PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 146, 249–253 (1974)

Stabilisation of Mouse and Human Interferons by Acid pH Against Inactivation Due to Shaking and Guanidine Hydrochloride (38080)

V. G. EDY, A. BILLIAU, M. JONIAU, AND P. DE SOMER

Rega Institute for Medical Research, University of Leuven, B-3000 Leuven, Belgium

Before human interferon can be considered usable for large-scale clinical trials and possible medical use, several problems associated with its production and application remain to be solved. Not least among these is the relatively low stability of interferon on storage and mechanical handling. Although most interferons are more stable than many other proteins upon exposure to high and low pH, they are readily inactivated by a wide range of other physical and chemical treatments (1, 2). It was shown in an earlier study (3) that human and mouse monolayer tissue culture interferons, whether virus- or polynucleotide-induced, were rapidly and almost completely inactivated by shaking. The activity of human leukocyte interferon was not affected by such treatment.

The activity of human leukocyte interferon can be destroyed by treatment other than shaking, e.g., on heating crude human leukocyte interferon at 70°C, 90% of the activity is lost within 30 min (4). Guanidine hydrochloride was found to be useful in the recovery of interferon from an inactive complex formed on heating crude concentrated human leukocyte interferon. There is some evidence (5) that the pH at which heat-treatment of mouse and human monolayer tissue culture interferons is carried out has a major effect on the extent of inactivation; at low pH the half-lives of mouse and human interferon activities at 56°C and 37°C are greatly extended.

The present paper reports an investigation into the effects of pH, shaking and guanidine hydrochloride on several types of interferon preparations, both human and mouse.

Materials and Methods. Interferon preparations. Human fibroblast interferon was prepared by poly(I)·poly(C)-induction of interferon-primed cells, followed by superinduction with metabolic inhibitors (6). The interferon was harvested in Eagle's minimal essential medium supplemented with nonessential amino acids and 2% heat-inactivated foetal bovine serum (EMEM + 2% FBS). Virus-induced human fibroblast interferon was prepared by induction with Newcastle disease virus (NDV) as previously described (7). Human leukocyte interferon was kindly supplied by Dr. K. Cantell, Central Public Health Laboratory, Helsinki, Finland. This was received in a concentrated form, and was diluted 100-fold with EMEM (without added serum) before use.

Mouse tissue culture interferon was prepared with superinduction of MO57/2 mouse monolayer cultures (8), and was harvested in EMEM + 2% FBS. NDV-induced mouse serum interferon was prepared by iv injection of NDV (Komarov strain) into male NMRI mice. After 8 hr the mice were bled, and the pooled sera held at pH 2.0 for 5 days to inactivate virus. This crude interferon preparation was diluted 100-fold with EMEM before use.

All the above interferon preparations had protein concentrations of between 0.5 and 2.0 mg/ml, as assayed by a modified Folin technique (9), using bovine serum albumin as a standard.

Interferon assays. Both human and

mouse interferon preparations were assayed by a dye-uptake method (10), in human or mouse embryo fibroblasts as appropriate, challenged with Mengo virus. Standard mouse and human interferon preparations were included in all the assays, and all titers are quoted in international research reference units (11).

Inactivation of interferon. Interferon preparations were inactivated by shaking basically as described (3). One milliliter samples of the interferon preparations were placed in 110 × 15 mm glass-stoppered glass tubes, which were rotated end-over-end at 50 rpm, at a temperature of 4°C. Under these conditions, the interferon preparations flowed from end to end of the tube, but very little or no foaming [known to inactivate interferon (12)] occurred.

To test the effect of guanidine hydrochloride on the activity of interferon preparations, solid guanidine hydrochloride (Serva Feinbiochemica GmbH & Co., Heidelberg, W. Germany) was added to the interferon solutions. Sodium hydroxide (2 *N*) was used to neutralise the solution following addition of guanidine hydrochloride, if necessary. After storage for 20–24 hr at 4°C, the interferon preparations were dialysed against phosphate buffered saline to remove the guanidine hydrochloride.

