tests observed in some experimentally induced non-immunologic anemias(12). It is possible that this phenomenon may have contributed to the positive reactions found with the azo dyes since the anemia in each case was followed by a good reticulocyte response. However, our demonstration of strongly positive Coombs' tests *in vitro* with trypan blue suggests that the agglutination of immature red blood cells was not the major factor contributing to the positive *in vivo* tests(1).

Summary. In an earlier investigation the activity of the diazo dye trypan blue in producing an anemia with positive Coombs' test was attributed primarily to its capacity for binding with protein and thus either altering the surface of erythrocytes or serving as a prosthetic group for the attachment of serum proteins. The present study of the activity of a number of structurally related compounds supports this hypothesis, for only those dyes which appeared in the serum in high concentration, with a large proportion of non-protein bound (free) dye, induced protein coating of the erythrocytes. In general, the active dyes had structural formulae similar to trypan blue and the diazo linkage, as well as the presence of the amino group, appeared to be essential.

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Differences in Cytopathic Effects of Adenovirus in Monkey Kidney Tissue Culture. (30353)

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Presence of a soluble group complement fixing antigen(1,2,3), absence of pathogenicity for laboratory animals(1), ether resistance (1), DNA nucleic acid configuration(3), 60-85 mμ size range(3), and production of characteristic cytopathic effect (CPE) in continuous cell cultures of epithelial origin(2) are characteristics which ostensibly place viruses in the adenovirus group. There are at present 28 recognized human types(2), 2 additional serotypes pending recognition(4), and numerous other adenovirus types of simian, bovine and perhaps avian origin(3).

Denny and Ginsberg(5) have suggested 2 subgroups based on neutralization kinetics, virus multiplication cycles and virus yields. Reports from Rosen(6) and Simon(7) indicate the presence of natural subgroups based on hemagglutination tests with monkey and rat erythrocytes.

The classical CPE of adenovirus in monkey kidney tissue culture was first described by Rowe and co-workers(1). This study included only types 1 to 6 and identical changes were described for all types. Rosen *et al*(8) reported differences in CPE in Rhesus monkey kidney cultures with types 1, 2, 3, and 5 as opposed to 9, 10, and 13, but details were not given. Rafajko(9) suggested a possible separation of adenovirus types 1 to 18 into three

TABLE I. Biography of Adenovirus Strains Used.

Adeno-virus type	Strain identifi-cation	Source	Passage history
1	Adenoid 71*	R. J. Huebner	HeLa/5, KB/9
1	C-2629	H. G. Cramblett	SSK/3†
1	C-3134	*Idem*	SSK/3
2	Adenoid 6*	R. J. Huebner	Adenoid, HES/2, HeLa/15, KB/10
2	11252	H. G. Cramblett	MK/1, SSK/2
2	12702	*Idem*	MK/1, SSK/2
2	C-346	"	SSK/4
3	G.B.*	R. J. Huebner	HeLa/7
4	R167*	*Idem*	HT/4,‡ HeLa/17, KB/9
5	Adenoid 75*	"	HeLa/4, KB/10
5	10492	H. G. Cramblett	MK/2, SSK/1
6	11060	*Idem*	SSK/3
6	Tonsil 99*	R. J. Huebner	HeLa/5, KB/6
7	Gomen*	ATCC	HeLa/12
7a	S-1058	R. J. Huebner	KB/4, HeLa/1, KB/6, HeLa/1, and KB/4
7	Pinckney	K. Schell	HEK/5, KB/5
8	Ijima	E. Jawetz	?/?, HeLa/11
8	Ishimine	*Idem*	?/?, HeLa/3
8	Koyama	"	?/?, HeLa/5
8	Trim*	R. J. Huebner	HeLa/11, KB/5
9	Hicks*	*Idem*	HeLa/3, KB/9
10	J. J.*	"	Pool of (a) KB/5, HeLa/1, (b) HeLa/7, KB/4
11	Slobitski*	"	HeLa/3, KB/8
12	Huie*	"	HEK/?, MK/?, KB or HeLa/12, KB/5
12	97838	K. Schell	HEK/3, KB/1, HEK/1, KB/1, HeLa/4
12	97844	*Idem*	HEK/3, KB/1, HEK/1, KB/1, HeLa/3
12	14510	"	KB/4, HEK/1, KB/1, HeLa/3
12	10534	"	KB/4, HEK/1, KB/1, HeLa/3
12	11476	"	HEK/7, HeLa/4
12	10531	"	MK/?, HEK/3, HeLa/4
12	10516	"	HEK/1, KB/2, HeLa/4
12	10096	"	?/?, HeLa/3
12	13316	"	HEK/1, KB/2, HeLa/3
12	15276	"	?/?, HEK/4
12	10904	"	?/?, HeLa/3
12	97313	"	?/?, HEK/1, HeLa/3
12	2196	"	?/?, KB/2, HEK/1
13	A. A.*	R. J. Huebner	HeLa/3, KB/4, HeLa/1, KB/5
14	DeWit*	*Idem*	Pool of: (2) HeLa/9, KB/8, (b) HeLa/9, KB/9
15	Ch. 38*	"	HeLa/2, KB/7
16	Ch. 79*	"	HeLa/1, KB/4, HeLa/1, KB/6
17	Ch. 22*	"	HeLa/1, KB/4, HeLa/1, KB/5
18	D. C.*	"	HeLa/4, KB/11

