thymocyte serum action would be expected to be less effective.

It is of interest that anti-LCM antibody production proceeded in RAMT serum treated animals, despite suppression of cellular responsiveness, confirming the dissociation of humoral and cellular immunity in LCM infection noted by Rowe *et al*(2). The reduced intensity of clinical and histologic signs following discontinuation of prolonged RAMT serum therapy may have been a consequence of circulating anti-LCM antibody which had reached an effective titer prior to the appearance of immunologically competent and potentially damaging lymphocytes. The outcome of LCM infection in mice, thus, may depend intimately on the balance between cellular and humoral factors responding to virus invasion.

Summary. Rabbit anti-mouse thymocyte serum significantly modified the course of mouse LCM virus infection. Clinical and histologic signs of infection were absent so long as therapy was regularly maintained, and the severity of the host response to the virus was reduced following discontinuation of prolonged therapy. Treatment in the early period of incubation was more protective than at later times.

Addendum. This work was presented in part before Am. Assn. of Immunologists, April 1967, Chicago, Ill.(11). Since it was submitted, Gledhill has reported preliminary data describing similar protective effects of anti-thymocyte serum in LCM infection(12).

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Growth and Cationic Stabilization of Group A Coxsackieviruses.* (32255)

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Coxsackieviruses were first isolated by Dalldorf and Sickles in 1947 by inoculation of newborn mice with fecal specimens from two children in Coxsackie, N. Y., who were suspected of having poliomyelitis(1). The first strains isolated produced lesions limited to muscles of the inoculated animals. In 1948, Melnick *et al*(2) isolated, from aseptic meningitis patients, a number of new types which produced lesions in a variety of other tissues of the newborn mouse. Because of their characteristic histopathology, the new types were classed as representative of

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the group B viruses, to distinguish them from the original group A viruses which produced only myositis(3).

The group B coxsackieviruses could be propagated *in vitro, e.g.,* monkey kidney cell cultures, but most group A coxsackieviruses did not grow in cell cultures and required suckling mice or hamsters for their propagation. However, in recent years, many of the group A coxsackieviruses, originally found to grow only in newborn mice and hamsters, have been adapted to grow and produce cytopathic effects in various cell cultures(4). Presently, all types but 1, 5, 6, 19 and 22 have been adapted to selected cell cultures (5).

In the course of preparation of reference enterovirus stocks at this WHO Virus Centre, we have studied the growth of the group A coxsackieviruses in a variety of cell cultures, in order to select those most susceptible. In addition, we have tested all 23 prototypes of coxsackievirus A for the property of $MgCl_2$ stabilization to thermal inactivation, for the previous work on the enteroviruses had not considered these types(6).

Materials and methods. Viruses. All 23 prototype strains of group A coxsackieviruses were obtained from Dr. Herbert A. Wenner, University of Kansas Medical Center, Kansas City, Kansas. Types 1, 2, 4, 5, 6, 19 and 22 were received as suspensions of infected suckling mouse torso and brain; types 7, 9, 14 and 16 as infected monkey kidney cell culture fluids; all other types plus the recently adapted types 2 and 4 were in the form of infected human amnion cell culture fluid. Echovirus type 9 strains (sometimes referred to as coxsackievirus type A23) were from the WHO Centre stock cultures; they were used as preparations that had been adapted to grow in newborn mice and in monkey kidney cells.

Cell cultures. Primary monkey kidney (MK), human embryonic kidney (HEK), human embryonic lung (HEL) and human amnion (HA) cell cultures in tubes were prepared according to standard methods(7,8). Human diploid (WI-38) and KB cells were grown in Eagle's medium B containing 10% calf serum. Maintenance medium was Melnick's medium B for MK cultures and Eagle's medium containing 5% calf serum for all other cell cultures. As recommended by Wenner(5), the pH of both maintenance media was raised to 7.5 and virus was adsorbed for 30 minutes at 37°C before addition of maintenance medium. At least 2-3 blind passages with undiluted cell culture fluid as inoculum (0.2 ml per tube) were attempted before negative results were recorded. For all titrations, 4 tube cultures were used for each 10-fold dilution. The specificity of cytopathic effects (CPE) was ascertained by testing against type-specific sera from the WHO Virus Centre stock and from the University of Kansas.

Mice. Suckling mice less than 48 hours old were used for propagation and titration of types 1, 5, 6, 19 and 22 (which have not yet been adapted to grow in cell cultures) as well as in comparative tests performed with coxsackievirus types A2 and A4 and echovirus type 9. All mouse inoculations were made by subcutaneous route with 0.05 ml of virus suspensions with at least one litter of satisfactory test mice per dilution.

