chicken lice, even though they may not be the true vectors. The positive neutralization tests with the cow serum would indicate, however, that some other arthropod, such as the mosquito, is also a vector in this area.

Likewise while the eastern equine virus was recovered from chicken mites in nature, as yet no experimental proof has been given that the virus lives, multiplies and is hereditarily transmitted in these hosts. Further work is being planned in this regard for both species of arthropods.

Summary. The virus of eastern equine encephalomyelitis has been recovered from chicken mites, *Dermanyssus gallinae* (De Geer), and from a mixture of chicken lice, *Menopon pallidum* Nitzsch and *Eomenacanthus stramineus* (Nitzsch), taken in nature in Tennessee. This is the first isolation of the eastern virus from chicken mites and chicken lice and the first time the virus has been found in this state. Neutralizing antibodies for this virus were present in the sera of a few chickens and one cow. Further confirmation is needed to prove the role of mites and especially of lice as true vectors of this virus.

We wish to express our appreciation to Dr. Amos Christie and Dr. J. C. Peterson of the Department of Pediatrics of the Vanderbilt University School of Medicine whose interest in the encephalitis problem in Tennessee initiated these studies. We wish also to acknowledge the assistance of the Tennessee State Health Department, particularly that of Dr. C. B. Tucker, State Epidemiologist.

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Development of Tuberculous Infection. *In vivo* Observations in the Rabbit Ear Chamber.*

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The rabbit each chamber, first described by Sandison[1] and later modified by Ebert, Florey, and Pullinger,[2] provides a technic for studying the dynamics of pathological changes in living tissue. In this paper results of studies on tuberculosis by this method are reported.

A thin layer of living vascularized tissue 40 to 50 μ thick can be inoculated with tubercle bacilli directly and examined by frequent daily observations in the unanesthetized animal over a period of weeks. Serial microscopic observations using the highest magnifications are possible, and a graphic record of the findings can be made with Kodachrome motion pictures. In this way it was possible to record the early development of tuberculous infection. The chamber method is particularly well suited for the study of changes in blood vessels, and striking observations have been made on the vascular reactions in the growing tuberculous lesion.

Materials. The Chamber: Originally observations were made using the modification developed by Ebert, Florey, and Pullinger,[2] but subsequently a simpler chamber was devised which was smaller and lighter, eliminating the necessity of the cumbersome ear splints and shields used to protect the larger chambers. It consists of a transparent lucite base with a mica coverslip supported by an aluminum ring connected with 3 small bolts (Fig. A).

The base is a highly polished round lucite plate 1 inch in diameter, 1/16 inch thick with a raised central table 1/4 inch in

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

1 Sandison, J. C., *Am. J. Anat.*, 1928, **41**, 447.

2 Ebert, R. H., Florey, H. W., and Pullinger, B. D., *J. Path. and Bact.*, 1939, **48**, 79.

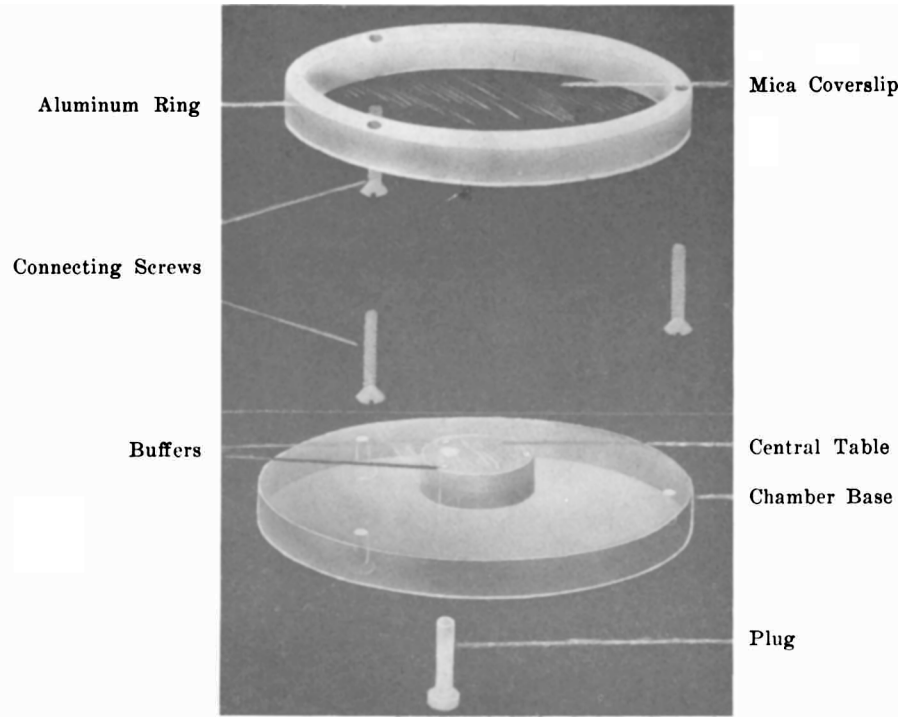

FIG. A.
Exploded diagram of the rabbit ear chamber.