* Prototype strains. † Stable skin line. ‡ Human trachea.

groups based on differences in cytopathic changes. Only one strain per type was used, however, and it remained to be demonstrated whether this phenomenon was strain or type specific. It is the purpose of this communication to describe differences in CPE noted when a variety of strains of adenovirus types 1 to 18 were inoculated into primary Rhesus and African Green tissue cultures and the possible significance of the results.

Methods and materials. Tissue culture. Seven-day-old primary Rhesus *(Macacca mulatta)* and African Green *(Cercopithecus aethiops)* monkey kidney tissue cultures in 16 × 125 mm roller tubes were used in these studies.* Prior to use, confluent cell monolayers were rinsed thrice with 2 ml of Hanks' balanced salt solution (BSS) and refed with 1 ml Basal Medium Eagle's(10) in Earle's BSS containing 1% agamma calf serum.† Penicillin and Polymixin B (100 units/ml) and Streptomycin (100 μg/ml) were incorporated in all media. Tubes were inoculated with 0.1 ml virus and incubated at 37°C in a stationary or rolling position. Observations were made and recorded daily.

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TABLE II. Characteristics of 3 Different Forms of Adenovirus CPE in Primary Rhesus Monkey Kidney Tissue Culture.

Group 1, General (types 9, 10, 13, 15, 17)	Group 2a, Peripheral (types 3, 7, 11, 14, 16)	Group 2b, Focal (types 1, 2, 4, 5, 6, 8, 12, 18)
a. Cells become swollen, spherical, increasingly refractile with clearly demarcated borders.	a. Cells at periphery become increasingly granular and thickened. Edge of the cell becomes smooth and clearly demarcated. Little rounding of cells.	a. Cells become rounded and granular and clump together.
b. Infected cells remain single or paired, darker than surrounding normal cells and distributed equally throughout cell sheet.	b. Cells proceed to round and group together in large clusters at periphery. Central portion of cell sheet remains relatively uninfected.	b. Clusters of infected cells are evident in foci *throughout* cell sheet.
c. As CPE progresses, cells form larger clumps and slough off cell sheet.	c. Some cytoplasmic stranding evident at periphery.	c. Much cytoplasmic stranding is evident. This is the outstanding feature.
d. There is *no* evidence of cytoplasmic stranding or "bridging."	d. With increased CPE, infection spreads throughout monolayer and large clumps of rounded, granular cells are evident. Eventually these retract from cell sheet giving a "web-like" appearance indistinguishable from group 2b.	d. Foci of infected cells retract from glass surface. Edges of retracted areas are laden with rounded, granular cells and demonstrate excessive cytoplasmic "bridging," giving a "web-like" appearance to infected cell sheet indistinguishable from group 2a.

