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Hemadsorption (Adsorption-Hemagglutination) Test for Viral Agents in Tissue Culture with Special Reference to Influenza. (23884)

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In a recent publication(1) we described "adsorption-hemagglutination" which occurs when appropriate erythrocytes are added directly to monkey kidney cell cultures infected with influenza A virus. The present report indicates some practical applications of this reaction, now designated as *hemadsorption,* in the diagnosis of influenza, mumps, and CA (croup-associated) virus infections. In addition, its usefulness with other viruses was investigated using prototype strains in tissue culture systems.

Materials. Several types of trypsinized monolayer cell cultures propagated in usual growth media were used. Most experiments employed monkey kidney (MK) cells prepared by modification of standard methods (2); chick embryo and monkey testicular cells were similarly processed; amnion cultures were made according to procedure developed in this laboratory(3); HeLa and bovine kidney cells were purchased from Microbiological Associates. Tissue culture (TC) tubes

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TABLE I. History of Virus Strains at Time of Hemadsorption Test.

Virus	Designation	Material tested	Source	No. of strains tested
Influenza	A/US/57	Throat swab	Unit*	33†
Mumps	26727 (urine)	MKTC	J. P. Utz	1
	FS-2064	Throat swab	Unit	1
CA (croup-associated)	Greer	MKTC	R. M. Chanock	1
	FS-CA	Throat swab	Unit	5
Sendai	Prototype	MKTC	R. M. Chanock	1
	Sendai-CDC	Egg	K. K. Takemoto	1
NDV (Newcastle)	F1234-13	Egg	L. Loomis	1
Vaccinia	MK 22	MKTC	J. Warren	1
Simian, SV 2,4,5,12,15	Prototypes	MKTC	C. P. Li	1 each
EMC	Entebbe	Mouse brain	L. Kilham	1
Herpes simplex	Zacchardi	HeLa TC	P. Halonen	1
Poliovirus 1,2,3	Prototypes	MKTC	Unit	1 each
Coxsackie A9, B1-B5	"	MKTC	Unit	"
ECHO 1-11	"	MKTC	K. Habel	"
Adenovirus 1,2,4,6,7,8	"	HeLa TC	W. P. Rowe	"

* *Unit* refers to the Laboratory Unit, Epidemiology Section, Lab. of Infect. Dis.
† 7 strains related to A/Md/1/55; 26 strains related to A/Jap/305/57.

were maintained in suitable media enriched with serum, but at time of inoculation were changed to Eagle's basal medium (4) with 2% fresh glutamine usually without serum. Histories of virus strains tested for hemadsorption are recorded in Table I. Influenza immune rooster serums were produced in our laboratory or obtained as standard merthiolated typing serums from International Influenza Center for Americas of PHS Communicable Disease Center. Dr. R. M. Chanock and Dr. J. P. Utz kindly supplied us with guinea pig and monkey antiserums used in CA virus and mumps identification. Citrated guinea pig, chicken, rhesus monkey, human O, and defibrinated sheep erythrocytes were washed 3 times and made to 0.4% suspension by volume in sterile physiological saline or balanced salt solution (BSS). Antibiotics were added to some batches.

Methods. Virus isolation procedure by hemadsorption test consisted of inoculating TC tubes with 0.1-0.2 ml of specimen material after replacing fluid with Eagle's basal medium. Tubes were incubated at 37°C and, at least with influenza field isolates, early demonstration of hemadsorption was enhanced by rotating them in a drum. After appropriate period of incubation 0.2 ml of erythrocyte suspension was added to each tube whether or not cytopathic effect (CPE) was observed. The tubes were left in inclined position 3 to 5 minutes, revolved to resuspend settled red blood cells, and examined under low magnification (×100-150). The hemadsorption test was read *negative* when unclumped erythrocytes were observed floating in supernatant without significant adsorption to TC cell sheet. Negative tubes were replaced in revolving drum and periodically examined 6-8 days. Because of inevitable lysis of erythrocytes on incubation they were replenished every 2 days. Test was read *positive* when there was evident adsorption of variable number of erythrocytes onto monolayer sheet in characteristic pattern described below. The tissue cell sheet was then scraped off and cellular debris, including erythrocytes, were homogenized in tissue grinder or mortar, using supernatant as diluent. One-tenth ml of homogenate was added to each of several monkey kidney tubes for passage. Because on second passage the CPE was invariably observed, typing was done after early cytopathic changes appeared. With mumps and CA viruses, the supernatant alone was used for passage and identification. We used one or both of following procedures to type the isolates. 1) *Hemadsorption-inhibition test.* After removing fluid from inoculated and control tubes they were washed once with BSS. Sixth-tenths ml of BSS were added

