normal growth rates, reproduction and longevity in rats(1). It seems likely that the changes in hepatic enzyme concentration noted in rats fed the synthetic liquid diets may be associated with increased endogenous synthesis of serine from carbohydrate sources. Therefore, normal growth rates may be achieved and sustained with marginal diets if considerable reserve capacity exists for the endogenous synthesis of necessary metabolites. The concentration of other biosynthetic enzymes and intermediary substrates may also vary considerably when synthetic amino acid diets adequate to maintain growth are substituted for protein containing diets. The effects of such refined diets on intermediary metabolism must be carefully defined and compared with diets of known protein content before interpretations are made on the effects of manipulating various constituents of the synthetic mixture(4).

These studies have further confirmed the responsiveness of the "phosphorylated" pathway of serine biosynthesis to dietary factors. Addition of serine and glycine to the liquid synthetic diet did not prevent the rise in enzyme levels associated with this diet. However, removal of serine and glycine from the liquid synthetic diet resulted in a further increase in 3-P-glycerate dehydrogenase level. This latter effect may result from further restriction of total amino acid intake or from a secondary deficiency of cysteine, an ultimate end product of serine metabolism. This latter interpretation is supported by data suggesting the importance of dietary cysteine in regulating enzyme concentration in the "phosphorylated" pathway of serine biosynthesis (3).

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Lysozymuria Induced in Rats by Nephrotoxic Agents. (31494)

T. BALAZS AND R. R. ROEPKE (Introduced by K.-F. Benitz)
Toxicology Research Section, Lederle Laboratories, American Cyanamid Co., Pearl River, N. Y.

Lysozymuria has been found in various renal diseases of man and has also been demonstrated after administration of mercuric chloride to rats(1,2). It has been suggested that measurement of this enzyme may become an important tool in the diagnosis of renal disease.

Since the pathogenesis of lysozymuria has not been established, experiments were conducted to investigate this problem in toxic tubular nephroses in rats.

Materials and methods. Male, albino rats of Sherman (Lederle) and Wistar (Royal Hart), strains, weighing 300-400 g, were used. Single doses of sodium chromate (Na_2CrO_4) subcutaneously, mercuric chloride ($HgCl_2$) and uranyl nitrate ($UO_2(NO_3)_2$) intraperitoneally were administered. The rats were placed in metabolism cages. Urine was collected in containers surrounded by dry ice and the volume was recorded. Concentrations of protein and glucose were estimated by a reagent strip (Combistix®)* and specific gravity by a Goldberg type refractometer.† Food and water were withheld for various periods to permit measurement of renal concentrating ability. Urine and kidney glutamic oxalacetic acid transaminase (GOT) was determined by the Sigma Frankel method(3). Urine, plasma and tissue lysozymes were assayed as described by Prockop and Davidson(2). For the determination of

* Ames Co., Elkhart, Ind.
† American Optical Co., Buffalo, N. Y.

TABLE I. Plasma, Kidney and Urine Lysozyme Contents Following a 30-Minute Infusion of Egg White Lysozyme (EWL) (0.1% Solution at 0.05 ml/Min).

Infusion	No. of rats	Time when rats killed after end of infusion (min)	Lysozyme: Plasma (μg/ml)	Lysozyme: Kidneys (μg)/2 kidneys	Lysozyme: Urine (μg) excreted
EWL	2	20	12	1800–2300	50– 60
EWL	2	60	8	1500–2000	350–400
Saline	2	20, 60	5	700–1250	<10

GOT in the kidney and lysozyme in the kidney and other tissues, the organs were homogenized in double distilled water (1:10 w/v) in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at about 800 $\times$ g for 10 minutes, then frozen and thawed. Renal clearances of creatinine, inulin and lysozyme were measured simultaneously in conscious rats. Under pentobarbital anesthesia a polyethylene catheter was inserted into the jugular vein. Priming doses of 1 mg of crystallized egg white lysozyme (EWL‡), 5 mg of creatinine and 0.075 μc ^{14}C-inulin (specific activity: 3.58 mc/g)§ were injected into this catheter and steady levels were maintained by continuous infusion at a rate of 6 mg/hour EWL, 30 mg/hour creatinine, and 0.45 μc/hour ^{14}C-inulin. Blood samples were obtained hourly from the orbital veins. Urine was collected hourly, at which time the bladder was emptied by pressure. Creatinine was determined by the method of Folin and Wu(4). Concentration of inulin was determined in a liquid scintillation spectrometer.

