of the nutrient medium, and the temperature, humidity and p_{CO_2}. Several media have been developed for this purpose. With the combination of the latest and best of these media (3) and the incubator described here, growth of euploid cells has been more rapid and reliable than under any other conditions of our experience.

Summary. 1. A device for continuously monitoring and regulating the carbon dioxide content of cell culture incubators is presented. 2. CO_2 concentration is determined by measuring the pH of a bicarbonate solution which is continuously equilibrated with gas samples drawn from the incubator. 3. Installation and operation of the device in a practical cell culture incubator is described.

1. Ham, R. G., Puck, T. T., *Methods in Enzymology,* Colowick, S. P., Kaplan, N. O., editors, Vol. 5, Academic Press, New York, 1962, p90.

2. Puck, T. T., *Squibb Centennial Lectures,* 1958-59, G. P. Putnam and Sons, New York; Puck, T. T., Cieciura, S. J., Robinson, A., *J. Exp. Med.,* 1958, v108, 945.

3. Ham, R. G., *Exp. Cell Research,* 1962, in press.

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Inhibitory Effect of Ammonium Sulfate on Columbia SK Virus Propagation in Mouse Ascites Tumor Cells *in vitro.* (27708)

EIICHI FURUSAWA AND WINDSOR CUTTING
Department of Medical Microbiology, Stanford University, Calif.

While screening antiviral substances by using an adapted strain of Columbia SK virus which can propagate efficiently in mouse ascites tumor cells(1,2,3,4), it was found that ammonium sulfate showed a definite inhibition against the cytopathic effect, hemagglutinin production, and replication of infectious virus. Several workers(5,6,7,8) have reported the inhibitory effect of ammonium ions on the influenza virus group in tissue cultures but have found no effect on other viruses. We now show that Columbia SK virus also is sensitive to this simple ion.

Materials and methods. A hemagglutination (HA)-positive strain and HA-negative strain of Columbia SK (Col SK) virus which are adapted to Ehrlich ascites cells were used in such cells. In some experiments, LCM virus (strain NY#621) which was isolated from a leukemic L_2C guinea pig by Dr. C. W. Jungeblut(9) was used, also in Ehrlich cells. After washing, 2 to 4 $\times$ 10^6 Ehrlich ascites cells harvested freshly from mice were suspended in 1 ml of culture medium per tube. The medium was composed 85% of Medium 199 and 15% of heat-inactivated (60°C, 30 min) Ehrlich ascites fluid from which fibrin had been removed by standing at 4°C for a week. Virus was added as 0.05 ml of a definite dilution of virus with Medium 199; ammonium compounds were added as 0.05 ml of aqueous solutions. The same volumes of Medium 199 and of distilled water were added to controls. The stationary cultures were incubated at 36°C. After various incubation times, cultures were shaken by hand to remove the cells from the glass wall, and then centrifuged. The supernatant was used for titration of infectivity and HA. The cells were resuspended in saline and stained with 1 drop of 0.4% of erythrosin which colors the damaged cells red but does not stain living cells. With this staining method, the cytopathic effect (CPE) of the virus and the toxicity of the chemicals could be detected quantitatively with a counting error within 10%. More than 75% loss of live cells, as compared with the controls, was recorded as 4+; 74% to 50% as 3+; 49% to 25% as 2+; and under 24% as 1+. For HA, a 0.2% suspension of sheep red cells in potassium barbital buffer was used(10). The infectivity was determined in culture tubes as previously described(3).

Results. 1. *Effect of ammonium salts on multiplication of virus with standard inoculum size.* An inoculum of 0.05 ml of 10^{-4} dilution, about 1000 tissue culture doses

TABLE I. Effect of Ammonium Ion on CPE, HA and Infectivity in the System of Columbia SK Virus and Ehrlich Ascites Cells *in vitro*.

Agent	Virus inoculum	Effect	Concentration of agent (μg) 5000	2500	1250	625	313	156	None
$(NH_4)_2SO_4$	None	CPE (No. of live cells)	98*	163	173	155	166	169	158
$(NH_4)_2SO_4$	10^3 TCD_{50}	CPE (No. of live cells)	99	160	167	94	30	7	5
		HA		<4	<4	160	1280	2560	2560
		Infectivity†		-3	-3	-5	-6	-7	-7
NH_4Cl	None	CPE (No. of live cells)	2*	58*	160	162	168	159	
NH_4Cl	10^3 TCD_{50}	CPE (No. of live cells)	3	44	162	91	32	5	
		HA			<4	160	1280	2560	

* Shows the toxicity of agent after 3 days of incubation; the numbers are indicated in 0.1 mm^3.