Viruses. Prototype strains of adenovirus types 1 to 18 were purchased from the American Type Culture Collection and passaged 3 times through KB tissue culture(11). These strains were obtained through the courtesy of Dr. Robert J. Huebner, Laboratory of Infectious Diseases, Nat. Inst. Health. Strains other than the prototype were obtained through the courtesy of Dr. Henry Cramblett, Dept. of Pediatrics, Children's Hospital, Columbus, Ohio; Dr. Klaus Schell, Dept. of Virus Research, Microbiological Associates, Inc., Bethesda, Md.; Dr. Ernest Herrmann, Section of Microbiology, Mayo Clinic, Rochester, Minn.; and Dr. Ernest Jawetz, University of California, Department of Microbiology, San Francisco.

Table I lists all strains used in this study, their source and passage history.

Prototype strains were tested in at least 6 different lots of Rhesus monkey kidney tissue culture before being placed into a particular group. All other strains were tested in no less than 2 different lots.

Virus infectivity titrations were carried out in 24-hour-old HeLa cells planted at 50,000 cells per ml in and maintained on Eagle's Minimum Essential Medium(13) in Earle's BSS with 5% agamma calf serum. Antibiotics were incorporated as previously described. Titrations were performed with serial 10-fold dilutions of virus, 4 tubes per dilution and 0.1 ml inoculum per tube. Tubes were read after 7 days and 50% endpoints calculated by the method of Reed and Muench(12). All titrations were repeated at least twice. Titers are expressed as $\log_{10}$ Tissue Culture Infective Doses—50% ($TCID_{50}$) per 0.1 ml.

Results. Rhesus kidney cultures were inoculated with adenovirus types 1 to 18 and microscopically observed daily. The quantity of virus inoculated per tube, in 0.1 ml varied from $10^{2.2}$ to $10^{7.0}$ HeLa $TCID_{50}$. At least 3 tubes were inoculated with each type.

Three different types or classes of CPE were detected, and all virus strains studied could be placed in one of the 3 groups. Table II summarizes the salient characteristics of the CPE for each group.

The characteristic "general" CPE of group 1 viruses readily distinguishes them from the other 2 groups. Fig. 1d and 1h illustrate typical group 1 CPE by adenovirus type 10, strain J.J., in Rhesus kidney culture. Infected cells become swollen, rounded, and refractile with clearly demarcated borders. They appear darker than surrounding normal cells and remain single or paired rather than in clusters and give the appearance of being loosely attached. The relatively even distri-