diameter projecting 1/16 inch above the base plate. A round hole 1/16 inch in diameter is drilled through the raised central table 1/16 inch off center to accommodate a fitted lucite plug. Three 1/16 inch holes are drilled at 120 degree intervals through the base plate, their centers being placed exactly 7/64 inch from the margin. The holes are countersunk to accommodate the beveled heads of the connecting screws. *The buffers*[2] are glued to the margin of the table surface on a radius passing through the connecting screws. They are cut from a thin sheet of lucite, and should be approximately 40 μ thick. *The plug* is machined from lucite and has highly polished ends. It has a flange on one end approximately 1/32 inch thick and 1/8 inch in diameter. It is approximately 1/16 inch in diameter and is tapered (very slightly) away from the flanged end so that when fully inserted it fits tightly. It is approximately 5/32 inch in overall length, being long enough to protrude 2/1000 inches above the surface of the central table when inserted.

The coverslip is made of selected clear mica split to a thickness of approximately 40 μ. It is supported by an aluminum ring 7/8 inch outside diameter and 11/16 inch inside diameter, thus being 3/32 inch wide. It is 1/16 inch thick and has 3 threaded holes placed centrally at 120 degree intervals to receive the connecting screws. These measurements allow the oil immersion objective to be brought into focus over the entire central table without striking the aluminum ring and yet afford sufficient strength with minimum weight. The mica is cut into a 7/8 inch circular sheet and glued to the aluminum ring with any firm adherent (we have found Permount satisfactory), and 3 holes are punched over the threaded holes in the aluminum ring. *The connecting screws* are brass 0-80 machine screws 3/16 inch long used to attach the base to the coverslip.

A specially designed movable stage similar to those previously described[2] is used to hold the ear chamber during observations.

Methods. Insertion of the Chamber: Un-

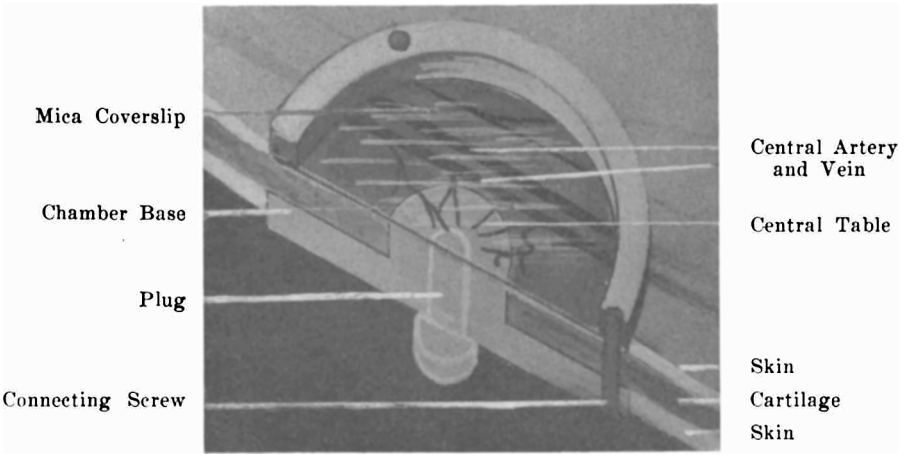

FIG. B.
Cross section diagram of a chamber in a rabbit's ear.

der aseptic conditions 4 holes are punched near the end of the pinna of a rabbit's ear—one central hole and 3 peripheral smaller holes. The skin is removed from both sides of the cartilage over a circular area including all of the holes. The chamber base is then placed with its raised central table protruding through the central hole, the 3 connecting bolts passing through the peripheral holes, and the coverslip is screwed in place on the opposite side of the cartilage. By careful adjustment of the connecting bolts the covslip is brought into contact with the 3 buffers on the surface of the central table. This leaves a sterile film of blood 40 to 50 μ thick overlying the central table visible through the mica coverslip into which vessels may grow from the vascular bed of the adjacent cartilage. Within 14 to 28 days the area becomes completely vascularized (Fig. B), and the vessels subsequently become differentiated into arterioles, venules, and capillaries.[3]

Inoculum. Bovine tubercule bacilli (Strain Ravenel RV) grown in Dubos liquid medium for one week were used in this experiment. After the culture was centrifuged and the supernatant liquid decanted, the bacilli were resuspended in normal saline; the weight of bacilli was calculated from turbidimetric readings of the original culture.