Results. The reversible denaturation of human leukocyte interferon by guanidine hydrochloride, and the use of this phenomenon in the reactivation of heat-inactivated interferon, have been described. If this procedure could be applied to other interferon preparations, and following other inactivations, then it could be of considerable importance in the purification of human and mouse interferons. As however, major stability differences between human leukocyte interferon and other human and mouse interferons have been reported (3, 4) the reversibility of guanidine hydrochloride denaturation of various interferon preparations has been tested.

It was found (Table I) that all interferons tested except human leukocyte interferon were inactivated by treatment with guanidine hydrochloride, maximal inactivation being caused by 4–6 *M* concentration. The stability of human leukocyte interferon to guanidine hydrochloride confirms the findings of Mogensen and Cantell (4). Inactivation of the sensitive interferon preparations was not complete; after treatment with 4–8 *M* guanidine hydrochloride a small fraction (generally about 1% but as high as 25% with some preparations) of the activity consistently remained. This occurred whatever the source of interferon (human and mouse tissue culture or mouse serum interferon), and was not affected by

TABLE I. Effect of Guanidine Hydrochloride on Various Interferon Preparations.[a]

Concentration of guanidine hydrochloride (*M*)	Titer (log_{10} units/ml)				
	Mouse serum interferon	Mouse tissue culture interferon	Poly (I) · poly (C) - induced human tissue culture interferon	NDV-induced human tissue culture interferon	Human leukocyte interferon
0	3.1	4.6	4.1	2.5	4.3
2	2.6	4.0	3.7	2.4	4.3
4	2.0	3.0	2.4	2.3	4.2
6	1.9	2.7	2.0	1.9	4.1
8	1.9	2.6	2.1	1.9	4.3

[a] Interferon preparations were as described in Materials and Methods. Solid guanidine hydrochloride was added to the desired concentrations, and the solutions neutralised where necessary by addition of sodium hydroxide. The samples were stored at 4°C for 24 hr, then dialysed against phosphate buffered saline to remove the denaturant, and finally assayed for interferon activity.

TABLE II. Effect of Shaking on the Activity of Various Interferon Preparations.[a]

Period of shaking (hr)	Titer (log_{10} units/ml)				
	Mouse serum interferon	Mouse tissue culture interferon	Poly (I) · poly (C) - induced human tissue culture interferon	NDV-induced human tissue culture interferon	Human leukocyte interferon
0	2.9	4.0	4.0	2.8	4.0
1	2.4	2.6	3.4	2.6	4.0
4	2.4	2.4	3.1	2.3	4.1
24	1.9	2.3	2.4	1.6	4.1
Stationary control	3.0	4.0	4.1	2.6	4.2

[a] One milliliter samples of the interferon preparations were shaken in stoppered glass tubes for the periods indicated, and then stored frozen until assayed for interferon activity.

the nature of the inducer [NDV or poly(I) · poly(C)] used in the production of the interferon preparation.

Human fibroblast interferon and all mouse interferon preparations tested have been found to be inactivated by shaking. However, human leukocyte interferon was totally resistant to even 24 hr shaking [Table II, also (3)]. Another preparation of human fibroblast interferon, the kind gift of Drs. E. Havell and J. Vilcek, was also found to be unstable on shaking, although inactivation was less extensive than that reported here for human fibroblast interferon prepared according to Billiau, Joniau, and De Somer (6). As described for the inactivation of interferon by guanidine hydrochloride, a small fraction of human fibroblast and mouse interferon was resistant to shaking for 24 hr.

The small amount of human fibroblast interferon activity that remained after shaking was completely stable to guanidine hydrochloride (Table III); likewise the remaining activity after guanidine hydrochloride treatment could be shaken for up to 24 hr with no loss in potency (Table IV). This remaining activity was sensitive to trypsin, was inactivated by heating at 56°C overnight, and was species specific, and was therefore probably interferon.

