TABLE I. Effect of B_6-Deficiency on Liver Protein, SD and $NADH_2D$.

Dietary regimen	No. rats	Protein, %	SD, units/mg protein	$NADH_2D$, M/kg protein/hr
B_6-deficient*	18	20.1 ± .38†	10.2 ± .55	25.5 ± .77‡
Paired wt control	18	19.7 ± .49	10.9 ± .31	29.3 ±1.21§
Ad libitum control	18	19.7 ± .35	10.1 ± .38	33.4 ± .98

* Data from non-fatty portions only. † S.E. of mean. ‡ $P<.02$ when compared with two control group. § $P<.02$ when compared with *ad libitum* group.

per liver protein. However, there was no reduction in staining for $NADH_2D$ by the histochemical method employed. This discrepancy may be because the histochemical method is not satisfactory for quantifying small differences in enzyme concentrations.

The cause of the reduction in liver $NADH_2D$ in B_6-deficiency is not immediately apparent. Burch *et al*(11), who demonstrated the reduction of this enzyme in a child who had died of kwashiorkor and in dying riboflavin-deficient rats, found it to be held very firmly by liver tissue and regarded it as a crucial flavin enzyme. Their results may indicate that a rather profound alteration in metabolism is the cause. Wainio and co-workers(12) reported that DPNH-cytochrome C reductase falls in protein deficient rats; however, Beaton *et al*(3) could find no evidence that B_6-deficiency impairs maintenance or synthesis of tissue protein in the albino rat.

Summary. In histochemical studies fatty change in the livers of Vit. B_6-deficient rats was associated with reduction in SD activity in the centrolobular portions. In homogenate assays of non-fatty portions of liver, Vit. B_6-deficiency had no effect on the SD activity. The deficiency significantly reduced the $NADH_2D$ activity, however, even after the nonspecific effect of undernutrition and fatty change had been eliminated.

We wish to acknowledge the helpful advice of Dr. Henry D. Moon, and the technical assistance of Miss Elizabeth Girdish and Mrs. Darlene Conn.

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Received July 25, 1963. P.S.E.B.M., 1964, v115.

Cell Culture Studies on Antiviral Agents: I. Action of Cytosine Arabinoside and Some Comparisons with 5-Iodo-2-Deoxyuridine.* (28834)

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An understanding of some of the mechanisms of cell-virus interaction has encouraged investigators searching for chemotherapeutic antiviral drugs. Recently herpes keratitis of of humans and rabbits has been successfully treated with 5-iodo-2-deoxyuridine (IUDR)

TABLE I. Cytotoxicity of CA and IUDR on Newly Planted Cells.

	HeLa		Rabbit kidney*		Rat kidney†		HEp-2	
	CA	IUDR	CA	IUDR	CA	IUDR	CA	IUDR
100 γ/ml	+	+	+	+	+	+	+	+
50	+	+	+	±	+	+	+	±
25	+	+	+	—	+	+	+	—
12.5	+	±	+	—	+	±	+	—
5	+	—	+	—	+	—	±	—
1	±	—	+	—	±	—	—	—
.5	—	—	±	—	—	—	—	—
.2	—	—	±	—	—	—	—	—
0	—	—	—	—	—	—	—	—

— = Cell growth appeared normal and comparable to control.
± = Definite signs of cell toxicity, lack of normal cell growth.
+ = No growth, definite cell toxicity.
* Cells subcultured twice from rabbit kidneys.
† Continuous cell line derived from 2-day-old rat kidneys.

by Kaufman(1) and with cytosine arabinoside (CA) by Underwood(2).

Several investigations of the *in vitro* antiviral action of cytosine arabinoside and some comparisons between cytosine arabinoside and 5-iodo-2-deoxyuridine are reported here.

