There was a continuous decline in both individual and combined estrogen concentrations from early proestrus to late estrus. Throughout pregnancy the levels of estradiol-17α and 16-epiestriol remained fairly constant. Estrone and estradiol-17β attained peak levels at 3-4 weeks and declined thereafter. Estriol was the major metabolite and by the last week of pregnancy showed a 43% increase over the initial level. All of the estrogens except estradiol-17α rose substantially during the first week post-partum.

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Multiple Molecular Species of Interferons of Mouse and of Rabbit Origin. (30783)

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Interferon(1-4) is a term which is often applied, collectively, to a variety of viral inhibitory substances of protein composition that are commonly formed by cells in response to stimulation by viruses and by other microbes or their components. Interferons commonly appear in the blood and tissues of animals and man in the course of viral and other infections and appear to provide a mechanism for recovery from viral disease which is separate and distinct from the specific immunological mechanisms. Exogenous interferons, administered prophylactically or therapeutically, presently show little promise in clinical medicine for preventing or treating viral disease. On the contrary, stimulation of endogenous interferon in the host itself by appropriate substances may contribute significantly to enhancement of host defenses. This emphasizes the need to characterize and to classify interferons, and to study their mechanisms for and mode of induction as a background to eventual application of the interference phenomenon to clinical medicine.

Investigations in this laboratory led to the first preparation of highly purified chick embryo interferon and to characterization of chick interferon induced by both RNA and DNA viruses(5,6). Continuing studies have been made of interferons induced in cells or animals of other species. The present report describes the characterization of interferons induced in mice, in mouse cell culture, and in rabbit cell culture by Newcastle Disease Virus (NDV). These studies are of significance in that they demonstrate 2 distinct kinds of interferon in mouse serum and different molecules with interferon activity in mouse and rabbit cell cultures infected with NDV virus.

Materials and methods. In vitro *assay for interferon activity.* The method was a modification of the procedure described previously(6). Serial 2-fold dilutions of the interferon sample were diluted in Eagle's medium containing 5% agamma calf serum and antibiotics and 1 ml amounts were added in quadruplicate to roller tube cultures of mouse

embryo. Following overnight incubation at 36°C, the culture fluid was replaced with 1 ml of the above medium containing 10 $TCID_{50}$ of vesicular stomatitis virus. Incubation was continued for 4 days and the cultures were observed microscopically for cytopathic change due to virus. The titer was the highest initial dilution of interferon sample which effected total suppression of viral cytopathic change. The test was modified to employ chick embryo or rabbit kidney cell cultures where indicated.

Preparation of interferons. In vivo *mouse serum.* A modification of the procedure described by Baron *et al*(7) was used. Groups of 20 to 25 ICR/Ha mice weighing 12-14 g each were injected intravenously with 0.2 ml of live Newcastle Disease Virus (NDV) purified and concentrated 5-fold from infected egg allantoic fluid by one cycle of low and high speed centrifugation. The mice were bled after 6 hours and the pooled sera were the source of interferon. *Mouse spleen cell culture.* Spleens from 15-20 g ICR/Ha mice were minced, digested with trypsin, separated from the fluid by a single cycle of low speed centrifugation, and employed in spinner culture using 1×10^7 cells per ml in Eagle's minimal essential medium containing 10% agamma calf serum and antibiotics. The cultures were infected with NDV virus at high dose level and incubated at 35°C for 18 hours after which the cells were removed by centrifugation. *Rabbit spleen culture.* Interferon was prepared in a manner similar to that for mouse spleen cell culture employing cells obtained from spleens of 6- to 8-week-old New Zealand rabbits. *L (MCN) cell culture.* The cells were suspended in Eagle's minimal essential medium with agamma calf serum and antibiotics at a concentration of 8.7×10^5 cells per ml and infected with a low multiplicity of NDV virus. The infected culture fluid was freed of cells by centrifugation and was the source of interferon. *Virus inactivation and partial purification of interferon.* The mouse serum diluted 1:5 with physiological saline solution or the undiluted infected culture fluids were cooled to 0°C in an ice bath and cold 2 N perchloric acid was added with stirring to a final concentration of 0.1 N. The precipitated protein including inactivated virus was removed by centrifugation and the supernate was adjusted to neutrality by addition of 2 N NaOH.