$MgCl_2$ stabilization tests(6). Three parts of each virus suspension, diluted 10-fold in either Melnick's medium B (for tests done in MK cells) or Eagle's medium (for tests done in human cells or mice), were mixed with one part 4 molar $MgCl_2$ and heated in a water bath at 50°C for one hour. Controls received sterile distilled H_2O in lieu of $MgCl_2$. After heating, virus suspensions were diluted 10-fold and titrated immediately in the appropriate system.

Results. Growth of group A coxsackieviruses. Four types of primary cell culture—MK, HEK, HEL, HA—and 2 continuous cell lines—WI-38 and KB—were tested for their support of the growth of the group A viruses as they were received from the University of Kansas. The stocks were thawed and titrated immediately. Negative results were recorded when no CPE could be observed after 2-3 blind passages. When CPE was observed, a further passage was made in order to obtain maximal titers. Virus harvests were kept frozen at —20°C until titrated in the same cell system. Virus types

TABLE I. Growth of Group A Coxsackieviruses in Various Cell Cultures or in Newborn Mice.

Virus type	MK		HEK		HEL		HA		WI-38		KB		In newborn mice:
	Max CPE (days)	Log TCD_{50} per ml	Max CPE (days)	Log TCD_{50} per ml	Max CPE (days)	Log TCD_{50} per ml	Max CPE (days)	Log TCD_{50} per ml	Max CPE (days)	Log TCD_{50} per ml	Max CPE (days)	Log TCD_{50} per ml	Log LD_{50} per 0.05 ml
1	0*		0		0		0		0		0		6.5
2	0		3	5.7	0		7	4.7	3	4.5	5	4.5	6.8
3	0		6	5.5	0		5	5.5	0		0		ND†
4	0		6	4.5	0		5	4.5	0		0		7.3
5	0		0		0		0		0		0		7.3
6	0		0		0		0		0		0		6.2
7	3	6.5	3	5.7	2	7.2	7	5.7	2	5.5	3	6.5	ND
8	0		0		0		3	5.2	0		0		ND
9	2	6.7	4	5.7	6	5.5	2	6.2	2	4.5	0		ND
10	5	3.7	6	3.7	0		3	5.2	0		0		ND
11	0		2	7.5	3	5.7	4	4.7	3	4.7	3	5.2	ND
12	0		0		0		4	5.2			0		ND
13	0		3	6.2	3	4.7	2	5.5	3	5.7	3	5.2	ND
14	7	4.5	8	5.7	7	5.2	3	4.7	3	6.5	5		ND
15	0		3	5.7	6	3.7	3	5.5	3	3.5	3	4.5	ND
16	6	4.2	4	5.7	4	5.7	3	4.5	3	5.2	0		ND
17	0		0		0		6	4.7			0		ND
18	0		2	5.5	2	6.2	2	5.5	3	5.7	3	6.7	ND
19	0		0		0		0				0		6.2
20	0		2	6.2	4	6.7	4	6.2	3	5.5	3	5.5	ND
21	0		2	6.7	2	5.5	2	5.5	2	6.5	3	4.5	ND
22	0		0		0		0				0		6.3
24	0		3	5.2	6	5.2	2	5.5	3	5.2	4	4.2	ND

* 0 indicates that no CPE could be observed after 2-3 serial passages.
† ND, not done.

TABLE II. Virus Titers Obtained in Cell Cultures Other Than That Used for Growth.

Coxsackie-virus type	Grown in	Log TCD_{50} per ml	Log TCD_{50} per ml in other cell cultures					
			MK	HEK	HEL	HA	WI-38	KB
A9	MK	6.7		6.5	5.5	6.5	5.5	
	HEK	5.7	6.5		5.5	5.7	5.2	
	HEL	5.5	6.2	5.7		5.7	5.5	
	HA	6.2	6.2	6.2	5.7		5.7	
	WI-38	4.5	6.2	5.7	5.2	5.7		
A15	HEK	5.7			4.2	5.5	4.2	4.7
	HEL	3.7		5.2		4.7	3.7	4.2
	HA	5.5		5.7	4.2		4.2	4.7
	WI-38	3.5		5.2	3.7	4.7		4.2
	KB	4.5		5.2	4.2	5.2	4.2	

failing to produce CPE in various cell cultures were passed and titrated in suckling mice. Table I presents the results of these tests. It can be seen that among primary cell cultures, human amnion cells best supported the growth of these viruses. Next in susceptibility were human embryonic kidney cultures, and close behind were human embryonic lung cells. WI-38 and KB cells were susceptible to 12 and 9 strains, respectively. Monkey kidney cells, on the other hand, were the least susceptible and did not support the growth of any of the types hitherto unadapted to them by other workers. The highest titers were generally obtained in cell cultures showing maximal CPE within the shortest time. Three blind passages did not produce any observable CPE with types that failed to produce CPE after 2 such passages.