Experimental. Virus. An isolate of herpes simplex virus, now in the seventh rabbit kidney passage, was obtained from the vesicular fluid of an oral herpetic lesion and designated MRS. Vaccinia virus was supplied through the courtesy of the Michigan State Dept. of Health, East Lansing, Mich. Both herpes and vaccinia virus grew in rabbit kidney cultures, produced characteristic cytopathic changes and were neutralized by specific immune sera. Pseudorabies, swine pox, fowl pox, B-virus, Newcastle disease, infectious bronchitis, Coe virus, PR-8 influenza, parainfluenza type 3 (HA-1) and adenovirus types 2, 3, 6, 8 and 10 were obtained from the American Type Culture Association.

Cell Cultures. The technique for preparation of rabbit kidney and other cell cultures for virus growth and titration has been described(3).

Cytotoxicity. Seven-day-old rabbit kidney cell monolayers were exposed to concentrations of cytosine arabinoside or 5-iodo-2-deoxyuridine from 0.5 to 100 γ/ml of growth medium for a period of 5 days. The cells were then fixed and stained with hematoxylin-eosin or observed by phase contrast microscopy for morphologic changes associated with cytotoxicity. With concentrations of CA of 10 γ/ml or less, no signs of toxicity were noted; however, at 50 γ/ml, some fragmentation and swelling of mitochondrial elements occurred. At 100 γ/ml of CA, considerable cellular damage was observed, represented by mitochondrial changes, increased granularity, frequent vacuolization, and abnormal mitotic figures. Gross cellular nuclear or mitochondrial alterations were not noted below 100 γ/ml of IUDR.

In contrast to the above data obtained with essentially "resting cells," it was found that considerably less CA or IUDR induced cytotoxic changes in newly planted cells. These results are tabulated in Table I. Addition of drug to a suspension of 100,000 cells at time of planting did not interfere with attachment to the glass surface, but interfered with multiplication (at cytotoxic chemical concentrations). With the exception of the HEp-2 cell line, a concentration of CA of 0.5 to 1 γ/ml was toxic; IUDR, on the other hand, showed cytotoxicity only at levels above 12.5 to 50 γ/ml. The cells did not recover after 8 to 12 hours of exposure and removal of the cytotoxic chemical.

From these data it was concluded that the presence of CA or IUDR in the medium feeding actively growing cells inhibited some cellular function necessary for replication. In contrast, cells in the "resting state" were affected to a lesser degree, probably due to the slower rate of uptake of the chemical and RNA-DNA-protein metabolism.

Antiviral spectrum of cytosine arabinoside.

TABLE II. Antiviral Spectrum of Cytosine Arabinoside.*

Virus	Host		Activity
Herpes simplex	Rabbit kidney cell cultures	Embryonic	Positive
		200 g	"
		Adult	"
	Rabbit skin cell cultures	Embryonic	"
	Rabbit corneal cell cultures	200 g	"
		Adult	"
	Mouse liver cell cultures	Embryonic	"
	Mouse kidney cell cultures	Embryonic	"
		Week-old	"
	Mouse skin cell cultures	Embryonic	"
	Rat skin cell cultures	Embryonic	"
	Rat kidney cell cultures	Embryonic	"
	Chicken kidney cell cultures	Embryonic	"
	Whole 9-day-old chicken embryo cell cultures	Embryonic	"
	Guinea pig kidney cell cultures	Embryonic	"
		Week-old	"
	Human kidney cell culture	Embryonic	"
	HeLa cell cultures		"
Vaccinia	Chicken skin cell cultures	Embryonic	"
	Chicken kidney cell culture	Embryonic	"
	Whole 9-day-old chicken embryo cell cultures	Embryonic	"
	Mouse liver cell cultures	Embryonic	"
	Mouse skin cell cultures	Embryonic	"
	Rat liver cell cultures	Embryonic	"
	Rat skin cell cultures	Embryonic	"
	HeLa cell cultures		"
Pseudorabies	Rabbit kidney cell cultures	200 g	"
	Rat skin cell cultures	Embryonic	"
Swine pox	Chicken kidney cell cultures	Embryonic	"
Fowl pox	Chicken kidney cell cultures	Embryonic	"
B-virus	Rabbit kidney cell cultures	200 g	"
Newcastle disease	Chicken kidney cell cultures	Embryonic	Negative
Infectious bronchitis of chickens	Chicken kidney cell cultures	Embryonic	"
Coe virus	HeLa cell cultures		"
	Mouse liver cell cultures	Embryonic	"
Influenza PR-8	Chicken kidney cell cultures	Embryonic	"
Hemadsorption type I	HEp-2 cell cultures		"
Adenovirus type 2, " 3, " 6, " 8, " 10	HeLa cell cultures and KB cell cultures		"