Biophysical characterization. Determination of molecular weight by gel filtration. This was according to methods described by Andrews *et al*(8), Whitaker(9), and Squire(10). Columns of 2×35 cm size were packed with hydrated Sephadex G-100 beads and slowly percolated for 2-3 days with 0.1 M tris-0.2 M NaCl, pH 8.0 buffer to achieve equilibration. One ml amounts of the interferons which had been dialyzed against the same tris-saline buffer solution were applied to the columns. The flow rate in the column was adjusted to 20 to 25 ml per hour and fractions were collected in 3 ml amounts. The fractions were assayed for interferon content and the molecular weight of each sample was calculated according to the formula $M^{1/3} = 130\ [1.447 - (V/Vo)^{1/3}]$ in which M is the molecular weight, V is the elution volume of the material tested and Vo is the void volume of the column. The method yielded values within 10% of reported molecular weights when tested with purified proteins. Further, the molecular weights obtained with interferons prepared from infected chick embryos and chick embryo cell cultures were in close agreement with values arrived at by the activity sedimentation coefficient method. *Determination of isoelectric point by elution pH from CM-Sephadex.* The procedure described previously was used(6,11). By this method, the samples were dialyzed overnight against 0.1 M sodium phosphate buffer, pH 6.0. One hundred ml of sample were applied to a 1.5×10 cm CM-Sephadex column equilibrated with the buffer and the interferon was eluted by successive addition of 5 ml amounts of 0.1 M phosphate with increasing increment of 0.1 pH unit. The effluent was collected in 5.0 ml fractions and the pH and interferon activity of each was measured. The pH of the fraction with peak activity was noted and the isoelectric point was calculated by adding 0.4 pH unit according to the formula which was developed(6,11).

Results. In vivo *mouse serum interferon.* The molecular weight of interferon prepared

from sera of mice inoculated intravenously with NDV virus was determined by the gel filtration method using Sephadex G-100. The curve presented in Fig. 1 shows that 2 molecular species of interferon were present. Component A had a molecular weight of 38,000

