zene resin, Dowex-1 was reported by Kraus and Moore(4). Quantitative separation of salts of these elements in pure solutions is relatively simple. However, the numerous elements and proteins of biological specimens required considerable hydrolysis in concentrated acids to prepare them for quantitative separation.

This procedure is very useful for specimens which have undergone neutron activation. The aluminum foil containers can be digested with the sample. This eliminates the error introduced when samples must be emptied from their containers. If the sample can be introduced directly to the flask the first concentrate HCl additions are unnecessary. The entire procedure requires approximately 16 hours to complete. It requires minimal attention and can be interrupted at any stage except following the last addition of HCl, when it must be immediately poured onto the column.

The separation of iron and cobalt from biological tissues has application in isotope studies when recovery of either or both elements is required. While the recovery reported here was accomplished from bovine plasma, the procedure is capable of this separation from high protein tissue such as packed red cells. Theoretically the technique will allow quantitative recovery of iron and cobalt in trace concentrations from any specimen that can be digested by concentrated acid.

Summary. Trace amounts of iron and cobalt are separated and quantitatively recovered from biological specimens using Dowex-1 columns. The procedure for digesting the protein and preparing the samples is given in detail. Recovery values for 72 samples using Co^{57} and Fe^{59} as tracers are tabulated. Excellent recovery and reproducibility are achieved.

1. Ogborn, R. E., Dunn, A. L., Blotcky, A. J., Johnson, G. F., James, L. R., *Am. J. Roentgenol.*, 1961, v85, 976.

2. Odeblad, E., Meurman, L., Ziliotto, D., *Acta Radiol.*, 1955, v44, 337.

3. Lowman, J. T., Krivit, W., *J. Lab. and Clin. Med.*, 1963, v61, 1042.

4. Kraus, K. A., Moore, G. E., *J. Am. Chem. Soc.*, 1953, v75, 1460.

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Action of 5-Fluorodeoxyuridine on Synthesis of Infectious Canine Hepatitis Virus in Dog Kidney Cells.* (28488)

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The DNA inhibitor, 5-fluorodeoxyuridine (FUDR)† interferes with the synthesis of thymine, probably at the level of thymidylate synthetase, and the inhibition is reversible with thymidine(1,2). FUDR or related compounds suppress synthesis of DNA viruses (3-7) but not RNA viruses(8-10). The effects of the drug on synthesis of infectious canine hepatitis (ICH) virus in dog kidney (DK) cells are described in this report. ICH virus, which is a member of the adenovirus group(11), multiplies in the nucleus where it stimulates formation of DNA(12,13).

Materials and methods. The procedures used in cultivation of DK cells and titration of ICH virus by the tube dilution method have been described(12). A 10^{-2} dilution of virus that titered at $10^{6.5}TCID_{50}$ per ml was used to infect the cultures and 2-hour adsorption periods were employed. The normal growth curve of the virus was determined by titrating at varying periods 2-42 hours after infection. The inhibitory effect of FUDR was studied by adding FUDR (10^{-3} M, 10^{-5} M, and 10^{-6} M) at varying periods 2-36 hours

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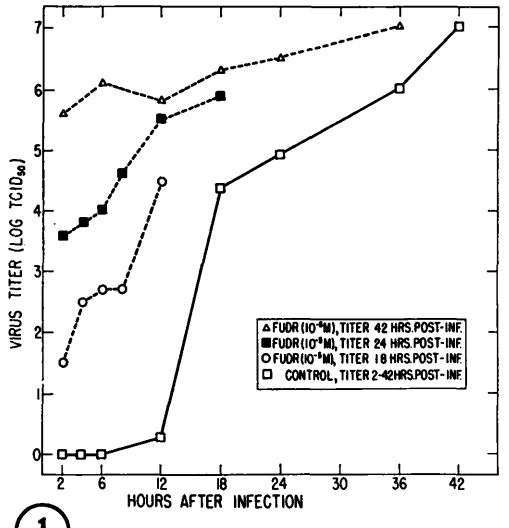

