction of VII and EII were observed. The induction of VII in the rabbit was inhibited by actinomycin D and puromycin; it was enhanced by high body temperature and it was relatively resistant to inhibition by corticosteroids (7, 9, 14). In contrast, the induction of EII was not inhibited by actinomycin D (7) or puromycin (10); it was not enhanced by elevation of body temperature (13), and the production of EII was easily inhibited by cortisol and enhanced by adrenalectomy (14). These studies together with those of Youngner and co-workers (17) on the induction of interferons by viruses and endotoxin in mice suggest strongly that in the intact animal the mechanism of induction of EII is distinct from that of VII. Different mechanisms of induction support the idea that different molecules may be induced.

With respect to the molecular differences between EII and VII, Hallum et al. (4) first reported that by gel filtration of mouse serum interferon the molecular weight of EII (89,000) was much larger than VII (25,000). Ke and co-workers (9) applied similar methods to study rabbit serum EII and VII and obtained somewhat different results. Both types of interferons were found to be heterogeneous. There was a minor high molecular weight peak (>100,000) in both EII and VII, and a larger peak whose molecular weight was 56,000 in the case of EII, and 40,000 in the case of VII. Recently, Smith and Wagner (15) further illustrated the heterogeneity of rabbit interferons with materials obtained from macrophages spontaneously or induced with virus or endotoxin.

Our attention has recently been redirected to the molecular dimensions of rabbit interferon by another finding. We found that VII was excreted much more readily than EII (8). Whereas the renal clearance rate was around 30.6 ml of plasma cleared per hr per kg of rabbit for VII, which was around one-fourth to one-fifth of the glomerular filtration rate (creatinine clearance), it was only 1.2 ml for EII. One possible explanation for this difference in renal excretion is the dissimilarity of

VII and EII in molecular size or configuration. The main peak of VII was somewhat smaller in molecular weight than the main peak of EII (9), but it was not clear whether this difference alone was sufficiently explanatory. It thus became important to study and compare the characteristics of these two interferons not only as they are found in the bloodstream but also after they have been excreted in the urine.

This paper presents further data on the stability of EII and VII and on the Sephadex gel filtration characteristics of these two types of interferons as found in the blood stream and in the urine.

## MATERIALS AND METHODS

*Viruses and cell cultures.* Stocks of Newcastle disease virus (NDV; Herts strain) and vesicular stomatitis virus (VSV; Indiana) were passed in 11-day-old chick embryos and in chick-embryo fibroblast cultures, respectively. To prepare the virus-inducer of interferon, allantoic fluids harvested from NDV-infected eggs were centrifuged at 81,000 × *g* in a Spinco model L ultracentrifuge for 1 hr, and resuspended in one-third of its original volume in phosphate-buffered saline (PBS; *p*H 7.2). A 1-ml amount contained about $10^9$ plaque-forming units (PFU) as titrated by plaque formation in chick fibroblasts (6).

To prepare rabbit cell cultures, embryos were obtained by cesarean section 23 days after conception. They were decapitated, eviscerated, and trypsinized (0.25% trypsin) as described for chick embryo cell cultures (6). Amounts of 30 ml of cell suspensions containing 500,000 cells/ml in a growth medium (designated LAH medium) consisting of 0.5% lactalbumin hydrolysate in Hanks balanced salt solution supplemented by 4% calf serum, 292 mg per liter of L-glutamine, and vitamins (3) were incubated at 37 C in 600-ml glass bottles. Primary rabbit embryo tube (13 × 100 mm) cultures were obtained by seeding 500,000 cells in 1-ml suspensions. Secondary petri plate cultures were obtained as follows. After 4 to 10 days of incubation, the primary monolayers were detached with 0.02% ethylenediaminetetraacetic acid and 0.25% trypsin solution in PBS, and $10^6$ cells in 5 ml of medium was seeded on 5-cm Falcon plastic plates. The plates were confluent after 3 days of incubation under an atmosphere containing 5% $CO_2$.

*Interferon titrations.* For the titration of rabbit interferons, two methods were generally used: cytopathic-effect (CPE) reduction and plaque reduction. These methods have been described and compared in a previous publication (11). The two methods are roughly comparable. The following is a resume of the procedures including modifications in techniques that have been introduced.