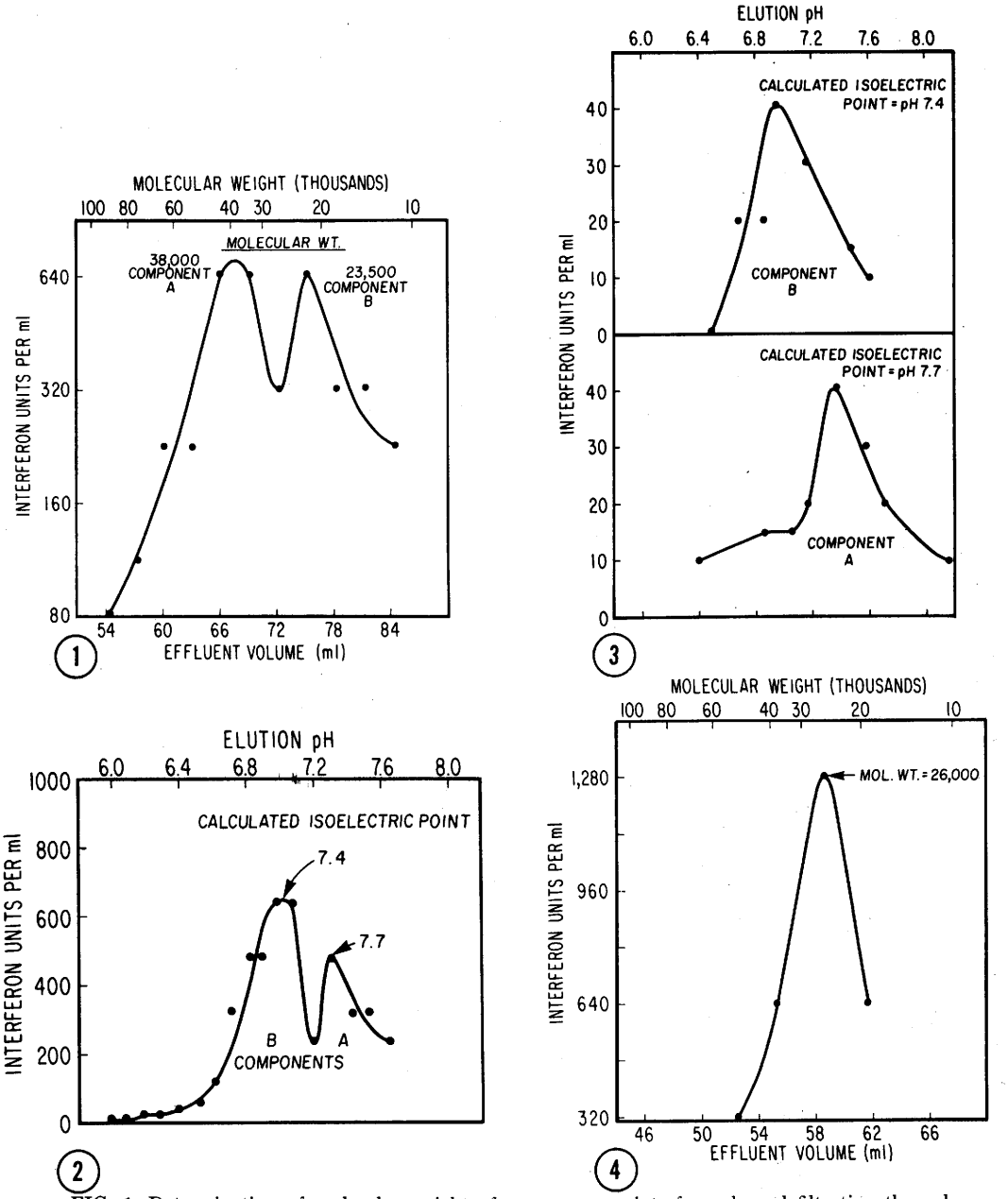


FIG. 1. Determination of molecular weight of mouse serum interferon by gel filtration through Sephadex G-100.

FIG. 2. Determination of isoelectric point of mouse serum interferon by elution pH from CM-Sephadex.

FIG. 3. Determination of isoelectric points components A & B (mol. wt.) of mouse serum interferon by elution pH from CM-Sephadex.

FIG. 4. Determination of molecular weight of mouse spleen cell culture interferon by gel filtration through Sephadex G-100.

TABLE I. Effect of Heat, pH, and Trypsin on Activity of Mouse Serum Interferon.

Treatment	Interferon activity in units per ml (% loss of activity) Component A	Component B
Temperature (1 hr, pH 7.0)		
23°C	320	320
37°C	160 (50%)	320
47°C	80 (75%)	320
56°C	60 (81%)	240 (25%)
66°C	10 (97%)	15 (95%)
76°C	< 10 (100%)	< 10 (100%)
pH (1 hr, 23°C)		
1	240	320
2	240	320
7	240	320
10	160 (33%)	320
11	120 (50%)	320
Trypsin (20 γ per ml) test, 5 hr, 23°C	< 20 (100%)	60 (87.5%)
Control, 5 hr,		
23°C	160	480
5°C	160	480

and component B, 23,500. Isoelectric point determinations of the same mouse serum interferon by the pH elution method also revealed 2 molecular species, one with a value of pH 7.4 and the other with a value of 7.7, as shown in Fig. 2. To determine the relationship between the fractions obtained by the two methods, the isoelectric points of the peak A and B fractions obtained by gel filtration through Sephadex were measured individually by the pH elution method. The findings are presented in Fig. 3. Fractions A and B, separated on Sephadex G-100, each showed different isoelectric points. From this, it was determined that component A of mouse serum interferon had a molecular weight of 38,000 and an isoelectric point of 7.7 while component B had a molecular weight of 23,500 and an isoelectric point of 7.4.

The peak fractions of interferon components A and B from mouse serum separated on G-100 Sephadex were tested for sensitivity to heat, pH, and trypsin. As shown in Table I, both components were readily destroyed by trypsin, indicating their protein composition. Component A was markedly heat labile, compared with chick interferon (5,6). Component B was more heat stable than component A but still somewhat less stable than chick interferon. Component B was stable over the entire range of pH values tested while component A lost 50% of its activity at pH 11.

Interferons characteristically display a high degree of species specificity with respect to the host species used to prepare and to assay interferon. Mouse serum interferon components A and B and embryonated hen's egg interferon induced by WS influenza virus(5) were tested for activity against vesicular stomatitis virus in mouse embryo, chick embryo and rabbit kidney cell cultures. As shown in Table II, all the interferons were active only in cells of the homologous species.

*Mouse spleen cell culture interferon.** Spinner cultures of mouse spleen cells were infected with NDV virus and the molecular weight and isoelectric point of the interferon were assayed. As shown in Fig. 4 and 5, the molecular weight was 26,000 and the isoelectric point was pH 7.0.