Technic of Inoculation. A red blood cell pipette (previously calibrated) with a heat-drawn tip was used to introduce the suspension of tubercle bacilli. The plug was removed aseptically,[2] and the fine tip of the pipette placed well into the plug hole; then 0.001 to 0.003 ml was slowly introduced. Although the method is crude, it allows for a rough approximation of the given figures and was found adequate for the purpose of this experiment.

Results. Four chambers were studied in detail from the time of tissue inoculation till the development of necrosis. A motion picture was taken of only one complete experiment over a period of 30 days, but in all experiments (with one exception discussed below) the results were essentially the same.

The Normal Chamber. The growth of new tissue onto the central table and the development of what Clark has called the "adult pattern" has been described in detail elsewhere.[3] The adult pattern refers to certain characteristics of appearance and behavior of mature connective tissue on the observation area after it has become stabilized. In summary, these characteristics are as follows: The vessels are well differentiated into arter-

[3] Clark, E. R., Hitschler, W. J., Kirby-Smith, H., Rex, R. O., and Smith, J. W., *Anat. Rec.*, 1931, **50**, 129.

ioles, venules, and capillaries. The muscular walls of the arterioles are clearly visible and are seen to contract rhythmically in the normal chamber. Contraction of the main arterioles supplying the tissue over the observation area causes temporary emptying of most of the vessels on the table. All vessels have a definite tonus, and the vascular endothelium is sharply defined. Usually only a portion of the vessels on the central table are filled with blood at any one time, and "plasma skimming"[4] is a common phenomenon. In the normal vessel the flow of red blood cells is streamlined if rapid enough, and white blood cells tend to be thrown toward the periphery of the stream. Often white blood cells roll along the endothelium at a much slower rate than that of the flow at the center of the vessel. If a physical or chemical agent causes injury to the vascular endothelium, it becomes "sticky", and instead of rolling along the endothelial wall leukocytes adhere to it. Sticking of leukocytes to endothelium is one of the earliest signs of inflammation in living tissue and precedes diapedesis of cells. A fairly heavy connective tissue stroma supports the vessels and no free fluid is seen. Extravascular cells are numerous in the growing chamber but are considerably reduced in numbers in the normal adult chamber. Histiocytes are seen scattered throughout the connective tissue and are particularly numerous along vessels. Adventitial cells which do not take up vital stains are seen along vessels. Fibroblasts and fibrocytes are not visible normally but stain faintly with supravital stains. Rare polymorphonuclear leukocytes and mononuclear cells are seen. Lymphatics are present in some chambers, but the lymph flow is very sluggish, and often only pulsation of fluid and cells can be seen. The "normal chamber" is an ideal which cannot be attained since accidental mechanical trauma may cause mild temporary inflammatory changes. On rare occasions small hemorrhages are seen, and there may be a temporary localized sticking of leukocytes to vascular endothelium. For the purpose of these experiments, however, chambers were chosen which showed a stabilized adult pattern and were as normal as seems to be possible.

Mechanical Trauma Due to Inoculation. The inflammatory changes associated with withdrawal of the plug, inoculating fluid, and reinserting the plug have been studied in detail in the past,[2] and although the extent of the trauma may vary considerably, the duration of change can be predicted with a fair degree of accuracy.

There is always some localized sticking of leukocytes in vessels near the plug margin, and localized collections of polymorphonuclear leukocytes and occasional mononuclears are visible for several days after pulling the plug. Localized stasis, thrombosis, and hemorrhage may occur, but vascular changes are temporary and there is a return to normal after 3 to 4 days. Small hemorrhages disappear in 3 to 5 days. In the 4 chambers studied in these experiments the traumatic changes varied from well localized cellular sticking to stasis and thrombosis of a few vessels and localized hemorrhage around the plug margin.

