Recovery and Identification of Interferon in the Rabbit Urine

It has been shown[1,2] that, after the i.v. inoculation of various viruses, a maximum level of circulating interferon is detectable after 4–8 h and then falls off very quickly. The presence of proteins in the urine[3–6] and the relatively small molecular weight of interferon[7] might explain the rapid disappearance of i.v. injected interferon from circulation[8,9], due, at least in part, to the excretion of this protein into the urine. This possibility was put forward by one of us (V.B.) during a discussion with Prof. M. Ho last June at Siena, and it was considered worth while investigating either from a biological or a practical point of view. Indeed the isolation of interferon could be more easily undertaken if this protein was found in the urine in large amounts and fairly free of contaminating serum proteins.

Albino rabbits weighing 2 kg were inoculated i.v. with about 10^8 plaque-forming units (pfu) of Sindbis virus. Control experiments were carried out either in rabbits injected with phosphate buffered saline (PBS), or in rabbits inoculated with virus, by taking the blood and urine samples immediately before the virus injection. Blood was collected from the ear vein and the urine by catheterization of the bladder at selected intervals during the experiment. Serum and urine for assay were immediately acidified with 2 *M* citric acid and dialysed at 4 °C, at pH 2.1, with constant stirring, against at least 40-fold larger volumes of 0.1 *M* citric acid for 4–5 days with 4 changes. Serum was then equilibrated with PBS, by dialysis at 4 °C, whereas the urine was exhaustively dialysed against distilled water and finally against 1 m*M* sodium phosphate buffer, pH 7.2. After dialysis serum and urine were found to be diluted about 2.7- and 1.2-fold respectively. Urine precipitates were removed by centrifugation at 2000 *g* for 60 min and freeze-dried.

Serum and urinary proteins were measured by UV-absorption at 280 nm and by biuret method[10] using a crystallized sample of bovine serum albumin as a standard. Interferon was titrated in baby rabbit kidney cell cultures by measuring viral inhibitory effect by plaque reduction method of vesicular stomatitis virus (VSV). At least 2 bottle cultures were used for each dilution of the interferon preparation.

Interferon titers of samples of rabbit serum and urine have been found to be consistently higher in urine than in serum, whereas no inhibitory activity could be detected in the controls.

The following experiments have been carried out in order to ascertain if such high inhibitory activity in urine could be ascribed to interferon:

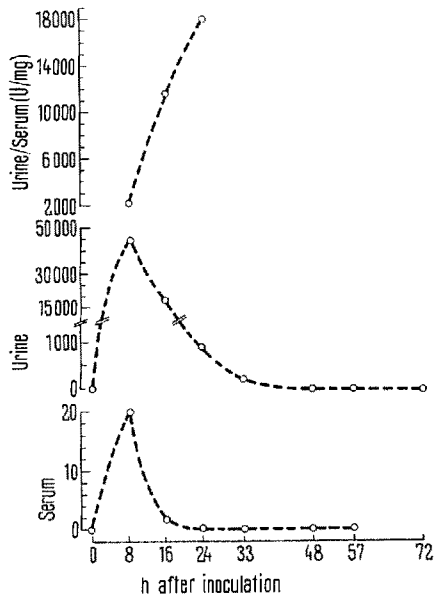
The urinary viral inhibitor was stable at pH 2 for up to 5 days at + 4 °C, it was non-dialysable (Visking tubing 23/32 in), and it was relatively stable at elevated temperatures. From the Table it appears to be partially resistant at 80 °C for 1 h, but it was destroyed by heating to 100 °C for 1 h. It was also highly susceptible to tryptic action, and centrifugation at 200,000 *g* for 2 h did not cause any decrease in antiviral activity of the supernatant. It had no direct inactivating activity on VSV since virus and urinary inhibitor, incubated together at 37 °C for 2 h, did not decrease viral infectivity. It showed species specificity, as it did not inhibit VSV in cell cultures derived from chick embryo or monkey kidney (strain 37 RC)[11], and it lacked viral specificity since it inhibited both Sindbis virus and VSV.

Having ascertained that the antiviral material in the urine had the same physicochemical and biological properties of serum interferon, it appeared interesting to study the time course appearance of urinary interferon.

The Figure shows that maximal antiviral activity in a group of 10 rabbits occurred both in serum and urine at

Stability of urinary interferon calculated as % plaque reduction. Interferon titre was 10,000 U/ml. Heating was effected at neutrality

Temperature (1 h)	Dilution 10^{-2}	10^{-3}
0 °C	100	100
40 °C	100	100
50 °C		100
60 °C	100	100
70 °C		100
80 °C	100	78
100 °C	0	0



Interferon titers, expressed as U/mg of protein in rabbit serum and urine collected at intervals after i.v. inoculation of 10^8 pfu of Sindbis virus. The Urine/Serum (U/mg) ratios are plotted at the top of the diagram.

[1] S. Baron and C. E. Buckler, Science *141*, 1061 (1963).
[2] Y. Kono and M. Ho, Virology *25*, 162 (1965).
[3] A. L. Sellers, H. C. Goodman, J. Marmorston and M. Smith, Am. J. Physiol. *163*, 662 (1950).
[4] G. H. Grant, J. clin. Path. *12*, 510 (1959).
[5] J. S. King and W. H. Boyce, *High Molecular weight Substances in Human Urine* (Charles C. Thomas, Springfield Ill. 1963), p. 39.
[6] B. L. Dinh, G. Hermann and P. Grabar, Bull. Soc. Chim. biol. *46*, 255 (1964).
[7] T. C. Merigan, C. A. Winget and C. B. Dixon, J. molec. Biol. *13*, 679 (1965).
[8] T. P. Subrahmanyan and C. A. Mims, Br. J. exp. Path. *47*, 168 (1966).
[9] N. B. Finter, Br. J. exp. Path. *47*, 361 (1966).
[10] A. G. Gornall, C. J. Bardawill and M. M. David, J. biol. Chem. *177*, 751 (1949).
[11] G. Rita, M. Russi and F. Dianzani, Atti Accad. Fisiocr. Siena *5*, 1367 (1957–58).