* Tests were run at drug concentrations of 50 γ/ml.

In Table II are summarized the data showing that CA was found active against a number of viruses and in a variety of cell systems. In the viruses tested, the activity was confined to DNA viruses of 100 mμ or larger. In the system employed, no significant reduction in titer was found when 100 γ/ml of CA were added to cultures infected with adenoviruses types 2, 3, 6, 8, or 10.

Fifty γ/ml of 5-iodo-2-deoxyuridine were active against herpes simplex, vaccinia, pseudorabies, swine and fowl pox, and B-virus in rabbit kidney cell systems.

Tests for contact antiviral activity. Samples of herpes simplex virus titering 2×10^6 PFU/ml or vaccinia virus titering 1×10^7 PFU/ml were mixed with 100 and 1000 γ/ml of either CA or IUDR in Hanks' balanced salt solution (BSS). The virus-chemical mixtures were incubated at 37°C for 30 minutes, then diluted 10^{-1} through 10^{-6} in BSS and 2 ml of each dilution were added to mono-

layers of rabbit kidney cells in 60 mm Petri plates. The plates were centrifuged in the #266 head of the International Centrifuge model UV at 2000 rpm for 30 minutes in a specially constructed rack adapted to the #384 cup.* After removing the fluids, the cell sheets were washed 4 times with BSS and the monolayers overlaid with agar. Plaque counts on non-treated controls subjected to the same manipulations compared favorably with those treated with CA or IUDR, indicating a lack of contact or *in vitro* virus inactivation.

To test the possibility that either CA or IUDR might act at the cell membrane to prevent cell-virus interaction, rabbit kidney cell plates were fed with 4 ml of a medium containing 100 γ/ml of either CA or IUDR and incubated for 4 hours at 37°C. Following incubation, the fluid was removed and 100 PFU of herpes simplex virus in 2 ml of BSS containing 100 γ of either CA or IUDR was added. The virus was deposited onto the cells by centrifugation. After centrifugation, the excess fluid was removed and the plates washed 3 times with BSS prior to overlaying. Plaque counts on control plates compared favorably with CA or IUDR-treated plates indicating no interference with virus attachment and penetration.

Electron micrographs of cell surfaces of cultures pretreated with CA or IUDR, prepared according to the procedure of Mathews and Buthala(4), revealed normal virus attachment, penetration and release, thus confirming the titration experiments described above.

Comparative antiviral potency of CA and IUDR in fluid cultures. The antiviral potencies of CA and IUDR were compared in rabbit kidney cells using herpes, vaccinia or pseudorabies viruses under fluid medium. Four thousand PFU of the appropriate virus were deposited onto rabbit kidney monolayers by centrifugation and either 100 γ/ml of IUDR or 10 γ/ml of CA added immediately to the 4 ml of feeder fluid. After 4 days of treatment, no viral cytopathogenicity was observed. Virus in the fluids from the treated cultures was centrifuged onto rabbit kidney monolayers, then overlaid with agar. After washing the treated monolayers 4 times, one-half of them were overlaid with agar for plaque formation. The remaining monolayers were disrupted by 2 cycles of freezing and thawing, centrifuged and the virus in the clear supernatant fluid deposited by centrifugation onto rabbit kidney monolayers and overlaid with agar. Plaque counts from one of 3 similar experiments are summarized in Table III.