The size of the inoculum varied from 0.000001 mg to 0.0015 mg. The larger inoculum was used in "injection chambers"[5] in which injections were made via a duct built into the lucite base. In this type of chamber part of the inoculum is unavoidably lost in the large injection duct.

Early Reaction. For descriptive purposes the response of the tissue in the observation area to the presence of tubercle bacilli has been divided into an early and a late reaction. The early response was minimal and lasted 9 to 23 days. During the first few days it was indistinguishable from the inflammatory change associated with pulling the plug.

In all cases the vascular reaction was exceedingly mild. (Fig. C2-4) Very slight dilation of vessels and localized cellular sticking was the essential reaction which developed during the initial period of 6 to 7 days;

[4] Krogh, A., *The Anatomy and Physiology of the Capillaries*, 1929, Yale University Press, New Haven.

[5] Ebert, R. H., Sanders, A. G., and Florey, H. W., *Brit. J. Exp. Path.*, 1940, **21**, 212.

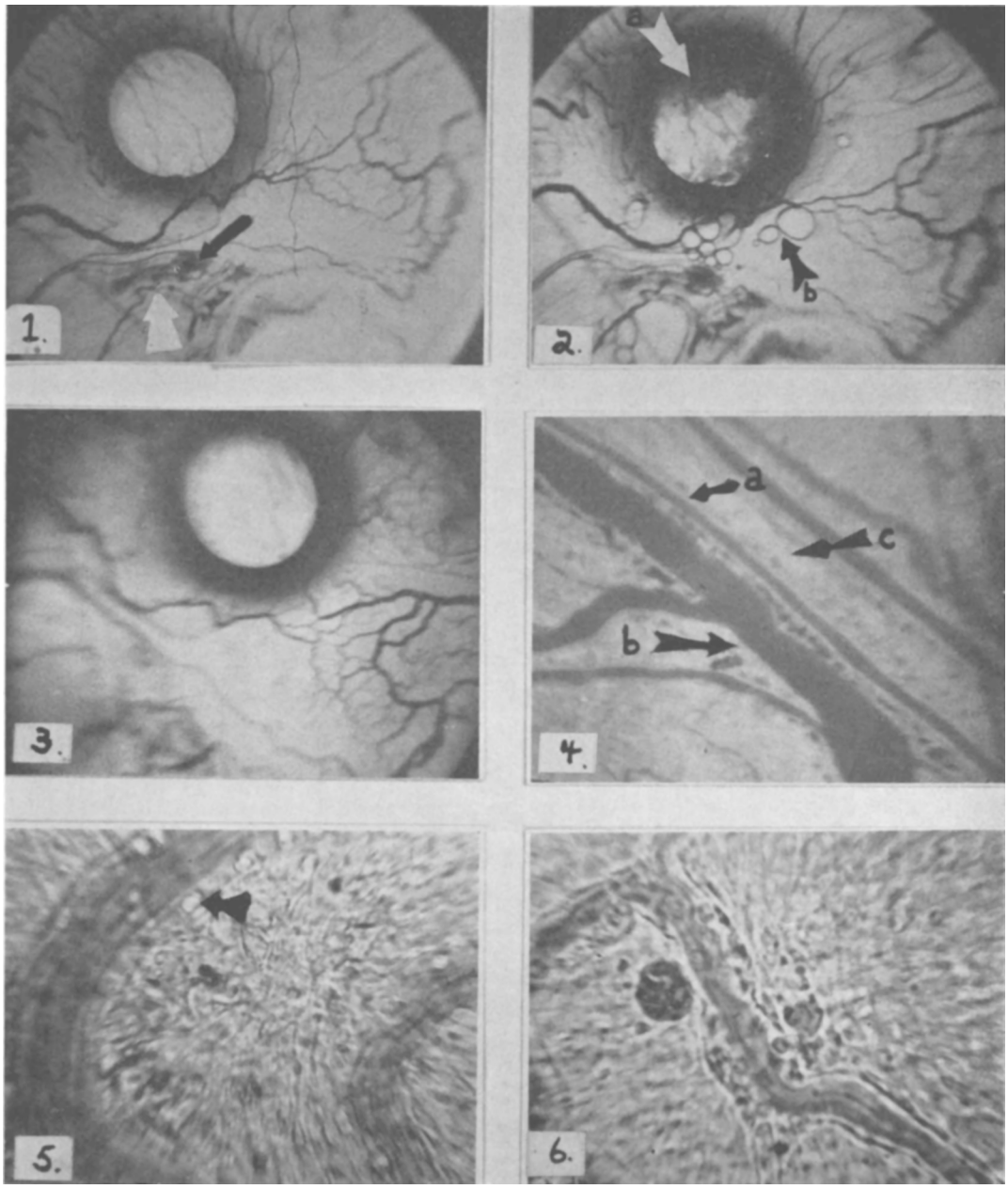

FIG. C.

