do similar rats that are operated on 2 weeks after zero time. 2. Performance of the uninephrectomy 2 weeks after uniadrenalectomy and adrenal enucleation results in significantly lower terminal mean blood pressure and correspondingly lower lesion severity indices. This suggests that the enucleation step proper is the crucial operation in bringing about the full severity of the hypertensive syndrome. 3. Uninephrectomy and uniadrenalectomy on day zero and contralateral enucleation of the compensatorily-hypertrophied adrenal 2 weeks later results in mean terminal blood pressure and lesion severity indices comparable to those rats in which these operations are carried out at zero time. Blood pressure only rises steeply after the hypertrophied adrenal gland is enucleated. 4. It is concluded that, while reduction of renal mass contributes greatly to the development of this form of experimental hypertension, enucleation of the adrenal is the decisive step. It is further concluded that this combination of operations early in infancy finds a more favorable internal environment for the development of severe hypertensive cardiovascular disease.

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Use of Tissue Culture Cultivated Toxoplasma in the Dye Test and for Storage.* (29900)

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The successful cultivation of *Toxoplasma gondii,* an obligate intracellular parasite, in tissue culture, has been described in several reports(1-6). Chernin and Weller(1,2) demonstrated that free organisms could be obtained in roller-tube cultures. This communication describes the use of roller-tube cultures of toxoplasma as a source of parasites for performance of the dye test(7) and for long-term storage of viable organisms.

Material and methods. Hep-2 cells were used throughout. The cells were maintained in 2.5% inactivated (30 minutes at 56°C) calf serum in medium 199 to which had been added 10% lactalbumin hydrolysate (LH) and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml, nystatin 50 U/ml).

The tissue cultures were inoculated initially with mouse peritoneal fluid infected with the RH strain of toxoplasma. The first 3 passages were maintained as stationary cultures at 35°C. They were transferred when the cytopathic effect (CPE) was marked and many toxoplasmas were present in wet mounts stained with methylene blue buffered at pH 11(7). Passage was accomplished by pooling several such fluids and transferring 0.1-0.2 ml aliquots to fresh culture tubes. Comparable uninoculated control tubes were maintained and passed in a similar manner. In these stationary cultures 13-17 days were required for the CPE to appear and more than 21 days for a significant number of free parasites to become recognizable.

One-half of the fourth passage tubes were placed in a roller drum revolving at 1/5 rpm. Since large numbers of organisms were observed to be floating free in the medium by the 7th or 8th days (with experience free toxoplasmas could be detected easily at

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100×), all subsequent passages were incubated in roller tubes. Exchanging the fluids with one ml of maintenance medium immediately before and on the third day following inoculation seemed to result in maximum multiplication of organisms. On occasion, an additional one ml of medium was added on the 6th day. This agrees with the experience of Chernin and Weller(2) and of Shimizu(6). The maintenance medium was adjusted to pH 7.4-7.6 with 4.4% $NaHCO_3$. Since incubation at 35°C resulted in greater numbers of free toxoplasmas and more marked CPE than at 33°C, the former temperature was adopted for routine use.

The effect of inoculum size on rate of appearance of free toxoplasmas in the medium was investigated as follows. Each of 3 sets of tubes was inoculated with different numbers of organisms. "A" received a large inoculum (0.2 ml of pooled medium containing $>$ 20 organisms per HPF), "B" a moderate one (0.2 ml containing 5-15 organisms per HPF), and "C" a small inoculum (0.2 ml containing $<$ 5 organisms per HPF). Total free parasite counts were performed on alternate days from randomly selected members of each set. The relative proportion of stained and unstained toxoplasma, also, was recorded. Unstained toxoplasmas averaged 8%, suggesting that most organisms were viable at the time of maximum multiplication. Stainability, however, may not be an absolute indicator of viability(7).

Dye test. Toxoplasmas were obtained for the dye test by rinsing each of several tubes 3 times with its own medium. After pooling, 0.2 ml aliquots were inoculated into each of the desired number of culture tubes. These were usually harvested at 7-8 days when there was marked degeneration of the cell sheet and large numbers of free parasites were identifiable at 100×. Harvesting was performed by rinsing each tube several times with its own fluid and pooling these in a 50 ml conical centrifuge tube. Following centrifugation in an International Size 2 centrifuge at 1200 rpm for 20 minutes, the supernate was discarded and replaced with an equal volume of a mixture of one part saline (0.85% NaCl) and 4 parts freshly thawed, activator serum(7). Heparin was not required. After gentle resuspension, 0.1 ml amounts were added to similar volumes of the previously diluted, inactivated test sera. Following incubation for one hour in a 37°C water bath, 0.05 ml of freshly prepared methylene blue buffered at pH 11 was added to each tube(7). The racks were shaken and returned to the water bath for 10 minutes to allow for maximum staining(8), following which they could be refrigerated until read. A known positive serum and a saline control were included in each test run.