8 h after Sindbis i.v. inoculation and afterwards fell off more quickly from serum than urine.

It appears likely, therefore, that urinary interferon derives directly from the plasma pool and its high specific activity is reached through the selective renal glomerular filtration. Indeed i.v. administered interferon has been detected in the kidney[8]. The urine/serum (U/mg) ratios suggest that some of the urinary interferon is derived, after 8 h, directly from the kidney and these data are consistent with the finding that inhibitory activity in the kidney is low and delayed[2]. Nevertheless the slower tailing off of the urinary interferon curve may also be accounted for by a small residue of urine in the bladder.

The possibility that urinary interferon could be due to a trauma of the bladder after catheterization has been excluded by the absence of blood in the urine and by collecting the urine only once, from 3 rabbits, 10 h after the virus inoculation. Again urinary interferon had a specific activity of 674 (U/mg) as compared to 4.7 in the serum of the same group of rabbits.

The data here reported suggest that levels of blood interferon are the result of complex equilibria of circulating interferon with tissues and body fluids and with simultaneous leakage of this protein into the urine. In this respect interferon behaves like lysozyme[12] or the sex-dependent α_2-globulin in the rat[13]. An important site of interferon breakdown may occur also in the intestine as it appears for serum albumin[14,15].

When this work was completed, we learnt that OH[16] has also found interferon in some body fluids and a small amount in urine. The discrepancy with our results may be due to the fact that OH-induced interferon production by typhoid endotoxin, and it is known[17] that this interferon has a higher molecular weight than the virus-induced one and is likely to be largely retained by the kidney filter.

From these results the conclusion is drawn that interferonuria represents an excellent condition for obtaining highly purified interferon. Clearly the dynamic and the metabolic fate of interferon will be more directly traced by isotopically labelled pure interferon[18,19].

Riassunto. È stato dimostrato che l'inoculazione endovenosa di virus Sindbis nel coniglio provoca formazione di interferone che viene liberato nel sangue ed escreto con le urine. L'interferone urinario sembra derivare quasi completamente dal siero, ha alti titoli ed ha le stesse proprietà fisico-chimiche e biologiche dell'interferone sierico.

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[12] G. C. PERRI, M. FAULK, E. SHAPIRO and W. L. MONEY, Proc. Soc. exp. Biol. Med. *115*, 189 (1964).
[13] A. K. ROY and O. W. NEUHAUS, Biochim. biophys. Acta *127*, 82 (1966).
[14] H. TARVER, F. B. ARMSTRONG, J. R. DEBRO and S. MARGEN, Ann. N.Y. Acad. Sci. *94*, 23 (1961).
[15] J. WETTERFORS, Acta med. scand. *176*, 787 (1964).
[16] J. O. OH, personal communication in Information Exchange NIH No. 6 (1966).
[17] Y. H. KE, M. Ho and T. C. MERIGAN, Nature *211*, 541 (1966).
[18] This work was supported by a grant from the Consiglio Nazionale delle Ricerche, Roma, Gruppo Nazionale di Medicina Sperimentale.
[19] It has been brought to the authors' attention that I. GRESSER et al. (Information Exchange Group No. 6, 221), P. DE SOMER et. al. (IEG. 253) and M. Ho and B. POSTIC (IEG. 256 and 266) have independently made observations similar to those here reported.

PRO EXPERIMENTIS

Whole Body Sagittal Technique in Mice Employing a New Bio-Autographic Method: Its Utility for the Evaluation of the Body Distribution of Antibacterial Drugs

Body distribution of antibacterial drugs is usually evaluated by measuring tissue levels using bacteriological or scintillometric methods: these, however, are troublesome and time consuming and require a large number of animals to obtain true average values. Progress was achieved by the autoradiographic method, using whole body sagittal sections of mice, which allows more rapid and accurate assay[1-5]. This method however requires time (generally not less than 4 months) to give reliable results; moreover, it allows a correct evaluation of distribution only in the period immediately following administration for the genesis of labelled active or inactive metabolites: therefore contemporaneous chromatographic trials are essential. For the purpose of obtaining a distribution pattern closer to the effective chemotherapeutic distribution, a method has been developed in our laboratory[6], based on the direct inoculation of thin, whole body sections of mice previously treated with antibacterial drugs, and on the direct observation of the live microbe by vital staining with tetrazolium salts.

Methods. Swiss albino mice, treated with antibacterial drugs and killed at various hours after the administration, are used; they are frozen on a Leitz microtome type 1300 and cut at $-10\,^{\circ}C$ into sections of 300 μ. Sections are

[1] S. ULLBERG, Prog. nucl. Energy *2*, 29 (1959).
[2] H. HANNGREN, E. HANSSON and S. ULLBERG, Antibiotics Chemother. *12*, 46 (1962).
[3] F. BENAZET and G. BOURAT, C. r. hebd. Séane. Acad. Sci., Paris *260*, 2622 (1965).
[4] H. HANNGREN, E. HANSSON and S. ULLBERG, Acta med. scand. *173*, 61 (1963).
[5] H. HANNGREN, E. HANSSON, N. SVARTZ and S. ULLBERG, Acta med. scand. *173*, 391 (1963).
[6] E. TUBARO and M. J. BULGINI, Nature *212*, 1314 (1966).