(i) For the CPE reduction method, 0.3 ml of twofold dilutions of a sample containing interferon is incubated in duplicate primary rabbit embryo tube cultures for 15 to 18 hr at 37 C. The tubes are then decanted and replenished with 1 ml of medium containing a suspension of 1,000 PFU of VSV. In 24 to 36 hr when control cultures are 75 to 100% destroyed, the tubes are read for (CPE). A titer is expressed as the reciprocal of the dilution that produces inhibition of CPE in 50% of the tubes (5).

(ii) For the plaque reduction method, 4 ml of twofold dilutions of interferon and control medium is inoculated in two to four plates and incubated for 15 to 18 hr at 37 C. After aspirating the inocula, about 50 to 100 PFU of VSV in 0.5 ml of LAH medium are adsorbed on each plate for 1 hr. After removal of the inocula, the plates are overlaid with 5 ml of LAH medium containing 1.2% agar (Difco). After 48 hr of incubation at 37 C, the agar overlay is flooded with 2 ml of 0.02% neutral red in PBS for 1 hr to permit the visualization of plaques. A 50% plaque reduction titer is obtained by plotting per cent plaque reduction of VSV against the dose of interferon on K-E 358-22 log probability paper, by the method introduced by Lindenmann and Gifford (12).

For the titration of interferon activity in effluent fractions obtained during gel filtration, the usual plaque reduction method was not done on each fraction because of the large number of fractions involved and insufficient volume of the fraction when interferon activity was low. The fractions were diluted in such a manner that 4 ml resulted in 20 to 80% reduction of 100 to 200 VSV plaques on two to four plates. The number of interferon units in 50% plaque reduction doses was estimated from an idealized dose-response curve constructed from past titrations. The regression line of this curve is redrawn showing the per cent plaque reduction on an arithmetic scale in Fig. 1. The theoretical validity for applying such a curve is based on the fact that, for interferon samples of varying purity, such curves are straight and parallel (12). To test the validity of this curve, a sample of interferon (VII) of known potency was arbitrarily chosen and titrated. The experimental points obtained from such a titration fell largely on this curve. The interferon titers estimated from this curve through use of single dilution titrations permit construction of an activity curve and location of peak positions when comparing a large number of fractions. They are meaningful primarily within the context of a single experiment.

The reproducibility of single dilution plaque inhibition assays of rabbit interferon has been examined by Smith and Wagner (15), who pointed out the desirability of several replicate plates. In our hands, through use of four replicate plates receiving 100 to 200 PFU, the 95% confidence limits were $\pm 0.5$ $\log_2$ unit, when 50 to 80% plaque reduction was observed. Between 20 and 50% plaque reduction, the 95% confidence limits were within $\pm 1$ $\log_2$ unit. Thus, the error in potency estimation is seldom greater than one twofold dilution for a single sample. The significance that can be attributed to a given sample assay is further strengthened by assays of adjacent samples when the sample is considered as a component of a well-defined peak.

*Collection of serum and urine interferons.* For the collection of serum and urine VII, albino rabbits weighing 2 to 4 lb (0.91 to 1.82 kg) were injected intravenously with $10^9$ PFU of NDV as previously described (13). Serum samples were collected by

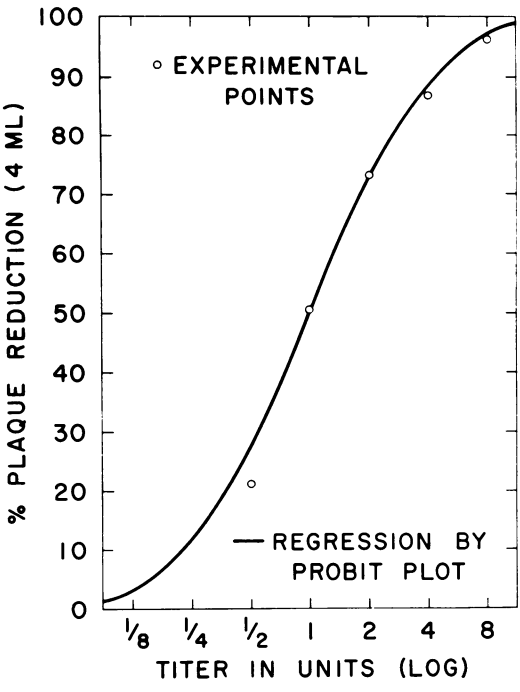


FIG. 1. *Relationship between plaque reduction and interferon titer.*

heart puncture 4 hr after injection. They usually titered at least 1:1,000, as measured by the CPE or plaque reduction methods. Urine was collected through a Foley no. 8 indwelling catheter over a period of at least 12 hr after virus inoculation. In some cases, 24-hr collections were made. Specimens visibly contaminated by blood were discarded. Interferon titers of such urine pools varied from 1:4,000 to 1:32,000, although individual specimens titered occasionally as high as 1:128,000.

