animals that does not operate or is depressed in the aroused state.

The author gratefully acknowledges the technical assistance of Teresa Plucinski and the Research Resources Laboratory of the University of Illinois Medical Center for the use of their cold-stress facilities.

1. Hawley, P. L., Aerospace Med. **39**, 144 (1968).

2. South, F. E. and House, W. A., *in* "Mammalian Hibernation III: Proceedings of the Third International Symposium on Natural Mammalian Hibernation," University of Toronto, 1965 (K. C. Fisher, *et al.*, eds.), p. 305. American Elsevier, New York (1967).

3. Soumalainen, P. and Soarikoski, P.-L., Experientia **23**, 451 (1967).

4. Prusiner, S., Williamson, J. R., Chance, B., and Paddle, B. M., Arch. Biochem. Biophys. **123**, 368 (1968).

5. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **189**, 265 (1951).

6. Davis, E. J. and Quastel, J. H., Canad. J. Biochem. Physiol. **42**, 1605 (1964).

Received June 3, 1968. P.S.E.B.M., 1968, Vol. 129.

Studies on the Physico-Chemical Inactivation of Rabbit Interferons* (33337)

YANG H. KE AND MONTO HO

Department of Epidemiology and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania 15213

In previous communications, we reported that rabbit serum interferons induced by virus and by endotoxin differed in their stability to heat and acid (1, 2). On the basis of molecular size as determined by Sephadex gel filtration, at least two types of virus-induced interferon (VII) and two types of endotoxin-induced interferon (EII) could be identified (2, 3). Both VII and EII contained a minor high molecular weight (>100,000) peak that was designated the "A" peak (VII-A and EII-A). The molecular weight of the main component of VII was 46,000 (VII-B), and that of the main component of EII was 54,000 (EII-B).

In this paper, we report further studies differentiating VII-A from VII-B and EII-A from EII-B on the basis of kinetics of inactivation at 56° and at pH 2. To pursue these differences along other lines, the stability of VII-A and VII-B in the presence of a number of reagents reactive against known chemical radicals, including periodate, was determined.

Materials and Methods. Details on viruses, tissue culture, interferon preparation, and assay techniques were as described elsewhere (2, 4) unless specified. Rabbit kidney cell cultures prepared from 2–3-week-old albino rabbits (about 300 g) were used as we found that they were on the average 8 times more sensitive to interferon than rabbit embryo cultures employed earlier (2).

Serums were obtained from rabbits after inoculation of Newcastle disease virus or *E. coli* endotoxin (Boivin, 2). Virus-induced interferon (VII) was fractionated by filtering 6-ml serum aliquots through a Sephadex G-100 column as previously described. Interferon activity peak regions were pooled and concentrated to 1.5–2.0 ml by dialysis against Carbowax 20 M (polyethylene glycol, manufactured by Union Carbide Corp., South Charleston, West Virginia). These pooled, concentrated semipurified fractions will be referred to as VII-A (m.w. >100,000; interferon activity 1000 units per 2.0 ml), and VII-B (m.w. 46,000; interferon activity 16,000 units per 2.0 ml, protein content 7.5 μg/unit). Endotoxin induced interferon (EII) was similarly processed and concentrated. The resulting EII-A (m.w.>100,000) titered about 400 units per 2.0 ml and EII-B (m.w. 54,000) titered 800 units per 2.0 ml.

For heat inactivation, 2 ml containing about 200 units of a designated interferon

* Supported by grant AI-02953 from the USPHS.

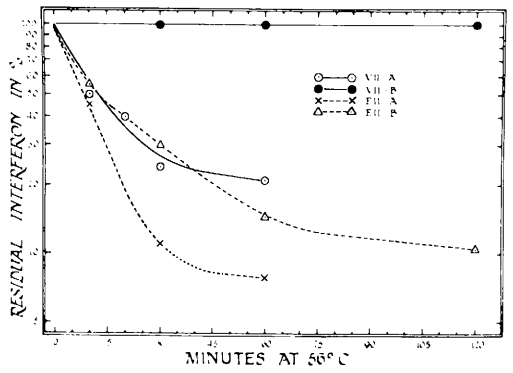

FIG. 1. Kinetics of inactivation of rabbit serum interferons at 56°.

suspended in LAH medium without bicarbonate, but containing 20% calf serum, were placed in separate stoppered tubes in a 56° waterbath. After each indicated interval, one tube was withdrawn and chilled in an ice bath until assayed.

