As a further check of the specificity of this lyophilized form of PBP, 10 patients from the University Hospital, who had been diagnosed as brucellosis cases and who had previously given a positive skin test with the old form of PBP, were again tested with this readily soluble form. All gave a positive skin reaction.

For negative control group, 20 healthy subjects, selected at random from the general population and with no history of brucellosis or symptoms usually associated with this condition, were also tested; all were negative to the lyophilized PBP.

Summary. A purified brucella protein, subjected to further purification and presented in a soluble lyophilized form, has been described. Weighed amounts are placed in glass vials, which, after the addition of a measured quantity of sterile saline buffered solution, produces a product ready for use. This procedure facilitates the use of the product in epidemiological investigation and as a routine diagnostic test.

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17201. Effects of Bone Extracts Injected into the Mouse Testis.*

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Bone will develop from bone marrow when it is transplanted under certain conditions. This subject has recently been reviewed by Levander[1] and Pfeiffer.[2] However, these two authors differ markedly in their interpretation of how bone is formed from the marrow transplant. Levander[1] has reported that when bone marrow is transplanted, all of the grafted cells disintegrate and mesenchymal cells from the surrounding tissue soon migrate into the graft and are transformed into chondroblasts and osteoblasts which form cartilage and bone. Pfeiffer[2] agrees with Levander that the hematogenic and myelogenic cells disappear from the graft but believes that when bone forms, the marrow reticulum cells of the graft survive and these cells develop into osteoblasts just as they do in the marrow cavity when medullary bone forms there as described by Bloom, Bloom and McLean[3] in the pigeon.

The evidence in the literature[4-7] that there is an extractable osteogenic substance would tend to support Levander's hypothesis. However, it has been questioned whether such a substance exists, and evidence that injury is the primary factor in causing bone to form when bone extracts are injected has been advanced by Heinen.[8] Apparently mesenchymal cells transform into osteoblasts in either case. It therefore becomes important to test whether the mesenchymal cells present in the area where bone developed in marrow grafts would produce bone under the same or greater injury than resulted from grafting, plus the extract from a similar or larger piece of marrow than was grafted.

Materials and methods. Mice of the Strong[9] A strain were used in this experiment because it has been shown that bone will develop in marrow grafts in the anterior chamber of the eye and in the testes of mice of