† Indicated with 10 log per 0.1 ml.

(TCD_{50}), of the 10th passage of HA-positive Col SK virus was made into culture tubes in which 0.05 ml of ammonium salt was added just before inoculation, and incubated for 3 days. Table I shows the results. Both the sulfate and chloride salts exhibit definite inhibitroy effects; sulfate seems to have a wider effective range (2500 to 625 μg) than chloride (1250 to 625 μg). As the ammonium sulfate showed a complete inhibitory effect at 1250 μg (one-fourth the toxic dose), it was used through the following experiments. 2. *Effect of ammonium sulfate on multiplication of virus with various inoculum sizes.* To determine the dose response to ammonium sulfate, a box titration of the inhibitory effect was performed. Different doses of the sulfate were inoculated with various dilutions of T14 virus in 5 groups. Table II shows the results after 3 days incubation. Each point was determined from 2 tubes. A satisfactory dose response was shown. It was found that 1250 μg of the sulfate could clearly suppress CPE and HA production of the 10^5 TCD_{50}(10^{-2} dilution) of virus inoculum, and doubtfully suppress the HA production of undiluted inoculum. 3. *Effect of ammonium sulfate on single cycle of virus growth.* Preliminary ex-

TABLE II. Inhibitory Effect and Dose Response of Ammonium Sulfate on Various Inoculum Sizes.

Virus dilution	Effect on	μg ammonium sulfate 2500	1250	625	313	156	Control
10^0	HA	320	1280	2560	2560	2560	2560
	CPE*	3+	4+	4+	4+	4+	4+
10^{-1}	HA	160	640	2560	2560	2560	2560
	CPE	2+	4+	4+	4+	4+	4+
10^{-2}	HA	40	160	640	2560	2560	2560
	CPE	1+	2+	3+	4+	4+	4+
10^{-3}	HA	20	20	160	640	2560	2560
	CPE	0	0	1+	3+	4+	4+
10^{-4}	HA	<10	<10	40	640	2560	2560
	CPE	0	0	1+	3+	4+	4+
10^{-5}	HA	<10	<10	40	360	2560	2560
	CPE	0	0	1+	3+	4+	4+
10^{-6}	HA	<10	<10	<10	160	640	2560
	CPE	0	0	0	2+	4+	4+
10^{-7}	HA	<10	<10	<10	<10	320	320
	CPE	0	0	0	0	3+	3+
10^{-8}	HA	<10	<10	<10	<10	<10	<10
	CPE	0	0	0	0	0	0

* As loss of cells.

TABLE III. Effect of Ammonium Sulfate on a Single Cycle of Virus Growth.

			Incubation time after washing infected cells							
		NH_4	3	5	7	9	11	24	48	72 hrs
Cells	HA	−	<4	<4	<4	64	128	256	128	64
		+	<4	<4	<4	<4	<4	32	16	8
	Infectivity	−	−2	−3	−5	−6	−6	−7	−6	−6
		+	−2	−2	−2	−2	−3	−6	−5	−5
	CPE	−	0	0	0	0	2+	3+	4+	4+
		+	0	0	0	0	0	0	2+	4+
Medium	HA	−	<4	<4	<4	125	512	2048	2048	2048
		+	<4	<4	<4	<4	<4	64	256	512