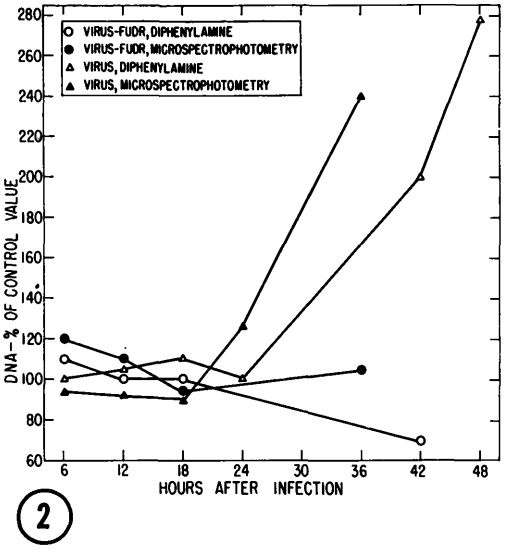

FIG. 1. Final titers of ICH virus at 18, 24, and 42 hours after addition of FUDR (10^{-5}M or 10^{-6}M). Each point in an "FUDR curve" indicates final yield of virus when FUDR was added at the time (indicated in abscissa) of the point itself. Each point in 18- and 24-hour "FUDR curves" is the mean of 2 separate determinations; each point in 42-hour curve is the mean of 3 determinations. The virus control curve represents titers of virus at varying times (indicated in abscissa) after infection. Each point is mean of 4 determinations. When final yields of virus were determined at 42 hours there was practically no suppression of viral synthesis by FUDR. Titers in 42-hour "FUDR curve" are approximately the same as the 42-hour virus control titer. Observe partial suppression of viral synthesis when final yields of virus were determined at 24 and 18 hours after infection and more suppression at 18 hours than at 24 hours. The later FUDR was added the less inhibition was obtained. "FUDR curves", representing DNA synthesis, precede the virus growth curve, and show that by 12 hours enough DNA was formed for maximum synthesis of infectious virus.

FIG. 2. Quantity of DNA in FUDR-inhibited and uninhibited cultures at varying times after infection. Virus (10^{-2} dilution of $10^{6.5}$TCID$_{50}$) was added at zero hour for 2-hour adsorption periods and DNA was determined by Fuelgen microspectrophotometry and diphenylamine methods. Quantities of DNA were calculated per 10^6 cells and are expressed as % of control value. Observe suppression of DNA synthesis at all stages of infection in FUDR-treated cultures and increase of DNA in uninhibited cultures.

after infection, and titrating for virus at 18, 24, 30, 42, and 54 hours. The reversal effect of thymidine was studied by adding thymidine (3×10^{-3} M or 3×10^{-5} M) with FUDR (10^{-5} M) at 2 hours and titrating at 18 and 24 hours after infection.

Results. (1) *Inhibitory effects of FUDR.* When FUDR (10^{-5} M or 10^{-6} M) was added at 2-12 hours, titers of ICH virus at 18 and 24 hours after infection were suppressed (Fig. 1). However, when final yields of virus were determined at 30, 42, or 54 hours after infection the titers were as high as in the virus controls (Table I). In all experiments, the later the drug was added the less inhibition was obtained. If the concentration of FUDR was increased to 10^{-3} M, inhibition was nearly complete as late as 54 hours after infection. Addition of thymidine with FUDR 2 hours after infection failed to reverse signifi-

TABLE I. Capacity of FUDR to Inhibit ICH Virus After 30, 42, and 54 Hours of Infection. FUDR (10^{-3}M and 10^{-5}M) was added 2 hr after infection and virus titers were determined at 30, 42, and 54 hr. When 10^{-5}M FUDR was added, final titers are high, indicating minimal inhibition of virus. However, when 10^{-3}M FUDR was added significant inhibition was found.