TABLE II. Hemadsorption Reaction with Influenza and Mumps Viruses.*

TC system	Monkey kidney					Human amnion		Monkey testis	Bovine kidney
Erythrocytes	Guinea pig	Chick	Sheep	Human O	Rhesus	Guinea pig	Chick	Guinea pig	Guinea pig
Influenza A	+	+	+	+	+†	0	0	0	0
Mumps	+	+	+	+	+	+	+	+	+

* + = Hemadsorption demonstrated. 0 = Hemadsorption not demonstrated, but no evidence of viral propagation.

† Not all field isolates positive.

to each tube to be serum treated and 0.8 ml BSS to uninoculated, virus control, and isolate control tubes. At least 2 tubes in each set were treated with appropriate antiserums in 0.2 ml amounts of 1:10 dilution. After 15 minutes incubation in slanted rack at room temperature each tube was treated with erythrocyte suspension, returned to the rack for about 3 minutes, and then examined under microscope. 2) *Neutralization-hemadsorption test.* Material from initial passage and serum were diluted 1:10 and 0.1 ml amounts of each was inoculated into several cell cultures. The tubes were incubated either until early CPE appeared in control tubes or for time estimated from first passage to allow viral propagation. Erythrocyte suspension was then added and tubes examined. Comparative studies of influenza isolates in embryonated eggs, employed technics in standard use at NIH (Eddy, B., unpublished). After 2 passages of each specimen in 10-11-day-old embryos, the fluids were tested for hemagglutination by the Salk pattern test(5). Positive specimens were titrated and typed by hemagglutination-inhibition using rooster immune serums treated with *V. cholerae* filtrate.

Results. Influenza. We demonstrated hemadsorption in MK cultures infected with influenza A virus using erythrocytes of guinea pig, chicken, sheep, human type O, and rhesus monkey origin (Table II). Attempts in other cell cultures including human amnion, monkey testis and bovine kidney have been unsuccessful apparently due to failure of influenza viruses to propagate in these TC systems. Tubes inoculated either with TC adapted strains or specimens from suspected cases were first examined for hemadsorption after 40 hours of incubation. Fig. 1a and 1b show a positive early reaction in different portions of the tube: the cell sheet is studded with adherent guinea pig erythrocytes in an easily recognizable characteristic pattern of *clusters,* oval or elongated *rosettes* (formed when individual or adjacent cells are surrounded by erythrocytes), and *chain formations* (Fig. 1a), some of which apparently follow lines of cleavage between MK cell groupings in *streamer* effect (Fig. 1b). These formations are commonly seen in tubes with limited viral propagation, but in later stages the entire sheet may be literally plastered with adsorbed red blood cells (Fig. 1c). At this time it is not uncommon to find visible *clumps, i.e.* irregular aggregates of truly hemagglutinated erythrocytes, in the fluid phase indicative of viral release from TC cells. For comparison, a similarly treated uninoculated MK cell culture of same age and a poliovirus-infected tube without hemadsorption are shown in Fig. 2a and 2b. The erythrocytes seen in these 2 photographs were actually slowly drifting across the field. CPE due to influenza field strains often did not appear on initial passage, although presence of hemadsorption in infected cultures indicated viral propagation in tissue cells. Because at this stage the nutrient fluid generally did not contain demonstrable virus, infected TC cells had to be included in the passage material. When specific CPE was observed on initial examination in a large enough number of tubes, the hemadsorption-inhibition test was done upon demonstration of hemadsorption in some cultures, resulting in positive identification of field strains in less than 2 days. Alkaline pH of TC medium exerts a marked effect on cultivation of influenza virus. Table III summarizes results of experiment in which the same known positive throat washing specimen was inoculated into 3 groups of MK tubes ad-

justed to pH 6.5, 7, and 8. Intermediate pH delayed viral propagation, while higher pH actually prevented demonstration of the virus in the field specimen. In addition, a MK-established influenza virus strain, previously isolated from this field specimen, was titrated at pH 6.5 and 8 and 100 times more virus was demonstrated at lower pH. This sug-