*L (MCN) cell culture interferon.** Fig. 6 and 7 show that the molecular weight and isoelectric point of interferon induced in L

TABLE II. Species Specificity of Mouse Serum Interferons in Tests with Vesicular Stomatitis Virus Challenge.

Cell culture used for interferon assay	Interferon titer* Mouse serum interferon component A	B	Embryonated chicken egg interferon
Mouse embryo	1:160	1:480	<1:20
Chick "	<1:20	<1:20	1:960
Rabbit kidney	<1:20	<1:20	<1:20

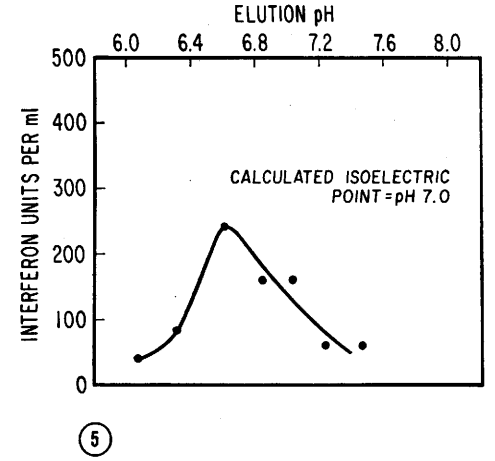
* Dilution of interferon preparation which gave 100% protection against a challenge of 10 $TCID_{50}$ of vesicular stomatitis virus.

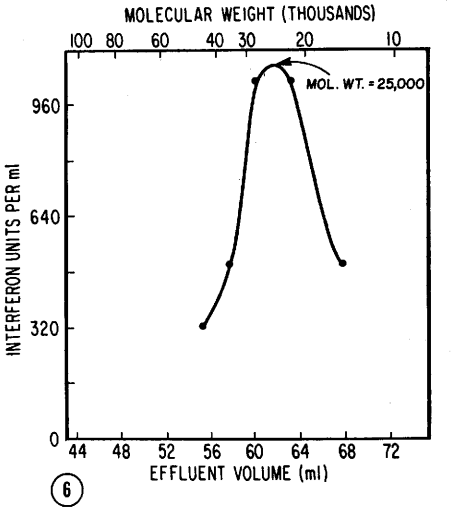
* These interferons were prepared by Dr. C. L. Baugh.

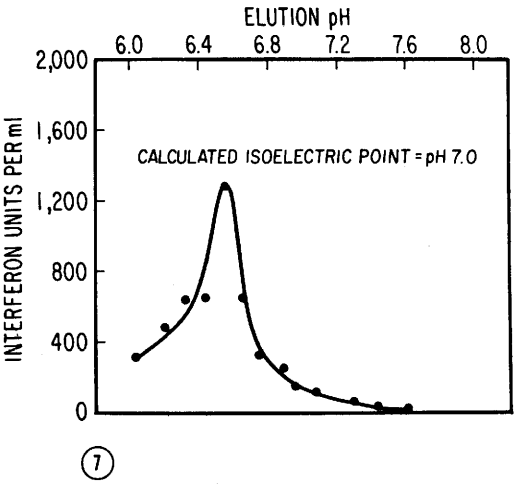
(MCN) cells by NDV virus were 25,000 and pH 7.0, respectively.

Rabbit spleen cell culture interferon. Interferon induced in rabbit spleen cell cultures by NDV virus showed a molecular weight of 33,000 and an isoelectric point of pH 7.4. See Fig. 8 and 9.

Discussion. Table III summarizes the observations of several workers in studies to characterize interferons and is included to simplify discussion. The findings in the present studies emphasize the diversity of molecular species of the viral inhibitory substances which are generally called interferons by virtue of their antiviral activity, protein nature, and host species specificity. The interferons which appeared in mouse serum following intravenous inoculation of NDV virus into mice were of 2 different kinds, *viz.*, component A with a molecular weight of 38,000 and iso-







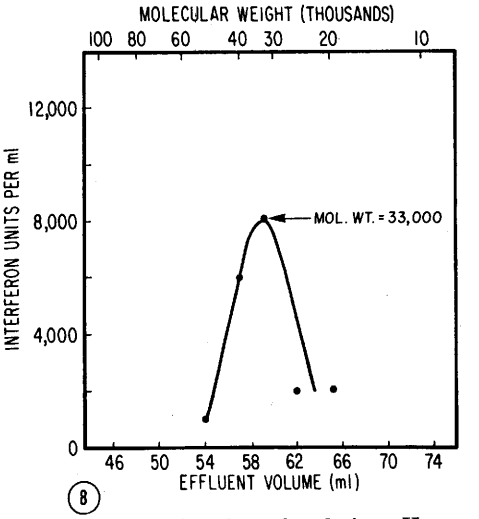


FIG. 5. Determination of isoelectric point of mouse spleen cell culture interferon by elution pH from CM-Sephadex.