FUDR*	Virus titer† of FUDR-treated virus, Hours			Control virus titer (no FUDR)
	30	42	54	
10^{-5}M	4.2			5.0
	3.5			
		5.2		7.0
		4.5		
			4.7	
			4.8	7.8
10^{-3}M			1.5	
			1.3	

* FUDR added 2 hours after infection.

† The inoculated virus (10^{-2} dilution of $10^{6.5}$ TCID$_{50}$) was added for 2-hour adsorption periods.

TABLE II. Effects of Thymidine on Capacity of FUDR to Inhibit ICH Virus. Thymidine was added with or without FUDR 2 hr after infection and titers of virus were determined at 18 and 24 hr after infection. Titers of virus in uninhibited controls are also shown. Thymidine had little effect in reversing the inhibitory effect of FUDR.

FUDR	Thymidine	Virus titers (Log $TCID_{50}$)—cultures with FUDR + thymidine at hours $_p$ virus 18	24	Virus titer—cultures with FUDR alone at hours $_p$ virus 18	24	Virus titer—control cultures
10^{-5}M	3×10^{-3}M	0 0		0		4.7
10^{-5}M	3×10^{-5}M	0.5 1.2		0		4.7
10^{-5}M	3×10^{-3}M		2.5 1.4		2.7	5.3

cantly the 18- and 24-hour inhibitory effects of FUDR (Table II).

(2) *Escape from effects of FUDR during prolonged incubation.* To explain lack of viral suppression in cultures incubated 30 hours or longer, the effect of prolonged incubation on degradation of the drug was considered. The following experiment was carried out: FUDR (10^{-5} M) was added to cultures of uninfected cells and virus-infected cells that were incubated at 37°C for 18 hours. The nutrient medium from these cultures containing FUDR was then added to newly infected cultures, which were incubated 24 hours, and titrated for virus. Total incubation time of FUDR was 42 hours. Control cultures with virus alone were titrated at 24 hours. The results (Table III) showed that 42 hours of incubation at 37°C had no effect on the 24-hour inhibitory capacity of FUDR. The slightly higher titers of virus in cultures preincubated with infected cells can be accounted for by the additional virus added.

To prove that DNA-suppressing capacity of FUDR was retained throughout the entire period of incubation, DNA in inhibited cultures was measured at varying periods 6-42 hours after infection and compared with DNA in uninhibited cultures. DNA was determined by Feulgen microspectrophotometry (13) and diphenylamine methods(12). The results showed that DNA never increased above the control value in FUDR-inhibited cultures, but increased 240-280% in uninhibited cultures (Fig. 2).

(3) *Time of DNA synthesis.* Since FUDR inhibits synthesis of DNA or precursor immediately, the only virus that forms after addition comes from DNA or precursor accumulated in the cell up to the time the drug is added. If FUDR is added early, before increased synthesis of DNA or precursor, the amount of virus is small; if added later, after synthesis of DNA or precursor, more virus will be found at the time of final titration (3). If a curve is plotted with the points representing final yields of virus at the times

TABLE III. Effects of Preincubation of FUDR on Capacity to Inhibit ICH Virus. FUDR was preincubated with infected or uninfected cells, then added to newly infected cultures that were incubated at 37°C for 24 hr. Total incubation of FUDR in each experiment was 42 hr. Infected controls with fresh FUDR and without FUDR are included. Each figure represents average of 2 determinations. Preincubation with infected or uninfected cells had no effect on inhibitory capacity of FUDR.

FUDR	Conditions of preincubation	Conditions of postincubation	24-hr virus titers (Log $TCID_{50}$) With preincubated FUDR	Virus control (no FUDR)*
10^{-5}M	37°C/18 hr with uninfected cells	37°C/24 hr with infected cells	1.7 1.4	5.3
10^{-5}M	37°C/18 hr with infected cells	37°C/24 hr with infected cells	2.5 3.2	

* For 24-hour virus titer with FUDR see Fig. 1.

of FUDR addition, the curve indicates synthesis of DNA or precursor since at each point the virus titer is related to the amount of DNA synthesized up to that point.

