suggest that the morphological features of tumors induced by adenoviruses and SV40 are not determined by T antigens. Additional studies of *in vitro* induction of neoplasia of hamster kidney with LLE46, other adenovirus-SV40 "hybrids," and mixtures of SV40 and adenoviruses will be necessary to clarify these points.

Summary. Newborn hamster kidney cell cultures were transformed *in vitro* by LLE46 virus, an adenovirus 7-SV40 hybrid, and by SV40. The cultures transformed by both viruses consisted of rapidly growing cuboidal and polygonal cells, although those in the LLE46 cultures were smaller and had less tendency to grow in clumps. Neither infectious adenovirus 7 nor SV40 could be recovered from the transformed cells. More than 90% of the cells in both the LLE46 and the SV40 cultures contained persistent intranuclear SV40 T antigen while no adenovirus 12 or 7 T antigens were demonstrable. Tumors were rapidly produced when both types of transformed cells were injected into newborn hamsters, and into irradiated young adult hamsters. As previously described, the tumors produced by the SV40 transformed cells were predominantly carcinomas with areas of tubular differentiation. Those produced by the LLE46 transformed cells, however, were predominantly undifferentiated and histologically similar to adenovirus 12 tumors. Although they did contain a few small foci resembling SV40 sarcomas, no tubular epithelial structures were seen.

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A Study of Antibodies Produced by Spleen Fragments *in vitro*.* (30812)

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The production of antibodies by spleen, lymph nodes and other tissues has been demonstrated in a variety of *in vitro* systems(1, 2). However, experimental demonstration of antibody production by cultured spleen has been less successful than by lymph node cultures even when the donor animal had been immunized by the intravenous injection of antigen(3,4). A comparison of the chemical and immunologic properties of antibodies produced *in vitro* with those produced *in vivo* has been difficult, mainly because the amounts produced *in vitro* are extremely minute. Such comparisons are important in order to de-

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TABLE I.

Culture No.	Immunization	Serum hemagglutinin titer	Serum content of precipitating antibody (mg protein/ml)	Precipitating antibody at hemagglutination end point (μg/ml)
R_3	50 mg H.S.A. in complete Freunds' adjuvant s.c. 3 mo before sacrifice. 2 mg H.S.A. in saline in hind foot pads 5 days before sacrifice.	1/2,560,000	1.62	.006
R_4	Same treatment as R_3. No secondary inj.	1/1,024,000	.92	.008
R_5	10 mg H.S.A. in complete Freunds' adjuvant in each foot pad 1 mo before sacrifice.	1/256,000	3.15	.0024

termine to what extent *in vitro* systems can be used as models for the study of antibody synthesis.

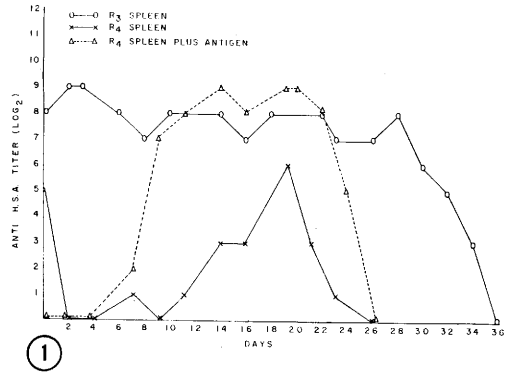
In the work to be reported, we have obtained antibody production by cultures of spleen fragments comparable in duration and amount to that reported by others for lymph node fragments(5). In addition, by purifying the antibodies by gel filtration we have been able to compare them in terms of molecular size and electrophoretic migration with humoral antibodies produced by the donor animals.

Materials and methods. Immunization. Adult male, white rabbits (3-5 lb) were immunized by the subcutaneous injection of human serum albumin (H.S.A.), which had been emulsified with an equal volume of complete Freund's adjuvant (Table I).

Antibody assay. The serum concentration of precipitating antibody to human serum albumin was determined by standard quantitative precipitin reactions(6). Passive hemagglutination tests were read in the usual manner, but the antigen was bound to rabbit erythrocytes with chromic chloride(7) rather than tannic acid.