As can be seen from Table III, 10 γ/ml of CA markedly inhibited virus production with herpes simplex, vaccinia and pseudorabies. The treatment fluid, overlaid-treated cells and the freeze-thaw-treated cells yielded a few infective units, accounted for by incomplete thermo-inactivation of the original inoculum.

In contrast, although 100 γ of IUDR retarded visible cytopathic changes in the treated monolayer, the treatment fluid from pseudorabies- and herpes simplex virus-infected cultures yielded virus titers indicative of multiplication. The agar-overlaid-treated

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* T_s (Time of sedimentation) can be written

1. $T_s = (60/4\pi^2)(1/Pi)[18/(\sigma-\rho)d^2]$

where η = viscosity
σ = density of particle
ρ = density of medium
$(\sigma-\rho) \cong 0.2$
d = diameter of solvated particle
Pi = precipitation index defined as $(RPM)^2/[(\log_e R_{max})-(\log_e R_{min})]$

60 mm Petri plate = 51.6 mm diameter bottom
= 2090 mm^2 area

Using 2 ml fluid the depth of fluid is (2000/2090) = 0.96 mm or 0.096 cm. Distance from spindle to top plate in centrifuge cup = 14.2 cm.

2. $Pi = (2000)^2/(\text{Log}_e 14.2 - \text{Log}_e 14.104) = (4 \times 10^6)/.0068 = 5.9 \times 10^8$

Substituting in equation 1:

$T_s = (137 \times 10^{12})/[Pi(d)^2]$ where d is expressed in $m\mu$

$T_s = (137 \times 10^{12})/(5.9 \times 10^8)(150)^2$ where diameter of herpes simplex virus is 150 $m\mu$

$T_s = 10.3$ min.

If diameter of virus is assumed to be 100 $m\mu$, then $T_s = 23.2$ min.

It has been consistent that centrifugation of herpes simplex virus for as short a time as 15 minutes results in 95 to 98% adsorption of virus to cells as evidenced by the lack of infective virus in the supernatant fluid.

monolayers and the freeze-thaw-treated monolayers confirmed the fact that 100 γ/ml of IUDR did not completely inhibit pseudorabies or herpes simplex multiplication.

The effect of IUDR and CA on vaccinia virus was comparable; the low titers recovered indicate little or no multiplication and possibly incomplete decay of the original inoculum.

It was observed in another series of experiments that if the treatment was continued for 6 days, no herpes simplex or pseudorabies could be isolated from CA-treated cells. However, in the IDUR-treated cells, evidence of cytopathic changes was seen late on the 4th day and the cells were completely destroyed by the 6th day.

The possibility was investigated that the "regrowth" of virus after 3 to 4 days in the presence of IUDR may have been due to depletion of the drug by cellular inactivation or production of metabolic products antagonistic to the antiviral activity. Cultures were infected with herpes simplex virus and immediately treated with various concentrations of IUDR from 1 to 1000 γ/ml. The drug level was maintained for 6 days by daily changes of the culture fluid. As observed above, there was a 3 to 4 day retardation of cytopathic changes following which the virus multiplied rapidly to destroy the cell sheet. From these data the "regrowth" of virus could not be accounted for by chemical depletion or by antagonistic degradation products accumulating in the culture medium.

Comparative antiviral potency of CA and IDUR in agar-overlaid cultures. At drug (IUDR and CA) concentrations from 1 γ to 10 γ/ml, virus "regrowth" prevented consistent virus inhibition studies in fluid cultures. Therefore, the method of plaque size suppression was employed to determine the minimum effective dose of the chemical in question capable of disrupting the normal virus-cell action. One ml of the appropriate drug concentration made up in overlay medium was mixed with 3 ml of agar-overlay medium and poured over a cell monolayer previously infected with 100 PFU of either vaccinia or herpes simplex virus which had

TABLE III. Comparison of CA and IUDR Antiviral Activity at 10 and 100 γ/ml, Respectively, in Fluid Cultures After 4 Days of Treatment.