Results. The effects of nephrotoxins on the 24-hour urine lysozyme and GOT levels and on the renal concentrating ability are shown in Fig. 1. No changes in any of these values were found at the low dose of Na_2CrO_4 except in one rat. Moderate increases in levels of GOT were accompanied by a marked lysozymuria at the middle and high doses. Hyposthenuria, marked proteinuria and slight glucosuria were also found at the highest dose. $HgCl_2$ at the 2 higher doses and $UO_2(NO_3)_2$ at all 3 dose levels induced a striking increase of GOT excretion, without a similar increase in lysozyme excretion, except in one rat at the high dose of $HgCl_2$. At the dose levels mentioned above polyuria, hyposthenuria and glucosuria were observed. The $UO_2(NO_3)_2$ groups showed a greater increase in proteinuria than the $HgCl_2$ groups.

The effects of a single dose of 20 mg/kg Na_2CrO_4 in 4 rats on the daily urine lysozyme and GOT levels and on urine specific gravity are shown in Fig. 2. This dose was repeated in 2 of these rats on the fourth day. Food and water were provided *ad libitum,* except for 6 hours, when renal concentrating ability was tested. Urine lysozyme increased 3 hours after administration of Na_2CrO_4. The peak excretion of both enzymes was found on the second day. Hyposthenuria was manifest. In the rats dosed with a single dose of Na_2CrO_4 GOT levels returned to normal on the sixth day, when lysozyme levels were still slightly elevated. The second dose of Na_2CrO_4 was followed by a marked lysozymuria: 3200 μg on the first, 5400 μg on the second, 2800 μg on the third day.‖

Rats injected with $HgCl_2$ or $UO_2(NO_3)_2$ were examined for excretions of urine lysozyme also 24-48 hours and 48-72 hours after dosing, respectively. Values were slightly higher than on the previous day, but did not reach the level of the Na_2CrO_4 group.

The effect of a 30-minute infusion of EWL (0.1% solution at a rate of 0.05 ml/minute) on the plasma, kidney and urine lysozyme contents is presented in Table I. The infusion resulted in an increase of renal lysozyme content and in a marked lysozymuria. Plasma lysozyme levels were elevated when the animals were killed 20 and 60 minutes after the end of infusions.

Renal and urinary GOT and lysozyme contents of 10 normal and 6 nephrotoxin dosed rats are shown in Fig. 3. Animals were killed 24 hours after treatments. Renal GOT content showed a significant decrease only in the

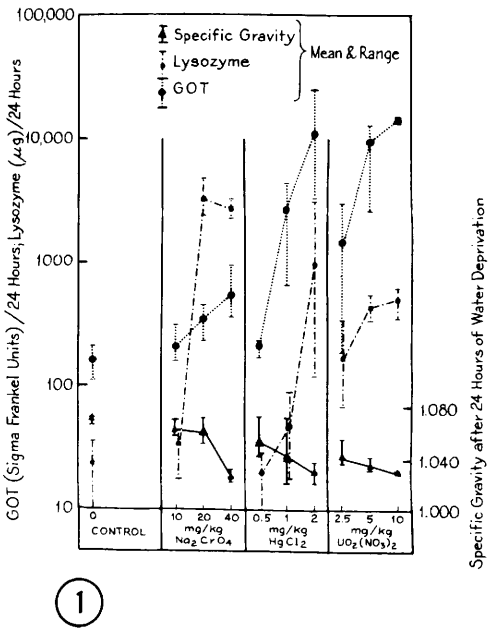
‡ Worthington Biochemical Co., Freehold, N. J.