Tissue culture. The culture medium consisted of 75% TC 199 (Difco), 20% pooled normal rabbit serum and 5% fetal calf serum. Cultures were prepared by exsanguinating the donor animal and removing the spleen under sterile precautions. The spleen was minced with a fine scissors into particles approximately 1-2 mm in diameter and 6 spleen fragments were placed into each plastic petri dish (Falcon), 60 mm in diameter. The fragments were then covered with a sterile pad of glass wool. The average wet weight of the total number of fragments in each dish was 6 mg. Cultures of popliteal lymph nodes were prepared identically. Three ml of the medium were placed in each petri dish and the medium was changed every 2 to 4 days. Cultures were maintained in an incubator at 37° in an atmosphere of 5% CO_2 in air. Each change of medium was assayed for anti-H.S.A. by the passive hemagglutination technique. When fragments were exposed to H.S.A. *in vitro,* they were incubated for 2 hours in medium containing 30 μg H.S.A./ml. The stimulated cultures and control cultures were then washed with antigen-free medium and incubation was continued using the normal medium.

Gel filtration studies. Sera were diluted 3-fold with 0.002 M Tris-HCl buffer containing 0.15 M NaCl (pH 7.4), applied to Sephadex G-200 columns and eluted with the same solvent. In most experiments 2 $\times$ 40 cm columns were used. The flow rate was approximately 30 ml per hour and 3 ml fractions were collected. Recovery from the column was calculated in terms of both protein [optical density (O.D.) units at 280 mμ] and hemagglutinating activity (hemagglutination titer of each fraction multiplied by the volume). The immunologically active peak from the Sephadex G-200 column was dialyzed against an ammonium sulfate solution of 50% saturation and the resulting globulin precipitate was recovered by centrifugation and then dissolved in 0.04 M acetate buffer of pH 5.5. The globulin solution was dialyzed overnight against the same buffer and then applied to a CM Sephadex



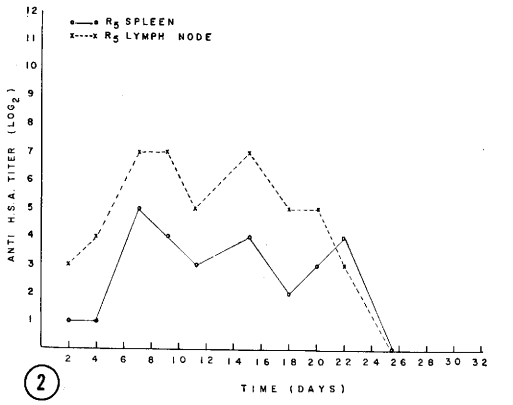


FIG. 1. Anti-H.S.A. titers of individual changes of culture media. R_4 Spleen is designated R_41 in text and R_4 Spleen Plus Antigen is designated R_43 in text.

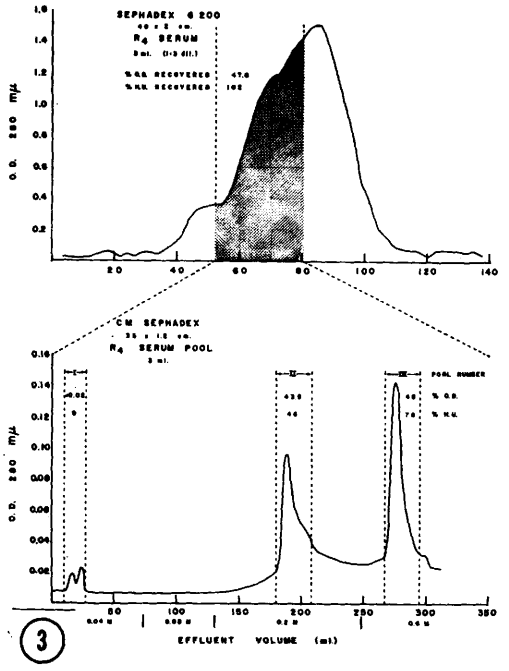
FIG. 2. A comparison of anti-H.S.A. titers in culture media from spleen and lymph node of the same animal (R5).

(C-50) column, 1.2 × 32 cm. The column was eluted stepwise by acetate buffer of pH 5.5 of increasing ionic strength. The eluates were tested for protein content and hemagglutinating activity as described above.