	Herpes simplex		Pseudorabies		Vaccinia	
Material tested for virus	CA	IUDR§	CA	IUDR	CA	IUDR
Treatment fluid*	1 PFU/4 ml	TNC PFU/4 ml	1 PFU/4 ml	TNC PFU/4 ml	23 PFU/4 ml	15 PFU/4 ml
Treated monolayers†	2 PFU/plate	TNC PFU/plate	1 PFU/plate	TNC PFU/plate	23 PFU/plate	132 PFU/plate
Extracts of treated monolayers‡	5 "	" "	2 "	" "	24 "	27 "

* Culture fluid containing drug.
† Treated monolayers washed and overlaid with agar.
‡ Treated monolayers washed and disrupted by freezing-thawing.
§ Plaques too numerous to count accurately—estimated at approximately 10,000.

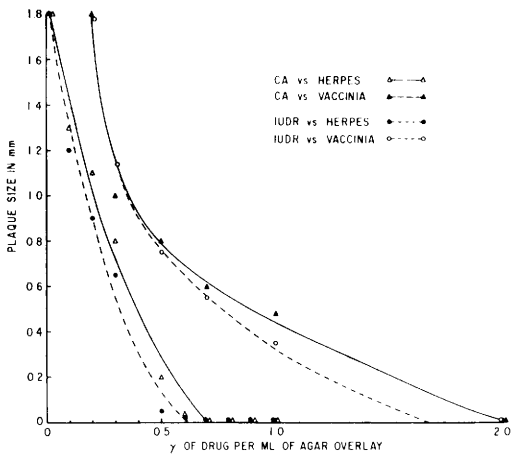

FIG. 1. Cell culture activity of cytosine arabinoside and iododeoxyuridine against herpes simplex and vaccinia virus as measured by plaque size suppression.

been centrifuged onto the cells not more than 20 minutes prior to overlay. After 4 days' incubation at 37°C, the agar-overlay medium was removed and the cell sheet air-dried. The number of plaques on a plate were counted and their average size determined by measurement through a 7 × ocular micrometer. In Fig. 1 the average plaque size is plotted. Plaque size decreased progressively with increased drug concentration. In general, the number of plaques remained constant over a considerable chemical concentration, but dropped rapidly at the highest concentrations.

In this system both CA and IUDR inhibited herpes simplex virus multiplication. In repetitive experiments, complete inhibition of virus multiplication was obtained at drug levels of 0.6 to 1 γ/ml. Both CA and IUDR were slightly less effective against vaccinia virus, requiring between 1.5 and 2.0 γ/ml to suppress plaque formation. Tests in which varied concentrations of CA and IUDR were mixed in the overlay medium failed to show more than an additive effect against either vaccinia or herpes simplex virus, *i.e.*, under the conditions of the test there was no evidence of synergistic activity.

Viral resistance to CA or IUDR. Incorporation of 1 to 1000 γ/ml of IUDR in cell culture fluid feeding monolayers of RK cells infected with herpes simplex virus retarded the appearance of the cytopathic changes for 3 to 4 days, but then the culture was rapidly destroyed. The possibility that this "regrowth" of virus might have been due to destruction of the IUDR was discounted when the same effect was noted despite daily replacement of drug and media. With CA, virus "regrowth" was observed only when the chemical concentration was below 5 γ/ml. When these experiments were repeated under agar, similar results were obtained and normal sized plaques of herpes virus growth were observed in IUDR-treated plates at levels of up to 1000 γ/ml.