For inactivation studies at pH 2, 6 ml of VII-A, VII-B, EII-A, or EII-B were diluted 1:2 in LAH medium without $NaHCO_3$ but containing 10% calf serum. To each sample, HCl (1.0 or 0.1 *N*) was added until pH 2.0 was reached as monitored by a pH meter. The samples were permitted to stand at 4°. After each indicated duration, 4-ml aliquots were withdrawn from the sample and immediately neutralized with NaOH before assay.

The general method used for testing the effect of various reagents on interferon was as follows: To 200 units or more of semipurified interferon in sterile distilled water, a stock solution of a reagent was added to desired concentration, and adjustment to the desired pH was made when necessary by adding NaOH or HCl as monitored by a pH meter. The reaction was permitted to proceed at 4° for the duration indicated, and then the samples were dialyzed successively in 20–50 vol of PBS and LAH medium for 18–24 hr. To control interferon samples against which the degree of inactivation was measured, sterile distilled water was added instead of the reagent solution. Otherwise, they were processed the same way. For periodate inactivation, sodium thioglycolate (100 m*M*) or ethylene glycol (1%) was added to the reaction mixture buffered at pH 7 or 4.5 to stop the reaction at the specified time prior to dialysis. To control samples, periodate and thioglycolate (or ethylene glycol) were added premixed in such ratio that upon addition of the mixture to interferon samples, the concentration of periodate and thioglycolate was the same as in the test samples.

Results. Effect of heat and acid on VII-A, VII-B, EII-A, and EII-B. The inactivation kinetics of the four types of interferon at 56° is shown in Fig. 1. VII-B, the major component of serum VII, was stable at 56°. On the other hand, the minor component VII-A was quite labile. There was a clear separation between these two types of VII. The two components of EII; i.e., EII-A and EII-B were both labile at 56°, but EII-A seemed to be inactivated at a faster rate (T_{50} = 10 *vs* 15 min).

Differences in inactivation between these four types of interferons were also evident at pH 2, although of a somewhat different pattern than at 56° (Fig. 2). There was relatively little difference in the inactivation of VII-A and VII-B, but as in the case of inactivation at 56°, VII-A appeared to be more labile. On the other hand and in contrast to the results of inactivation at 56°, EII-A and EII-B were better differentiated by inactivation at pH 2. Taken together, it would appear from these results that all four types of interferon, except for VII-A and EII-B, are distinguishable on the basis of inactivation kinetics. VII-A and EII-B, however, are clearly separable by molecular weight (2).

Effect of chemical reagents on VII-A and

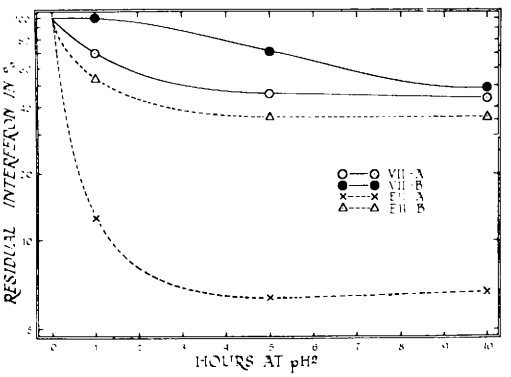

FIG. 2. Kinetics of inactivation of rabbit serum interferons at pH 2.

TABLE I. Effect of Chemical Reagents on Virus-Induced Rabbit Serum Interferons.[a]

Reagent	Concentration (m*M*)	Reaction conditions: pH	Reaction conditions: Duration (hr)	Type of interferon	Residual activity (%)	Groups primarily attacked
Benzoylchloride	25	7	3	VII-A	100	$-NH_2$
	25	7	24	VII-A	100	
	25	7	3	VII-B	100	
	25	7	24	VII-B	100	
2-Mercaptoethanol	200	7	3	VII-A	100	–S–S–
	200	7	24	VII-A	100	
	200	7	3	VII-B	100	
	200	7	24	VII-B	100	
Potassium cyanate	50	7	3	VII-A	100	–SH
	50	7	24	VII-A	100	
	50	7	3	VII-B	100	
	50	7	24	VII-B	100	
Cyanogen bromide	16.5	5	3	VII-A	58	γ–S–methyl of
	16.5	5	24	VII-A	10	methionine
	16.5	5	3	VII-B	82	
	16.5	5	24	VII-B	20	
Sodium thioglycolate	1000	5	3	VII-A	41	–S–S–
	1000	5	24	VII-A	31	
	1000	5	3	VII-B	19	
	1000	5	24	VII-B	10	
	100	7	24	VII-B	100	
Sodium meta periodate	33	7	3	VII-A	<1	vicinal $-OH_f$ =O,
	33	7	24	VII-A	<1	$-NH_2$ groups
	33	7	3	VII-B	<2	
	33	7	24	VII-B	<1	
	5	7	1	VII-B	3	
	1	7	1	VII-B	7	
	5	4.5 (darkness)	1	VII-B	5	
	1	4.5 (darkness)	1	VII-B	10	
Sodium iodate	5	7	1	VII-B	100	
Sodium formate	5	7	1	VII-B	100	
Formaldehyde	5	7	1	VII-B	100	

[a] The general method of inactivation described in *Materials and Methods* was used. The temperature of the reactions was 4°.