To maximize the production of EII, rabbits were shorn to reduce body temperature before they were injected intravenously with 20 $\mu$g of *E. coli* endotoxin (Boivin) suspended in PBS (13). Serum was collected by heart puncture 2 hr after injection. Urine was collected in the same manner as urine VII over an 8 to 12 hr period. The titers obtained from the serum and urine pools were 1:512 and 1:32, respectively.

Although no infectious virus was detected in the serum of animals that had received virus, sera were routinely heated in a 56 C water bath for 15 min, and treated for 1 hr at 37 C with 5% rabbit anti-NDV serum to eliminate undetected infectious virus which may possess interfering activity.

The urine specimens containing either VII or EII were centrifuged at 15,000 $\times$ *g* for 30 min, and dialyzed against PBS for 24 hr before use. In the case of urine EII, it was further concentrated to 1:30 to 1:50 of its initial volume through use of Carbowax (polyethylene glycol, manufactured by Union Carbide Corp., South Charleston, W.Va.), and then centrifuged again at 15,000 $\times$ *g* for 30 min. Routinely, the concentrated urine EII was used as soon as it was processed. Occasionally, it was necessary to store it at $-70$ C for a period of no more than 2 weeks which did not result in significant loss of activity. The urine VII was stable at $-70$ C for several months.

Some characteristics of urine VII have already been described (8). It was nondialyzable, trypsin-sensitive, heat- (56 C) and acid-stable, species-specific, inhibited both VSV and Sindbis virus, but did not inactivate them directly. Urine EII prepared in the fashion described above failed to inactivate VSV directly at 37 C for 1 hr. The antiviral activity of both urine VII and EII could be partially inhibited by actinomycin D. These properties are consistent with the type of intracellular viral inhibitor that is interferon (16).

*Sephadex gel filtration.* Sephadex G-100 (Pharmacia Fine Chemcals, Inc., Uppsala, Sweden) was packed in a column with a diameter of 2.5 cm and a bed height of 95 cm (bed volume = 475 ml). The column was equilibrated at 4 C with 0.15 M PBS (*p*H 6.6), at a flow rate of 20 ml/hr regulated by maintaining the hydraulic head of the buffer reservoir at 30 cm. Samples of 6 ml each or less were applied to the top of the column by hydraulic flow through capillary plastic tubing. Effluents from the column by downward flow were collected in 6-ml fractions by means of a volume-operated fraction collector.

To calibrate the column, the method of Andrews (1) was followed with minor modifications. The void volume (*Vo*) of the column was determined with dextran blue (molecular weight $\simeq 2 \times 10^6$). The total aqueous volume (*Vt*) was determined by filling the column with water and subtracting the volume occupied by the gel matrix (2). The following marker proteins of known molecular weight (in parentheses) were used routinely to monitor the performance of the column: human $\gamma$-globulin ($\simeq$160,000), bovine serum albumin ($\simeq$69,000), and equine cytochrome *c* ($\simeq$15,600). Other proteins that were also occasionally used to calibrate the column include ovalbumin ($\simeq$45,000) and ribonuclease ($\simeq$13,400). Sodium azide (65), a compound used as antibacterial disinfectant for the column, was found satisfactory for determining the volume of the total aqueous phase (*Vt*) of the column.

Amounts of 20 mg of each of the marker proteins were dissolved in 6 ml of PBS and were filtered through a 0.45-$\mu$ membrane filter (Millipore Corp., Bedford, Mass.) before being layered on the column. Protein concentrations in effluent fractions were determined by optical density in a Beckman DU spectrophotometer operated at a wavelength of 280 m$\mu$. Throughout this study, the elution pattern of these proteins as well as various interferon samples were reproducible on repeated determinations, and the optical density peaks did not vary more than one fraction (Fig. 2).