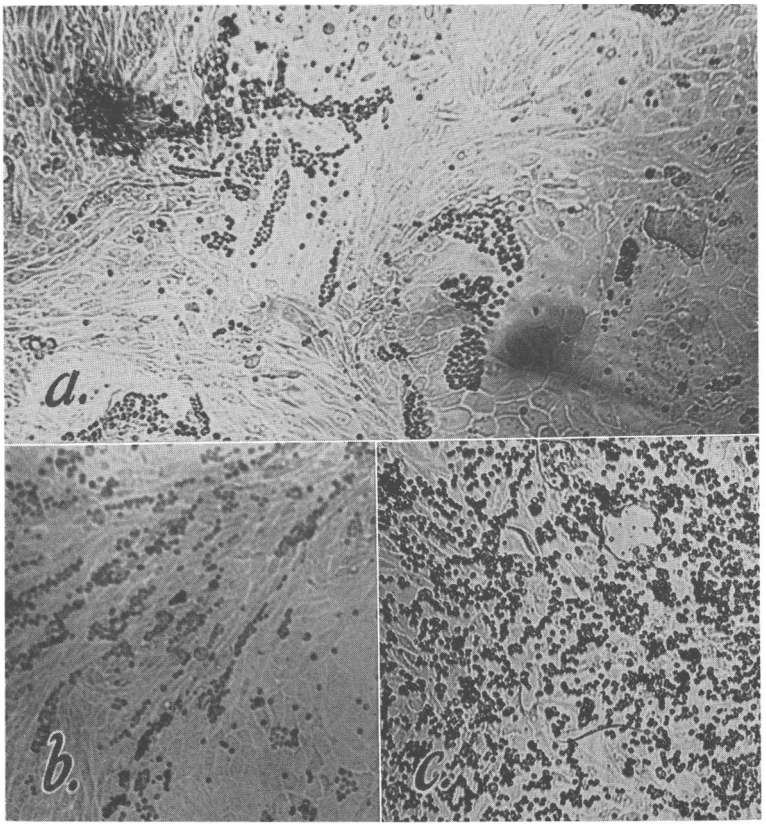

FIG. 1. Influenza A virus in MK culture. Hemadsorption with guinea pig erythrocytes at 40 and 100 hr (×105). *a.* Early effect (before CPE): clusters, chains, rosettes. *b.* Same tube, another field: streamers. *c.* Late effect (after CPE).

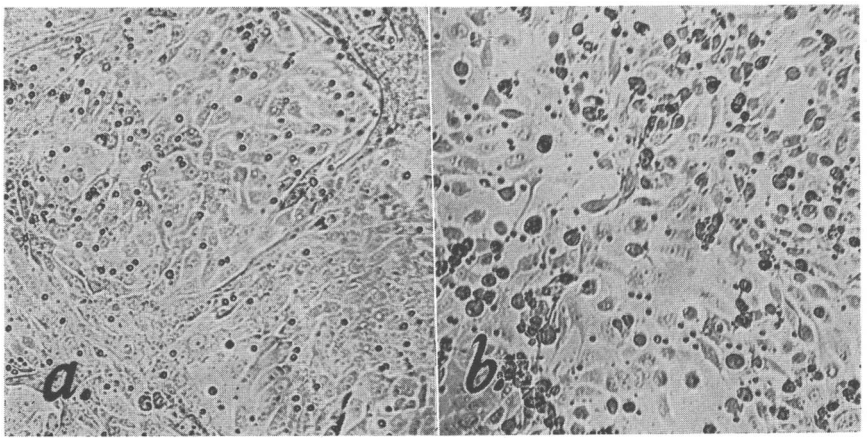

FIG. 2. MK cultures *without* hemadsorption of guinea pig erythrocytes (×105). *a.* Uninoculated control. *b.* Poliovirus, type 1 (early CPE).

TABLE III. Effect of pH of MK Maintenance Medium on Detection of Influenza Virus in Throat Washing Specimen by Hemadsorption Test.

	Days after inoculation		
	2	4	7
pH 6.5	+	+	+
7.0	0	+	+
8.0	0	0	0

+ = Hemadsorption demonstrated.
0 = " not demonstrated.

TABLE IV. Isolation of 1957 Influenza A Strains by Hemadsorption and Egg Technics.

Specimen group	No. of specimens	Hemadsorption positive	Embryonated egg positive	Total positive specimens
I	23	8	8	8
II	54	11	2	12*
III	16	2	0	2
Total	93	21	10	22*

* Includes 1 specimen negative in TC but positive in eggs.

gests that as much as 2 logs of infectious virus contained in aliquot of field specimen can be suppressed in TC by unfavorable pH.