periments showed that a toxic dose of ammonium sulfate (5000 μg) did not inactivate 1000 TCD_{50} of virus when incubated for 2 hours at 36°C or overnight at 4°C in culture medium without cells. Also, it did not interfere with the adsorption of virus to Ehrlich ascites cells or HA titration with sheep red cells, findings similar to those of Jensen(6). Therefore single cycle experiments were done to confirm the intracellular level of the inhibitory effect. Washed cells (adequate for 24 tubes) in 1 ml of Medium 199 were mixed with 1 ml of an undiluted T17 virus (HA = 2048) and incubated for 30 min at 36°C, then washed 3 times with saline, suspended in culture medium, and subdivided into tubes with, or without, 1250 μg of ammonium sulfate. At various incubation times, cultures were examined for CPE, HA and infectivity. Table III shows the results. In the controls, virus synthesis could be detected 5 hours after infection in the cells, then the titer increased rapidly at 7, 9, and 11 hours and reached a maximum 24 hours after infection; HA production at each point confirmed clearly the process of the one step growth. The lack of further increase of the virus titer and HA at the 2nd and 3rd day shows that most of the cells were already infected by the original inoculum and that there was little second cycle of virus growth. Thus it was demonstrated that ammonium sulfate clearly suppressed the intracellular virus multiplication when it was added 30 minutes after infection. 4. *Effect of ammonium sulfate added at various latent periods of single cycle growth.* For comparison with Eaton and Scala's report(8) that ammonium phosphate given later than 1 hour after infection was not effective on the system of influenza virus and Krebs 2 ascites cells, addition of ammonium sulfate at various latent periods in our system was tested. Each tube was infected with 0.1 ml of undiluted T20 virus (HA = 1280), incubated for 2 hours at 36°C, then washed 3 times with saline, and resuspended in fresh medium and incubated for 24 hours. Ammonium sulfate (1250 μg) was added at 2, 3, 3.5 and 4 hours after infection. As controls, ammonium sulfate was added to 2 groups at time of infection; in one the sulfate was replaced after the washing step. Table IV shows the results after 24 hours. It was found that ammonium sulfate was completely effective at 2 hours and partially effective at 4 hours (the later stage of the latent period). 5. *Effect of ammonium sulfate on CPE of HA-negative strain of Col SK and LCM viruses.* The HA-positive virulent strain of Col SK virus was compared with 2 strains of HA-negative viruses.

TABLE IV. Effect of Ammonium Sulfate Added After Various Latent Periods on Virus Multiplication.*

		Time NH_4 added after infection					
	No NH_4	0	2	3	3.5	4 hrs	With NH_4 until washing time
HA	640	20	20	40	40	80	80
CPE	3+	0	0	0	1+	2+	2+

* Each tube was infected with 0.1 ml of undiluted T20 virus (HA=1280), incubated for 2 hrs at 36°C, then washed 3 times with saline; values read 24 hrs later.

TABLE V. Effect of Ammonium Sulfate on CPE in HA-negative Col SK Virus and LCM Virus.

	Col SK (1000 TCD_{50})				LCM (1000 TCD_{50})		
	HA-positive		HA-negative		HA-negative		
Inoculum	T10*	T20	T30	T67	T1	T8	T10
Virus alone	4+	4+	4+	4+	4+	4+	4+
" with NH_4	0	0	4+	4+	4+	4+	4+

* T10 etc. indicate the 10th passage etc. of virus.

One was the HA-negative avirulent strain of Col SK virus which was derived from the HA-positive strain of Col SK virus after many passages in Sarcoma 180 ascites cells *in vitro* as reported previously(4). The other was LCM (strain NY#621), which was found to propagate with CPE in Ehrlich and Sarcoma 180 ascites cells *in vitro* from the first generation without any adaptation. About 1000 TCD_{50} of these viruses, and HA-positive Col SK virus as the control, were inoculated with or without 1250 μg of ammonium sulfate. Table V shows the result after 3 days of incubation. Unexpectedly, these HA-negative viruses were resistant to ammonium sulfate, and CPE appeared without any inhibition. Additional experiments also showed that ammonium sulfate also could not suppress replication of infective viruses even when 10 to 100 TCD_{50} of these HA-negative viruses were used.

Discussion. Eaton and Scala(5,8) and Jensen, Force and Unger(6,7) reported independently the inhibitory effect of ammonium ions on influenza virus propagation in *in vitro* systems. We report that Col SK is also sensitive to these ions. Jensen *et al.* used chloride, but in our system sulfate was superior to chloride and showed a wider effective range. Supplemental experiments showed that sodium sulfate was not inhibitory and that therefore the effect of ammonium sulfate was due to the ammonium ion. The degree of inhibitory effect of the sulfate in our system was similar to that shown by them, except in the single cycle experiment. They(8) reported that addition of ammonium phosphate at 1 hour after infection caused no inhibition in the system of influenza PR8 and Krebs 2 ascites cells *in vitro*. By contrast, in our system ammonium sulfate was effective until 4 hours after infection, which was a late stage of the latent period. The different results may be due to the somewhat different system and doses. They used 0.01% of ammonium phosphate in the incomplete replication cycle, while we used 0.125% of sulfate (10 times more) in the complete replication cycle. In our system the latter dosage was about ¼ the toxic level; no cell damage was produced during 5 days of incubation. It is of interest that the HA-negative Col SK and LCM viruses were both resistant to ammonium sulfate. The relationship between HA producing property of the virus and sensitivity to ammonium ions is obviously an interesting field. Several trials to obtain an HA-positive ammonium-resistant strain of Col SK virus were performed. At present, after 6 continuous passages with 1250 μg of ammonium sulfate, the virus still has HA-producing property and is fully sensitive to the sulfate. Eggers and Tamm(11) reported that a hydroxybenzylbenzimidazole (HBB) - insusceptible variant of Coxsackie A9 virus was obtained after only 2 passages in the presence of HBB. Tanami and Pollard(12) reported development of a strain of psittacosis virus partially resistant to 5-fluorouracil after several passages.