For the construction of the standard curve (Fig. 9), the logarithm of the molecular weights of the protein standards were plotted against their "partition coefficients" (*Kd*) which are defined by the following formula (*see* reference 2): $Kd = (Ve - Vo)/Vi$, where *Ve* is the elution volume of the protein, *Vo* is the void volume, and *Vi* is the so-called inner volume of the gel,

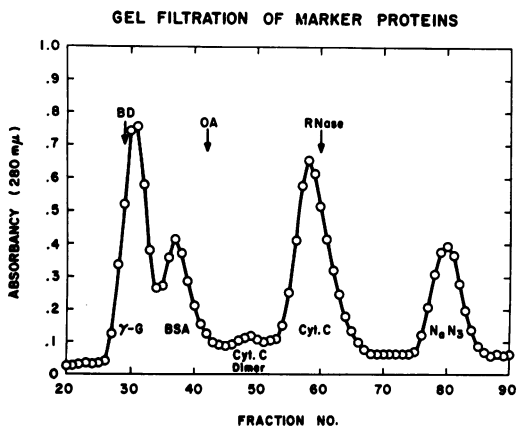


FIG. 2. *Calibration of Sephadex G-100 column by filtration of compounds of known molecular weight. Absorbancy at wavelength 280 mμ (A280) was determined for each effluent fraction to find elution volume for the following proteins: human γ-globulin (γ-G), bovine serum albumin (BSA), equine cytochrome c (cyt. c), and its dimer (cyt. c dimer), and sodium azide ($NaN_3$). The elution volumes of dextran blue (BD), ovalbumin (OA), and ribonuclease (RNase), which were determined separately, are indicated by the arrows.*

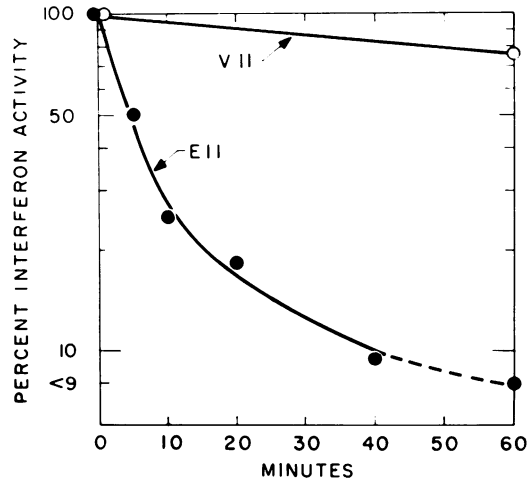


FIG. 3. *Effect of heating at 56 C on VII and EII.*

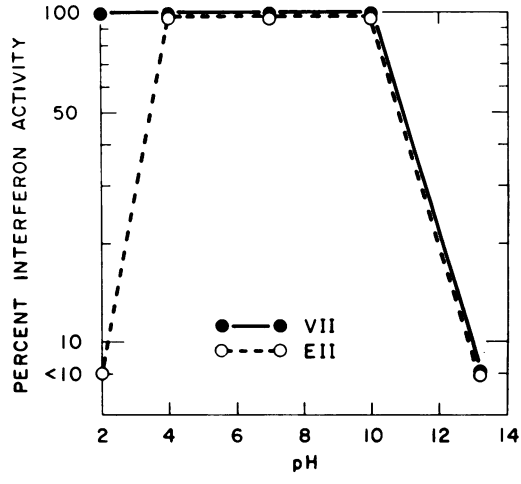


FIG. 4. *Effect of pH treatment at 4 C on VII and EII.*

which is equal to $Vt - Vo$, $Vt$ being the total volume of the aqueous phase.

The curve obtained resembled a sigmoid curve which is fairly linear over a molecular weight range from 15,600 (cytochrome *c*) to 69,000 (bovine serum albumin). The molecular weight of unknown proteins, such as interferons, could be estimated by locating their *Kd* values on the standard curve and finding the corresponding molecular weights.

## RESULTS

*Kinetics of heat inactivation of serum interferons.* Samples of 1 ml of undiluted serum VII and EII in 13 × 100 mm tubes were heated in a 56 C water bath. At intervals indicated in Fig. 3, one tube was taken and chilled in an ice bath to stop the heating effect. After all samples were collected, they were assayed for interferon by the CPE reduction method. There was very little loss of VII activity after heating for 60 min (Fig. 3), which is consistent with earlier findings of Kono and Ho (11). In contrast, the activity of EII diminished sharply, but not entirely exponentially throughout the period of inactivation. This finding confirms our earlier findings that EII is heat-labile (5), and it is consistent with EII being heterogeneous in molecular composition (9). Such heterogeneity may account for the observed deviation from a first order reaction. The inactivation pattern of serum VII which had not been preheated was not significantly different.