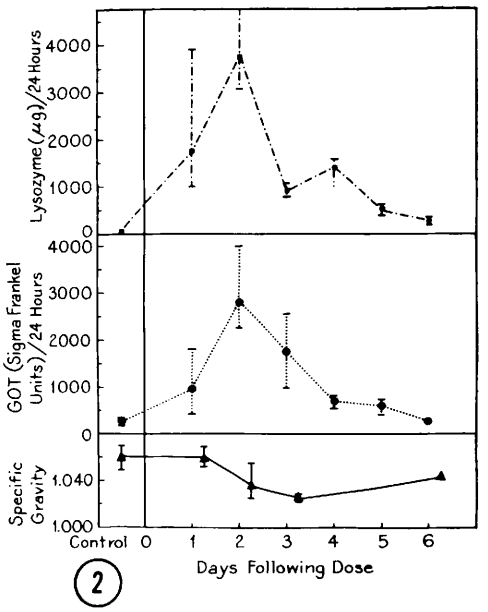
§ New England Nuclear Corp., Boston, Mass.

‖ Mean values of 2 rats.

3 $HgCl_2$ treated rats. This decrease was sufficient to account for the increase in urinary GOT. By contrast, the 24-hour lysozyme excretion of the Na_2CrO_4 treated rats exceeded the lysozyme content of the kidneys. Lysozyme contents of kidney homogenates, frozen and thawed 10 times or treated ultrasonically for 1-10 minutes were found to be identical with the values reported in Fig. 3 and with those of previous authors(5).

Plasma and urine lysozyme levels of normal and nephrotoxin treated and plasma levels of nephrectomized rats are shown in Table II. A slight increase of plasma lysozyme level was found in the Na_2CrO_4 treated rats whereas a marked increase was found in the nephrec-





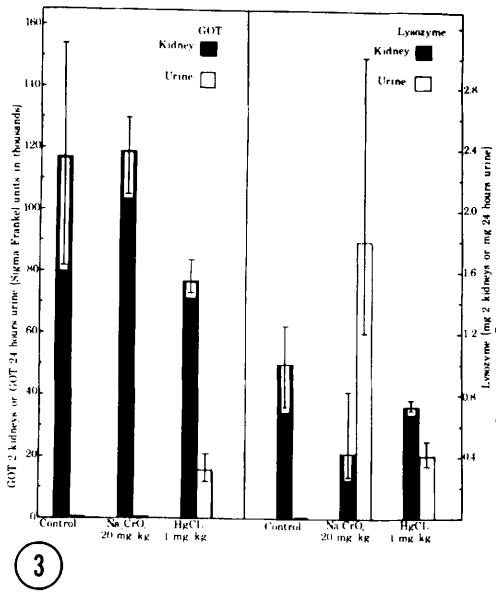


FIG. 1. Effects of a single administration of nephrotoxins on 24-hr excretion of lysozyme and GOT and on renal concentrating ability in rats. (4 rats were used for each dose level; urine collected 0-24 hr in Na_2CrO_4 and $HgCl_2$ groups and 24-48 hr in $UO_2(NO_3)_2$ group.)

FIG. 2. Effects of a single dose of Na_2CrO_4 (20 mg/kg) on daily urine lysozyme and GOT excretion and on renal concentrating ability (6 hr of water deprivation) in rats. Values indicate mean and ranges of 4 animals on 1-3 days, 2 animals thereafter. Two controls were examined daily.

FIG. 3. Renal and urinary GOT and lysozyme contents 24 hr after administration of nephrotoxins. (Values represent means and ranges of 10 control, 3 Na_2CrO_4 and 3 $HgCl_2$ dosed rats.)