Culture media were processed in a similar manner except that, as an initial step, the media shown to have anti-H.S.A. activity were pooled (the first change of medium was not included since it might have contained preformed antibody) and dialyzed against a half-saturated ammonium sulfate solution. The precipitated globulins were recovered, dissolved to 10-fold concentration in Tris-buffered saline and then treated as described for sera. A larger Sephadex G-200 column (4 × 45 cm) was used to process these concentrated globulin pools, but in order to make precise comparisons, some media and sera were also processed on an identical G-200 column without first concentrating the medium. The active peak from the G-200 column was pooled and concentrated either by lyophilization or by ammonium sulfate precipitation. The samples were dissolved in 0.04 M acetate buffer (pH 5.5) and then dialyzed against this same buffer. After dialysis, the samples were applied to CM Sephadex (C-50) columns as described for serum. For study by agar electrophoresis and immunoelectrophoresis, the active peaks from the columns were dialyzed against barbital buffer, pH 8.2, ionic strength 0.1.

Results. The immunization records of 3 rabbits whose serum antibody content, hemagglutination end points and spleen cultures have been studied, are listed in Table I. Note that in the case of R_5 serum, there was more precipitating antibody and less hemagglutinating antibody than in that of the other rabbits. This is not uncommon with early antibody(8). The results of hemagglutination assays of each change of medium from these cultures are shown in Fig. 1 and 2. Fig. 2 also indicates the comparative hemagglutination assays of culture media of lymph node and spleen from rabbit No. 5. Hemagglutination inhibition studies showed specific inhibition by H.S.A. of positive media and sera at comparable concentrations of antibody and antigen. It can be seen that the peak of antibody synthesis in R_4 spleen cultures was reached at the same time in both those exposed to antigen and those not exposed to antigen *in vitro*. The reason for the delay in peak antibody synthesis in culture is not clear, but this delay has been observed by others under a variety of culture conditions(4,5,9).

The optical density profiles shown in Fig. 3 for R_4 serum and in Fig. 4 for R_43 medium are also representative of the elution patterns obtained with R_3 and R_5 serum and spleen cultures. In the case of R_5 lymph node media and R_41 media (spleen culture not exposed to antigen *in vitro*), however, the effluent from CM Sephadex columns produced only 2 immunologically active peaks (as was the case with all sera). The protein in the R_5 spleen media emerged from the CM Sephadex column in 3 peaks (with peaks II and III



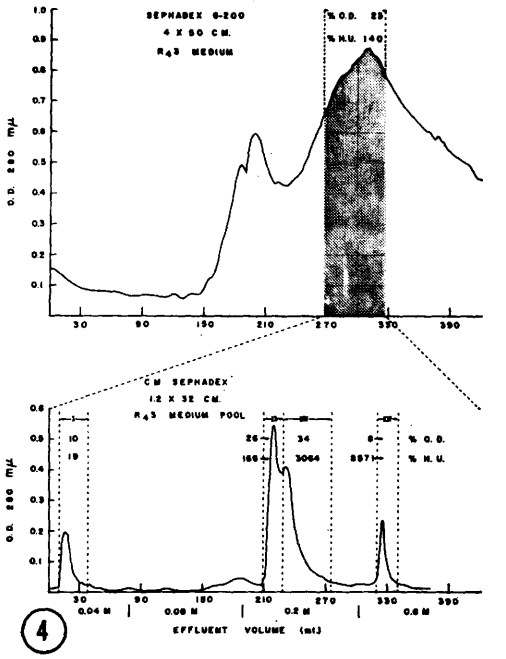


FIG. 3. Optical density profiles of R_4 serum eluted from Sephadex G-200 and CM Sephadex columns as indicated. H.U. refers to hemagglutinating units (determined by multiplying the titer of each eluate by its volume). O.D. Units were obtained by multiplying the optical density of each fraction at 280 mμ by its volume. Three ml of a 1:3 dilution of serum were applied to the Sephadex G-200 column and 3 ml of the concentrated active pool (shaded area) were applied to the CM Sephadex column.

FIG. 4. Optical density profiles of R_43 medium eluted from Sephadex G-200 and CM Sephadex columns as indicated. Ten ml of redissolved, concentrated globulins were applied to the Sephadex G-200 column. Seven ml of the active pool (shaded area) were applied to the CM Sephadex column.

incompletely separated) as did the media from R_3 and R_43.