An important demonstration of practical usefulness of hemadsorption test for influenza virus comes from its efficacy as compared to embryonated egg procedures. Table IV summarizes results of inoculation of both isolation systems with specimens from 3 outbreaks of Asian influenza in the United States. The first group of 23 was collected and stored under optimal conditions of CO_2 ice and 8 viral strains isolated from the same 8 specimens by both technics. Using hemadsorption test 6 of 8 strains were detected at 40 hours (the other 2 were demonstrated at 24 and 72 hours). The second and third groups of specimens, although properly collected, were subjected to thawing and freezing and storage at −20°C, undoubtedly with some loss of the infective virus. From these groups 13 strains were isolated and typed by hemadsorption reaction and only 2 by egg technic. In group II there was a single egg positive specimen missed in TC, but 10 other TC isolates were not detected in eggs. Thus, of 93 specimens tested at same time by both methods, 21 were found positive by TC-hemadsorption and only 10 by egg-HA systems.

We have never failed to type a strain of influenza virus by this technic. The reaction is apparently specific and heterologous immune serums produced in roosters, monkey, rabbits, and guinea pigs against other types of influenza. Coxsackie A and B, mumps, ECHO, CA, and polioviruses did not inhibit it.

Mumps. Studies with mumps virus were restricted to 2 strains. A recent isolate from patient's urine by Utz and coworkers(6) on propagation in MK cells produced strongly positive hemadsorption. The other strain was isolated and typed by hemadsorption reaction from routine throat swab of clinical case. This virus was reisolated 3 times in MK and amnion cultures and repeatedly identified as mumps by neutralization-hemadsorption and hemadsorption-inhibition tests. Fig. 3b shows early characteristic hemadsorption pattern when no CPE due to mumps virus could be detected, (Fig. 3a) and Fig. 3d shows effect 24 hours later when probable CPE was noted (Fig. 3c). Unlike influenza virus, mumps readily propagated in human amnion (maintained in Eagle's medium with 4% horse serum). It also propagated in monkey testicular and bovine kidney cell cultures as evidenced by development of hemadsorption after a period of negativity (Table II). Hemadsorption test may be of considerable advantage in rapid laboratory diagnosis of mumps.

Croup-Associated (CA) Virus. A MK passaged prototype strain (Greer) produced cytopathic effects and hemadsorption with guinea pig cells in MK, human amnion, and HeLa cells. Five cytopathogenic viruses isolated in MK cultures from throat swabs collected from patients with undifferentiated respiratory illness in 1955, were identified as CA virus by hemadsorption-inhibition (using normal, mumps, Sendai, and CA guinea pig antiserums). Hemadsorption reaction appears to be convenient and reliable method for identification of this virus.

Other pathogenic viruses.‡ Prototype strains of other viruses pathogenic for human beings were tested for hemadsorption in appropriate TC systems and those belonging to

‡ Exploratory experiments at Rockefeller Foundation Virus Laboratories suggest that hemadsorption may occur with certain arthropod-borne viruses.