To test the virus resistance hypothesis, several well-isolated plaques of herpes simplex virus were picked from rabbit kidney plates containing 500 γ/ml of IUDR and inoculated into cultures of rabbit kidney cells containing 500 γ/ml of IUDR; after 48 hours the cultures showed destruction by the virus and the virus was again harvested and plaqued in the presence of 500 γ/ml of IUDR. This alternate picking of plaques and culturing in the presence of IUDR was repeated 10 times. After 10 passages, the virus reached a titer of 7×10^7 PFU/ml of fluid when grown in the presence of 1000 γ/ml of IUDR or in the absence of IUDR and exhibited no extended lag period as observed on the first passage.

The 10th IUDR passed herpes simplex virus was passaged 6 additional times without IUDR in the culture medium. At each passage the virus was titered in the presence of IUDR (500 γ/ml) and in its absence. At each passage the titers were between 1.2 and 2.3×10^7 PFU/ml regardless of whether IUDR was included or excluded from the titration medium. These data indicate a relative stability of IUDR resistance and exclude the possibility that the resistant virus required IUDR for growth.

Inclusion of CA in the agar at levels of 2 to 5 γ/ml inhibited most herpes simplex virus growth. Occasionally a plaque of small size developed which, when isolated and placed in 2 to 5 γ/ml of CA in fluid cultures, produced titers of 1×10^4 to 1×10^6 PFU/ml. Repeated passages of plaque to fluid in the presence of 5 γ/ml of CA appeared to in-

TABLE IV. Frequency of CA or IUDR Resistance with Herpes Virus: Numbers of Resistant Plaques Formed from an Inoculum of 100,000 PFU per Plate.

Virus strains	Drug concentrations, γ/ml agar	Plaque counts	
		CA	IUDR
McKrea	100	0*	125†
	10	30	472
	5	TNC	800
Virtue	100	0	110
	10	92	582
	5	TNC	560
Stone	100	0	67
	10	0	104
	5	18	170
M. M.	100	0	236
	10	0	260
	5	2	263
MRS	100	0	19
	10	0	33
	5	1	25
Clapp	100	0	125
	10	25	TNC
	5	TNC	TNC
RDH	100	0	12
	10	0	18
	5	13	37

* Plaques on CA-treated plates ranged from 0.1 to 0.5 mm in diameter.

† Plaques on IUDR-treated plates ranged from 1.0 to 2.0 mm in diameter. Control plate plaques, 1.5-2.0 mm in diameter.

crease the degree of CA adaptation. However, inclusion of 10 γ/ml of CA inhibited the growth of the virus culture.

Having confirmed our earlier observations that IUDR-resistant herpes simplex virus could be obtained with ease, and that CA-resistant virus was probable, tests were conducted to determine whether there was cross-resistance between CA and IUDR. It was found that resistance to one did not confer resistance to the other.

To determine whether the marked resistance development to IUDR was a general phenomenon with all herpes simplex viruses, these studies were expanded to include several laboratory strains and other clinical isolates. Three additional laboratory strains of herpes† (McKrea, Stone and Virtue) and 3 clinical isolates (Clapp, M. M. and RDH) isolated in our own laboratory from oral herpetic lesions were tested. After a single passage in rabbit kidney cells, each virus pool was titered and 100,000 PFU spun onto monolayers of RK cells in 60 mm Petri plates. Each plate was overlayed with agar containing graded amounts of either CA or IUDR.

† Supplied by Dr. H. Kaufman.

The plaque counts summarized in Table IV indicate the relative ease of obtaining resistant plaques to IUDR. Plaques picked from the IUDR plates were passed into cultures containing 1000 γ/ml of IUDR. All of the cultures were rapidly destroyed and the virus titer ranged from 1×10^7 to 9×10^7 PFU/ml. Treatment with CA on the other hand produced few plaques of extremely small size, mainly at 5 γ/ml of drug. Plaques picked from the CA plates regrew only at 5 γ/ml and not at 10 γ/ml, confirming our earlier observations. Each of the virus pools were tested with herpes simplex specific immune serum and were completely neutralized.