*Stability of pH of VII and EII.* Samples of 2 ml of serum VII and EII were first dialyzed at 4 C for 24 hr against 400 ml of LAH medium without serum and bicarbonate but adjusted to the desired *p*H with HCl or NaOH, and then for 12 hr in medium at *p*H 7.0. The specimens were titrated as above; results are presented in Fig. 4. Both VII and EII were stable at *p*H 4, 7, and 10, and both were inactivated at *p*H 13. However, whereas VII withstood *p*H 2 treatment for at least 24 hr, the EII activity was destroyed almost completely.

*Sephadex gel analysis of serum and urine interferons.* Serum and urine obtained from rabbits inoculated with NDV or endotoxin were filtered through a Sephadex G-100 column after they had been prepared as described in Materials and Methods. All runs were done at least twice and

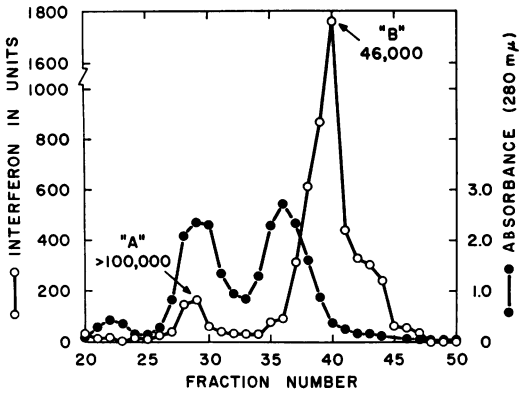


FIG. 5. *Sephadex G-100 gel filtration of VII from rabbit serum.*

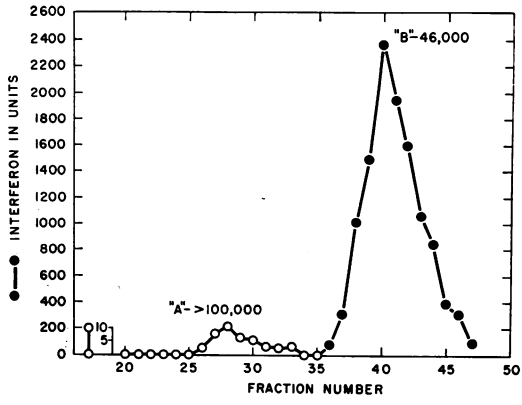


FIG. 6. *Sephadex G-100 gel filtration of VII rabbit urine.*

TABLE 1. *Molecular weights of rabbit interferons as determined by gel filtration on Sephadex G-100 column*

| Source of interferon | Inducer | Kd[a] | Estimated molecular weight |
|---|---|---|---|
| Serum (A)..... | NDV | .020 | >100,000 |
| Serum (B)..... | NDV | .260 | 46,000 |
| Urine (A)..... | NDV | .010 | >100,000 |
| Urine (B)..... | NDV | .260 | 46,000 |
| Serum (A)..... | Endotoxin | .030 | >100,000 |
| Serum (B)..... | Endotoxin | .220 | 54,000 |
| Urine (A)..... | Endotoxin | —[b] | —[b] |
| Urine (B)..... | Endotoxin | .330 | 35,000 |

[a] Partition coefficient $Kd = Ve - Vo/Vt - Vo$ (*see* Materials and Methods).
[b] Undetectable.

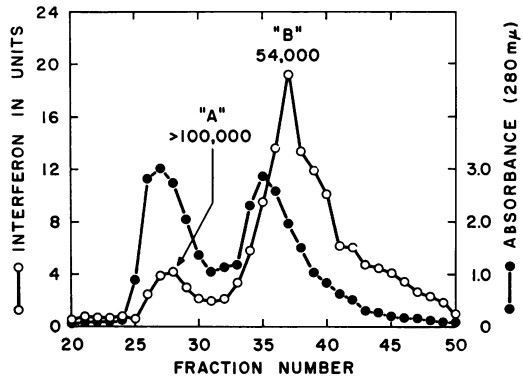


FIG. 7. *Sephadex G-100 gel filtration of EII from rabbit serum.*

were reproducible from one run to the other. Representative runs are presented in Fig. 5 to 8. The molecular weights of the biologically active peaks were determined on a standard curve (Fig. 9) according to their *Kd* values (*see* Materials and Methods). These values are presented in tabular form along with the molecular weights in Table 1.

