differences of fibrinogen(13), and Lewis has noted quantitative differences of various coagulation factors(14). Although we can show no procoagulant activity of catfish plasma when tested in deficient human or canine systems, these studies cannot be interpreted unequivocally to indicate the absence of similar but species-specific procoagulant factors. It does not seem possible to arrive at a definitive conclusion by adding plasma from one species to a clotting system which is capable of detecting "accelerators" of another species.

Summary. The blood of the fresh water catfish (*Ameiurus nebulosus*) clots rapidly. There appears to be species specificity of the prothrombin-thromboplastin reaction with both the one- and two-stage methods. Catfish brain thromboplastin causes rapid clotting of catfish plasma, but acts as a partial thromboplastin with canine plasma. Catfish plasma does not correct the long partial thromboplastin time of human plasmas deficient in factor V, factor VIII, factor IX, factor X, or factor XII.

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Characterization of Chick Embryo Interferon Induced by a DNA Virus. (29870)

G. P. LAMPSON, A. A. TYTELL, M. M. NEMES AND M. R. HILLEMAN

Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pa.

Interferon(1) is elaborated by animal cells in response to stimulation by viral and perhaps other substances. First definitive purification and biochemical characterization of an interferon was accomplished in this laboratory(2,3), employing interferon elaborated by chick embryo infected with an RNA virus, influenza A (RNA-interferon). The present report extends these biochemical studies to include the characterization of interferon elaborated from chick embryo cells by a DNA virus, herpes simplex. Additionally, development of technology for more precise estimate of isoelectric points for interferon is presented. The essential qualitative identity of the interferons induced by these two widely different viruses in cells of the same host species supports the present concept that interferon is a host-specific substance synthesized under the host genetic code rather than under the viral code.

Materials and methods. In vitro *assay for interferon activity.* The method(2) used previously was modified slightly. By the present procedure, serial 2-fold dilutions of the interferon sample were diluted in medium 199 containing 1% calf serum and antibiotics and 1 ml amounts were added in quadruplicate to monolayer primary cell cultures of whole chick embryo. After overnight incubation at

36°C, the interferon was removed and was replaced with 1 ml of the same culture medium containing 100 $TCID_{50}$ of Sindbis virus strain 171 DA obtained from Dr. J. Enders. The cultures were incubated at 36°C for 3 days at which time the controls showed total destruction of cells. The titer was the highest initial dilution of interferon sample which gave total suppression of viral cytopathic effect. One unit of interferon was 1 ml of the greatest dilution of substance which effected total suppression of Sindbis virus in the assay. *Preparation of DNA virus-induced interferon (DNA-interferon).* Monolayer primary cell cultures in Blake bottles were inoculated with 3 to 10 $TCID_{50}$ of the McIntyre strain of herpes simplex (DNA) virus and incubated at 36°C. The cells were maintained in medium 199 with 1% calf serum during virus growth and interferon production. The culture fluids were harvested after 4 days' incubation at 36°C at which time about half of the cells showed cytopathic degeneration. The crude fluids, clarified by low speed centrifugation, contained about 50 units of interferon per ml and served as the starting material for purification. *Partial purification of DNA-interferon.* The DNA-interferon was partially purified from the crude fluid by the method described previously(2). This consisted essentially of perchloric acid precipitation to remove virus and extraneous protein, concentration and purification by precipitation with zinc acetate at pH 7.4, employment of a single chromatographic separation on carboxymethyl (CM) cellulose, and no final electrophoretic step. Characterization was made using fractions from the CM-cellulose column with highest specific activity. Total purification was not necessary for purpose of the biophysical characterization. Insofar as possible, purification was performed using polyethylene containers in order to preclude adsorption of the active substance to glass.