The development of tuberculous infection illustrated by frames taken from a kodachrome motion picture of a single experiment.

1. 18×. One day prior to inoculation. The tissue is essentially normal except for a small hemorrhage produced by accidental trauma (see arrows).

2. 18×. Ten minutes after the inoculation of 0.0004 mg of virulent bovine tubercle bacilli (Strain Ravenel RV). There is hemorrhage around the plug (a) caused by the procedure. Note the air bubbles below the plug (b) introduced when the plug was reinserted. There is little vascular damage.

3. 18×. Third day after the inoculation. The hemorrhage around the plug has cleared and the air bubbles have disappeared. The vessels are normal.

4. 100×. Third day. An arteriole (a), venule (b), and lymphatic (c) appear normal.

5. 400×. Tenth day. This is the first vascular reaction seen. There is sticking of white cells to vascular endothelium (arrow). A tuberculin test (1:10 O.T.) set on the tenth day was read as weakly positive on the twelfth day (a 1:10 O.T. test on the sixth day was negative).

6. 400×. Eleventh day. There is marked sticking of white cells to vascular endothelium.

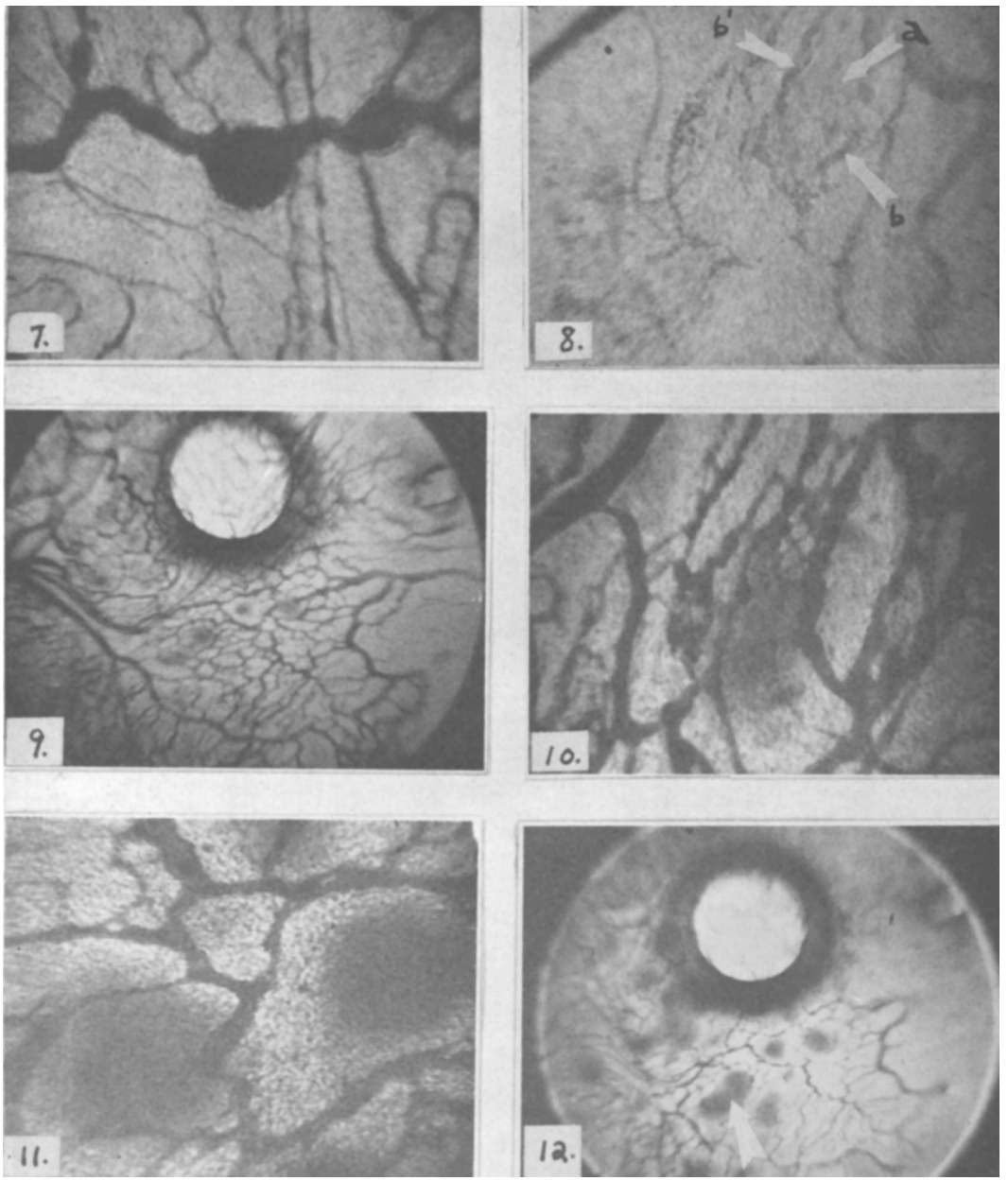

FIG. C (continued).

7. 100×. Eleventh day. There is aneurysm formation in a venule due to vascular endothelial damage.

8. 100×. Fifteenth day. A small area of infiltrate can be seen (a) and adjacent to it are 2 small thrombosed vessels (b and b'). These were the first thromboses seen.

9. 18×. Seventeenth day. There is marked dilatation of all vessels and several areas of increased density can be seen centrally located. These are regions of dense cellular infiltration.

10. 100×. Eighteenth day. A small area of cellular infiltration surrounded by multiple dilated vessels.

11. 100×. Twentieth day. Two areas of infiltrate surrounded by dilated markedly damaged vessels. The blood flow in these vessels was sluggish and pulsating and there was hemoconcentration.

12. 18×. Twenty-first day. There has been an increase in the size and number of tuberculous infiltrates. Most of the vessels on the right side of the table and over the plug are thrombosed.

