in agar diffusion experiments and even in immunoelectrophoresis. All 3 classes of immunoglobulins were readily detected. In all instances 7S γ-globulin proved to be the dominant antigen, but 19S γ-globulin and β_{2A}-globulin were readily demonstrated. These results correspond closely to the studies of McDuffie *et al.*(9) on anti-A antibodies. They described 3 types of antibodies, following immunization with isolated anti-A, which relate to the 3 immunoglobulins found in the present study. Their undetermined component probably was the β_{2A}-globulin.

The intermediate sedimenting isoagglutinins previously described(6) proved to be largely polymers of what from the antigenic standpoint appeared to be ordinary 7S γ-globulin. These findings will be described elsewhere(10). The question of the exact s-rate of the reactive β_{2A}-globulins was not readily determined. In the acid eluates the major portion sedimented with an s-rate of approximately 7S but the possibility exists that this treatment may have caused some dissociation.

Summary. 1. The specific precipitates prepared with A substance from 6 out of 8 high-titer anti-A antisera showed the presence of β_{2A}-globulin. This was demonstrated with 3 different antisera specific for this component, utilizing eluates prepared by excess A substance and also by acid extraction. 2. Antisera made against eluates of specific precipitates contained antibodies specific for normal β_{2A}-globulins. 3. All 3 classes of immunoglobulins were encountered in the specific precipitates of the majority of the sera. Antibodies corresponding in antigenic type to ordinary 7S γ-globulins, made up the bulk of the precipitates studied.

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Concentration of Adenoviruses by Isoelectric Precipitation. (28342)

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Various methods for virus concentration were examined during efforts to increase antigenic potency of adenovirus vaccines. Such conventional methods as centrifugation, dialysis, and precipitation in salt solutions of varying saturation resulted in unsatisfactory virus concentration or in inclusion of large amounts of non-viral protein. However, it was observed that virus could be precipitated selectively by proper adjustment of pH with high virus recovery on resuspension. Steinman and Murtaugh(1) already have reported that a simian adenovirus and its complement-fixing antigen could be concentrated from culture fluids by this technique. These observations have now been extended to adenovirus types of human origin which are candidates for inclusion in vaccines.

Methods. Adenovirus strains used for most experiments were Types 1 (Ind. 1), 2 (Ind. 2), 3 (J.F.), 5 (Ad. 75), and 7 (L.L.) from Dr. R. J. Huebner, Nat. Inst. of Health, Bethesda, Md., and Type 4 (R-Wallace line Q) from Lt. Col. E. Buescher, Walter Reed

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TABLE I. Optimal pH for Precipitation of Adenoviruses.

Virus type	No. of exp	pH range
1	1	3.2 ± .2
2	1	3.0 ± .5
3	1	3.2 ± .2
4	1	3.1 ± .3
5	1	3.25 ± .25
7	4	3.15 ± .25

Army Institute for Research, Washington, D. C. Viruses were grown at 36°C in primary cynomolgus monkey kidney tissue cultures in Eagle's basal medium with Earle's salt solution (EBME) or Medium 199 without serum. Harvest fluids were clarified before use by filtration or low-speed centrifugation. Experimental vaccines containing 1:4000 formalin were made in general accordance with the Minimum Requirements for Adenovirus Vaccines, U. S. Public Health Service, 1957.

Infectivity titrations were made in 2 to 4 tubes of monkey kidney cultures incubated at 36°C in stationary racks and using 2- or 10-fold dilution increments. Cytopathic endpoints were recorded on the seventh day post inoculation and are reported as $TCID_{50}$/ml. Antigen extinction tests in guinea pigs and serum neutralization tests in monkey kidney tube cultures were performed essentially as described by Binn and Hilleman(2). Potency of the vaccines was computed by the Reed and Muench formula(3), and expressed as the negative $\log_{10}$ of the vaccine dilution, which converted 50% of the animals to a seropositive status.