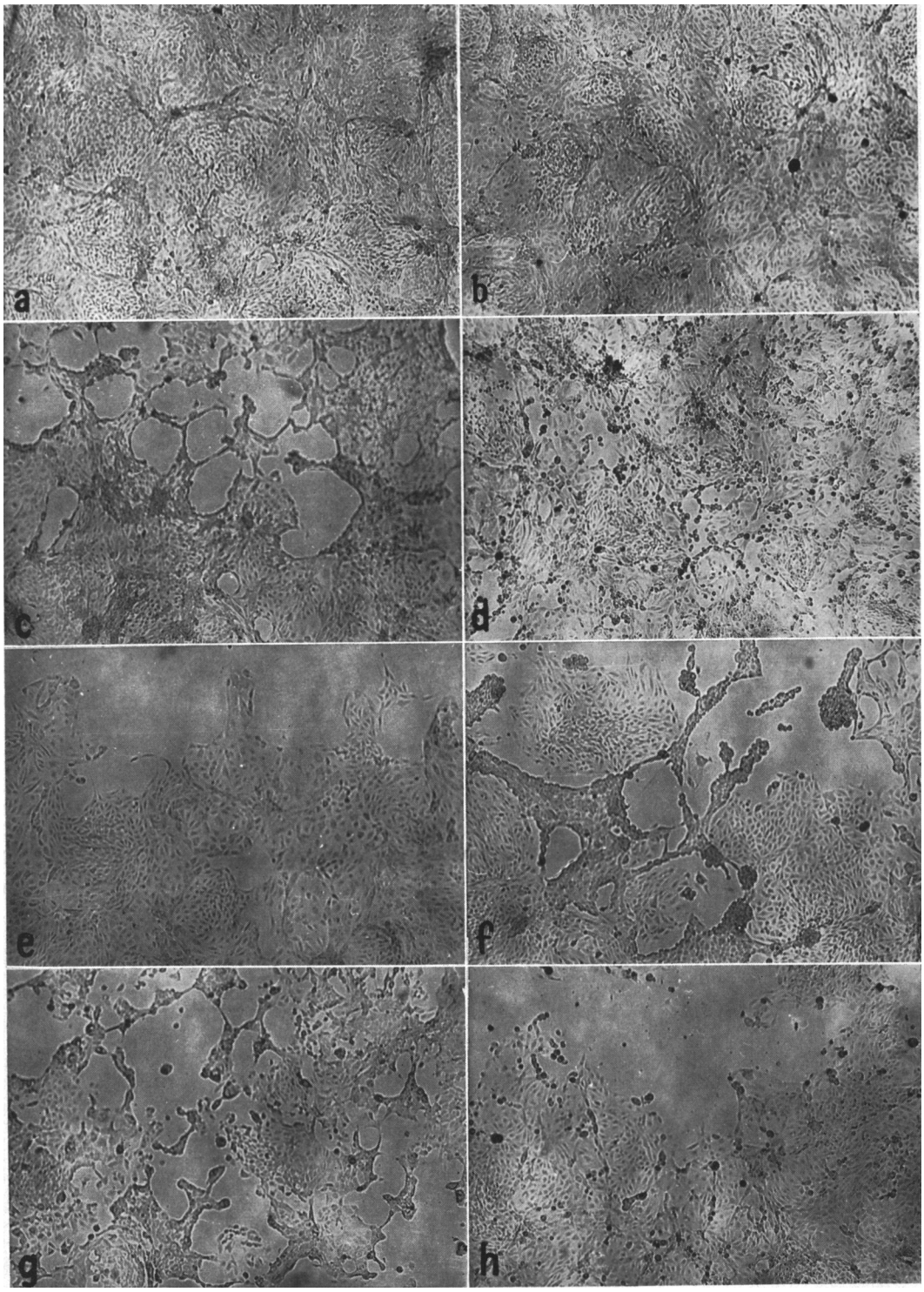

FIG. 1. Unstained primary Rhesus monkey kidney cells, 9 days old, 28 ×: (a) uninoculated control, center of monolayer; (b) adenovirus type 7 (Pinckney), 48 hr post inoculation,

center of monolayer; (c) adenovirus type 12 (Huie), 48 hr post inoculation, center of monolayer; (d) adenovirus type 10 (J.J.), 48 hr post inoculation, center of monolayer; (e) uninoculated control, periphery; (f) adenovirus type 7 (Pinckney), 48 hr post inoculation, periphery; (g) adenovirus type 12 (Huie), 48 hr post inoculation, periphery; (h) adenovirus type 10 (J.J.), 48 hr post inoculation, periphery.

bution of infected cells throughout the culture is striking. When early CPE is detected, little evidence of infection is noticed at the periphery of the cell sheet. No cytoplasmic stranding is evident. This is in the direct contrast to Groups 2a and 2b. Viruses of types 9, 10, 13, 15, and 17 make up Group 1 "general" (Table II).

Differences in CPE between Groups 2a and 2b were more subtle and required closer scrutiny although both were easily distinguished from Group 1. It was necessary to observe carefully the cell cultures daily, since the period during early CPE, *i.e.*, 1 to 2+, allowed for maximum differentiation. Group 2a "peripheral" CPE produced by type 7, strain Pinckney, in Rhesus kidney cultures can be seen in Fig. 1b and 1f. There appears to be a definite predilection by the virus for the periphery of the cell monolayer. Cells along the edge of the sheet increase in granularity and the border becomes smooth and sharply demarcated. As the infection progresses, cells begin to group along the periphery in large clusters with some evidence of cytoplasmic stranding. Interestingly, while the early changes occur in cells at the periphery, little if any indication of virus infection occurs in the central portion of the culture (Fig. 1b). This is in direct contrast with changes seen by viruses of Groups 1 and 2b. Progression of CPE by Group 2a viruses eventually renders them indistinguishable from Group 2b. Adenoviruses placed into this group include types 3, 7, 7a, 11, 14, and 16.