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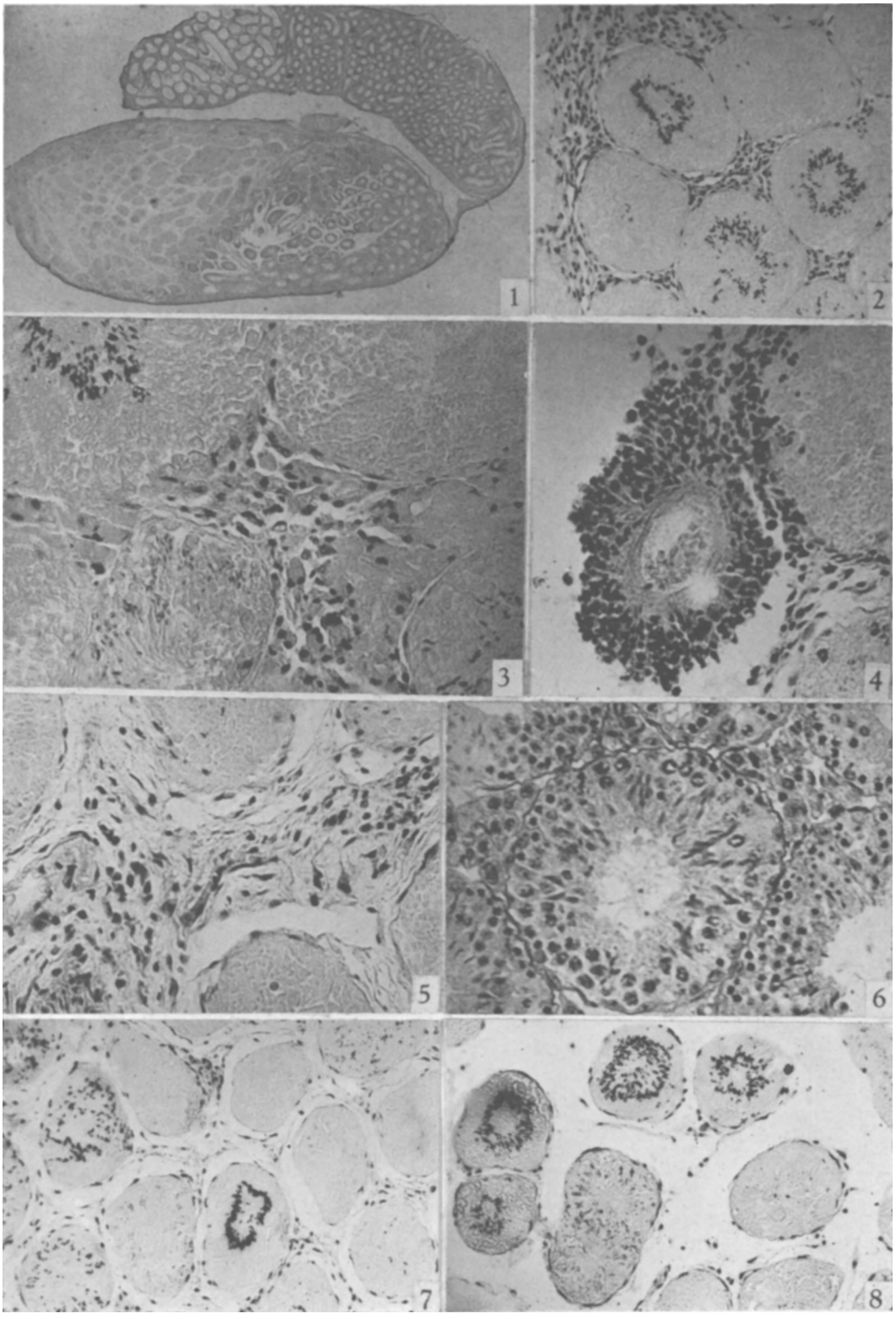
this strain.[2] Of these two sites, the testis was used since it contains numerous primitive mesenchymal cells. It nicely delimits the area affected, and it does not contain or allow any contact with the type of connective tissue associated with ligaments or bone attachments. The bone extract was made according to the procedure of Annersten,[5] with slight modification to adapt it to the mouse. All of the long bones, the pelves, the shoulder girdles, the calvarium and any other bones that could be readily freed from the surrounding tissue were removed from 6 or 7 mice. These bones, weighing 5 to 8 g were ground with a mortar and pestle and extracted with 15 to 20 cc of HCl-alcohol (100 cc of 95% alcohol and 5 cc of 0.1N HCl). The ground bone was placed in a glass stoppered tube. The alcohol was added and stirred. The mixture was then placed in a refrigerator at +1°C for 48 hours and stirred frequently. It was then centrifuged. The supernatant liquid was placed in an appropriate container and evaporated to one-quarter its former volume by passing a stream of warm air over it. The extract became opalescent and a fine lipid film formed around the edges of the surface. No attempt was made to analyze the content of this extract. However, Annersten[4] gives a report on the analysis of his extracts of rabbit bones. The solution was next diluted with an equal volume of isotonic salt solution and injected immediately. If injections were not to be made at once, the dilution was not done until just before injection. The extract was stored in the refrigerator at +1°C.

The extract was injected by means of a 1 cc tuberculin syringe equipped with a 25 gauge needle. The mice were anesthetized, and the left testis was exteriorized through a suprapubic midline incision. The testis was grasped by the gubernaculum, and the hypodermic needle inserted at the posterior pole of the testis, parallel to its long axis. As much extract as could be retained (about 0.05 cc) was injected. If too much solution was injected, the internal pressure caused herniation of the seminal tubules at the point of entrance of the hypodermic needle. The testis was then returned to the abdominal cavity and the incision sutured. Thirty-three male mice were used. The animals were autopsied at 15 days, and at 3, 4, 5 and 6 weeks after the extract was injected. The testes were preserved in Bouin's fluid, sectioned in paraffin (7 μ) and stained with Harris' hematoxylin and triosin.