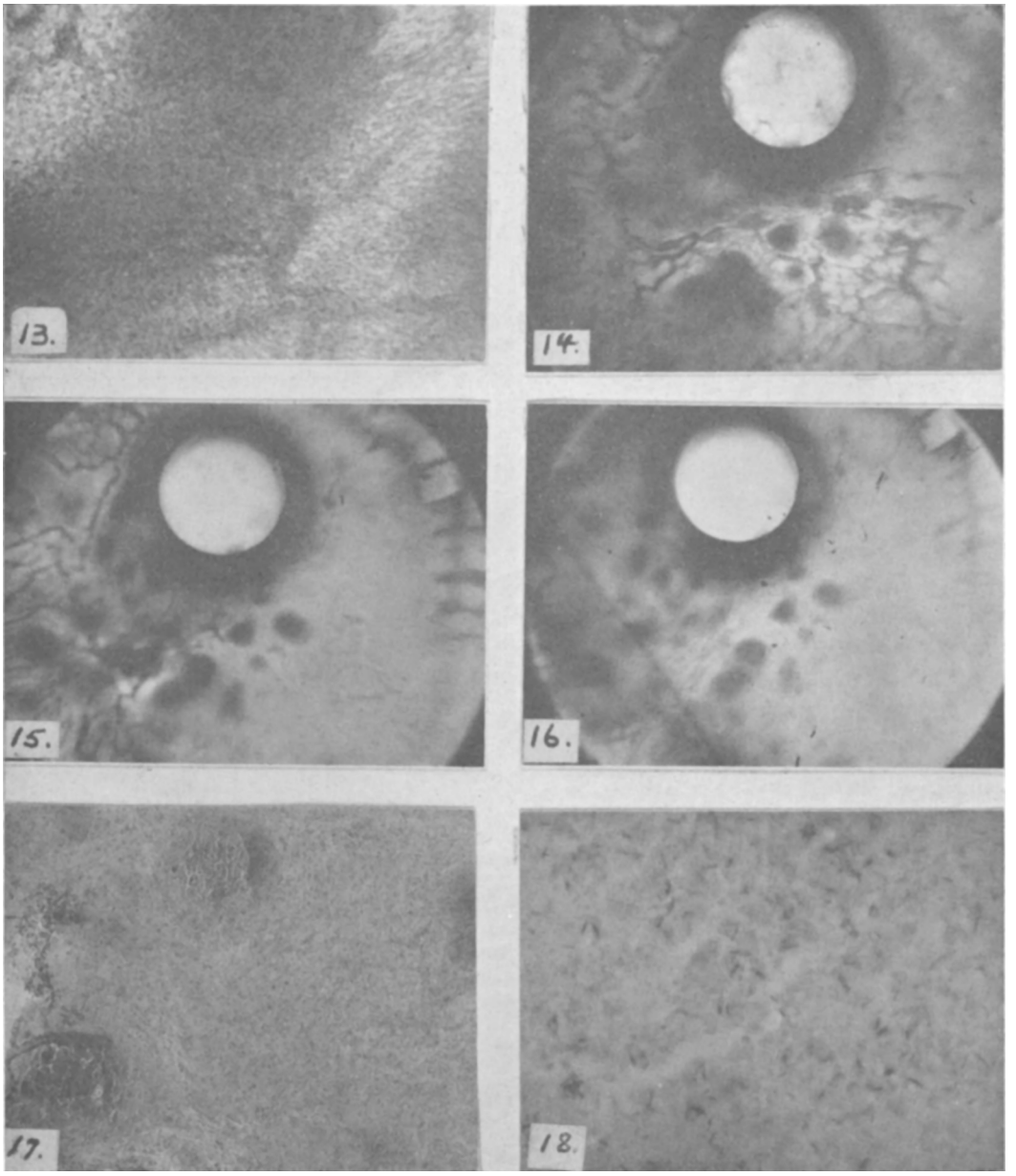

FIG. C (continued).

13. 100×. Twenty-first day. This is the same area photographed in Fig. C-11 and pointed to by the arrow in Fig. C-12. The dense regions have become confluent and there has been complete thrombosis of the vessels surrounding the infiltrates.

14. 18×. Twenty-third day. There is increased confluence of the dense areas of tuberculous infiltrates and extension of thrombosis.

15. 18×. Twenty-fifth day. The thrombosed vessels on the right side of the table seen in Fig. C-14 have disappeared and the area is necrotic. Only a small vascular area remains along the left border of the observation table. The remaining vessels are dilated and show evidence of damage.

16. 18×. Twenty-ninth day. There has been almost complete destruction of all the vessels on the table. The animal was sacrificed immediately after this photograph was taken and the tissue fixed by injection of the central artery with alcohol.

17. 100×. A hematoxylin and eosin section showing tuberculous caseation. The dense areas are the same as those seen *in vivo*.

18. 950×. Numerous acid-fast bacilli are seen. This is a section through the region of density seen in Fig. C-13. All areas of tuberculous infiltration were loaded with tubercle bacilli. Only rare bacilli were found between these areas.

these changes were so mild that in the early experiments it was doubted that infection had actually occurred. In one case reinoculation was carried out on the 5th day because the tissue apparently had returned to normal. In two cases localized free fluid persisted after the inoculation with tubercle bacilli. In one case this fluid persisted till the development of the "late reaction", and in the other it disappeared 8 days before the "late reaction" developed.

In 2 experiments there was very little cellular exudate—not much more than could be accounted for by the mechanical trauma. In the 2 cases in which fluid was seen mononuclear cells and macrophages were found in the fluid, and in these experiments as well as one other localized collections of macrophages were observed after the 9th day.