Focal cytopathic changes in Rhesus kidney cultures illustrated by type 12, strain Huie (Fig. 1c and 1g), are typical of Group 2b adenoviruses. Cells become granular, rounded, and clump together. Clusters of infected cells are evident at the periphery and in foci throughout the cell monolayer, particularly in areas where small explants are present. Extensive cytoplasmic stranding is prominent along the edges of the sheet and in central areas where portions of the cell sheet have retracted from the glass surface, giving a reticular or "web-like" appearance to the culture. Viruses in this group include types 1, 2, 4, 5, 6, 8, 12 and 18.

Since differing quantities ($10^{2.2}$ to $10^{7.0}$ $TCID_{50}$) of adenovirus had been inoculated into cultures it was necessary to determine if the differences in CPE were related to the quantity of inoculum. Therefore, 3 tubes were each inoculated with 100 HeLa $TCID_{50}$ of various adenovirus types from the 3 groups. Tubes were coded and randomly distributed in a stationary rack with uninoculated controls and incubated at 37°C. Cultures were read "blind" 24 and 48 hours later by an individual other than the person inoculating the tubes. Results from a typical experiment are seen in Table III. Ten cultures were inoculated with 4 different "general" types, *i.e.*, 9, 10, 15, and 17. Of these all were correctly identified. Types 3, 14, and 16 represented the "peripheral" group and 8 of 9 tubes were classified correctly. The single tube missed was recorded as focal or peripheral. More difficulty was encountered with the "focal" group where 3 of 12 tubes were missed. As can be seen, however, in all instances the in-

TABLE III. Adenovirus Cytopathic Effect in Coded Monkey Kidney Roller Tubes Inoculated with 100 $TCID_{50}$ of Virus.

Adenovirus type	CPE group	Tubes read/Tubes scored correctly
1	2b (Focal)	3/3
2	2b (Focal)	2/3*
3	2a (Peripheral)	3/3
5	2b (Focal)	2/3*
9	1 (General)	2/2
10	1 (General)	3/3
12	2b (Focal)	2/3*
14	2a (Peripheral)	2/3†
15	1 (General)	2/2
16	2a (Peripheral)	3/3
17	1 (General)	3/3
	Total	27/31

* Tube missed, scored as peripheral.

† Tube missed, scored as peripheral or focal.

Seven-day-old Rhesus (*Macacca mulatta*) monkey kidney cultures inoculated with 0.1 ml of adenovirus (100 $TCID_{50}$). Tubes were coded and read blind at 24 and 48 hr for general, peripheral or focal type cytopathic effect.

TABLE IV. Selective Neutralization of Adenovirus Cytopathic Effect When Viruses from Different Groups Are Mixed.

Adenovirus type inoculated	Antiserum present	Cytopathic effect observed	
		24 hr	48 hr
7a	None	1+ Peripheral	2-3+ Peripheral
9	"	1+ General	1+ General
7a + 9	"	1+ Peripheral	2-3+ Peripheral
7a	7a	Negative	Negative
7a	9	1-2+ Peripheral	2-3+ Peripheral
9	9	Negative	Negative
9	7a	1+ General	1+ General
7a + 9	7a	Negative	1+ General
7a + 9	9	1+ Peripheral	2-3+ Peripheral
7a + 9	7a + 9	Negative	Negative

Seven-day-old Rhesus (*Macacca mulatta*) monkey kidney roller tubes were inoculated with 0.2 ml of virus or virus-serum mixture. All combinations were incubated 30 min at room temperature prior to inoculation. Where no antisera were added, Hanks' BSS was substituted.

correctly scored tubes were mistakenly graded as peripheral. Similar experiments were repeated many times in different lots of Rhesus kidney cultures over a period of more than 2 years.