Observations. In gross appearance the treated testes are somewhat less than 2/3 their normal volume. If the seminal tubules herniate, or some of the acid-alcohol extract leaks out, there is a tendency for the testis to adhere to surrounding tissue. Adhesion usually occurs in the open inguinal canal or in the region around the abdominal incision. The area that is affected by the extract can be readily seen in gross observation, since the tubules appear as chalky white structures showing through the tunica albuginea. The remainder of the testis is the normal pinkish gray color without the tubular nature of the testis being apparent. The extract seems to fix the tissue somewhat in about 2/3 of the testis (Fig. 1). At 2 weeks there are no lumina in the tubules of the treated area. The cell membranes are no longer apparent, and the coalescence of the cells produces a homogeneous light pink-staining granular mass. Apparently the nuclei of the basal layers of cells disappear, but the chromatin material of the spermatids and spermatozoa is grouped concentrically at the centers of the tubules. Nuclear membranes are not seen, yet the chromatin material is very dense and compact, without evident coalescence, and retains its hematoxylin-staining affinity. The connective tissue forming the tubule walls appears desiccated, but otherwise does not show much alteration. The interstitial tissue is composed of connective tissue, Leydig cells and occasional macrophages and lymphocytes. The cytoplasm of the connective tissue cells and Leydig cells takes the same light pink stain as does the material within the tubules. Some of the Leydig cells have the characteristic interstitial cell nuclei, while in others the nuclei are becoming pycnotic. The nuclei of the fibroblasts are in most instances pycnotic. Lymphocytes are not yet numerous, and the blood vessels show only slight evidences of future hyalinization. Macrophages are only occasionally seen.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All tissues were fixed in Bouin's fluid, sectioned at 7 μ and stained with Harris' hematoxylin and triosin.

FIG. 1. Longitudinal section through entire testis and epididymis following injection of 0.05 cc of an acid alcoholic extract of bone into the testis. The portion to the left is the treated area and that to the right is the normal testicular tissue. Note the sharp dividing line between the infarct and the normal area. × 12.

FIG. 2. A section of the treated area of a testis 20 days after injection of an acid alcoholic extract of bone. This section is near the normal part of the testis and shows the predominance of fibroblastic elements in the interstitial tissue of this area and the disappearance of all normal cellular structure in the seminiferous tubules, except for the darkly staining chromatin material of the spermatids and spermatocytes which is arranged concentrically in the center of the tubules. × 110.

FIG. 3. Same testis as in Fig. 2. A section near the center of the treated area. Note the rather normal appearing Leydig cell between the 2 tubules at the extreme right and the numerous cells in the interstitial tissue, some of which may be altered Leydig cells but most of which are undoubtedly macrophages. See also altered protoplasm, lack of chromatin material and even, faintly staining appearance of all spermatogenic cells. The chromatin material of the spermatids and spermatozoa still stains very strongly, however. × 220.

FIG. 4. A cross section of an arteriole at periphery of infarct in a testis 20 days after injection of bone extract, showing marked alteration of these vessels and presence of numerous plasma cells and lymphocytes around them. The remaining interstitial tissue appears greatly dehydrated but not otherwise much altered. × 220.

FIG. 5. Interstitial tissue 4 weeks after injection of bone extract. Note little change in the cells. They are extremely pycnotic, and the fibers appear denser. The small blood vessels show none of the changes seen at periphery. All of tissue has been killed and fixed by the alcohol and remains in this condition with no evidence of removal of dead tissue. × 220.

FIG. 6. The normal portion of a testis that had received acid alcoholic extract of bone 6 weeks before. The treated area of the same testis is shown in Fig. 8. × 220.

FIG. 7. Treated area of a testis 5 weeks after injection of bone extract. There is little change from earlier stages except that there is more interstitial edema. The tubules are smaller and there are possibly fewer chromatin remnants of the spermatids and spermatozoa in the tubules. × 110.