Late Reaction. In contrast to the relatively mild early reaction there occurred sometime between the 10th and 24th day a much more explosive sort of tissue response. The change was sudden and progressive. It began with marked sticking of white cells to the vascular endothelium (Fig. C5, 6) accompanied by considerable infiltration of white cells into the tissue. This was followed by progressive dilation of vessels until they seemed to have lost all tonus (Fig. C-9,10). Associated with this dilation the vessels became tortuous, and in one case small aneurysmal dilations occurred in the wall of 2 venules (Fig. C-7). The vascular endothelium no longer appeared as sharply defined as it did in the normal vessels. As was mentioned above, only a portion of the vessels in the normal chamber appeared filled with blood at any one time but now all vessels were filled and blood flow became sluggish and pulsating. Agglutination of red blood cells was noted, but sludging was not as prominent as has been described by Knisely and associates in other disorders.[6,7] Simultaneously with these vascular changes there occurred a considerable increase of the amount of exudate (Fig. C-8), and free fluid was seen in all chambers. For the most part the exudate was composed of monocytes, lymphocytes, and macrophages but some polymorphonuclear leukocytes were seen. Occasionally giant cells could be distinguished. There was considerable increase in the size of the localized collections of cells mentioned in the early reaction, and these areas became so packed that individual cells were seen with difficulty. Stasis and localized thrombosis of small venules surrounding these dense collections of cells then developed (Fig. C8-11), and this phase was rapidly followed by extensive thrombosis of surrounding vessels (Fig. C12-16).