Isoelectric point determination. A method for approximating the isoelectric points of interferons was developed based on elution profiles of interferon at various pH from CM-sephadex compared with those of purified proteins of known isoelectric point. By

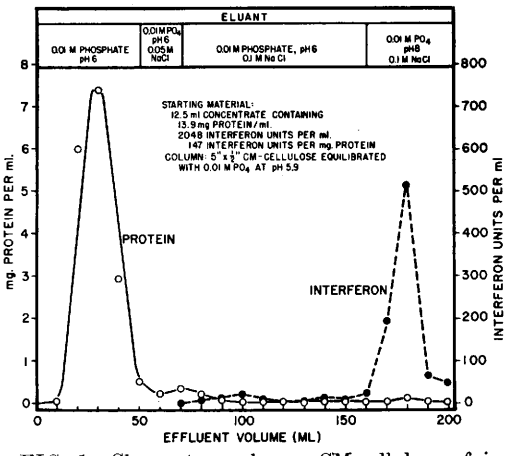

FIG. 1. Chromatography on CM-cellulose of interferon concentrate.

such method, 1 ml of a 1.0% solution of a known *purified protein* in 0.1 M phosphate buffer, pH 6.0, was applied to a 1.5 × 10 cm column of CM-sephadex (C-50) which had been equilibrated with 0.1 M phosphate buffer, pH 6.0. A semi-continuous pH gradient was applied by adding, successively, 5.0 ml amounts of 0.1 M phosphate buffer with increasing increment of 0.2 pH unit. The effluent from the column was collected in 5.0 ml samples. The optical density at 280 mμ, and the pH of each sample were measured. *Interferons* were applied in similar manner to columns of CM-sephadex and elution was accomplished by the same procedure. It was necessary, however, to apply a larger volume of pH 6.0 buffer to the column before the pH gradient was started so that the majority of impurities contained in the partially purified interferon preparation could be removed. *Other methods.* Protein was measured by the method of Lowry *et al*(4). The additional biochemical, and biophysical procedures employed are described in the appropriate sections in the text.

Results. Partial purification of DNA-interferon. A summary of data relating to a typical purification of DNA-interferon through the first CM-cellulose chromatographic step is shown in Fig. 1 and in Table I. The DNA-interferon elution pattern shown in Fig. 1 is essentially identical with that presented(2) earlier for interferon induced by influenza A virus (RNA-interferon). The crude DNA-

TABLE I. Summary of Typical Partial Purification of DNA Virus-Induced Interferon.

Purification step	Volume (ml)	Mg protein per ml	Interferon units per ml	Interferon units/mg protein	Fold-purification
Crude interferon (clarified)	800	.77	48	62	—
Perchloric acid clarified, zinc concentrate	12.5	13.9	2048	147	2.4
CM-cellulose eluate fraction (Fig. 1)					
3	10	7.4	0	0	—
10	"	.055	0	435	7.0
14	"	.024	16	660	10.6
15	"	.024	16	660	10.6
16	"	.029	24	820	13.2
17	"	.064	192	3000	49.0
18	"	.110	512	4660	75.1
19	"	.029	64	2200	35.5
20	"	.018	48	2700	43.5

interferon in the present study contained 62 units of activity per mg protein compared with 52 units per mg of RNA-interferon previously reported(2). The perchloric acid-zinc precipitation steps effected a 2.4-fold purification of DNA-interferon compared with 16-fold purification recorded for RNA-interferon. The single most active fraction of DNA-interferon from the CM-cellulose column contained 4,660 units of interferon per mg protein and represented a 75-fold purification compared with 50,500 units per mg protein and 970-fold purification for RNA-interferon handled in a similar way. The most active fractions of DNA-interferon which were combined, *i.e.*, fractions 16 to 20, yielded a total of 8,400 interferon units, or 22% of the 38,400 units contained in the 800 ml of starting material. At the similar step in purification, the yield of RNA-interferon was 18%, a comparable value. The lesser degree of purification of DNA-interferon compared with RNA-interferon might have been related to the difference in original composition, *i.e.*, tissue culture fluid compared with egg allantoic fluid, or with other phenomena such as possible presence of proteolytic enzymes in the DNA-interferon which reduced specific activity.