Storage of parasites. The preservation of live toxoplasma during storage presents a problem. Live parasites persist for about 14 days in infected mouse brains suspended in Tyrode's solution(9) or 10% rabbit serum saline. Others have used infected mouse peritoneal exudate suspended in a variety of fluid media(10,11). Eyles, Coleman and Cavanaugh(12) also demonstrated that storage for up to 209 days was possible following slow freezing of the organisms in glycerol.

Accordingly, it was decided to investigate tissue culture adapted toxoplasmas as a source of organisms for storage. Toxoplasmas which had had at least 10 serial tissue culture passages were utilized for these tests. Parasites were obtained as described above, but resuspended in phosphate buffered saline (PBS), pH 7.2. This suspension was added in 2 ml amounts to 8 ml of each of the following: a) 1, 5, 7.5, 10 and 20% heat inactivated, dye test negative, rabbit serum in PBS; b) inactivated calf serum (CS), 2.5 and 20% in medium 199 with 10% LH; c) PBS and 199 with 10% LH as controls. After gentle mixing, the tubes were transferred to a 4°C refrigerator for storage. Dye counts were made on days 1, 3, 5, 7, 10, 15, 25 and 30 to determine the proportion of stained to unstained parasites. Subinoculations were performed on the 1st, 10th, 20th and 30th days of storage by removing 0.6 ml from each tube type and inoculating 0.3 ml into each of two Hep-2 tubes. The latter were examined daily for the appearance of CPE and free organisms.

Addition of glycerol or sorbitol(13) in different concentrations to the various diluents

was without apparent benefit. Sorbitol seemed to produce excellent results, but repeated attempts to subinoculate from such tubes were unsuccessful. In this instance, at least, stainability and viability were not equivalent.

When it was learned that the best survival was obtained in the 2.5% CS-199 mixture (the growth medium in which the toxoplasma had been cultivated), it was decided to investigate storage in the tissue culture tubes themselves. For this purpose, 60 Hep-2 culture tubes were inoculated with 0.5 ml amounts of suspensions containing > 20 toxoplasmas per HPF. After incubation for 24 hours in a roller drum at 35°C, the fluid medium in half of the tubes was replaced with fresh 2.5% CS-199, while the other half received the same with 10% glycerine. They were then transferred to a 4°C refrigerator. Two tubes from each set were withdrawn after 4, 7, 14, 21, 28, 42, 56, 90, 120, 180 and 360 days of storage. These were scraped gently with a pipette to dislodge the adherent cells and then spun for 30 minutes at 1000 rpm in an International Size 2 certrifuge. The supernate was decanted and the sediment resuspended in one ml of fresh medium, inoculated into Hep-2 culture tubes and incubated in a roller drum at 35°C. They were observed daily for the appearance of CPE and free toxoplasma. The medium was exchanged at 3-day intervals.

Duplicates of the above were treated in the same manner, except that after the 24-hour incubation period, they were transferred to wire baskets and packed tightly with cotton-batting. The baskets were placed in a —13°C freezer for 24 hours and then transferred to a dry ice chest. Two tubes from each series were withdrawn on the same days as the refrigerated sets, thawed slowly at room temperature and passaged as described.

Results. Inoculum size. The rate of appearance of free organisms in the tissue culture fluids was markedly affected by the size of the inoculum. With the large dose (0.2 ml with > 20 parasites per HPF), a marked increase in numbers occurred between the fourth and sixth days, but the maximum was achieved on the seventh day. The moderate inoculum cultures multiplied more slowly. The maximum was observed on day 10, but this only approximated the numbers seen in the large inoculum tubes on day 6. The smallest inoculum proved to be inadequate for here on day 14 the number of free parasites had just about reached the levels observed with the large and moderate inocula on days 5 and 9, respectively. Thus, the most desirable concentration for passage appears to be from fluids containing approximately 20 or more parasites per HPF.

Storage trials. The data obtained from the storage tests are tabulated in Table I. These agree with the findings reported by Eyles *et al*(12) in that viable organisms were recovered after storage for as long as 360 days in the dry ice. We did not employ a time-delay freezer, although 2-step freezing was used. Whether this is a requirement cannot be stated. The cultures stored for 360 days became positive after one blind passage after 21 days. There is a suggestion, but only that, that addition of 10% glycerine offered an advantage for storage beyond 120 days.

Of equal interest is the observation that viable toxoplasmas persist for at least 90 days in their own tissue culture tubes stored at 4°C and perhaps, for as long as 120 days when 10% glycerine is added to the medium prior to refrigeration. In both the refrigerated and frozen cultures, the incubation period on subsequent transfer lengthens significantly with the duration of storage. This probably reflects the "die off" rate. If this is the correct explanation, then the 42-56-day storage interval may be fairly critical so that passages probably should be made at 60-day intervals.

Discussion. Cook and Jacobs(3) suggested 2 reasons for the decreased reproductive rate of toxoplasma at a low temperature: 1) reduction in multiplication rate of the organisms; 2) interaction of the reduced metabolism of the host cell with the decreased rate of multiplication of the parasite. Our findings do not seem to support the latter alternative for it is unlikely that the metabolism of the Hep-2 cells was markedly reduced at 33°C since the decrease in pH of the medium was identical at 33°C and 35°C. The explanation may be that not only do the organisms multiply more slowly at the lower tempera-

TABLE I. Comparative Persistence of Viable Toxoplasma Stored in 2 Media at 4°C and Frozen in Dry Ice.