All concentration experiments were performed at 5°C unless otherwise indicated. In the first attempts to determine isoelectric points, 10 ml or larger aliquots of virus were acidified with 1N HCl in descending steps of 0.2 to 0.5 pH unit. Precipitated virus was recovered by centrifugation at 2500-3000 rpm for 30 minutes and resuspended by vigorous pipetting in one-tenth volume EBME or 199 Medium at pH 7.4. In later experiments, precipitate from 0.1 to 14.7 liters of acidified virus suspension was recovered by passage through Celite columns. These columns were built from an autoclaved slurry in water on a sintered glass or similar porous support, and usually were overlaid with one to two cm of washed sea sand. Best recovery of virus was obtained when filtration vacuum did not extend 15 inches of Hg, and when the columns were not sucked dry. Entrapped virus was eluted by passing pH 7.4 buffer or medium through the columns at room temperature. Additional details are described with the various experiments.

Results. Concentration of live virus. In 9 experiments utilizing low-speed centrifugation, optimal sedimentation of infectious particles of the 6 adenovirus types was obtained following acidification to a pH approximating 3.15 (Table I). Not all virus was recovered by centrifugation, as the titer remaining in supernatant fluids was approximately one-fifth of that in the original suspension. Virtually the same recovery of infectious virus was obtained over a zone ±0.4 pH unit of the optimum.

In an effort to improve recovery of virus, variations in temperature and time of processing were investigated. Forty ml portions of an acidified virus pool were allowed to stand at 5°C or 25°C for intervals up to 24 hours before centrifugation. As can be seen from Table 2, recovery of virus was not increased by prolonged maintenance at a low pH at either temperature. Somewhat reduced yields of concentrated virus were obtained at 25°C.

In view of the small amounts of sediment to be collected, the observed virus losses during centrifugation, and the unsuitability of multiple centrifugations for the processing of large volumes of fluid, Celite columns were investigated as a means for trapping of acidified virus. Subsequent eluting with smaller volumes of alkaline medium effected concentration.

From examination of fluids in a series of such experiments, it became evident that the total number of infective units in eluted concentrates was essentially equal to that of the input. Curiously, the filtrates also contained low titers of virus and some were found not readily elutable and detected only when the columns were dismantled and broken into slurries. We are reminded thereby that the

TABLE II. Effect of Time and Temperature on Titer of Type 7 Adenovirus Infectivity Following Precipitation at pH 3.1 and Low-Speed Centrifugation.

	Log $TCID_{50}$/ml			
	Precipitation at 5°C		Precipitation at 25°C	
Hr after pH adjustment	Supernate	Sediment (10×)	Supernate	Sediment (10×)
0	3.0	4.4	—	—
1	3.9	4.4	2.4	3.9
3	2.9	4.3	3.4	3.7
6	2.7	4.0	2.9	3.9
24	2.3	4.4	3.3	4.0
Untreated virus	3.5			

infectivity assay measures infective units each of which may contain a variable number of infective centers or particles. Examples of typical results are shown in Table III. Data in that table also indicate that isoelectric precipitation is rapidly completed and that prompt filtration yields maximum recovery of virus.

Celite porosity was not found to be a critical factor in the trapping or eluting of precipitated virus. In spite of variations in flow rate up to 10-fold through these materials, similar results were obtained with 4 grades of Celite (Johns Manville #535, #503, #501, and Hyflo).

Concentration of antigen in inactivated vaccine. A demonstration of the efficiency of isoelectric precipitation and Celite filtration was obtained in several studies with formalin-inactivated adenovirus vaccines (Table IV). Lots of Types 4 or 7 vaccine were acidified, adsorbed, and eluted from Celite columns. The capacity of original vaccines to immunize guinea pigs was compared with that of filtrates and eluted concentrates. Observed antigen extinction titers of the concentrates closely approximated the expected titers, while the filtrates were essentially devoid of antigenic activity.

TABLE III. Effect of Time at pH 3.0 on Adsorbtion and Elution of Infective Units of Adenovirus Type 7 from a Celite Column.