With respect to serum interferons, both VII and EII show a minor high molecular ("A") peak and a major low molecular ("B") peak (Fig. 5 and 7). The molecular weights of the "A" peaks are greater than 100,000. There is some difference with respect to the low molecular weight peaks: in the case of VII, it is 46,000, and in the case of EII, it is 54,000 daltons. These results are consistent with what we reported earlier, although the precise figures for the low molecular weight peaks were somewhat different (9); they were reported as 40,000 and 56,000, respectively

A partial explanation for this difference is that, in the earlier work, the total volume of the aqueous phase (*Vt*) was calculated on the basis of phenol red, which has been found to be partially adsorbed by the gel in the column and is inappropriate for such a calculation.

The VII pattern found in the urine (Fig. 6) reflects faithfully that found in the serum, with the exception that there is proportionately even less of the high molecular weight moiety than in the serum. The ratio of the peak titers of fractions "B" to "A" is 88 in the case of serum VII and 225 in the case of urine VII. This difference may represent the greater ease with which the lower molecular weight material ("B") is filtered through the renal glomeruli. There is no evidence that the molecular size of the "A" and "B" peaks had been altered in the kidney. The "B" peaks are eluted precisely at the same spot in both serum and urine, representing a molecular weight of 46,000.

In contrast to the above, the pattern of urine

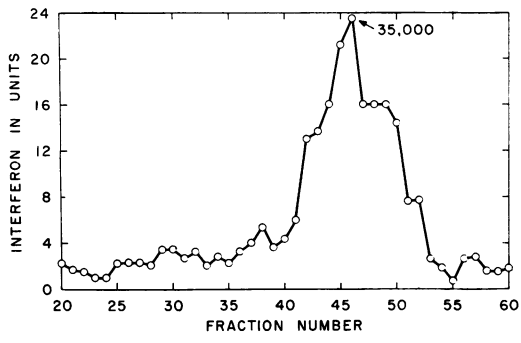


FIG. 8. *Sephadex G-100 gel filtration of EII from rabbit urine.*

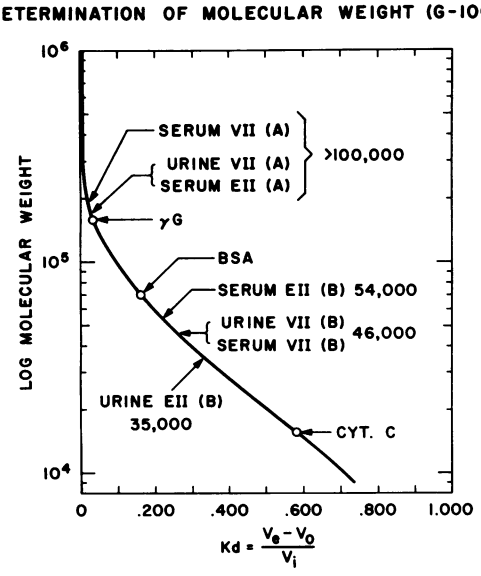


FIG. 9. *Determination of molecular weight of interferons by gel filtration through Sephadex G-100 column on a standard curve constructed as described in Materials and Methods.*

EII is quite different from that of serum EII (Fig. 7 and 8). First of all, there is no evidence of the "A" or high molecular weight peak in the urine. This may be explained by the low activity of this peak and the difficulty with which it is excreted. Secondly, there is an alteration in the "B" peak (Fig. 8). Instead of being eluted at the point representing a molecular weight of 54,000, it is eluted from urine at the fraction which represents a molecular weight of 35,000, the lowest molecular weight for a rabbit interferon we have encountered.

## DISCUSSION

The present work confirms our earlier report that, with respect to gel filtration characteristics, rabbit serum interferons are heterogeneous in at least two dimensions (9). Firstly, after inoculation of either virus or endotoxin, the interferon obtained consists of molecules of two sizes. Secondly, the pattern of molecular weights of interferon differs with the inducer. To these two dimensions of heterogeneity is now added a third one, i.e., at least in the case of EII, the pattern of interferons may also vary with its source in the body.

The heterogeneity of rabbit interferons has also been recently demonstrated by Smith and Wagner (15). Their data indicate that rabbit macrophages produce two primary types of interferons, one with a molecular weight of 45,000 that is synthesized in greater amounts after viral induction, and a 37,000 component synthesized after induction with endotoxin. In serum interferon obtained from animals inoculated with virus, however, a different major constituent with a molecular weight of 51,000 was found. Almost all types of interferon contained a minor high molecular weight component (>134,000) in varying amounts, and many other less definite peaks were described from their gel filtration data.