VII-B. The effect of various chemical reagents on VII-A and VII-B is presented in Table I. It is apparent that at the concentrations used, benzoylchloride, 2-mercaptoethanol, and potassium cyanate had no effect on either VII-A or VII-B. Cyanogen bromide (16.5 m*M*) and very high concentrations of sodium thioglycolate (1000 m*M*) were partially active. These results possibly indicate that the γ-sulfmethyl group of methionine may be important in the interferon molecule, but whether disulfide bonds are essential is dubious in view of the lack of effect of 100 m*M* thioglycolate and 200 m*M* 2-mercaptoethanol. The most active agent found was sodium periodate. Both VII-A and VII-B were almost entirely inactivated by 33 m*M* in 3 hr. Furthermore, VII-B was inactivated by 1 m*M* for 1 hr at pH 7 as well as at pH 4.5 in darkness. In general, the degree of inactivation studied over 1–50 m*M* range of periodate was somewhat less in darkness and at

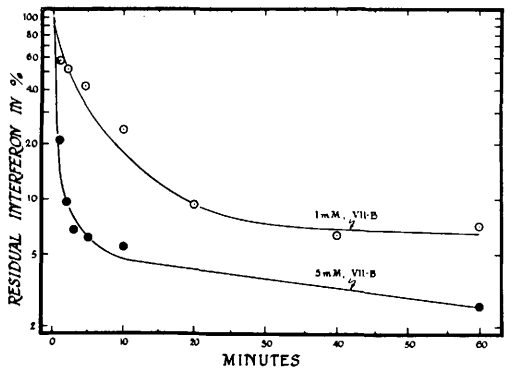

FIG. 3. Kinetics of inactivation of interferon with periodate at 1 and 5 m*M*.

pH 4.5 than at pH 7 (Ke and Ho, unpublished data). VII-A was not studied in these respects. No inactivation of interferon was observed after treatment of VII-B with 5 m*M* sodium iodate, sodium formate, or formaldehyde for 1 hr at pH 7. This suggests that the effect of periodate observed was not due to the secondary effect of the products of the initial periodate oxidation.

The kinetics of inactivation of VII-B by periodate at 5 m*M* and 1 m*M* were studied (Fig. 3). The general shape of the two curves does not conform to a first-order reaction throughout. This may indicate a second-order reaction as had been described for periodate oxidation of carbohydrates (5), or it may be due to heterogeneity of the reactant. For example, certain susceptible sites on the interferon molecule may be more easily oxidized than others. At 5 m*M* concentrations of periodate, VII-B was more rapidly inactivated than at 1 m*M*.

Discussion. The present work elaborates further on the heterogeneous characteristics of rabbit interferons. Previously, it was shown that virus and endotoxin-induced interferons differed markedly in their response to heat and acid treatment (1, 2). We now show that the high molecular weight (>100,000) type of serum virus-induced interferon (VII-A) is much more labile at 56° and pH 2 than the main component (VII-B, mol wt. = 46,000), which is consistent with the findings of Smith and Wagner (6). We postulated earlier that perhaps the two high-molecular weight components, i.e., VII-A and EII-A, may be related, but this appears unlikely because their kinetics of inactivation at pH 2 are so different.

Lindenmann and co-workers (7) found that urea, sodium metaperiodate, and sodium iodoacetate had no effect on chick interferon, suggesting that hydrogen bonding, oxidizable carbohydrate, and sulfhydryl groups were not involved in its biological activity. Fantes and O'Neill (8) subjected partly purified chick interferon to a variety of chemical treatments and concluded that one of several amino, disulfide, and γ-sulfmethyl groups of methionine were essential for interferon activity. The present work with benzoylchloride, 2-mercaptoethanol, and potassium cyanate suggests that amino, disulfide, and sulfhydryl groups are not essential in rabbit interferon activity, although the inactivating effect of high concentration of thioglycolate suggests that disulfide bonds may be essential. The effect of cyanogen bromide suggests that as in the case of chick interferon, methionine may be an essential component of rabbit interferon. Nagano and co-workers (9) found that 50 m*M* periodate inactivated an interferon obtained from vaccinia-infected rabbit skin. And Fantes reported more recently that periodate (5 m*M*, 22°) completely inactivated highly purified chick interferon in 5 hr (10). We have confirmed this finding not only with purified chick interferon, but also with crude allantoic fluid interferon (Ho and Ke, unpublished results). It is possible that the negative results of Lindenmann and co-workers were due to inadequate concentrations of periodate, since they used only 0.01, 0.1, and 1.0 m*M* for 1 hr.