To determine if the property of CPE differences were unique for Rhesus kidney cultures, African Green monkey kidney cultures were inoculated as previously described with representative types from each group. Results were equivocal although most types in group 1 "general" could be delineated from 2a or 2b and vice-versa. Difficulty, however, was encountered in separating Group 2a or 2b. Primary Rhesus kidney cultures, therefore, appear superior to African Green in differentiating adenovirus CPE.

Differences in CPE in stationary and rolled cultures was next examined. Representative viruses from each of the 3 groups were inoculated into Rhesus kidney cultures and incubated stationary and rolling (1/5 rpm) at 37°C. CPE characteristics of a particular group were observed in virus inoculated cultures whether incubated in a stationary or rolling position. However, control and inoculated cultures normally could be maintained in better condition when rolled.

Ability to neutralize selectively with type specific antiserum the CPE attributable to one virus type when viruses from 2 different groups were mixed was examined and results are presented in Table IV. As can be seen, type specific antiserum prevents CPE caused only by the homologous virus when adenovirus from 2 different groups is inoculated together. When types 7a and 9 were admixed with type 7a antiserum, only "general" CPE was evident. If type 9 antiserum was added to a mixture of the 2 viruses, typical "peripheral" CPE was observed. Interestingly, peripheral CPE appeared dominant when 7a and 9 were inoculated together without antiserum. Whether or not this is due to interference should be investigated. Most likely, it is dictated by the larger amount of infectious type 7a virus than to any interference phenomenon.

Twenty-five strains (Table I) in addition to the prototype strains were studied. In every instance the CPE grouping was identical to that of the prototype. Since adenovirus type 8 was the only type in which the CPE classification did not agree with Rosen's grouping based on hemagglutination, 3 additional strains (Ijima, Koyama and Ishimine) were studied. All 3 exhibited CPE typical of the prototype Trim strain, *i.e.*, Group 2b "focal."

Adenovirus type 12 was the type most thoroughly studied. Fifteen strains in addition to the prototype Huie were examined. Without exception, all demonstrated type 2b "focal" CPE identical with Huie.

Discussion. It has been clearly demonstrated that with the 43 strains used in this study, adenovirus types 1 to 18 differ in the type of CPE elicited in Rhesus and less obviously in African Green primary monkey kidney tissue cultures. From these results, it was possible and desirable to classify the first 18 adenovirus types into 2 major groups, 1 and 2, with the second group subdivided into *a*

and *b*. When one examines closely the types placed in each of these groups (Table II) it is obvious that all but type 8 are arranged similarly to the scheme proposed by Rosen (6) based on hemagglutination, *i.e.*, Group 1 hemagglutinates rat erythrocytes (RBC) completely, Group 2a hemagglutinates Rhesus monkey RBC completely, Group 2b hemagglutinates rat RBC partially or does not hemagglutinate. Although type 8 hemagglutinates rat RBC completely, it usually gives CPE illustrative of Group 2b. This was true not only for the prototype but for the 3 additional strains studied. No explanation for this single exception is available, although additional type 8 strains are undergoing investigation.

With the exception of type 4, Denny and Ginsberg(5) have submitted a similar classification based on biological properties, *e.g.*, nuclear changes, neutralization kinetics, etc. It appears, therefore, that the type of CPE elicited in primary Rhesus kidney culture is perhaps another biological property which allows adenoviruses to be subgrouped.

It remains to be demonstrated whether the property of CPE variation is peculiar only to the strains tested or true for all strains of a given type. The type in which the most strains were examined (type 12) seemed to indicate that the type of CPE in monkey kidney culture is a property of all strains of a given type. Characteristic CPE of adenovirus types appears to be more manifest in primary Rhesus rather than African Green tissue culture as exemplified by the sometimes equivocal results in African Green cultures. Typical CPE is detected in Rhesus cultures whether in a stationary or rolling position. Likewise, the kind of CPE is not dictated by the quantity of virus inoculated but rather seems to be a property of the virus. Additional evidence is obtained by the selective neutralization of one type of CPE when viruses of 2 different groups were inoculated with specific antiserum.