FIG. 6. Determination of molecular weight of L (MCN) cell interferon by gel filtration through Sephadex G-100.

FIG. 7. Determination of isoelectric point of L (MCN) cell interferon by elution pH from CM-Sephadex.

Fig. 8. Determination of molecular weight of rabbit spleen cell culture interferon by gel filtration through Sephadex G-100.

TABLE III. Comparison, Molecular Weights and Isoelectric Points of Interferons of Diverse Origin Based on Data from Selected Reports.

Host origin			Interferon		
Species	System	Inducing substance	Molecular weight	Isoelectric point	Literature reference
Mouse	Serum (*in vivo*)	NDV virus			Present report
	Component A		38,000	7.7	" "
	" B		23,500	7.4	" "
	Spleen—cell culture	" "	26,000	7.0	" "
	L (MCN)—cell culture	" "	25,000	7.0	" "
	Mouse fibroblast—cell culture	Chickungunya virus	26,000	—	Merigan, T. C.(12)
	L " "	NDV virus	31,000	—	Hallum, J. *et al*†
	Mouse serum	" "	26,000	—	" " " "
	" "	Endotoxin (*E. coli*)	89,000	—	" " " "
	" "	Statolon	90,000	—	Merigan, T. C.†
	" "	*B. abortus*	54,000 & 77,000	—	Hallum, J. *et al*†
Chick	Embryo	WS influenza virus (RNA)	38,000*	6.9–7.1	Lampson, G. P. *et al*(5)
	" cell culture	Herpes simplex virus (DNA)	36,000	7.0	" " " " " (6)
	"	WS influenza virus	38,000	—	Merigan, T. C.(12)
	"	WS " "	26,000	—	Kreuz, L. E. *et al*(13)
	"	Mel " "	33,000	6.0–6.6	Jungwirth, C. *et al*(14) Bodo, G. *et al*(15)
	"	WS " "	30,000	—	Burke, D. C. *et al*†
	"	Influenza B/Eng virus	—	6.8–6.9	Fantes, K. H.†
Human	Foreskin cell culture	NDV virus	26,000	—	Petralli, J. K. *et al*†
	Leukocyte " "	Sendai virus	25,000	—	Falcoff, E. *et al*†
Monkey	Renal " "	Influenza A virus	13,000–25,000	—	Rotem, Z. *et al*(16)
Rabbit	Spleen " "	NDV virus	33,000	7.4	Present report

* Performed subsequent to publication by gel filtration.
† Personal communications as reported by the Interferon Information Exchange.

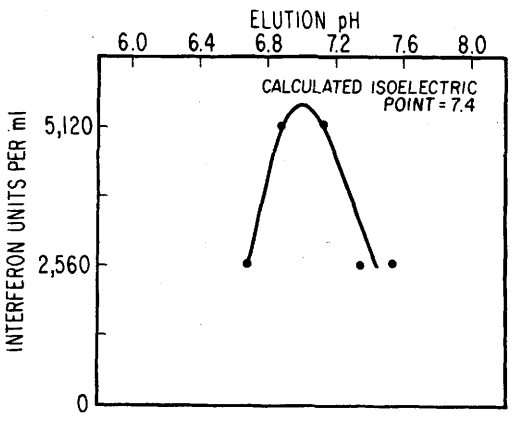


FIG. 9. Determination of isoelectric point of rabbit spleen cell culture interferon by elution pH from CM-Sephadex.

electric point of pH 7.7, and component B with values of 23,500 and pH 7.4, respectively. Interferons induced in mouse spleen and L (MCN) cell cultures were essentially alike based on similar molecular weights, *i.e.*, 26,000 and 25,000 and identical isoelectric point. The interferons induced in mouse cell cultures resembled component B of mouse serum interferon in regard to molecular weight (26,000, 25,000 *vs* 23,500) but the isoelectric point of component B was 0.4 pH unit higher. Both components were protein as demonstrated by sensitivity to trypsin and were host species-specific. The molecular weight of interferon induced by NDV in rabbit spleen cell culture was intermediate, *viz.*, 33,000 between that of components A and B (38,000 and 23,500) of mouse serum but the isoelectric point was identical with that of component B, *i.e.*, pH 7.4. The mouse serum interferons, especially component A, appeared to be somewhat less heat stable than chick interferon and component A was slightly less stable at high pH than recorded(5,6) previously for chick interferon. The greater heat sensitivity of the mouse interferon compared with chick was noted previously by Merigan (12).