Although the presence of a carbohydrate is suggested in many reports on purified interferon, the possible role of such a carbohydrate in interferon has not received much discussion. Burke (11) reported that there was 1.6% carbohydrate expressed as percentage of protein concentration in his preparation. Lampson and co-workers (12) found that highly purified chick interferon contained a "trace" amount of carbohydrate, but α-amylase had no effect on interferon activity. Williams and Phillips found that in one

highly purified electrophoretically homogeneous sample of chick interferon, glucosamine was present (in 13). Nagano, Okazaki, and co-workers (14, 15) separated by phosphate buffer (pH 5.8) concentration gradient elution of rabbit skin interferon on TEAE-cellulose a carbohydrate moiety that inhibited vaccinia virus only in rabbit skin, but not in cell cultures. It is not known whether this interesting moiety has any relationship to interferon. In any case, chemical analyses of so-called purified interferons are of doubtful validity because it is unlikely that interferon has been 100% purified by any group (in 16), and the reported carbohydrate content could be due to impurities.

The consumption of periodate is known to be stoicheometric with the oxidation of α-glycolic structures predominant in carbohydrates, and the periodate reaction has long considered to be specific for α-glycolic structures (17). Reactivity of periodate with a substance is often taken as a clue to its being a polyol or carbohydrate. However, periodate has been found to oxidize many other structures (18–20). Particularly important was the finding that certain amino acid residues of glycoproteins and free amino acids were oxidized in addition to the carbohydrate (18, 20).

In conclusion, we cannot postulate on the basis of the periodate reaction alone that a carbohydrate moiety is a necessary component of the interferon molecule, but this remains a possibility. One attractive feature of this idea is that it may spur the search for a possible virus-inhibiting common denominator to the myriads of interferons now identified. Other reagents more specific for carbohydrates should be tested for their effect on interferon. We have tested one glycosidase, lysozyme, and found it to be ineffective against rabbit interferon. Also, as mentioned earlier, Lampson and co-workers (12) found that α-amylase had no effect on chick interferon. The final proof that a carbohydrate is essential for interferon is direct chemical detection, which must await the absolute purification of interferon.

Summary. The kinetics of inactivation at 56° and at pH 2 were different for the high- and low-molecular weight components of virus-induced and endotoxin-induced rabbit interferons. The two types of virus-induced interferons were not affected by benzoylchloride or potassium cyanate, suggesting that amino or sulfhydryl groups are not essential for their interferon activity. The integrity of disulfide bonds also seems unnecessary as indicated by the lack of effect of 2-mercaptoethanol and 100 m*M* thioglycolate, although 1000 m*M* of the latter compound partially inactivated the interferons. The effect of cyanogen bromide indicated a requirement for the γ-sulfmethyl group of methionine. Sodium metaperiodate in low concentrations rapidly inactivated purified rabbit interferons as well as crude and purified chick interferon. Although the action of periodate may suggest an essential carbohydrate moiety in the interferon molecule, this action may be equally due to an effect on protein.

We would like to thank Misses Lucille Ople and M. K. Breinig for their excellent technical assistance, and Dr. John A. Armstrong for his stimulating discussions.

1. Ho, M., Science **146**, 1472 (1964).
2. Ke, Y. H. and Ho, M., J. Virol. **1**, 883 (1967).
3. Ke, Y. H., Ho, M., and Merigan, T. C., Nature **211**, 541 (1966).
4. Ho, M. and Breinig, M. K., J. Immunol. **89**, 177 (1962).
5. Price, C. C. and Kroll, H., J. Am. Chem. Soc. **60**, 2726 (1938).
6. Smith, T. J. and Wagner, R. R., J. Exptl. Med. **125**, 579 (1967).
7. Lindenmann, J., Burke, D. C., and Isaacs, A., Brit. J. Exptl. Pathol. **38**, 551 (1957).
8. Fantes, K. H. and O'Neill, C. F., Nature **203**, 1048 (1964).
9. Nagano, Y., Kojima, Y., and Suzuki, T., Compt. Rend. Soc. Biol. **154**, 2166 (1960).
10. Fantes, K. H. and Furminger, I. G. S., Nature **216**, 71 (1967).
11. Burke, D. C., Biochem. J. **78**, 556 (1961).
12. Lampson, G. P., Tytell, A. A., Nemes, M. M., and Hilleman, M. R., Proc. Soc. Exptl. Biol. Med. **112**, 468 (1963).
13. Fantes, K. H., *in* "Interferons" (N. B. Finter, ed.), North-Holland Publ., Amsterdam (1963).
14. Nagano, Y., Japan. J. Exptl. Med. **37**, 183 (1967).