Low pH has been shown to protect human and mouse interferons against heat-inacti-

TABLE III. Guanidine Hydrochloride-Stability of Shake-Stable Human Fibroblast Interferon.[a]

Concentration of guanidine hydrochloride (*M*)	Titer (log_{10} units/ml)
0	1.5
2	1.5
4	1.2
6	1.4
8	1.5

[a] Human fibroblast interferon (initial titer $10^{4.3}$ units/ml) was shaken for 24 hr. Solid guanidine hydrochloride was then added to the desired concentrations, and the samples stored at 4°C for 24 hr. Guanidine hydrochloride was removed by dialysis against phosphate buffered saline, and the samples were then assayed for interferon activity.

TABLE IV. Shake-Stability of Guanidine Hydrochloride-Stable Human Fibroblast Interferon.[a]

Period of shaking (hr)	Titer (log_{10} units/ml)
0	2.5
1	2.6
4	2.5
24	2.6
Stationary control	2.1

[a] Human fibroblast interferon (initial titer $10^{4.3}$ units/ml) was made 4 *M* with guanidine hydrochloride, stored at 4°C for 24 hr, and then dialysed against phosphate buffered saline. One milliliter samples were shaken in stoppered glass tubes for the periods indicated, and then frozen until assayed for interferon activity.

TABLE V. Effect of pH on Inactivation of Human and Mouse Interferons by Shaking.[a]

Period of shaking (hr)	Titer (log_{10} units/ml)			
	Human		Mouse	
	pH 3.5	pH 7.0	pH 3.5	pH 7.0
0	4.3	4.0	4.3	4.0
1	4.5	3.6	4.2	2.5
4	4.3	2.8	4.1	1.9
24	4.3	2.1	4.2	<1.9
Stationary control	4.4	4.2	4.2	4.0

[a] Interferon preparations [from poly (I) · poly (C) - induced human fibroblasts or MO57/2 mouse cells] were dialysed against citrate–HCl buffer pH 3.5 or citrate–phosphate buffer pH 7.0. One milliliter samples were then shaken for the periods indicated, then dialysed against phosphate buffered saline, and finally assayed for interferon activity.

TABLE VI. Effect of pH on Inactivation of Human Interferon by Guanidine Hydrochloride.[a]

Concentration of guanidine hydrochloride (*M*)	Titer (log_{10} units/ml)		
	pH 7.0	pH 3.5	pH 2.0
0	3.1	3.0	3.0
2	2.9	3.0	2.5
4	1.5	2.3	2.9
6	1.3	2.2	3.0
8	1.3	2.2	N.T.

[a] Human interferon from poly (I) · poly (C) - induced fibroblasts was dialysed against citrate–HCl buffer, pH 2.0 or pH 3.5, or against citrate–phosphate buffer, pH 7.0. Solid guanidine hydrochloride was added to the above concentrations, and the samples stored at 4°C for 24 hr. After dialysis against phosphate buffered saline, the samples were assayed for interferon.

N.T. = not tested.

vation (5). Therefore, the effects of shaking and of guanidine hydrochloride treatment at low pH on the activity of human and mouse tissue culture interferons were tested. Interferon, shaken at pH 3.5, retained full activity after even 24 hr (Table V). Similarly, at low pH guanidine hydrochloride had greatly reduced effect on the activity of human tissue culture interferon preparations (Table VI).

Discussion. Interferons have been found to differ greatly in their stability to various treatments (1, 2), *e.g.*, human leukocyte and human fibroblast interferons have very different resistance to shaking (3) and heating (4). In the present work stability differences have also been found on treatment of interferon with guanidine hydrochloride, which was found to inactivate human and mouse monolayer tissue culture interferons, poly(I)·poly(C) or virus-induced, and virus-induced mouse serum interferon, but not virus-induced human leukocyte interferon. As both polynucleotide- and virus-induced interferons are inactivated, it is clear that the stability of the interferon does not depend on the nature of the inducer.