The presence of 2 molecular species in a single specimen of interferon has been noted previously in only 2 other systems. The first system was that of chick embryo interferon induced by WS virus in which a small amount of a second component was detected by chromatography on CM-cellulose(5). The second system demonstrating 2 interferons was that of Hallum and associates† who found interferons with molecular weights of 54,000 and 77,000 in sera from mice inoculated with *Brucella abortus*. It is of possible significance that diversity of molecular constitution within a single sample has been demonstrated thus far only with interferons derived from intact host animals and not with interferons prepared in cell cultures. This may reflect a greater diversity of kinds of cells producing interferon in the host animal than in cell cultures.

The range in molecular size of interferons induced in the intact host or in cell cultures of animals of various species is generally within 25-38,000. By contrast, interferons induced by other microbial agents (endotoxin, Statolon, *B. abortus*) are larger, 54-90,000. Ho *et al*(17) and Youngner *et al*† have presented evidence to suggest that the inhibitory substance which is rapidly evoked by endotoxin is preformed since its formation does not require the transcriptive function of host cell DNA or protein synthesis by the cell. Interferons induced by viruses and by *Brucella abortus* do require host cell function and are evidently synthesized by the cell after stimulation. The requirement in Statolon induced synthesis is unknown.

Summary. Interferons found present in mouse serum following intravenous injection of NDV virus were of 2 different kinds. Component A had a molecular weight of 38,000 and an isoelectric point of 7.7 while component B had a molecular weight of 23,500 and an isoelectric point of 7.4. Interferons induced in mouse spleen and L (MCN) cell cultures by the same virus had molecular weights of 26,000 and 25,000, respectively, and an isoelectric point of 7.0. Rabbit spleen cell culture interferon had a molecular weight of 33,000 and an isoelectric point of 7.4. A second molecular moiety which appears in chick embryo interferon induced by WS influenza virus was found to have a molecular weight of approximately 50,000 in contrast to 38,000 for the more

† Personal communication as reported in the Interferon Information Exchange.

predominant component. The mouse serum interferons appeared less heat stable than chick interferon and component A more sensitive to high pH. The characteristics of these interferons were compared with the properties of the spectrum of interferons produced in various animal species by various microbial agents.

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Histamine Liberation by Gastric Mucosa of Pylorus-Ligated Rats Damaged by Acetic or Salicylic Acids.* (30784)

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When the oxyntic gland area of the gastric mucosa is damaged by irrigation with acetic or salicylic acids, there is an increased output of water, plasma proteins, glucose, sodium and potassium from the mucosa(1,2,3). Stimulation of acid secretion and hyperemia may also occur. Many of the effects of damage could be the result of intramucosal liberation of histamine.

The gastric mucosa contains a large amount of histamine, some in the region of the oxyntic cells and some in the mast cells(4), and it has frequently been suggested (*e.g.*, 5,6) that histamine is liberated within the mucosa during stressful or damaging situations. If histamine liberation occurs during damage, the total histamine content of the mucosa should fall, and histamine should be present in the interstitial fluid. Since other constituents of interstitial fluid appear in gastric contents after damage, one would expect that if histamine is also dissolved in the interstitial fluid, it too would be detectable in gastric contents. In the experiments reported here we show that when the stomach of the pylorus-ligated rat is damaged with acetic or salicylic acids, the histamine content of the mucosa decreases and that histamine appears in the gastric contents.

Materials and methods. Male and female rats weighing between 150 and 260 g were fasted overnight without restraint. A rat was anesthetized with sodium pentobarbital, and anesthesia was maintained for the duration of the experiment. The stomach was exposed by midline incision and gently ligated at pyloric and gastroesophageal junctions. The stomach was rinsed twice with 154 mM NaCl injected and removed through the forestomach. Then one of four solutions was injected at volume equal to 15% of body weight. The abdomen was closed by clips, and the fluid was left in the stomach 90 min after which the animal was decapitated and the stomach immediately removed.

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