Although most of the prototype strains tested had undergone numerous laboratory passages, some strains had relatively few passages since their isolation. Experiments are in progress to determine whether the property of CPE difference is stable or can be altered by laboratory selection. It has been reported by Nasz(14) that some adenoviruses have been passaged 3 years in the laboratory with no deviations in the type of CPE caused in primary human amnion cultures.

Nasz(14) has described two distinct kinds of adenovirus CPE in primary human amnion tissue culture. His "type 3" CPE resemble our Group 2b and "type 5" Group 1. It is interesting that although most of the strains of a single type gave identical CPE, there were examples of differences among strains within a single type. We have not observed strain differences in our monkey kidney systems. Until we study the effects of many more strains it will be impossible to ascertain whether the observations of Nasz are true for monkey kidney as well as human amnion cultures. That both results are plausible and acceptable is logical since different tissue culture systems were being examined and many of the types giving a specific CPE in human amnion cultures gave a completely different picture in monkey kidney.

Summary. Prototype strains for human adenovirus types 1 to 18 have been inoculated into primary Rhesus *(Macacca mulatta)* and African Green *(Cercopithecus aethiops)* monkey kidney cultures and give rise to two distinct types and a less obvious type of CPE. Rhesus kidney cultures give better resolution than do African Green. Types 1 to 18 are placed into 3 groups on the basis of the kind of CPE elicited and with a single exception these groups are identical to those suggested by Rosen(6) based on hemagglutination. Fourteen strains from type 12 were tested and all gave identical CPE, indicating that differences are probably type rather than strain oriented. Viruses from 2 different groups can be mixed with specific antiserum for one of the pair and the antiserum will selectively inhibit the CPE of only the homologous virus.

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Influence of Diphenylhydantoin on Spontaneous Release of Ovulating Hormone in the Adult Rat. (30354)

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A number of pharmacological agents are capable of blocking spontaneous ovulation in the rat when given prior to the "critical period"(1). With the rats used in this study the "critical period," that is, the period of neurohumoral stimulation of the anterior pituitary gland for the release of ovulating hormone, occurs between 14:00 and 16:00 on the afternoon of proestrus(2). Atropine, pentobarbital (Nembutal), chlorpromazine, morphine or reserpine blockade of spontaneous ovulation in the rat can be overcome by applying an "electrolytic" stimulus to the medial preoptic area(3,4, unpublished). This information suggests that these neuropharmacological agents exert their inhibitory effect on gonadotropin release primarily by way of their influence on the central nervous system. The present study is concerned with the influence of diphenylhydantoin (DPH), an anticonvulsant, on spontaneous ovulation in the rat. Experimental evidence has been presented which indicates that diphenylhydantoin interferes with gonadotropin release in the rabbit (see *Discussion*) and with reflex adrenocorticotropin release in the rat(5) and man(6). The possible mechanisms by which diphenylhydantoin alters the central nervous system to exert its anticonvulsant activity as well as its peripheral sites of action have been recently reviewed(7,8).

Material and methods. Adult albino rats from an inbred Osborne-Mendel derivative strain weighing between 180 and 255 g were used. The animals were maintained on Purina lab chow and water *ad libitum*. With the animals existing under the regimen of 14 hours of light and 10 hours of darkness, the "critical period" occurred between 14:00 and 16:00, that is, 8 to 10 hours following the onset of light(1). The experimental animals were used on the day of proestrus with 2 previous 4-day cycles being a prerequisite for their selection.

Diphenylhydantoin (DPH) was dissolved in a solvent consisting of 40% propylene glycol and 10% alcohol in water and adjusted to pH 12 with sodium hydroxide. DPH was dissolved in the solvent at a concentration of 50 mg per cc. Single intraperitoneal injections were made either just prior to or about 2½ hours before the "critical period."

A stock solution of NIH-LH-B_1† (bovine LH) was prepared with physiological saline and stored in a frozen condition until needed. Appropriate dilutions were made with physiological saline and the hormone was injected subcutaneously. A standard dose of bovine LH (8 μg/100 g body weight) is capable of consistently inducing the full ovulatory re-

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