Inactivation by shaking or guanidine hydrochloride did not occur in acid medium (pH 2 and 3.5). A similar protective effect has been described in studies on the heat-sensitivity of mouse serum interferon, mouse and human monolayer tissue culture interferons and human leukocyte interferon (5).

In neutral medium a small fraction (generally about 1% of initial activity) resisted inactivation by either shaking or guanidine hydrochloride. Moreover, the residual activity from guanidine hydrochloride inactivation was completely resistant to shaking and vice versa.

The following possibilities could explain the existence of this residual activity. (i) The resistant residue may represent a small amount of leukocyte-type interferon present in all preparations. This seems unlikely, since the resistant fraction is a fairly constant proportion of the initial activity regardless of the source of the interferon or the inducer used in its preparation. (ii) After denaturation by either shaking or guanidine hydrochloride, the interferon molecules refold or reassemble at random. A small number regain in this way the biologically active configuration. (iii) After inactivation by either shaking or guanidine hydrochloride, all the molecules of interferon are in such a configuration that they possess greatly reduced biological activity.

At present there is no firm evidence to exclude either of these two latter hypotheses.

It has been suggested that low pH protects interferon against heat-inactivation by preventing the formation of random hydrogen bonds during the cooling of the interferon preparation (5). This explanation might also be applied to the data on the pH protection observed on the shaking- and guanidine hydrochloride-inactivation of interferons. The possibility that guanidine hydrochloride is inactive at low pH can be discounted (13).

Summary. The inactivation of poly(I)·poly(C)-induced and virus-induced human fibroblast interferon, virus-induced human leukocyte interferon, poly(I)·poly(C)-induced and virus-induced mouse tissue culture interferon and virus-induced mouse serum interferon by shaking and by guanidine hydrochloride was studied. Only human leukocyte interferon was completely resistant to these treatments. All other interferons examined were largely inactivated, but a small amount of each preparation was resistant to inactivation. The residual activity from either treatment was completely resistant to the other.

Inactivation by both shaking and guanidine hydrochloride was prevented in acid (pH 2 and 3.5) conditions. Possible explanations for these observations are considered.

The authors are indebted to Francine Cornette and Machteld Vrolickx for excellent technical assistance. This investigation was supported by a Grant from the Belgian A.S.L.K. Cancer Foundation.

1. Fantes, K. H., *in* "Interferons and Interferon Inducers" (N. B. Finter, ed.), 2nd Ed., p. 171. North Holland, Amsterdam (1973).

2. Ng, M. H., and Vilcek, J., Advan. Protein Chem. **26**, 173 (1972).

3. De Somer, P., Joniau, M., Edy, V. G., and Billiau, A., In vitro. in press (1974).

4. Mogensen, K. E., and Cantell, K., Acta Pathol. Microbiol. Scand. **B81**, 382 (1973).

5. Marshall, L. W., Pitha, P. M., and Carter, W. E., Virology **48**, 607 (1972).

6. Billiau, A., Joniau, M., and De Somer, P., J. Gen. Virol. **19**, 1 (1973).

7. Billiau, A., Joniau, M., and De Somer, P., Proc. Soc. Exp. Biol. Med. **140**, 485 (1972).

8. Edy, V. G., Billiau, A., and De Somer, P., Appl. Microbiol. **26**, 434 (1973).

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

10. Finter, N. B., J. Gen. Virol. **5**, 419 (1969).

11. International Symposium on Standardisation of Interferon and Interferon Inducers. London, 1969. Symp. Ser. Immunobiol. Standard. **14**, 326 (1970).

12. Fantes, K. H., J. Gen. Physiol. **56**, 113S (1970).

13. Tanford, C., Advan. Protein Chem. **23**, 121 (1968).

Received Nov. 26, 1973. P.S.E.B.M., 1974, Vol. 146.