Characterization of DNA-interferon. Molecular weight. The partially purified interferon was insufficient in amount or in purity to permit measurement of sedimentation coefficient by optical methods. Hence, activity sedimentation measurements were carried out and the molecular weight of the interferon was computed according to Hogeboom and Kuff(5). For the determination, the interferon contained in 2% sucrose solution was layered on an equal volume of the same interferon in 3% sucrose solution in a 5 ml centrifuge tube and the interfaces were mixed gently. For reference determination, purified egg white lysozyme (Nutritional Biochemicals Corp.) was prepared in 0.25% solution in 2% and in 3% sucrose solution and a gradient was established in the same manner. The samples were spun for 3½ hours at 39,400 rpm in the SW-39 rotor of a Spinco model L centrifuge. At the end of the run, the contents of the 2 tubes were partitioned into equal "upper" and "lower" volumes using a tube slicer. The upper layer of the tube containing interferon and the original untreated interferon were assayed for antiviral activity employing serial 1.2-fold dilution and 8 tubes per dilution. The upper layer and original lysozyme samples were diluted 1:1 and the optical densities at 280 mμ were determined. The sedimentation coefficient for the interferon was calculated to be 3.1 S giving a molecular weight of 36,000. The similar calculation for lysozyme gave a coefficient of 1.7 S and a molecular weight of 14,500. The latter value corresponds well to the published value(6) of 14,400 ± 100 for egg white lysozyme and serves to validate the value of 36,000 obtained for the interferon.

Heat, pH and trypsin sensitivity of DNA-

TABLE II. Effect of Heat, pH and Trypsin on Activity of DNA-Interferon.

Treatment	Interferon activity (units/ml) Treatment	Loss in activity (%)
Temperature (pH 7.0)		
30 min		
23°C	64	—
50	48	25
60	48	25
70	32	50
85	24	63
60 min		
25°C	512	0
56	512	0
66	512	0
76	256	50
86	96	81
pH (1 hr at 23°C)		
1.0	256	0
2.0	256	0
7.0	256	0
10.0	256	0
11.0	192	25
Trypsin (4 hr 37°C)		
Trypsin	<16	>94
Control—no trypsin		
5°C stored	256	
37°C "	256	

interferon. The chromatographed DNA-interferon was used. Table II shows that there was significant loss of activity on heating at 70°C or higher temperature. The activity was remarkably stable in the entire pH range tested and the substance was no longer active following treatment with 0.001% solution of crystalline trypsin at pH 7.0.

Isoelectric point. Ionophoresis. Ionophoretic experiments were previously carried out (2) for RNA-interferon using cellulose acetate paper and the isoelectric point was estimated to be approximately pH 8.0. The precision of measurement was improved somewhat by use of Sepraphore* type cellulose acetate paper which minimized endosmosis and adsorption of the interferon to the paper. Studies of purified proteins of known isoelectric point revealed anomalies and it was possible, therefore, to fix the isoelectric point of DNA-interferon only as near neutral, *i.e.*, somewhere between pH 6.0 and 8.0. *CM-sephadex elution profile.* The elution profiles for the DNA- and RNA-interferons and of the proteins† of known isoelectric point (7-13) are shown in Fig. 2, and Table IV summarizes the calculations which were carried out. By the method employed, the pH of the peak activity fraction ranged from 0.4 to 0.6 pH units below the known values obtained by other methods. The average difference was 0.4 and this factor was added to the peak activity—pH values for DNA- and RNA-interferons. The calculated isoelectric points for DNA- and RNA-interferon were pH 7.0 and 6.9-7.1, respectively, and these are fully in accord with the data which indicate that interferon is neither highly basic nor highly acidic but more nearly neutral.