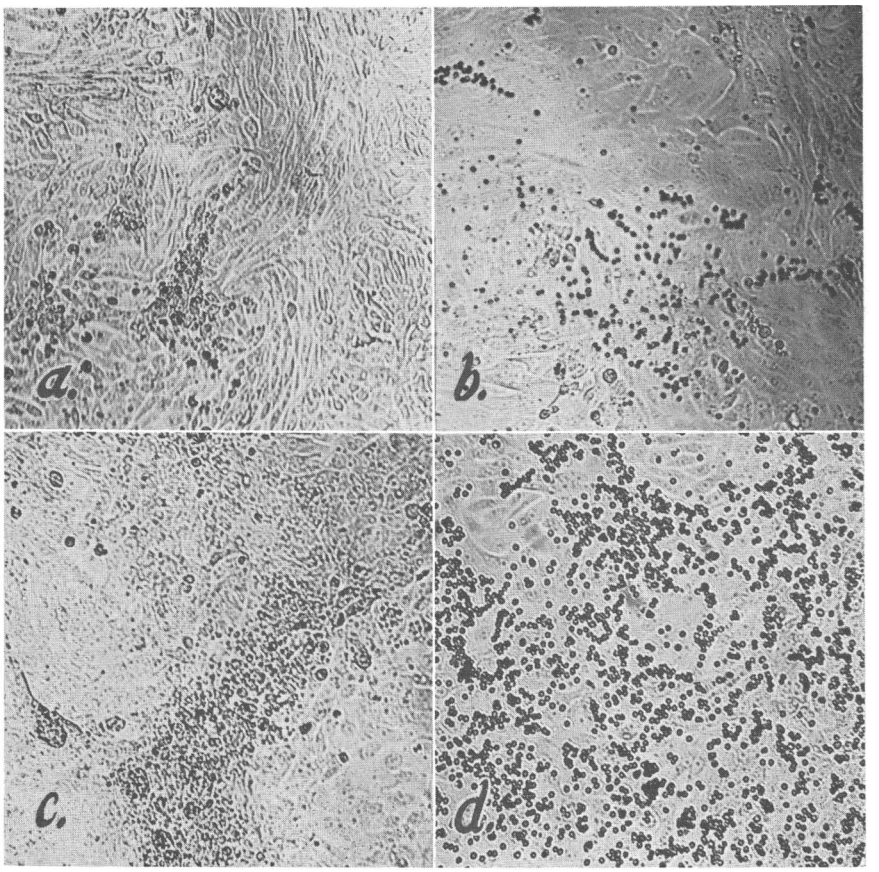

FIG. 3. Mumps virus in MK culture. Hemadsorption with guinea pig erythrocytes at 48 and 72 hr (×105). *a.* 48 hr: no CPE. *b.* Same with erythrocytes added. *c.* 72 hr: probable CPE. *d.* Same with erythrocytes added.

hemagglutinating group were generally positive. Table I summarizes origin and history of these strains. *Sendai.* Two strains from different sources after passage in MK produced hemadsorption with both guinea pig and chicken cells. *Newcastle disease virus (NDV).* An egg-adapted strain was propagated in chick embryo monolayer cultures as shown by hemadsorption with chicken and guinea pig erythrocytes for each of 5 serial passages. *Vaccinia.* MK adapted vaccinia strain produced heavy hemadsorption with erythrocytes from 2 individual chickens but not with guinea pig or sheep cells. The virus was propagated for 5 passages in chick embryo TC with invariably positive hemadsorption with chicken cells from the same 2 birds. *Encephalomyocarditis* (EMC). No viral propagation could be demonstrated upon repeated inoculation of EMC-infected mouse brain into MK and chick embryo TC. Hemadsorption was always absent, although on addition of brain inoculum suggestive clumping of sheep erythrocytes occurred in virus-laden nutrient fluid. This was not observed with guinea pig or chicken cells. *Other prototype virus strains propagated in MK cultures.* Poliovirus types 1, 2, 3, and herpes simplex virus propagated in MK cell cultures failed to cause hemadsorption with guinea pig, chicken, and sheep red cells. Coxsackie A9, B1 through B5, and ECHO virus types 1 through 11, were also negative with guinea pig and human erythrocytes. Attempts with adenovirus types 1, 2, 4, 6, 7, and 8 were equally unsuccessful with guinea pig and sheep cells. *Simian viruses.* Occasionally, upon incubation of certain batches of uninfected control tubes with added erythrocytes, we found that red cells adhered to the monolayer MK cell

sheet. Because characteristic patterns of tight clusters, rosettes, and streamers were missing, we named this appearance *sticking* of erythrocytes to distinguish it from the typical pattern of hemadsorption. Sticking seemed to be particularly troublesome with certain lots of trypsinized MK cells and we suspected it to be due to simian orphan viruses. To elucidate this problem we studied the behavior of Hull's virus types SV 2, 4, 5, 12, and 15 reported by him to cause hemagglutination under variable conditions(7). We repeatedly passaged prototype strains with production of characteristic CPE and regularly checked the cultures for presence, intensity, and character of reactions with guinea pig, chicken, human O, and rhesus monkey erythrocytes. Our results with type SV4 were consistently negative regardless of species of erythrocytes used or conditions provided. The other prototype strains gave erratic results on many trials, ranging from negative to reactions resembling familiar hemadsorption patterns. The results apparently depended on such factors as incubation temperature (4°, 22°, 37°), whether readings were taken after a few minutes, several hours, or overnight contact of erythrocytes with the TC sheet, and whether contact was constant in a stationary rack or intermittent in revolving drum. Adherence of erythrocytes to MK cells caused by hemagglutinating simian viruses and the sticking observed in our uninoculated cultures were distinguishable from what we described as hemadsorption. However, it is possible that some simian viruses pre-existing in MK cells might produce the same type of pattern as with other hemagglutinating viruses.