In the present study when "FUDR curves" were plotted for final virus yields at 18 and 24 hours after infection, they showed synthesis of DNA or precursor shortly after infection, with sufficient amount produced by 12 hours for maximum synthesis of infectious virus (Fig. 1).

Discussion. In this study FUDR (10^{-5} M or 10^{-6} M) caused suppression of viral synthesis in cultures titered at 18 or 24 hours, but failed to cause suppression in cultures titered at 30 hours or longer. The final titers of virus in these prolonged cultures were nearly as high as those in virus controls. Flanagan and Ginsberg(7) reported similar results with types 4 and 5 adenovirus in HeLa cell cultures. They had inhibition at 32 hours, but lack of inhibition at 40 hours or longer.

It was shown that lack of inhibition of viral synthesis after 30 hours was not due to degradation of FUDR from temperature of incubation, or loss of DNA-suppressing ability.

Previous studies(12,14) of ICH virus-infected cultures by phase-contrast, and Giemsa and acridine orange stains have shown that DNA first appears in the nucleus as small granules in the ground substance. These enlarge, congregate in the center, and combine to form an inclusion body. The chromatin and nucleoli move to the margin of the nucleus in a halo when the inclusion body forms. With fluorescent antibody, virus is observed in the nuclear granules, inclusion body, and marginated chromatin. Apparently, only a small part of the newly synthesized DNA is needed for viral production for there is a great excess in the mature inclusion body that is unrelated to virus.

Studies of FUDR-inhibited cultures by similar methods show entirely different cellular changes(14). At no time do nuclear granules or inclusion bodies form when FUDR is added before or 2 hours after infection, and the chromatin does not marginate. Of particular interest, in cultures of this type examined 42 hours after infection, are cells with small, pleomorphic, and often vacuolated nuclei. The nuclear chromatin in these cells is thickened and stains brightly for DNA with acridine orange and reacts positively for virus with fluorescent antibody.

Thus, it appears from this and previous studies that when DNA precursors are unavailable due to the action of FUDR, after a time, the virus is able to utilize chromosomal DNA for multiplication. This accounts for synthesis of virus without increase in DNA, the changes observed in the chromatin and nuclei, and the presence of virus within the chromatin.

There is no evidence that RNA is involved in this process since RNA does not increase in cells in inhibited cultures(14). Also, it seems unlikely that an abnormal DNA is formed for synthesis of infectious virus.

Summary. FUDR suppressed synthesis of ICH virus in DK cells after 18 or 24 hours of incubation, but failed to suppress the virus when the cultures were incubated for 30 hours or longer, unless the concentration of the drug was increased. Viral inhibition by FUDR was not reversible with thymidine. The lack of inhibition in cultures incubated for longer periods was not due to degradation of FUDR or loss in DNA-suppressing capacity. DNA synthesis began shortly after infection, and by 12 hours enough DNA was formed for maximum synthesis of infectious virus.

1. Heidelberger, C., Chaudhuri, N. K., Danneberg, P., Mooren, D., Griesbach, L., Duschinsky, R., Schnitzer, R. J., Pleven, E., Scheiner, J., *Nature,* 1957, v179, 663.

