THE TRANSPLANTATION OF BONE

An Experimental Study

A thesis submitted for the degree of Doctor of Medicine by John Chalmers, M.B., Ch.B.(Ed.)1950, F.R.C.S.Eng.

We can help the sick and the maimed in only one way - by aiding and abetting the natural defensive and recuperative mechanisms of the human body. Our methods must be based on a knowledge of these mechanisms.

Sir Arthur Keith (1919)

From: "Menders of the Maimed"

Hodder & Stoughton, London.

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INTRODUCTION

There can be few medical problems on which more has been written than the transplantation of bone. A review article as long ago as 1919 referred to more than 600 papers on this subject and since that time contributions in this field have continued to be published at an almost overwhelming rate. One would hesitate to add further to this mass of information, were it not that when faced with the important practical question of selecting the most satisfactory alternative to bone autograft for clinical use, no answer could be found.

Certainly the problem has not lacked investigation; the difficulty lay, rather, in the number of "best" alternatives which had their enthusiastic advocates. Differences in experimental technique and in methods of assessing a graft's progress had resulted in a wealth of conflicting opinion.

Chapter III of this thesis describes an attempt to evaluate different bone grafting materials in order to assess their relative clinical value. In devising a biological test for a bone graft the author has tried to eliminate some of the sources of uncertainty which had led to the conflicting conclusions of previous workers. Among these had been the use of test situations which did not

correspond to any clinical bone grafting procedure; the use of inexact methods for measuring a graft's progress; or the evaluation of some aspect of a graft's behaviour which was not relevant to its clinical function.

The materials submitted to this biological test were all prepared from homogenous bone, as a review of the literature had indicated that these were likely to be of most value. However, the fact that homografts of bone could be usefully accepted by the host in itself raised further questions.

In recent years great advances have been made in the understanding of the nature of the reaction of a host to transplants of tissue of homogenous origin. In general, such transplants fail because of the development of an immune response by the host against the transplant. The success of bone homografts in the light of this new knowledge had been largely unexplained.

A further study was, therefore, undertaken to determine whether the apparently privileged behaviour of bone homografts was due to a fundamental difference between bone and other tissues with respect to homograft immunity. The experimental work on this part of the study is described in Chapters I and II of the thesis.

The author has been most fortunate in the generous help and advice which he has received from many people.

Professor J. I. P. James, at whose suggestion this investigation was first undertaken, has throughout given much encouragement by his interest.

Dr. H. A. Sissons collaborated in the work described in Chapter III. This association has been most valuable and enjoyable; his wide knowledge in bone physiology and pathology and in experimental method has always been freely shared. The work of Chapters I and III was carried out in his laboratory at the Institute of Orthopaedics.

The study which forms the basis of Chapter II was carried out in the laboratory of the Department of Orthopaedics at the University of Illinois. The author is most grateful to Professor R. D. Ray for his warm hospitality and enthusiastic teaching.

Technical assistance was given by many. I would like to acknowledge particularly the help given by my wife, by Miss Louise Stewart and Miss Lorna Lea in the laborious and exacting task of measuring the progress of the bone grafts by the technique described in Chapter III.

I am most grateful to Mr. T. C.Dodds, his assistant, Mr. James Paul, and the other members of the University of Edinburgh Department of Medical Illustration, for their skill and co-operation in the preparation of the illustrations.

The author was supported by a financial grant from the Medical Research Council during 1956 - 57, and by a British Post-Graduate Medical Federation Travelling Fellowship during 1958.

Edwards High Vacuum Limited assisted in the construction and operation of the freeze-drying unit used in the experiments.

The Isotope Division of the Atomic Energy Research Establishment carried out the irradiation of the bone specimens.

The Council of the Royal College of Surgeons of England kindly gave facilities for the experimental work on dogs to be carried out at the Buckstone Browne Farm.

To all these people and institutions, the author is deeply grateful. Without their help this work could not have been accomplished.

The results of certain of the experiments described in Chapter I and of a small aspect of Chapter III have been published in recent communications, (Chalmers, 1959; Chalmers, et al. 1960).