No. days stored	Medium	4°C		Frozen	
		Tubes pos/ Tubes inoc	Days req for growth (avg)	Tubes pos/ Tubes inoc	Days req for growth (avg)
4	TC*	2/2	13.5	1/1	14
	TCG†	2/2	13	2/2	14.5
7	TC	1/1‡	15	2/2	16.5
	TCG	2/2	14.5	2/2	16
14	TC	2/2	16.5	1/2	18
	TCG	1/2	15	2/2	17.5
21	TC	2/2	17.5	2/2	18
	TCG	1/1	17	1/2	18
28	TC	2/2	18	2/2	19.5
	TCG	2/2	18	2/2	19
42	TC	1/2	19	2/2	20.5
	TCG	1/2	20	1/2	21
56	TC	2/2	21	2/2	22
	TCG	2/2	20.5	2/2	22.5
90	TC	1/2	25	1/2	24
	TCG	2/2	24	1/2	25
120	TC	0/2	—	2/2	24.5
	TCG	2/2	26.5	2/2	25.5
180	TC	—	—	1/2	28
	TCG	0/2	—	2/2	28
360	TC	—	—	1/2	13§
	TCG	—	—	2/2	15§

* TC = Tissue culture medium (2.5% CS in 199).
† TCG = TC with 10% glycerin.
‡ A denominator of 1 means that the second tube was discarded because of contamination.
§ = Second passage (21 days in the first blind passage).

ture, but that they become encysted and so fail to destroy the cells.

In agreement with Chernin and Weller(2) we noted a marked increase in number of toxoplasma between the seventh and eighth days of incubation. The appearance time could be shortened noticeably by increasing the concentration of parasites in the inoculum or lengthened markedly, by decreasing it.

While the cytopathic effect generally is quite severe by the seventh or eighth days, at which time nearly all of the culture cells are lysed, on several occasions it was possible to harvest toxoplasmas twice from the same tubes. This was apparently the result of an exceptionally heavy growth of Hep-2 cells. In such cultures there were numerous organisms free in the medium on the seventh day, but only a minimum number of Hep-2 cells had been disrupted. The fluid was pipetted off without rinsing the sides of the culture tubes and used in dye tests. One ml of fresh maintenance medium was added quickly to each tube which was then returned to the roller drum. Four days later, the CPE was complete and the medium again was laden with parasites which were collected and used in other tests.

More than 1500 animal and human sera have been examined for dye test antibodies with parasites obtained from tissue cultures such as have been described. No changes in either the multiplication rate or their behavior in the dye test were noted in this adapted strain of toxoplasma after 28 consecutive tissue culture passages.

The use of tissue culture cultivated toxoplasma for the dye test and other purposes offers several advantages over infected mouse peritoneal exudate. The preparations are "cleaner," heparin is not needed and there is probably a lower risk of accidental infection for laboratory personnel. The tubes can be handled with ease and the entire process of inoculation, changing of the medium and harvesting of the organisms requires relatively little effort. The number of parasites in each test field is sufficient to permit both rapid and accurate counting.

The suggested method for storage of viable

parasites in tissue cultures also offers advantages over previously available procedures in that all of the equipment required is readily available in any laboratory. This relatively simple method can be utilized by laboratories which may have to suspend toxoplasma tests temporarily since after one passage, organisms can be returned to the refrigerator for an additional period of storage.

Summary. *Toxoplasma gondii* cultivated in roller tube cultures of Hep-2 cells produce large numbers of free, extracellular parasites for the performance of dye tests. The successful storage of toxoplasma in refrigerated tissue culture preparations for up to 120 days and in the frozen state for as long as 360 days is described.

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Pathogenicity of Specific Glycopeptide Antigen in Farmers Lung.* (29901)

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We have previously described(1,2) the clinical, physiological and pathological features of Farmers Lung. Characteristically, the typical clinical syndrome consists of chills, fever, cough and shortness of breath 4 to 8 hours after exposure to moldy hay or forage and subsequent development of a diffuse granulomatous interstitial pneumonitis. Recently Kobayashi(3) and Pepys(4) have demonstrated that the serum of patients with this disease reacts with antigens extracted from moldy hay to form specific precipitin lines in agar gel immunodiffusion. This observation, however, does not prove a causal relationship between exposure to moldy hay and subsequent development of pulmonary disease.

To establish that such a cause and effect relationship does exist, a provocative challenge test utilizing these antigens was employed in a group of patients with Farmers Lung and a comparable control group. The clinical response was observed, and pulmonary function studies were performed on both groups following this aerosol inhalation challenge.

Materials and methods. Antigens were extracted from a sample of moldy hay which was known to have caused the typical clinical syndrome in one of our patients in the following manner. After defatting with acetone, the sample was extracted with 8% trichloroacetic acid (TCA). Precipitable fractions were obtained by treating the TCA soluble

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