FIG. 8. Treated area from the same testis as shown in Fig. 6. It is similar to Fig. 7 except for the greater interstitial edema. At 6 weeks there is no evidence that the killed and preserved cells are being cleared out of the area. × 110.

At 3 weeks the contents of the seminiferous tubules are essentially the same as at 2 weeks (Fig. 2). The protoplasm is, however, somewhat more homogeneous. In the intertubular spaces there are fewer cells with definitive interstitial cell nuclei, but cells with pycnotic nuclei which might formerly have been interstitial cells of Leydig are fairly numerous (Fig. 3). They are, however, almost completely absent from the center of the treated area. The fibroblasts show little if any change from that seen at 2 weeks. A few polymorphonucleated cells are present at the periphery of the treated area. Also in this region are a few lymphocytes. The vascular condition has changed very little from that at 2 weeks. Hyalinization of the blood vessels is beginning at the periphery, and lymphocytes are becoming more numerous around these blood vessels (Fig. 4). At the dividing line between the treated and normal areas an occasional seminiferous tubule shows the characteristic clumping of the spermatocytes that occurs when the seminiferous tubules are damaged. This type of degeneration in the tubules is never seen except at the periphery of the treated area, and is secondary to the primary damage.

By 4 weeks the tubules are further shrunken in size. The protoplasm appears to be more homogeneous. There is less chromatin material visible, and many cross sections of tubules show none at all. There is much more intertubular edema, although this varies considerably from animal to animal. The nuclei of the fibroblasts are quite pycnotic (Fig. 5), and only rarely can a cell be found which could have been an interstitial cell of Leydig, even in the peripheral zone of the affected area. There is a somewhat marked hyalinization of the blood vessels, but it is only the vessels at the periphery of the treated area that show this response. Lymphocytes are much more numerous, especially near the blood vessels, and plasma cells are now present. Macrophages are not noticeable at this time. The amount of connective tissue in the interstitial spaces is about normal, but it ap-

pears dehydrated, and the large empty spaces due to further shrinkage of the tubules make it appear less abundant.

From the fourth to the sixth week the changes already described become more extreme (Fig. 7 and 8). There is further shrinkage of the tubules and disappearance of chromatin material, although certain tubules resemble those at 2 or 3 weeks, except for the smaller size. Usually only connective tissue which has been coagulated by the injected alcohol is seen in the intertubular spaces. The fibers often show evidence of disintegration. The spaces between the tubules, and between the tubules and connective tissue become greater. Apparently the shrinkage of the tubules is much greater than that of the connective tissue. In the central area only an occasional cell that may be a degenerating interstitial cell or a macrophage is seen. If these are living macrophages, there is no evidence of their phagocytic activity.

The vascular changes seen in the treated testes seem to be associated with the peripheral area, and the presence of lymphocytes and plasma cells is limited primarily to this region. Presumably the lymphocytes come in from the blood stream, but the plasma cells may possibly be formed in the area. There is no evidence whether the plasma cells arise from lymphocytes or not. They are, however, limited to the same areas. There seems to be no evidence of undamaged fibroblasts or primitive mesenchymal cells in the treated area. The vascular and lymphocyte response in the peripheral region occurs whether it is adjacent to normal seminiferous tubules or to the tunica albuginea. The tubules in the area outside of that exposed to the extract are essentially normal (Fig. 6) except for a slightly cryptorchid appearance which may be due to the fact that the testis is only about 2/3 its normal size and probably has resided in the abdominal cavity most of the time. The interstitial tissue and Leydig cells appear to be increased somewhat in this area. This is probably due to the reduced tubular volume.

Discussion. The fact that bone did not develop in the mouse testis when acid alcoholic extract of bone was injected, even though the amount of extract injected represented a volume of marrow many times that which was transplanted to the mouse testis and which formed bone,[2] strongly suggests that the marrow graft contributes something besides a possible extractable osteogenic substance, presumably the growing marrow reticulum cells, which it seems probable become osteoblasts and form bone.