As shown in Fig. 3, when serum from an immunized animal was fractionated on G-200 and then rechromatographed on CM Sephadex, the recovery of antibody (hemagglutinating activity) was essentially quantitative (122%, Fig. 3), with most of the antibody appearing in the peak eluted by 0.6 M acetate. When the same sequence was applied to globulin fractions obtained from the culture medium of spleen cells exposed to antigen *in vitro* (R_43), however, the activity recovered from the CM Sephadex column was increased more than 100-fold (Fig. 4); again, most of the antibody emerged with buffer of higher ionic strength. A similar enhancement was also obtained when medium from spleen cells of animals in which a secondary response had been elicited *in vivo* (R_3) was fractionated, but no enhancement was observed with R_5 or R_41 media (primary antibody production, no antigen added). The antibody recovered in the experiment described in Fig. 3 was destroyed by immersion in boiling water for 3 minutes, was not lost (or otherwise affected) when dialyzed against isotonic saline or buffers (pH 7.4-8.2), and could be prevented from agglutinating H.S.A.-coated rabbit red cells by H.S.A., but not by other proteins. The fractions containing the immunologic activity were shown to be gamma globulins by electrophoresis and immunoelectrophoresis (developed with a commercial goat anti-rabbit serum). There is, therefore, no reason to suppose that the results were artifacts. Recombination of the peaks, however, did not mask the immunologic activity of the individual peaks.

Since the Sephadex G-200 studies on sera and medium were not done on identical columns, the active peaks had slightly dif-

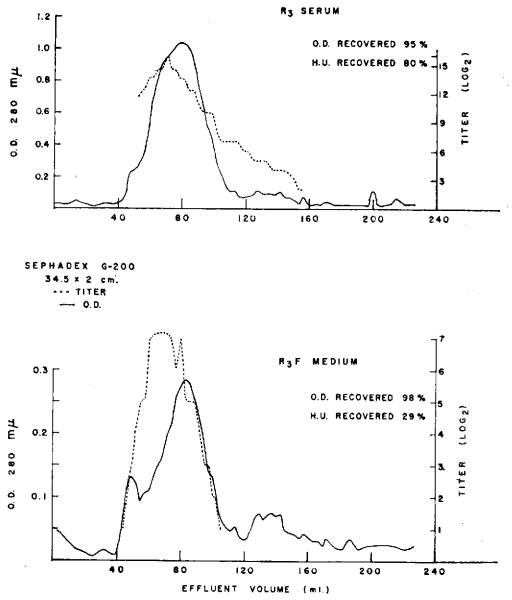


FIG. 5. Optical density profiles of R_3 serum and R_3F medium eluted from an identical Sephadex G-200 column. The identity of the elution volumes of the immunologically active peaks is apparent.

ferent elution volumes (calculated as fractions of bed volume). Therefore, R_3 serum and R_3 medium (R_3F) were processed on the same Sephadex G-200 column, the details of which are shown in Fig. 5. The column had a bed volume of 108 ml and the peak of antibody activity for the media and sera was eluted with identical volumes. These peaks were also found to be identical in elution volume with a standard gamma globulin solution. The void volume of the column was shown to be 54 ml by the use of a 0.2% solution of high molecular weight blue dextran, approximately the point at which the inactive first peak, presumably macroglobulin, was eluted. In this respect, therefore, the antibodies both in serum and medium behaved like 7S globulins.

Summary and conclusion. Cultures of spleen fragments from rabbits which had been immunized by subcutaneous injection of H.S.A. in complete Freund's adjuvant were shown to be capable of continuing antibody synthesis and producing a secondary response on exposure to antigen *in vitro*. Gel filtration studies of humoral and *in vitro* antibody on Sephadex G-200 columns showed that they were of similar molecular weight. Additional purification on CM Sephadex columns indicated that serum antibody could be further separated into 2 fractions having antibody activity. The *in vitro* antibody also separated into 2 fractions at the same ionic strength of eluting buffer, but in the case of 2 media [one from cultures having a secondary response initiated *in vivo* (R_3) and one initiated *in vitro* (R_43)] a third active peak was partially separated by eluting with 0.2 M buffer and striking enhancement of antibody was achieved. The cause of this enhancement has not as yet been identified, but these studies suggest that a distinct population of antibody molecules is produced in the secondary response *in vitro*, which can be detected only after CM Sephadex filtration.

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