2. Rich, M. A., Saslaw, L., Eidinoff, M. L., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 791.

3. Salzman, N. P., *Virology,* 1960, v10, 150.

4. Tanami, Y., Pollard, M., Starr, T. J., *ibid.,* 1961, v15, 22.

5. Kaplan, A. S., Ben-Porat, T., *ibid.,* 1961, v13, 78.

6. Chang, R. S., Humes, M., *J. Exp. Med.,* 1962, v115, 937.

7. Flanagan, J. F., Ginsberg, H. S., *ibid.,* 1962, v116, 141.

8. Weelock, E. F., Tamm, I., *ibid.,* 1961, v114, 617.

9. Simon, E. H., *Virology,* 1961, v13, 105.

10. Rich, M. A., Perez, A. G., Eidinoff, M. L., *ibid.*, 1962, v16, 98.

11. Kapsenberg, J. G., PROC. SOC. EXP. BIOL. AND MED., 1959, v101, 611.

12. Moulton, J. E., Frazier, L. M., *Virology*, 1961, v15, 91.

13. Soo, S. F., Moulton, J. E., *Am. J. Vet. Res.*, 1963, v24, 150.

14. Moulton, J. E., Frazier, L. M., *ibid.*, 1963, in press.

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Sensitivity of Irradiated Mice to Bacterial Endotoxin. (28489)

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In the course of studies on the mobilization of granulocytes with bacterial endotoxin it was observed that extremely small doses given on the third day after lethal irradiation killed mice that would otherwise have lived for several additional days(1). Greatly increased sensitivity to endotoxin has been reported in a number of circumstances, notably adrenalectomy(2), reticuloendothelial blockade(3), vaccination with BCG(4) serial zymosan injections(5), and elevated ambient temperature(6). The mechanisms of endotoxin hypersensitivity are not well understood. None of the suggested possibilities would have led us to anticipate the effect observed in irradiated mice.

In the present studies we attempt to quantify and localize the response with respect to *a*, radiation dose; *b*, time after irradiation, and *c*, organ or system responsible for the hypersensitivity reaction.

Methods. The mice used were (BALB/c $\times$ DBA/2)F_1 females 12 to 20 weeks old, maintained during the experiment in individual cages. The temperature in the animal room ranged from 76 to 80°F. Except where shielding was involved, irradiation was from a Co^{60} source. In the shielding experiments radiation was delivered from 2 opposing Westinghouse Quadraconex tubes operating at 200 KV, 15 ma, and filtered with 0.25 mm Cu and 1 mm Al. Lead shields were ⅛ inch thick. A preparation of *S. typhosa* endotoxin* was used, injected intraperitoneally. The end point used was death in less than 72 hours after challenge. LD_{50} values, with 95% fiducial limits, were estimated by probit analysis and are given as μg per 20 g mouse.

Results. 1. *Effect of radiation dose on endotoxin LD_{50} 3 days after irradiation.* Counting the animals that died in less than 72 hours after challenge, the LD_{50} of the endotoxin preparation in control mice was 480 (440-530) μg per 20 g mouse. When the challenge was given on the third day after irradiation the endotoxin LD_{50} values shown in Fig. 1 were observed. There was no evidence of increased sensitivity following an exposure of 300 rads but after 600 rads the endotoxin LD_{50} dropped to 310 μg and to 80 μg after 820 rads. Between 400 and 900 rads the endotoxin LD_{50} decreased at the rate of about 100 μg per 100 rads. After a dose of 1000 rads the endotoxin LD_{50} was estimated to be 0.5 (0.3-1.1) μg per 20 g mouse. The radiation LD_{50} for death during the same interval, (*i.e.*, 6 days after irradiation) was 1170 (1140-1210) rads in mice given no endotoxin. This value is plotted in Fig. 1 to correspond with an endotoxin LD_{50} of zero.

2. *Effect of time of irradiation on endotoxin LD_{50}.* In our original mobilization experiments it was noted that sensitivity to endotoxin was greater 3 days than 2 days after irradiation(1). Using doses of 850 and 890 rads the effect on endotoxin toxicity was studied for challenges made 2, 4 or 5 days as compared to 3 days after irradiation. Results of these experiments are shown in Fig. 2. The third day was clearly the most criti-

* Prepared and generously supplied by Difco Laboratories, Detroit, Mich.