In the literature on the transplantation of bone marrow the following facts are evident: 1. Autoplastic transplants of bone marrow are fairly successful in forming bone.[1,10–14] 2. Homoplastic transplants are seldom successful[10,11,15,16] and then only in young animals, and with large amounts of marrow.[1,17] 3. In inbred strains of mice implantation of tiny fragments of marrow cause the development of bone.[2] 4. The degree of resistance to grafting which is present in the grafting site is of extreme importance; where resistance is high, bone has not formed when marrow is transplanted.[2] All of these conditions are related to the ability of grafted cells to grow in their new environment. It seems, therefore, that bone develops in marrow transplants only under conditions that are favorable for transplantation. Since all workers agree that the myelogenic and hematogenic cells do not survive grafting, it must be that the marrow reticulum cells do survive as suggested by Pfeiffer[2] and are the cells which Levander[1] believes are mesenchymal cells which have migrated into the area.

While it seems quite clear from the above evidence that marrow reticulum cells form

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bone when bone marrow is transplanted, just as they have been shown to do in their normal position,[3] the evidence is equally convincing that bone is caused to form in many areas where marrow reticulum cells are not present. The osteoblasts and chondroblasts in the latter areas must come from the primitive mesenchymal cells. However, in spite of the obvious similarity of the primitive mesenchymal cells and the marrow reticulum cells new problems become evident. The injection of an acid alcoholic extract of bone into muscle has been reported to cause the primitive mesenchymal cells to form bone,[1,4-6] yet the testis of the mouse contains large numbers of primitive mesenchymal cells which failed to respond to acid alcoholic extract of bone. In the present experiment all the tissue in the treated area is killed. It represents a subtotal infarction, and apparently the damaged tissue is gradually removed by autolytic enzymes brought in by the tissue fluids which penetrate the area. No cells remain in the treated area that could form bone. If bone should form under these circumstances it would have to be from cells outside the treated area or from cells which migrate into the area by way of the blood stream or by connective tissue ingrowth from the periphery. It would not be expected that any injected osteogenic substance would remain in the area for as long as these experiments ran. Therefore, if bone should develop later, it could hardly be attributed to the injected osteogenic substance. It is also evident that the injury produced by injection of the bone extract, which Heinen[2] believes is responsible for bone formation under these conditions, did not cause bone to develop in the testis. It is true that we did not pretreat the area with 40% alcohol as Annersten[4,5] did, but with the limited area within the testis and the extreme damage to the tissue, it was felt that the effect of alcohol pretreatment would only be to further limit the amount of extract that could be retained. Moreover, Bertelsen[6] has claimed that this pretreatment is not necessary.

The fact that the presence of alcoholic extract of bone and the considerable injury produced by its presence has not caused bone to form in the mouse testis, while marrow transplants readily produce bone there, suggests that in the mouse the primitive mesenchymal cells of the testis are not easily stimulated to form bone. Therefore, the mouse testis is not a good place to test either whether any extractable osteogenic substance exists, as postulated by Annersten[4,5] and Bertelsen,[6] or whether injury to the tissue brought about by the extract is the cause of the response when bone formation follows the injection of alcoholic extracts into muscle.[2] The rabbit, which has been used in the experiments testing the presence or absence of an osteogenic factor, on the other hand is notorious for the ease with which ossification occurs following injury, a fact which has been becoming increasingly evident since the experimental production of ectopic bone in the rabbit by Sacerdotti and Frattin.[18] It seems probable that the present failure to obtain bone formation is related to the use of the mouse as an experimental animal and the testis as the injection site. It was not thought feasible to test the bone extract on the muscle of the mouse since it would be unlikely that appreciably more extract could be injected here than into the testis without affecting the connective tissue involved in tendon or muscle insertion.

Summary. An acid alcoholic extract of bone did not cause the development of bone when it was injected into the testes of 33 male mice of the Strong A strain. It caused extreme damage to about 2/3 of the testis, producing a subtotal infarction of the area. Since fragments of bone marrow produce bone when transplanted to the testis, whereas extracts from much larger amounts of marrow and bone do not cause the development of bone, it is concluded that the marrow reticulum cells survive grafting and produce the bone.

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