TABLE II. Plasma and Urine Lysozyme Contents in Rats Treated with a Single Dose of Nephrotoxin and Plasma Lysozyme Contents in Nephrectomized Rats.

Treatment	Dose (mg/kg)	No. of rats	Plasma lysozyme at 24 hr (μg/ml) Mean	Range	24 hr urine lysozyme (μg) Mean	Range
Normal		10	5.6	4–10	<10*	<10–36
Sham operated		4	6.2	4–10	<10*	<10
$HgCl_2$	1	3	5.3	5– 6	54	40– 90
Na_2CrO_4	20	4	12.1	9–15	3240	2400–4880
Nephrectomized		7	70	64–78	—	
Nephrectomized + Na_2CrO_4	20	2	70	68–72	—	

* Median value.

tomized animals. Administration of Na_2CrO_4 had no effect on serum levels of lysozyme in nephrectomized rats.

Heart, lung, spleen, stomach, intestines, pancreas, liver, testes, bladder, lymph glands and bone marrow were examined for lysozyme activities. Marked activity was found only in lung (480-1000 μg/g), in bone marrow (240-480 μg/g), in spleen (140-180 μg/g) and in liver (80-90 μg/g).

The normal production rate of lysozyme in rats was estimated by measuring the rate of rise in plasma-lysozyme following nephrectomy and the apparent volume of distribution (AVD) of lysozyme.

Plasma-lysozyme levels showed a linear increase of 4.4-7.5 μg/ml/hour (mean 5.5 μg) during the first 7 hours following nephrectomy.

The AVD of lysozyme was determined by measuring plasma concentrations of lysozyme following an intravenous injection of a large dose (1.2-3.6 mg) of egg white lysozyme in nephrectomized rats. The falling plasma concentrations reached a relatively steady level in about an hour. The ratio of the injected dose to the product of blood concentration and body weight gave the AVD of lysozyme as a percentage of body weight. This was found to be 15% (13-17%). Thus the production rate of lysozyme in a 400 g rat $= 5.5 \times (.15 \times 400) = 330$ μg/hour.

Data of lysozyme and creatinine clearances in normal, Na_2CrO_4 and $HgCl_2$ treated rats are summarized in Table III. The variability in each group was probably due to the difference in urine flow(6). The ratio of lysozyme-to-creatinine clearance increased in the Na_2CrO_4 group, but did not change significantly in the $HgCl_2$ group. Clearance of inulin was found to be similar to that of creatinine in control and in the two Na_2CrO_4 treated rats which were examined. Tubular reabsorption capacity (Tm) and percentage tubular reabsorption of lysozyme were calculated on the assumption that the glomerular filtration rate of lysozyme is equal to that of creatinine or inulin. The data indicated a decrease in tubular reabsorption of lysozyme in rats dosed with Na_2CrO_4.

The results of our experiments implied that the rate of inactivation of lysozyme by the kidney in the normal rat is equal to the rate of production elsewhere in the body. However, attempts to show inactivation of either endogenous or exogeneous lysozyme by kidney slices and homogenates were not successful.

Plasma lysozyme levels 24 hours after nephrectomy were found to be lower than expected from the initial rate of increase in nephrectomized rats. This finding suggests that there is also an extrarenal inactivation or elimination of the enzyme in rats, which becomes apparent at high plasma concentration of lysozyme.

Discussion. Na_2CrO_4 given in a subnephrotoxic dose did not cause lysozymuria. $HgCl_2$ only at the highest dose level caused marked lysozymuria. No change was observed in the activity of EWL *in vitro* when aliquots of aqueous Na_2CrO_4 solution or urine samples of rats dosed with $HgCl_2$ were added (unpublished data). These findings indicated that these compounds in no way interfered with the assay of lysozyme.