Summary. An inhibitory effect of ammonium sulfate on Col SK virus propagation in *in vitro* cultures of mouse ascites tumor cells has been described. The effect was observed on virus synthesis, HA production, and CPE. Single cycle virus growth experiments showed that ammonium sulfate was apparently effective even at the later stage of the latent period. The effect was limited to the HA-positive strain of Col SK virus; the HA-negative strain of Col SK virus and a neurotropic virus (LCM, strain NY#621) were completely resistant to the ammonium ion in the same system.

1. Furusawa, E., Cutting, W., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 618.
2. ———, *ibid.*, 1960, v103, 851.
3. ———, *ibid.*, 1961, v108, 245.
4. ———, *ibid.*, 1962, v109, 417.
5. Eaton, M. D., Scala, A. R., *Virology*, 1961, v13, 300.
6. Jensen, E. M., Force, E. E., Unger, J. B., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 447.
7. Jensen, E. M., Liu, O. C., *ibid.*, 1961, v107, 834.
8. Eaton, M. D., Scala, A. R., *ibid.*, 1961, v108, 766.
9. Jungeblut, C. W., personal communication.
10. Horvath, B., Jungeblut, C. W., *J. Immunol.*, 1952, v68, 627.
11. Eggers, H. J., Tamm, I., *J. Exp. Med.*, 1961, v113, 657.
12. Tanami, Y., Pollard, M., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 648.

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Augmentation of Oxygen Consumption of Rat Brain *in vitro* by Various Carbohydrate Intermediates. (27709)

A. LIVINGSTON, C. H. WILLIAMS AND F. W. BARNES, JR.
Psychophysiologic Research Laboratory, V. A. Hospital, Perry Point, Md., Dept. of Medicine, Johns Hopkins Medical Institutions and Hospital for Women of Maryland, Baltimore

When during other studies it was unexpectedly discovered that addition of citrate ion to rat brain slices *in vitro* produced as much as a 27% increase in their rate of oxygen consumption, no previous report of a similar phenomenon could be found except that of Krebs and Johnson(1) who noted that citrate increased the respiratory rate of pigeon breast muscle *in vitro*. Lipsett and Crescitelli(2) found that citrate prevented the increase in oxygen consumption obtained by adding 0.1 M KCl to rat brain slices in a dextrose substrate. Therefore the above findings on rat brain were investigated *in vitro* further; this paper concerns preliminary results showing some of the interrelated effects of ions and various substrates.

Approach and methods. The standard technic of the Warburg method was used. Time elapsing between decapitation of rats and beginning of equilibration was 25 minutes (± 5) for both brain slices and brei. Concentrations of constituents in the flasks and the errors of the differences of the means are given in the tables. The pH at the start was 6.8-7.0 and changed about 0.4 unit during experimental runs. The lack of effect of a nonmetabolized carbohydrate, mannitol, was shown by the finding that oxygen consumption (μl per mg fresh wt/hr) of 0.0166 M dextrose alone was 1.26 while that of 0.0166 M dextrose plus 0.0416 M mannitol was 1.29.

Results. In the first experiment (Table I, A) the effect of citrate at .0416 molar concentration in the presence of only one substrate, dextrose, was a considerable enhancement of O_2 consumption of brain slices and brei (27% and 51%, respectively). Optimal concentration values of dextrose alone and of combinations of dextrose and citrate are shown in Table I (B and C). Similar enhancement was also shown clearly for the brains of several species, mouse, cat, rabbit, guinea pig and dog, at optimal concentrations found for the rat (Table II). When 3 rat organs other than brain were tested, enhancement was found in heart and kidney, but not in liver (Table II).

We investigated how much increase in O_2 consumption resulted from addition of Krebs-cycle intermediates with and without dextrose (Fig. 1, 2). In Krebs-Ringer phosphate medium only lactate gave a greater respiratory rate than dextrose alone, while pyruvate, oxalacetate, and ketoglutarate gave the same, and malate, fumarate, and citrate gave less (Fig. 1C). In the presence of dextrose, however, only citrate gave enhancement (Fig. 2B) in Krebs-Ringer medium.

If phosphate ion concentration was varied, the substrate effects were different. When