Biologic specificity. Primary cell cultures of the variety shown in Table III were treated overnight with chromatographed DNA- or RNA-interferon, after which the interferon was removed and replaced with 1 ml of

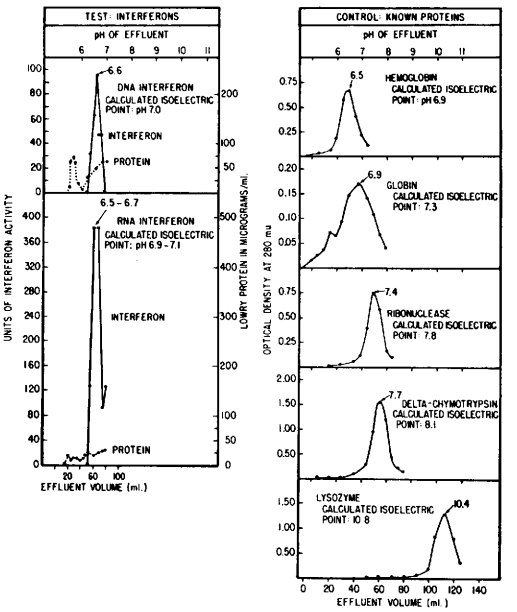

FIG. 2. pH elution profiles of DNA- and RNA-interferon of chick embryo origin compared with those of control purified proteins.

* Sepraphore, Gelman Instrument Co., Ann Arbor, Mich.

† Hemoglobin, 2 X crystallized, Nutritional Biochemicals Co.; Globin, purified, Nutritional Biochemicals Co.; Ribonuclease, crystallized, chromatographically pure. Mann Research Labs.; delta Chymotrypsin, 3 X crystallized, Nutritional Biochemicals Co.; Lysozyme, 3 X crystallized, Sigma Chemical Co.

TABLE III. Comparison of Activities of DNA-Interferon and RNA-Interferon* of Chick Embryo Origin Assayed in Various Host Cell Systems Using Vesicular Stomatitis Virus as Challenge.

Host cell species	Interferon-titer	
	DNA-interferon	RNA-interferon*
Chick embryo	1:1536	1:1024
Mouse "	<1:16	<1:16
Grivet kidney	<1:16	<1:16
Bovine "	<1:16	<1:16
Rabbit "	<1:16	<1:16

* Interferon present in allantoic fluid of embryonated hen's eggs infected with influenza A (WS strain) virus.

medium 199 containing 1% calf serum and 100 TCD_{50} of a strain of vesicular stomatitis virus. This virus strain was obtained originally from Dr. Werner Henle and was passed one time, prior to use, in cells of the homologous cell systems shown in the Table. Strict host species specificity was shown. Activity of both DNA- and RNA-interferon against vesicular stomatitis virus was discernible only in homologous chick embryo cells.

Discussion. These findings indicate that chick cell interferon induced by a DNA virus is a protein of low molecular weight (about 36,000) which is trypsin sensitive, relatively heat stable (70°C) and is stable over a wide range of pH (pH 1 to 11). The substance displays a high degree of host species specificity when tested in a variety of cells in culture and exhibits an isoelectric point around neutral, based on the profile of elution by pH gradient from CM-sephadex. The qualities are quite consistent with those reported earlier(2) for highly purified chick interferon induced by RNA-type influenza A virus. The described properties for chick DNA-interferon are not in disagreement with the findings recorded in a limited characterization of a DNA-interferon induced in mouse cells by vaccinia virus(14).

Contemporary reports(15,16), which state that the molecular weight of chick interferon is still controversial, fail to take cognizance of the decisive information published previously. A summary of available data is given in Table V and is listed chronologically with time of appearance.

The original molecular weight determination (63,000) made by Burke(17) was based on sedimentation in a centrifugal field employing optical rather than the essential activity measurement of the sedimentation boundary. It has been estimated(2) that Burke's interferon was largely impurity and that sedimentation of extraneous material was being observed. The purported supporting data given by Porterfield(18) was not considered contributory because of the strong adsorption of interferon to agar which rendered the data meaningless. By contrast, all measurements of molecular weight determined by assay of biologic activity are consistent with a molecular weight in the general area of 30,000.

In our previous report(2), the isoelectric point of chick RNA-interferon was estimated to be around pH 8.0 based on studies of ionophoretic mobility on cellulose acetate paper. The reliability of such determination was improved by use of Sepraphore cellulose

TABLE IV. Estimate of Isoelectric Point of DNA- and RNA-Virus Induced Chick Embryo Interferon Based on pH Elution Profiles Compared with Those of Known Proteins.