TABLE III. Renal Clearance of Lysozyme and Creatinine and Tubular Reabsorption Capacity of Lysozyme in Normal and Nephrotoxin-Treated Rats.*

Parameter	Controls		Na_2CrO_4, 20 mg/kg		$HgCl_2$, 1 mg/kg	
	Mean†	Range	Mean†	Range	Mean‡	Range
Urine flow (ml/min)	.029	.003- .06	.020	.004- .040	.04	.025- .06
Clearance (C) of lysozyme (ml/min)	.25	.08 - .46	.15	.023- .21	.26	.15 - .38
(C) of creatinine (ml/min)	1.9	.55 - 2.81	.33	.107- .52	2.6	1.9 - 3.3
(C) of lysozyme/ (C) of creatinine	.13	.09 - .17	.45	.23 - .77	.10	.08 - .11
Tubular reabsorption capacity of lysozyme (μg/min)	154	66 -210	22	2.4 -35	220	210 -231
% tubular reabsorption of lysozyme	86	85 - 90	32	7.7 -61	90	88.5 - 92

* Renal clearance values determined 24-48 hr after a single dose of nephrotoxin.
† Mean of 4 animals.
‡ Mean of 2 animals.

The finding that there is a renal accumulation and urinary excretion of EWL after its infusion is in accord with that of previous authors who found that rats whose endogenous lysozyme production was enhanced by growth of transplanted tumors, showed an increase in kidney and urine lysozyme content(5).

Results of renal clearance measurements suggested a tubular reabsorption of lysozyme in normal rats and indicated its decreased reabsorption in Na_2CrO_4 treated rats. The massive lysozymuria induced by this nephrotoxin can be explained on this basis. Daily extrarenal production rate of lysozyme (about 8 mg) exceeded the maximal daily excretion of this enzyme in Na_2CrO_4 treated rats. A 20-50% reduction in the tubular reabsorption could account for the levels of lysozyme excretion of these animals.

Prockop and Davidson(2) induced glomerular damage in rats by the administration of anti-kidney serum. This resulted in a massive proteinuria with only a very slight increase in lysozyme excretion. They found no correlation between proteinuria and lysozymuria in man.

The agents administered in the present study are known to cause tubular lesions at relatively specific sites of the proximal convoluted tubules (PCT). Na_2CrO_4 affects the first, $UO_2(NO_3)_2$ the middle and $HgCl_2$ the terminal portion of the PCT(7,8). However, high doses of $HgCl_2$ might involve even the first portion of the PCT(9).

Na_2CrO_4 induced a disproportionately high lysozymuria but no marked glucosuria, since the site of glucose reabsorption is the middle portion of the PCT which is not much affected by this agent. By contrast, lower doses of $UO_2(NO_3)_2$ and $HgCl_2$ induced marked glucosuria without significant lysozymuria. The primary site of the Na_2CrO_4 lesion appears to coincide with that of the lysozyme reabsorption.

Recent investigations demonstrated by histochemical methods that renal tubular reabsorption of horse-radish peroxidase takes place mainly in the cells of the PCT of the outer cortex (*i.e.*, the proximal portion of the PCT). The terminal portion of PCT showed a low reabsorption of horse-radish peroxidase (10). This enzyme accumulated in acid phosphatase-bearing particles, the lysosomes, which are known to contain also the lysozyme in the rat kidney(11). There appears to be a similarity in the renal handling of horse-radish peroxidase and lysozyme.

Summary. The urinary excretion of lysozyme was investigated in rats following administration of nephrotoxins. Signs of $HgCl_2$ and $UO_2(NO_3)_2$ induced nephroses were accompanied by a moderate lysozymuria. Na_2CrO_4 caused a disproportionately high ex-

cretion of this enzyme for 3 to 4 days, exceeding normal renal lysozyme content.

Endogenous plasma lysozyme levels increased in nephrectomized rats, indicating an extrarenal source and a renal inactivation of this enzyme. Simultaneous measurements of renal clearances of exogenous lysozyme and creatinine demonstrated a decreased reabsorption of lysozyme in Na_2CrO_4, but a normal one in $HgCl_2$ dosed rats. The data imply a decreased tubular reabsorption of lysozyme in the pathogenesis of lysozymuria.