The sequence of events in a thrombosing vessel could be followed in the greatest detail. First, there appeared to be hemoconcentration in the dilated venule; then red cells became so densely packed that the margins of individual cells were no longer visible. Some pulsation continued for a time in the degenerating vessel, and it was common to see a thrombosing vessel branching from another venule in which flow was still present. Gradually the endothelium became less distinct and finally the vessel disappeared. The rate at which these changes occurred varied. In some chambers it occurred rapidly in about 6 to 7 days; in others, more gradually over a period of 19 days—but in every case except one the result was the same: progressive thrombosis of blood vessels until almost all the visible tissue was avascular and necrotic (Fig. C-16). In the one exception the explosive reaction began on the 15th day and gradually progressed till the 22nd day after inoculation. At this time there was very marked dilation, considerable exudate, and several areas of venous thrombosis. Between the 22nd and 27th days the process remained relatively stationary, and from the 28th till the 35th day there was a gradual return to normal with considerable reabsorption of the exudate. A tuberculin test on the 46th day with 1:10 OT was negative.

Tuberculin tests at intervals were carried out in only one rabbit. In this animal the

[6] Knisely, M. H., Eliot, T. S., and Bloch, E. H., *Arch. Surg.*, 1945, **51**, 220.

[7] Knisely, M. H., Stratman-Thomas, W. K., Eliot, T. S., and Bloch, E. H., *J. Nat. Malaria Soc.*, 1945, **4**, 285.

first tuberculin test (1:10 OT) on the 6th day was negative. The second, on the 10th day was weakly positive; a third on the 27th day was strongly positive. The marked tissue response began on the 10th day simultaneously with the development of skin hypersensitivity and progressed to almost complete dissolution of the visible tissues on the 29th day (Fig. C-16).

Stained Sections. At the end of the experiments the observation area and surrounding tissue was fixed in alcohol or ice-cold acetone, and serial sections were made with both hematoxylin and eosin and acid-fast stains. In 3 cases almost the entire area of tissue over the central table was necrotic (Fig. C-17) and numerous acid-fast bacilli were found in the areas of dense infiltrate noted in the living animal (Fig. C-18). Scattered acid-fast bacilli were found elsewhere, but nowhere were they as numerous as in the areas of heavy infiltration. Tubercles consisting of areas of central necrosis, and containing acid-fast bacilli, surrounded by epithelioid cells and giant cells were found in the connective tissue adjacent to the central table. In the fourth case the tissue was removed from the animal in which the chamber had shown a return toward normal. In these sections epithelioid cells and giant cells were found but no acid-fast bacilli could be discovered after careful search.

Discussion. The most striking observation made in the course of these experiments was the vascular reaction to the presence of tubercle bacilli in the connective tissue. Although tuberculin testing was done systematically in only one experiment, the exact correlation of developing skin hypersensitivity and vascular changes in this one case seems more than chance occurrence. During the early stages the reaction was little more than what one would expect from a benign foreign body, but the development of hypersensitivity ushered in a series of destructive changes in the vascular tree of the observation area. It is impossible to say if these changes in vascular endothelium were primary or secondary, but regardless of their exact origin they certainly play an important part in the development of tissue destruction. In effect we observed a series of small infarcts. It should be remembered that the thrombosis described as occurring was in relatively small vessels—none larger than 30 to 40 μ in diameter normally (although some were considerably more dilated at the time of thrombosis). In other words, this was microscopic thrombosis and did not involve any vessel of appreciable size.

One hesitates to infer that similar changes occur in all tissue and in all animals as a part of the pathogenesis of the caseous tubercle. It was a striking fact, however, that in the stained sections one found no indication of the dramatic vascular changes which had occurred in the living animal and could see nothing but necrotic tissue surrounded by tuberculous tissue.

Summary. A new use of the rabbit ear chamber technic is described.

Microscopic studies of the tissue changes in developing tuberculous infection within the chamber were made. The early reaction to the presence of living tubercle bacilli was minimal. The late reaction was an explosive, necrotizing response. In the one rabbit systematically skin tested with Old Tuberculin this late reaction began coincidentally with the development of skin hypersensitivity. Progressive vascular damage ending in venous thrombosis played an important role in tissue destruction.

The experimental technic and microscopic observations described have been recorded in Kodachrome motion pictures. It is suggested that this technic may be used for microscopic study of the dynamics of tissue changes in other pathological conditions.

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