Comparison of virus titers obtained in cell cultures other than those used for growth. Harvests of types A9 and 15 in MK (type A9 only), HEK, HEL, HA, WI-38 and KB (type A15 only) were tested for infectivity titer in all other cell cultures that supported their growth. Results are summarized in Table II. Both types showed variations greater than 2 logs in infectivity titer of the harvest when grown in the various cell cultures. However, the titers obtained upon assay of the various harvests in the same cell system varied by no more than 0.8 log—and usually less than 0.5 log—regardless of the type of culture in which the virus had been grown initially. Thus, under these experimental conditions, variations in cell susceptibility rather than in the virus play a major role in determining the infectivity titers of various virus stocks.

$MgCl_2$ stabilization to thermal inactivation. Aliquots of all 23 prototype stock viruses, stabilized with 1 M $MgCl_2$ were heated as described above, and then titrated along with control samples without $MgCl_2$. All preparations were tested in the same cell system used for the propagation of the stock viruses. Types 1, 5, 6, 19 and 22 were tested in suckling mice as none of the cell cultures used in this study supported the growth of these viruses. Types 11 and 13 as aliquots of HEL-grown stock viruses (which showed titers about 2 logs lower than those of HEK-grown virus stocks), were tested in HEL cell cultures in order to observe $MgCl_2$ stabilization in a system known to give sub-optimal infectivity titers. With other types, either HEK or WI-38 cells were used. Results are presented in Table III. It can be seen that molar $MgCl_2$ protected all prototype strains from thermal inactivation (50°C for one hour) except those derived from suckling mouse tissues.

Types 1, 5, 6, 19 and 22 used as suckling mouse tissue extracts and titrated in suckling mice were not inactivated by heating at 50°C for one hour without $MgCl_2$. Further 10-fold dilution of these viruses did not cause any change in their thermal resistance. At this point, it was thought that such resistance to thermal inactivation might be peculiar to types requiring suckling mice for their propagation. However, when stocks of coxsackievirus types A2 and A4 grown in suckling mice were subjected to heating in the absence of $MgCl_2$, they were not significantly inactivated even when diluted 10^{-2}, although the

TABLE III. Effect of Molar $MgCl_2$ on Thermal Stability of Group A Coxsackieviruses (50°C for One Hour).

Coxsackie-virus type	Log TCD_{50} (per ml) or LD_{50} (per 0.05 ml)			
	Stock virus	Stock virus after heating in H_2O	Stock virus after heating in $MgCl_2$	Test system
A1	6.5	6.5	6.5	*Suckling
2	3.7	<1	3.5	HA
3	4.5	<1	4.2	HA
4	4.7	<1	5.2	HA
5	7.3	7.3	6.5	Suckling
6	6.2	6.0	6.3	"
7	6.5	<1	6.7	MK
8	5.5	<1	5.2	HA
9	6.7	<1	6.5	MK
10	5.7	<1	5.5	HA
11	5.7	<1	5.5	HEL
12	4.5	<1	4.7	HA
13	4.2	<1	4.7	HEL
14	5.7	<1	5.2	HEK
15	5.7	<1	5.5	HEK
16	5.7	<1	5.7	WI-38
17	4.2	<1	3.7	HA
18	5.7	<1	6.2	HEK
19	6.2	5.7	6.0	Suckling
20	6.7	<1	6.2	HEL
21	6.7	<1	6.7	HEL
22	6.3	6.0	5.8	Suckling
24	5.2	<1	4.7	WI-38

* Suckling mice.

tissue-culture-grown stocks of these same types were inactivated by heat unless $MgCl_2$ was present (Table IV). Parallel tests were also carried out with another enterovirus, echovirus 9, using stocks grown in suckling mice and in tissue culture, and similar results were obtained. In another experiment, one part of MK-grown coxsackievirus type A9 was mixed with 9 parts of 20% suspension of normal newborn mouse tissues and kept at 4°C overnight. This preparation as well as aliquots of control MK-grown type A9 virus were simultaneously tested for $MgCl_2$ stabilization. It was found that mouse tissue suspension gave complete protection against thermal inactivation under the conditions of the test. The results of this experiment are also included in Table IV.