Since IUDR was shown to be considerably less toxic to the cell culture system, a rapid producer of resistant virus population, and CA shown to produce few, if any, resistant plaques at 10 γ/ml, a study of the combination of 100 γ/ml of IUDR and 10 γ/ml of CA was undertaken.

The experiment, from which data are shown in Table IV, was repeated except that the agar overlay contained 100 γ/ml of IUDR and 10 γ/ml of CA. In addition, the virus shown to be resistant to 1000 γ/ml of IUDR was also included and overlaid with the same drug concentration. After 7 days no plaques could be detected and no virus was isolated from the sonicated cells on a single subculture; however, after 3 blind passages in the absence of chemical, virus was evident. The meaning of these results is not clear; however, it might indicate a "masked virus" state or the partial inhibition of virus growth due to the drug carried over with each passage. Control plates containing only 100 γ/ml of IUDR were destroyed within 3 days.

From these data it appears that treatment of herpes simplex infection with IUDR even at high drug levels may result in a virus

population resistant to the drug. To circumvent this, low levels of CA, together with a high level of IUDR, may be indicated. However, the use of CA alone at low levels, *i.e.*, 10 to 20 γ/ml, appears to be non-toxic on established rabbit kidney cell monolayers and capable of eliminating all of the infectious virus.

Determinations of blood levels of CA and IUDR. The plaque suppression technique described above provides an extremely sensitive method for assessing *in vitro* antiviral activity. The application of this procedure should provide an assay procedure for determining drug concentrations in serum or body fluids. Preliminary studies revealed that a substantial number of human serum samples contain high concentrations of herpes simplex virus antibodies which when incorporated into the agar overlay, even in high dilutions, caused considerable reduction in herpes simplex plaque size and number. Therefore, a method for precipitating serum proteins, leaving CA or IUDR in solution, was investigated. One hundred γ/ml of CA or IUDR were mixed with ACD-treated herpes immune rabbit or human blood, as well as with herpes immune rabbit or human serum, and incubated at 37°C for 1 hour. Following incubation the blood cells were separated by centrifugation from the whole blood and an aliquot of plasma and serum from each sample was then diluted and mixed with overlay medium as described above. The remainder of the plasma and serum samples were treated with equal volumes of acetone at 45°C and the precipitates which formed during the 20 minutes at 45°C were separated by centrifugation at 400 × *g* for 10 minutes. The clear supernatant fluids were withdrawn and the acetone boiled off. After adjusting the final volume to that of the original, samples from each precipitated serum were diluted as for the serum or plasma and included in the overlay.

The following points were established: (1) After acetone precipitation of the antibody-rich serum, it was possible to recover all of the added CA or IUDR and to detect drug levels of 1 γ/ml or less; (2) As essentially all of the CA and IUDR was recovered, the acetone treatment did not destroy the drug or induce binding to the precipitate; (3) There was little or no protein binding or erythrocyte absorption of the drug in whole blood; (4) It is clear that this technique can be used for blood level determination of both CA and IUDR and should be applicable for the assay of any antiviral agent with *in vitro* activity.

In addition, levels of CA and IUDR of less than 1 γ/ml were detected in tissue extracts of liver, brain, spleen, and kidney.

Summary. Cytosine arabinoside (CA) was shown to have antiviral activity in cell culture against the DNA viruses of herpes simplex, B-virus, pseudorabies, vaccinia, swine pox and fowl pox, but inactive against several adenoviruses and RNA viruses.

Comparative tests were run with 5-iodo-2-deoxyuridine (IUDR) and CA. Cytotoxicity *in vitro* was more marked with CA. Newly planted cells showed toxicity at 0.5 to 1 γ/ml of CA, contrasted to 12.5 to 50 γ/ml of IUDR. In established monolayers, CA toxicity was not acute below 100 γ/ml and IUDR below 1000 γ/ml.