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Cholesterol Esters in Myelin and the Component Fatty Acids.* (31495)

FRANKLIN YOUNG** AND F. H. HULCHER (Introduced by Camillo Artom)

Department of Preventive Medicine and Genetics and Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, N.C.

It is generally held that almost all cholesterol in brain occurs in the free form and that cholesterol esters when present constitute only a small proportion of total cholesterol(1-3). Two to five percent of the total cholesterol was reported to be esterified in human brain in early life(4). No detailed study has been made of the fatty acids of cholesterol esters in brain. O'Brien *et al*(5) have found traces of cholesterol esters in human myelin. Even in the accompanying paper(6) in which the fatty acid and fatty aldehyde composition of various lipid fractions in myelin is described, no such study has been made of cholesterol esters. The present paper reports the isolation and identification of cholesterol esters of bovine myelin and describes the fatty acid composition of these esters.

Materials and methods. Two samples of bovine myelin were prepared by a procedure previously described(7). From each sample, lipids were extracted, washed, dried(8) and fractionated by thin layer chromatography on Silica gel HR.† The 20 × 20 cm plates were covered with a layer of adsorbent 0.5 mm thick. Fifty milligrams of lipid extract were applied as a streak to each plate. The solvent system was hexane‡:diethyl ether:glacial acetic acid, 73:25:2 (v/v/v)(9). All solvents used in this study except diethyl ether were re-distilled. The fraction which traveled at the same rate as the cholesterol stearate standard was eluted with chloroform:petroleum ether (1:9). The methyl esters of the fatty acids in the cholesterol ester fraction were prepared(10) and analyzed in a dual-column gas-liquid chromatograph. The 6-foot, 1/8-inch stainless steel columns were packed with 15% (w/w) diethylene glycol succinate polyester on acid-washed Chromosorb W.§

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** Present address: College of Tropical Agriculture, Hawaii Agricultural Experiment Station, University of Hawaii, Honolulu, Hawaii.

† Obtained from Brinkmann Instruments, Inc., Westbury, N. Y.

‡ Hexane is Skellysolve B obtained from Skelly Oil Co., Kansas City, Mo.

§ Applied Science Laboratories, Inc., State College, Pa.

TABLE I. Infrared Spectra of Free Cholesterol and Cholesterol Ester Fractions.

Chemical group	Wave length microns	Cholesterol	
		Free	Ester
-OH stretching vibration	2.95	+	—
C–CH_3 " "	3.45	+	+
C=O " "	5.75	—	+
C=C " "	6.00	+	+
C=C of unsaturated fatty acid	6.25	—	+
CH_2 bending vibration	6.80	+	+
CH_3 " "	7.25	+	+
=C–O– " "	8.00	—	+
C–O–C vibration of esters	8.60	—	+
–C–O– vibration	9.47, 9.67	+	+
C–OH vibration of secondary cyclic alcohol	10.15	+	—
Δ^5 sterol	11.92, 12.52	+	+

The column temperature was programmed from 155°C to 210°C at a rate of 2°/min. Helium, at a flow rate of 40 ml/min was the carrier gas. A disc integrator-equipped recorder was used to quantitate the peak areas of the fatty acids. Duplicate chromatograms were run on each sample. The fatty acids were identified by the relative retention ratio method (methyl palmitate as 1.00) (11), with fatty acid standards.

In another experiment total sterol esters were isolated by silicic acid-celite (1:1, w/w) column chromatography with chloroform: petroleum ether (1:9) as the eluant(8,12). After drying, the weight of the isolated fraction was determined.