Substance tested	Measured pH of fraction showing peak activity	Established isoelectric point		Deviation of measured value from established isoelectric point	Calculated† CM-Sephadex isoelectric point
		pH	Lit refs		
DNA-interferon	6.6				7.0
RNA-interferon*	6.5–6.7				6.9–7.1
Hemoglobin	6.5	6.9	7	+ .4	6.9
Globin	6.9	7.5	8	+ .6	7.3
Ribonuclease	7.4	7.8	9–11	+ .4	7.8
Δ Chymotrypsin	7.7	8.1	12	+ .4	8.1
Lysozyme	10.4	10.5–11.0	12, 13	+ .4	10.8
				Avg: + .4	

* Interferon B prepared as described previously(2).
† Sum of observed value plus average deviation of + 0.4.

TABLE V. Summary. Biophysical Properties of Interferons of Diverse Origin.

Interferon preparation				Interferon properties				
Host species	Virus used RNA	Virus used DNA	Degree of purification	Molecular weight Method	Molecular weight Value	Electrophoretic properties Nature	Electrophoretic properties Finding	Reference
Chick	Influenza A	—	Partially pur'd	Schlieren – sedimentation	63,000	Acidic	DEAE cellulose – retained at pH 5 but not at 4.5	Burke(17)
"	" "	—	" "	Activity – Agar diffusion	<80,000			Porterfield *et al*(18)
Chick	Influenza A	—	Highly pur'd	Activity – sedimentation	20–34,000	Sl basic	No migration at pH 8.0*	Lampson *et al*(2)
Chick	Chikungunya	—	Crude	Activity – sedimentation	13–25,000			Rotem *et al*(19)
Mouse	NDV	—	"	" "	"			"
"	Influenza A	—	"	" "	"			"
Monkey	" "	—	"	" "	"			"
Chick	Influenza A	—	Crude	Activity – sedimentation	25,000			Kreuz *et al*(20)
"	" "	—	Partially pur'd	" "	34,000			"
Chick	Influenza A	—	Partially pur'd	Activity – Sephadex G-100	40–45,000	Acidic	Anode migration, pH 7.6	Phillips *et al*(15)
"	" "	—	" "	" – sedimentation	38,000			"
Chick	Influenza A	—	Partially pur'd	Activity – Sephadex G-100	33,000			Jungwirth *et al*(21)
Chick	Influenza A	—	Highly pur'd	Activity – sedimentation	38,000	Basic	Cathode migration, pH 8.6	Merigan(16)
"	Chikungunya	—	" "	" "	"		" " " "	"
Mouse	"	—	" "	" "	26,000		" " " "	"
"	NDV	—	" "	" "	"		" " " "	"
Chick	Influenza A	—	Partially pur'd	— —	—	Acidic	Free electroph, Isoelect pt 6.0-6.6	Bodo *et al*(22)
Chick	—	Herpes simplex	Partially pur'd	Activity – sedimentation	36,000	Neutral	CM-Sephadex, pH 7.0	Present report
"	Influenza A	—	" "	— —	—	"	" " " " 6.9-7.1	" "

* Cellulose acetate paper (Sepraphore), endosmosis insignificant, adsorption minimal.

acetate paper which minimized interferon adsorption and water flow. Careful study of a variety of proteins of known isoelectric point revealed, however, such degree of anomaly as to prevent fixing the isoelectric points of DNA- and RNA-interferon more precisely than in the range of pH 6 to 8. The newly developed method for isoelectric point determination based on pH elution profile from CM-sephadex provides a more precise method for isoelectric point determination than achieved heretofore. The elution peaks obtained for each of the control proteins were, on the average, 0.4 pH units less than the known isoelectric point as determined by free electrophoresis. Hence, such correction factor was applied to the values obtained for the DNA- and RNA-interferons. Support for the validity of such correction is provided (23) in the correlation between the elution diagrams of various hemoglobins from carboxymethyl cellulose and the isoelectric point as determined by free electrophoresis. In the latter example, there was a constant correction factor of approximately 0.4-0.6 pH unit. The principle for operation of the elution method is based on electrostatic repulsion of the negatively charged protein from the negatively charged column at any pH which approaches or exceeds the isoelectric point of the protein. One marked advantage in the elution method is the provision for comparison of behavior of several proteins by a uniform procedure under identical conditions of ionic strength and buffer composition.