CHAPTER I

Homograft Immunity in Bone Grafting

It is now well known that homografts of most tissues survive for a limited period and then die because of the development of a state of immunity in the host against the graft. This phenomenon has prevented the general use of homografts as "spare parts" in medical practice with the exception of a few tissues namely blood, cartilage, cornea, blood vessels and bone.

Bone homografts have enjoyed a wide popularity since the report by Macewen in 1881 of a remarkable case in which he restored the entire humeral diaphysis in a boy by means of a series of fresh bone homografts. There is no doubting the clinical success of bone homografts, and they are now used more extensively than any other solid tissue homograft in surgical practice.

They "work" but precisely how they work is still uncertain. From the fact that killed bone grafts may serve a useful function it is clear that cell survival is not essential. This could account for the success of bone homografts while homografts of other tissues which can only perform a useful function if they remain viable, fail.

The rôle of bone in homograft immunity has received little attention.

Hutchison (1952) compared fresh bone autografts and homografts in various sites in the rabbit. He described a growth of new bone around the autograft which he considered arose from both graft and host tissue, although he does not make clear how he was able to distinguish between these contributions. He noticed the appearance of some new bone around the homografts from 10 to 42 days after transplantation. This bone subsequently died. He stated that this bone was 'layed down by osteoblasts differentiated from the host connective tissue cells' but again does not give evidence for this statement.

Bonfiglio, Jeter and Smith, (1955) compared the healing of fractures in auto- and homografts of ulnae in rabbits. They found that while the autografts fractures all united there was union in only a third of the homograft fractures. As these grafts were placed orthotopically it is difficult to assess the part played by the host tissues in the healing of the fractures. These workers also demonstrated complement fixation anti-bodies in the serum of rabbits which had previously been injected with extracts of homologous bone plus Freunds adjuvant, although serum anti-body could not be demonstrated following a bone homograft alone.

In similar experiments, Curtiss, Powell and Herndon, (1959) were unable to detect the presence of circulating anti-bodies; although in a previous paper from the same

laboratory Curtiss, Chase and Herndon (1956), studying the degree and type of union as judged histologically of grafts of cancellous bone to defects in dogs' ulnae, found autografts to behave more favourable than homografts. Blood group compatability between host and donor did not affect the progress of the homografts.

While these studies demonstrated differences in behaviour between autograft and homograft of bone which might be attributable to the development of an immune reaction against the latter, much remained to be learned concerning such a reaction when involving bone.

To investigate this the following study was planned.

The effect of fresh and killed bone homografts on the host was to be studied, using the rejection times of subsequent skin homografts from the same donors as a measure of the immune reaction to the bone homograft.

The effect of the host on the bone homograft was to be investigated by following the progress of such grafts histologically in both normal and pre-immunised hosts, comparing them with similar bone autografts.

Method and Materials

Table 1 lists the series of experiments.

TABLE 1

(1)	Bone autograft.
(2)	Bone homograft in normal rats.
(3)	Freeze-dried bone homograft.
(4)	Skin homograft three weeks after fresh bone homograft from the same donor.
(5)	Skin homograft three weeks after freeze-dried bone homograft from the same donor.
(6)	(a) Bone homograft three weeks after skin homograft from the same donor.
	(b) Bone homograft four months after skin homograft from the same donor.
(7)	Bone homograft four weeks after bone homograft from the same donor.
(8)	Freeze-dried bone homograft four weeks after skin homograft from the same donor.

The experiments were carried out on rats 60 to 100 days old.

Homografts were exchanged between two strains - Wistar albino and hooded rats - to reduce the chance of compatability between host and donor.

The bone grafts were obtained from the proximal half of one ilium and had an average weight of 38 milligrams. After all soft tissue had been scraped off they were chipped into fragments about a millimetre in their longest diameter.

The graft was placed in a slit made in the lumbar paravertebral muscles. An extra-skeletal site was used so that the progress of the graft would not be complicated by the inclusion of skeletal tissue from the host.

Freeze-dried bone was prepared by continuous evacuation over phosphorus pentoxide at -30 degrees centigrade for two weeks. This achieved drying to 1 per cent of the original water content.