Comparative antiviral potency of CA and IUDR against herpes virus *in vitro* fluid cultures showed CA to be much more active; however, in agar using plaque suppression, both chemicals gave identical endpoints.

A single passage of herpes simplex virus in the presence of 500 γ/ml of IUDR resulted in a virus culture resistant to IUDR. The virus after 10 passages in the presence of 500 γ/ml of IUDR was found to have a similar titer in the presence or absence of IUDR. The 6th IUDR free passage of the 10th IUDR passed virus reached similar titers in the presence or absence of IUDR.

Herpes simplex virus growth in CA occurred only at low drug levels. Virus populations unaffected by 1000 γ/ml of IUDR were sensitive to 10 γ/ml of CA. A combination of 100 γ/ml of IUDR and 10 γ/ml of CA completely inhibited virus growth, *ergo,* no resistant virus.

Minuscule amounts of CA or IUDR could be detected in whole blood, serum or extracts of several tissues by the plaque suppression method. Concentrations of 0.2 γ/ml could be

detected in human serum containing a high titer of herpes simplex antibodies if the serum was first precipitated with warm acetone. The test appeared to be applicable to blood or tissue level studies of any antiviral agent that shows significant *in vitro* activity.

Grateful acknowledgement is made to Dr. J. H. Hunter who synthesized cytosine arabinoside and to Dr. B. P. Sagik for many helpful suggestions.

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Received September 30, 1963. P.S.E.B.M., 1964, v115.

Isolation of Immunologically Competent Lymphocytes from Sensitized Mouse Spleens.* (28835)

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Large numbers of lymphocytes may be obtained from mouse spleens for immunologic studies. Although a number of methods have been reported for isolation of lymphocytes from blood and serosal exudates, these have not been satisfactory when applied to suspensions of splenic cells. This report is concerned with a simple method for preparation of highly purified suspensions of lymphocytes from splenic tissue. The red blood cells are removed by lysis and the lymphocytes are separated from other nucleated cells by centrifugation. Lymphocytes obtained by this procedure maintain their viability and immunologic competence.

Methods and results. Lymphocyte suspensions. Mice were sacrificed by cervical dislocation; the spleens were removed aseptically and minced immediately in a Petri dish at room temperature. Minced tissues from 2 to 3 spleens were suspended in 5 ml of tissue culture medium 199 and disrupted further by pipetting. This suspension of splenic cells contained many lymphocytes, as well as other nucleated cells and a variable number of erythrocytes. The suspension was filtered through 2 layers of lint-free fine gauze to remove the residual particles of tissue.

The suspension was centrifuged and the supernatant was discarded. Ten milliliters of unbuffered 0.35% saline was added to the packed cells and the cells were resuspended; this resulted in lysis of the erythrocytes. After one minute 1.2 ml of 5% saline was added to restore physiologic tonicity. The above procedure was repeated. The cell suspension, consisting of nucleated cells, was washed 3 times by resuspension in medium 199 and centrifugation. The supernatant was discarded and the cell mass placed into 12 cm segments of untreated polyethylene tubing (1.2 mm internal diameter) with a Pasteur pipette. One end of the tubing was closed by tying it into a knot with a small hemostat. The plastic tubes, tied-ends down, were put into open segments of glass tubing (12.0 × 0.8 cm) for support and placed into centrifuge cups. The tubes were centrifuged at 275 *g* for 10 minutes. The lower one-third of the packed cells in the plastic tubing, containing lymphocytes with an admixture of other nucleated cells, was cut off and discarded. The upper two-thirds of the cell pack consisted almost entirely of lymphocytes. An average of 96% (range 90-99%) of the cells were small or medium lymphocytes. The remaining cells were macrophages and plasma cells. Myeloid cells were rare in these preparations. An average yield of 40 million lymphocytes was obtained from each spleen.

Viability. The viability of the lymphocytes was established by a dye-exclusion test(1) as well as by motility studies. A drop of

* Supported in part by USPHS grants and Am. Cancer Soc.