Samples	0 hr	24 hr
Original virus (1000 ml)	2.6*	—
After pH adjustment	—	2.6
Filtrate through columns	1.7	1.5
First 10 ml eluted	3.4	3.9
Second 10 " "	4.5	3.9
Third 10 " "	3.8	3.3
Fourth 10 " "	3.0	2.6
Fifth 10 " "	2.9	2.7
Suspended filter	2.4	2.0
	Log_{10} total infectious units	
Input	5.6	5.6
Eluted	5.7	5.3
Recoverable, non-eluted	4.1	3.7
Lost in filtrate	4.7	4.5

Aloe No. 29217-D Celite, 3 cm wide by 1.5 cm deep, overlaid with an equal volume of sand. Eluted with EBME, pH 7.4. Filter finally broken up and suspended in 50 ml of EBME.

* $TCID_{50}$/ml.

TABLE 4. Concentration of Guinea Pig Immunizing Antigens in Adenovirus Vaccines by Precipitation at pH 3.1 and Column Filtration.

Vaccine		Log antigen-extinction titer			
				Concentrate	
Type	Liters	Original	Filtrate	Theoretical	Observed
4	1	2.1	—	4.0	3.5
4	9.8	2.3	0	4.3	4.4
7	14.7	2.4	.08	4.4	3.5
7	9.5	1.4	.08	3.4	4.0
7	8.5	1.0	0	3.0	3.5

Aloe Celite columns 3 to 4 cm wide by 1½ to 2 cm deep. Eluted with EBME at pH 7.4 and flow rates about 80 ml/min.

The 5 experiments involved vaccine volumes of approximately 1, 10, and 15 liters. The 3 vaccines prepared from 10-liter volumes were more antigenic than either of the single vaccines prepared from 1 or 15 liters. These findings suggest that an optimum volume for passage through a column exists, but do not define it precisely. Nevertheless, it is evident that the method is a relatively simple and effective procedure for concentrating immunizing antigens of adenoviruses.

Discussion. The work of Ginsberg(4) led to the conclusion that adenoviruses were rela-

tively acid resistant. Successful concentration by precipitation at low pH values is, therefore, not surprising. The demonstration that several adenovirus types, including one from a simian source(1), have essentially the same isoelectric point, pH 3.15 ± 0.15, indicates a general similarity in the physical nature and chemical composition of these agents. It will be of interest to learn whether all viruses now included in the adenovirus group have such an isoelectric point and so possibly be established as a taxonomic criterion.

It is evident that the methods described here can be used to concentrate large volumes of adenovirus in a closed, continuous flow system. The technique might be applied, therefore, to problems concerning purification of virus for physical and chemical examination or possibly to concentrate virus from clinical specimens. However, our primary consideration was for preparation of vaccine components. The greatest concentration factor attempted was 100-fold, although considerably greater concentration probably could be achieved either in a primary step or in sequential precipitations and elutions. The extent to which these procedures will be applied to vaccine production is likely to be limited by the practical and economical factors.

Concentration of inactivated vaccine seemed slightly more efficient than that of live virus, but this probably relates to differences in sensitivity of assay methods. Nevertheless, it is tempting to speculate that formalin treatment results in improved aggregation of viral units under optimal isoelectric conditions. This is not unreasonable since other virus preparations, such as influenza or polio, when treated with formalin will sediment more readily in gravitational fields. It is also remotely possible that isoelectric precipitation results in an altered physicochemical state of the inactivated virus so that their antigenicity is increased. Such conclusions, however, must await the development of greater accuracy in titrations of infectivity and antigenic potency.

Summary and conclusions. The isoelectric optimum of 6 types of human adenoviruses in undialyzed monkey kidney harvest fluids has been established as pH 3.15 ± 0.15. Live and formalin-inactivated viruses were concentrated efficiently by elution of isoelectric precipitates from Celite columns. These observations appear to have several practical applications, as in the preparation of more potent vaccines.

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Production of Hemagglutinin by Dengue Virus in HeLa Cells. (28343)

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Attempts at demonstrating *in vitro* formation of hemagglutinins by arthropod-borne viruses have been numerous(1,2,3,4,5), both in cultures of stable cell lines and in primary tissue cultures. Dengue virus, however, has been absent from the list of agents thus characterized.

In this laboratory, dengue virus has been adapted to HeLa cells with concomitant cytopathic effect (CPE)(6). In the course of the further studies reported here, representative strains of types 1, 2, 3 and 4 have been passed serially over a period of 2 years, and

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