It is, of course, difficult to compare directly their data and ours, since much of theirs is concerned with macrophage interferon obtained in cell cultures, which may or may not be related to our VII and EII obtained from animals. The one exception is that both they and we studied serum VII. We found that the main serum VII component had a molecular weight of 46,000, a figure which is closer to their main macrophage interferon component, but which is different from their main serum VII component (51,000). It is entirely possible that, in the fractionation of both serum VII and EII, we were unable to resolve components with lower molecular weights than indicated by our "B" peaks, as there is a broad shoulder to the right (Fig. 5 and 7). To characterize this region further, one should separate and concentrate its constituent fractions and perhaps rechromatograph on a gel column with lower porosity. However, lack of resolution in this area cannot explain our inability to find a main peak with a molecular weight of 51,000 in VII. It is also possible that our different findings are within the range of experimental or computative error, and that they are complementary.

One of our most interesting findings is that the molecular weight of the main peak in urine EII (35,000) is much smaller than the main peak of the corresponding serum material (54,000). In this regard, it is intriguing that Smith and Wagner (15) found that macrophage EII con-

tains a primary peak with the molecular weight of 37,000; they postulate that other constituents may be biologically active polymers of this basic unit. This explanation may apply to our finding that interferons obtained from two different body fluids are heterogeneous and dissimilar. Possibly the main serum EII peak (54,000) represents a polymer or conjugate of a basic unit which is broken down or dissociated before it is excreted. One might speculate that the larger unit found in the serum is not readily excreted, although the fact that the "A" peak (>100,000) seen with serum VII is detected to some extent in the urine indicates that the rabbit kidney is not entirely impermeable to proteins with molecular weights greater than 54,000. Therefore, we cannot entirely exclude the possibility that the "A" peak of EII is excreted to some extent, but it is not detectable under our experimental conditions.

Other possibilities which might explain the difference between urine and serum EII are: (i) preferential excretion of a 35,000 molecular weight species which is undetectable or masked in the serum material; (ii) a breakdown or alteration of EII in the renal tubular structures, in the bladder or by the urine itself; and (iii) renal production of urine EII. Some of these possibilities may be tested experimentally.

Filtration characteristics are but one criterion of the molecular heterogeneity of rabbit interferons. Another is the marked difference in physical stability. We originally reported that serum EII was more labile to acid and to heat (5). Smith and Wagner (15) also found that macrophage interferon induced by endotoxin in vitro was more labile than VII. They found, in addition, that, in the case of VII, the high molecular weight peak (>134,000, similar to our "A" peak) proved to be more labile at 56 C and at *p*H 2 than the main peak. Recently we have confirmed this with our material. In this report, we again show that crude EII and VII are markedly different in their stability. However, since the "A" peaks are minor components of both EII and VII, this difference in stability may be assumed to lie in the main "B" peaks. If true, it would mean that, although the two "B" peaks do not differ greatly in molecular size (46,000 versus 54,000), they may represent basically different molecules. Preliminary results on inactivation of "B" peaks seem to indicate that this is the case.

On the basis of the above considerations, one might postulate two basic types of interferon molecules induced in the intact rabbit. One is represented by the "B" peak of serum VII. The molecular weight of this molecule is 51,000 according to Smith and Wagner (15), and 46,000 according to our present work. It is more stable physicochemically, it may be more readily excreted, and it may represent the synthesized as opposed to the preformed type of interferon (7, 17). The second basic type is represented by the main peak in urine EII (molecular weight = 35,000). The main "B" peak of serum EII may be a conjugate of this material. This type is more labile; it cannot be excreted in the complexed form (possibly its shape may be more elongated than the first type), and it may represent the preformed as opposed to the synthesized type of interferon. Very possibly, the "A" peaks (molecular weight >100,000) of both serum EII and VII represent polymers or conjugates of this type. This postulate is open to verification by an analysis of the stability, mechanism of action, and other physicochemical properties of the various peaks in question.

ACKNOWLEDGMENTS

We thank Lucille Ople, Mary Kay Breinig, and Ann Henry for excellent technical assistance, and John A. Armstrong for helpful discussions and criticisms.

This investigation was supported by Public Health Service research grant AI-02953 from the National Institute of Allergy and Infectious Diseases.

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