Results and discussion. Cholesterol esters separated by thin layer chromatography or column chromatography constituted 4% or 3.6% respectively of the total lipids by weight. The isolated fraction gave a positive Liebermann-Burchard reaction for cholesterol(13). Infrared spectroscopy (Table I) showed that the fraction contained cholesterol and unsaturated fatty acids which were esterified as shown by the adsorption peak at a wave length of 5.75 μ. There was no adsorption band for the hydroxy group (2.95 μ) usually obtained in the infrared analysis of free cholesterol and no band was found for N-CH_3 indicating the absence of choline-containing phospholipids in this fraction.

The gas liquid chromatographic analyses showed the presence of at least 18 fatty acids with a 14-carbon chain or longer. This variety of fatty acids of cholesterol esters in bovine myelin exceeds that of the same lipid fraction in bovine plasma(14). Table II presents the relative percentages of the fatty acids and the order in which they appeared on the chromatogram. The confirmation of the identities of 18:3 (number of carbons:number of double bonds) and 20:4 fatty acids as well as the positive identification of other unknown fatty acids by other methods must await the availability of samples larger than those presently obtainable from the gas chromatographic effluent. Since Johnson *et al*(1) reported that in cat, dog, and beaver cholesterol esters were present only in white matter, but were present in both white and gray matter of one of the two human samples, studies with myelin should be extended to species other than bovine. Alterations in certain lipid fractions of the brain and in their fatty acid composition in various diseases have been reported recently(15-18). It is possible that brain cholesterol esters and their fatty acid composition may be altered also in pathological conditions.

Summary. Cholesterol esters were isolated from purified bovine myelin by thin layer and column chromatography. Liebermann-Burchard reaction and infrared spectroscopy were used to identify the isolated fraction.

TABLE II. Fatty Acid Composition of Cholesterol Esters of Bovine Myelin.

Fatty acid*	Relative retention ratio†	Relative percentage
14:0		.4
15:0		.2
	.87	.4
16:0		7.3
16:1		.9
17:0		.4
	1.47	1.1
18:0		17.3
18:1		48.6
18:2		.9
18:3		5.6
	3.18	1.4
	3.42	.6
	3.58	1.6
20:4		7.0
	4.76	1.0
	5.27	3.6
	6.32	1.7

* No. of carbons:No. of double bonds.
† Relative retention ratio of the unknown fatty acid (methyl palmitate as 1.00).

Component fatty acids of the cholesterol esters were quantitated by gas-liquid chromatography.

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Autointerference of Rabies Virus in Chick Embryo Fibroblasts.* (31496)

KAMESABURO YOSHINO, SADAKO TANIGUCHI, AND KAZUJI ARAI

Department of Bacteriology, Yokohama City University School of Medicine, Minamiku, Yokohama, Japan

In our previous study(1), HEP Flury strain of rabies virus(2) was found capable of forming plaques in primary chick embryo fibroblasts, and the titration of infectivity by this plaque technique has replaced the more laborious baby mouse(2) and one-day egg LD titrations(3). While applying this plaque assay to materials containing a high concentration of virus, we noticed that monolayers receiving an undiluted seed virus suspension formed no plaques. Our investigation of the nature of this phenomenon led to the finding that interferon production was responsible for it. These results are reported here.

Materials and methods. Diluent. The virus diluent was yolk-saline(1), which was 0.01 M phosphate buffered saline of pH 7.2 containing fresh egg-yolk at 0.1%. Just prior to use, penicillin and streptomycin were added at 500 μ/ml and 100 γ/ml, respectively.

Viruses. Standard 7-day egg passage of HEP Flury strain of rabies virus(2) was used at the 259th to 263rd passage levels. Brains of infected embryos were emulsified to make a 20% emulsion in yolk-saline, to serve as seed virus. In one case, the same strain serially passaged in chick embryo fibroblasts(4) was tested; a pool of culture fluids constituted a seed. Small plaque mutant of Western equine encephalitis (WEE) virus(5) and Sindbis virus were at 15th and 51st chick embryo cell passages, respectively, when used. Dairen I strain of vaccinia virus was received from the National Institute of Health of Japan as

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