Discussion. Significant advances in the propagation of group A coxsackieviruses have been made in recent years(3,5,8). All group A coxsackieviruses except types 1, 5, 6, 19 and 22 had been already adapted by other workers to grow in cell cultures before the present study was undertaken. Three serial passages of the remaining 5 types in MK, HEK, HEL, HA, WI-38 and KB cell cultures at this laboratory failed to produce any observable CPE. With the other 18 prototypes, however, we were able to confirm the results obtained by other workers and extend the host cell range of these viruses to certain other easily obtainable cell cultures. A comparative study of the infectivity titers of harvests of a single virus type but grown in different cell cultures indicated that susceptibility of cell cultures used for growth and for titration plays a major role in determining the infectivity titer. Although titers varied markedly in harvests obtained in different cell systems, little variation in titer of the harvests was observed in assays carried out in one cell system.

The property of $MgCl_2$ stabilization to thermal inactivation was established for all A coxsackieviruses so far adapted to grow in cell culture. However, types that grew in suckling mice were not inactivated, even at 10^{-2} dilutions, by heating at 50°C for an

TABLE IV. Protective Effect of Suckling Mouse Tissues on Thermal Sensitivity of Coxsackieviruses A2, A4, and A9, and Echovirus Type 9.

Virus type	Log LD_{50} (per 0.05 ml) or TCD_{50} (per ml)					
	Virus grown or suspended in suckling mouse tissues*			Virus grown in cell culture		
	Untreated	Heated in H_2O	Heated in $MgCl_2$	Untreated	Heated in H_2O	Heated in $MgCl_2$
Coxsackievirus A2	6.8	6.2	6.0	3.7	<1	3.5
" A4	7.3	7.2	7.3	4.7	<1	5.2
" A9	7.2	6.7	6.5	7.2	<1	6.5
Echovirus 9	5	4.5	4.6	5.5	<1	5.5

* Coxsackievirus type A9 was suspended in normal suckling mouse tissues; all others were grown in suckling mice.

hour in the absence of molar $MgCl_2$. This protective effect was shown to be due to the presence of suckling mouse tissue extract and was not an inherent property of these types. Coxsackievirus types A2 and A4 and echovirus type 9 as infected suckling mouse tissue extracts and as infected cell culture fluids were tested for $MgCl_2$ stabilization. It was found that in contrast to suckling mouse tissue materials, the viruses in cell culture fluids were readily inactivated by the heat treatment in the absence of molar $MgCl_2$, but stabilized in the presence of the salt. Furthermore, MK-grown coxsackievirus type A9 could be protected against thermal inactivation by suspending it in normal newborn mouse tissue extract.

Summary. The susceptibility of primary cultures from monkey kidney, human embryonic kidney, human embryonic lung, and human amnion cells, and of WI-38 and KB cells, to 23 prototype strains of group A coxsackieviruses was studied. All types except A1, 5, 6, 19 and 22 could be propagated in one or more of the various cell cultures used. The property of $MgCl_2$ stabilization was established for all 18 prototype viruses that can be grown in tissue culture.

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Mutation in *Drosophila* by Methylazoxymethanol, the Aglycone of Cycasin.* (32256)

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Cycasin, methylazoxymethanol-beta-glucoside, is a naturally occurring compound that has been isolated from cycad plants(1). Cycasin has been found to be toxic and carcinogenic when fed to conventional laboratory rats but not when fed to germfree rats(2). The evidence indicates that in germfree rats beta-glucosidase is not present to hydrolyze cycasin and release the aglycone, methylazoxymethanol (MAM), which is toxic and carcinogenic in rats(3,4), induces reverse mutation in *Salmonella*(5), and is an active alkylating agent(6). Cycasin is radiomimetic in *Allium* (onion) seedlings(7) which show beta-glucosidase activity. The present study reports the results of tests for mutagenesis in *Drosophila* using the induction of sex-linked recessive lethals in males from feeding cycasin, MAM, and MAM-acetate.

Material and methods. Tests for sex-linked recessive lethals in *Drosophila melanogaster* were carried out using the Muller-5 technique. Wild type males which were subjected to chemical treatment were taken from the Canton-S strain. *Drosophila* were reared on the corn meal, molasses, dried yeast, agar, propionate medium of Sandler. The medium was inoculated with the live propionate-resistant Y-2 yeast strain of Wagner.

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