Use of hemadsorption reaction in serologic studies. Paired serums from 4 patients with known 8 to 16-fold rise of hemagglutination-inhibiting antibodies against Asian strain of influenza virus were heated at 56°C for 30 minutes, but were not subjected to other special treatment to remove inhibitors. Serial serum dilutions were tested by neutralization-hemadsorption test against approximately 500 $TCID_{50}$ of a TC adapted cytopathogenic strain of Asian influenza virus. Demonstrable antibody rise as measured by prevention of eventual hemadsorption was similar to that shown by standard hemagglutination-inhibition test. The hemadsorption-inhibition procedure, which we preferred for typing isolates with potent immune animal serums, was less satisfactory because repeated checking of virus-inoculated control tubes for hemadsorption was necessary to determine optimal time for performing inhibition test. When incubation proceeded to a point of recognizable CPE excessive amounts of virus were usually present.

Discussion. Demonstration of specific erythrocyte adsorption by several important hemagglutinating viruses propagated in TC systems provides a practical approach to diagnostic and fundamental studies of these agents. Because hemadsorption depends on direct visualization, it is detectable in presence of minimal hemagglutinins associated with TC cells, which results in greater sensitivity than with procedures based on development of CPE or the usual hemagglutination by virus suspensions from TC supernates or egg fluids. The technic was simple, rapid, and specific. Hemadsorption reaction is a useful technic for identification not only of viruses which produce otherwise undetectable hemagglutinins in TC fluids but also of agents which may propagate without apparent damage to TC cells. Undoubtedly it will find application in fundamental studies of virus-cell relationship.

Our continuing experience with this reaction prompted us to adopt the name of *hemadsorption* in place of *adsorption-hemagglutination* proposed initially(1). Indeed, even though the phenomenon undoubtedly is due to hemagglutinin production within infected cell, the particular practical advantages of this reaction are due to selective erythrocyte adsorption onto monolayer surface of cultured tissue cells rather than three-dimensional clumping of erythrocytes, that is, true hemagglutination.

Summary. The phenomenon of hemadsorption is dependent upon selective attachment of erythrocytes onto the monolayer surface of tissue culture cells. It is demonstrated by addition of erythrocytes to a tissue culture system in which propagation of a hemagglutinin-producing virus has occurred. We used

this reaction as a means of virus detection in isolation, titration, and identification of influenza A, mumps, and CA (croup-associated) viruses. Prototype strains of 2 other myxoviruses (Sendai and NDV) and vaccinia produced hemadsorption in appropriate cell cultures. Prototype strains of common nonhemagglutinating viruses (herpes, Coxsackie, ECHO, adeno- and polio-viruses) gave negative results. Under some conditions hemagglutinating simian agents, which may be encountered in monkey kidney cell cultures, caused adherence of erythrocytes to the tissue cell sheet, but the characteristic patterns seen with myxoviruses were not observed. We found hemadsorption to be a simple, direct, and reliable test offering a new approach to diagnosis of some viral infections. It may also provide an investigative tool for research on virus-cell relationships.

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Influence of Female Sex Hormones in Experimental Teratogenesis.* (23885)

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Studies of congenital malformations deserve increased emphasis because of their importance as a significant health problem. Many investigators have demonstrated experimental production of mammalian congenital defects by alteration of maternal environment. The methods have included: nutritional deficiences(1-4), vitamin deficiencies (5-7), radiation(8-10), hypoxia(11-12), and more recently, cortisone(13-15). Present data indicate that under certain conditions estrogens are also teratogenic.

Materials and methods. Virgin female mice of the Webster-Swiss strain, weighing around 25 g, were employed. They were kept at least 2 weeks after shipment, and used after reaching the indicated weight. They were fed Rockland mouse diet in pellet form.‡ We employed 2 series: (A) Estrone ("Theelin") in peanut oil (1 mg/cc); and (B) Estradiol Benzoate in sesame oil (1 mg/cc). As a control for group (1), nothing was given to a comparable number of mice; and for group (2), a similar volume of sesame oil was administered. Injections were given between 10:00 and 11:00 a.m., except those on eleventh day of gestation, done at 5:00 p.m. During administration No. 22 gauge needles were inserted subcutaneously from median part of back into subcutaneous layer of left lateral breast region. This eliminated any spillage of injected material through the puncture wound. Preliminary experiments were performed upon a large number of mice to decide upon a proper dose of estrone and estradiol benzoate. 0.2 mg daily was the dosage level at which the incidence of cleft palate was highest, and the

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