15. Okazaki, H., Japan. J. Exptl. Med. 37, 159 (1967).
16. Wolstenholme, G. E. W. and O'Connor, M. ed., CIBA Symp. on Interferon, Churchill, London (1968).
17. Malaprade, M. L., Bull. Soc. Chim. France 43, 683 (1928).
18. Clamp, J. R. and Hough, L., Biochem. J. 94, 17 (1965).
19. Dixon, H. B. F., Biochem. J. 83, 91 (1962).
20. Lee, Y. C. and Montgomery, R., Arch. Biochem. Biophys. 95, 263 (1961).

Received June 5, 1968. P.S.E.B.M., 1968, Vol. 129.

Studies of the Interaction of Phenylbutazone, Oxyphenbutazone and Methandrostenolone in Man* (33338)

EIGILL F. HVIDBERG,[1] PETER G. DAYTON,[2] J. M. READ III, AND COLON H. WILSON

Divisions of Clinical Pharmacology and Rheumatology, Department of Medicine and Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322

It was previously reported (1) that methandrostenolone caused an increase of plasma levels of oxyphenbutazone in human subjects. To extend these investigations, similar studies were carried out with phenylbutazone, of which oxyphenbutazone is a major metabolite in man (2). In the present work an interaction was observed among phenylbutazone, oxyphenbutazone, and methandrostenolone.

Materials and Methods. Eight ambulatory subjects (2 with osteoarthritis and 6 with rheumatoid arthritis) were selected from a population treated at an arthritis clinic. All medication was stopped at least 1 week prior to the study. Two types of studies were undertaken. In one (half-life study) a single oral dose of oxyphenbutazone (Tandearil) or phenylbutazone (Butazolidin) was administered. The dose of the former was 400 mg, the latter 400–600 mg. Plasma concentrations were determined at suitable intervals up to 1 week after dose. In the other type of study, oxyphenbutazone or phenylbutazone were given continuously over a period of several days; the daily oral dose of oxyphenbutazone was 300 mg; that of phenylbutazone, 400 mg, except for subject 3 who received 300 mg. The doses were divided as follows: 100 mg at 8 a.m., 1 p.m. and 7 p.m.; or 100 mg 8 a.m., 1 p.m., and 200 mg at 7 p.m. Blood was drawn at 11 a.m. Methandrostenolone (Dianabol) was given in oral doses of 2.5 mg each at 8 a.m. and 7 p.m.

In the absence of phenylbutazone, oxyphenbutazone concentrations in plasma, albumin, or buffer solutions were determined by a previously published procedure (2). Phenylbutazone was measured either by a published procedure (3) or by a modification which involved, after the initial extraction from acid, two washes of the organic phase with 15 ml of 0.15 *N* HCl. When oxyphenbutazone was determined in the same sample used for the phenylbutazone assay, after the heptane extractions (3), 2 ml of the 3.5 ml aqueous acidic phase was extracted (2) and correction was made for the loss (24% of oxyphenbutazone) by the heptane extractions. Equilibrium dialysis experiments were performed at 37° as described (4). In brief, 5 ml of plasma or 5% (w/v) albumin solution was added to each bag and 1 ml of buffer, pH 7.4 was added inside; 17 ml of buffer were the outside phase. Human plasma (albumin 4.5 g/100 ml, globulin 2.6 g/100 ml) was diluted in the same manner. Methandrostenolone (23 μg) was added to the dialysis bag in 0.15 ml of ethanol. The same amount

* Supported by NIH grant GM 14270 and Vet. Rehab. Admin. Grant RT-6; presented in preliminary form at the 1968 Federation Meeting, Atlantic City.

[1] Merck International Fellow in Clinical Pharmacology; present address Department of Pharmacology, University of Copenhagen.

[2] Reprint requests should be addressed to Dr. Dayton.