Recent findings by Davies(24) show that crude interferon prepared in bovine cells may be complexed with albumin which is an acidic protein. Such combination might explain the purported acidic quality of interferon reported by Burke(17) based on studies of adsorption and elution of interferon from DEAE-cellulose. Complexing with albumin might also explain the anodic migration obtained by Phillips *et al*(15) on electrophoretic analysis. The rather strongly basic nature of interferon, as would be implied by Merigan's(16) data is inconsistent with present findings. Merigan makes no mention of measurement or control of water flow in his system which might have moved the interferon toward the cathode. The elution pattern of interferons from CM-sephadex established the isoelectric point as similar to that for hemoglobin (pH 6.9). Bodo and Jungwirth(22) recorded an isoelectric point of pH 6.0 to 6.6 by zonal density gradient electrophoresis in tris-maleate buffers. It has been demonstrated that the ionophoretic mobilities of many proteins are strongly dependent on buffer composition and ionic strength.

The present data indicate that DNA as well as RNA viruses stimulate production of interferons of similar chemical and biophysical quality and are consistent with the concept that interferon is synthesized by the host cell employing the host, rather than the viral, genetic code. It is of some interest that recent reports(25-28) indicate that the activity of interferon in inhibiting virus in cells depends not only on cell RNA synthesis but on protein synthesis as well. Sonnabend(28) has noted depression of normal host cell RNA production by interferon and has postulated that interferon induces the synthesis in cells of an unstable enzyme which degrades newly synthesized host as well as viral RNA. This theory implies no direct action of interferon on the virus and provides a basis for explaining the virus-generic activity of interferon.

Summary. Biochemical, biophysical and biological characterization was carried out previously for highly purified chick embryo interferon induced by an RNA virus, influenza A (RNA-interferon). The present report summarizes the findings in studies to characterize partially purified chick embryo interferon induced by a DNA virus, herpes simplex (DNA-interferon). DNA-interferon was shown to be a protein of low molecular weight (about 36,000) which is trypsin sensitive, relatively heat stable (70°C) and is stable over a wide range of pH (pH 1 to 11). The findings by others are reviewed and analyzed. Development of a new method for measuring isoelectric point based on pH elution profile from CM-sephadex established the isoelectric point for DNA- and RNA-interferon at pH near neutral. Both DNA- and RNA-interferon were active against ves-

icular stomatitis virus in the homologous chick cell culture system but showed no activity against this virus in mouse embryo, grivet kidney, bovine kidney or rabbit kidney cell cultures. The remarkable similarity of chick DNA-interferon to RNA-interferon, even though induced by basically different agents, is consistent with the concept that interferon is synthesized by resident host mechanisms rather than under the viral genetic code.

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Vasopressin Effect on Urinary Concentration in Rats with Hereditary Hypothalamic Diabetes Insipidus (Brattleboro Strain).* (29871)

AVERY R. HARRINGTON† AND HEINZ VALTIN‡ (Introduced by R. E. Gosselin)
Department of Physiology, Dartmouth Medical School, Hanover, N. H.

Patients with hypothalamic or neurohypophysial diabetes insipidus (D.I.) respond to administration of exogenous vasopressin with increased concentration of the urine; such a response, in fact, distinguishes these conditions from nephrogenic diabetes insipidus. However, even with administration of vasopressin many patients with hypothalamic or neurohypophysial D.I. fail to achieve the urine osmolalities which normals attain(1). Rats with surgically induced D.I. do not concentrate the urine normally when given exogenous vasopressin in acute experiments(2). The same is true of rats with hereditary hypothalamic D.I., which probably can synthesize no vasopressin whatsoever(3,4).

We have recently discovered that the sub-

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