Unless otherwise stated thirty-two rats were used in each series. Two rats were killed every second day until the eighteenth day, then at four, six and eight weeks and thereafter at monthly intervals to six months (in a few instances to one year). The number of animals at a particular period was sometimes increased when confirmation of a specific point was found necessary. The details of each Series are given in Tables 2, 3, and 6 - 9.

When the animals were killed a block of muscle containing the bone graft was excised and fixed in formalin. Paraffin sections were prepared after decalcification with neutral E.D.T.A.* and were stained with haematoxylin and eosin.

Skin grafts were carried out with minor modifications of

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*Ethylene diamine tetra-acetic acid. The decalcifying solution is prepared by dissolving 250 grammes of the disodium salt of E.D.T.A. in 1,750 millimetres of distilled water and adjusting the pH to 7.0 by the addition of sodium hydroxide.

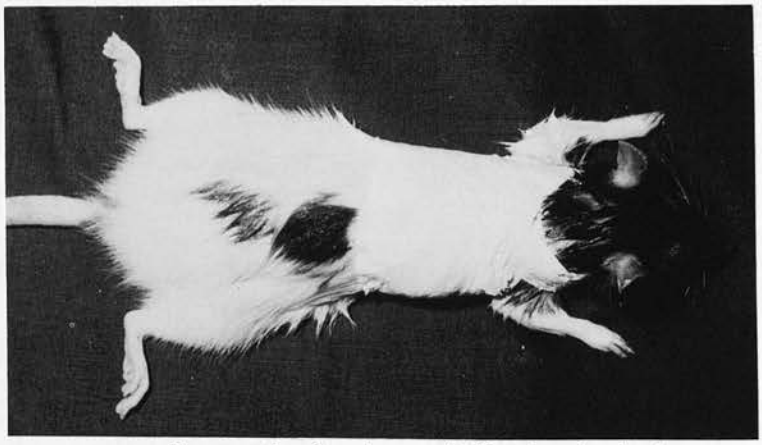
the technique described for "fitted grafts" by Billingham and Medawar (1951) and Woodruff and Simpson (1955). A circular piece of skin about a centimetre in diameter was dissected off the panniculus carnosus of the lateral chest wall and sutured to a defect created by the removal of a similar graft on another rat. The graft was protected by a dressing of vaseline gauze and a plaster-of-Paris jacket, (Fig. 1).

The skin grafts were inspected daily after the sixth day in the case of first set grafts and after the 4th day in second set grafts. The time of rejection of the graft was determined by the development of induration or by an alteration from the normal pink to a red or brown colour, (Fig.2). Usually, these changes occurred at about the same time, but whichever showed earlier was taken as the end point of survival. It was found necessary to replace the plaster jacket after each inspection if mechanical interference with the graft was to be avoided.

Survival times of first and second set skin homografts

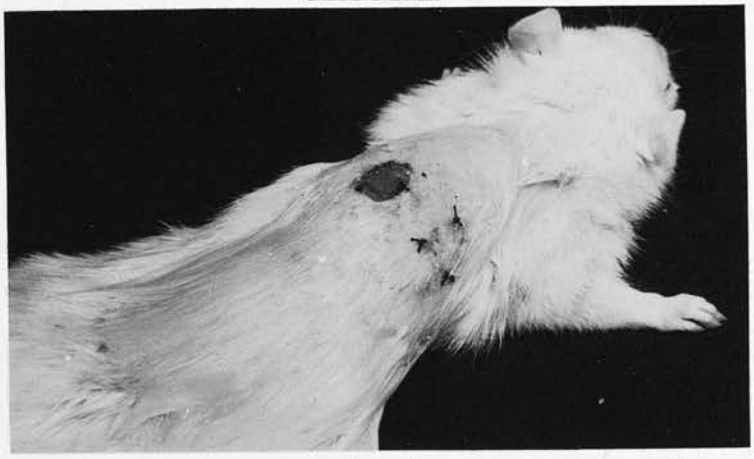
In order to establish the normal survival times of first and second set skin homografts under these experimental conditions, ninety-three first set homografts were carried out giving a mean survival time of 10.9 days (Fig. 3).

Figure 1



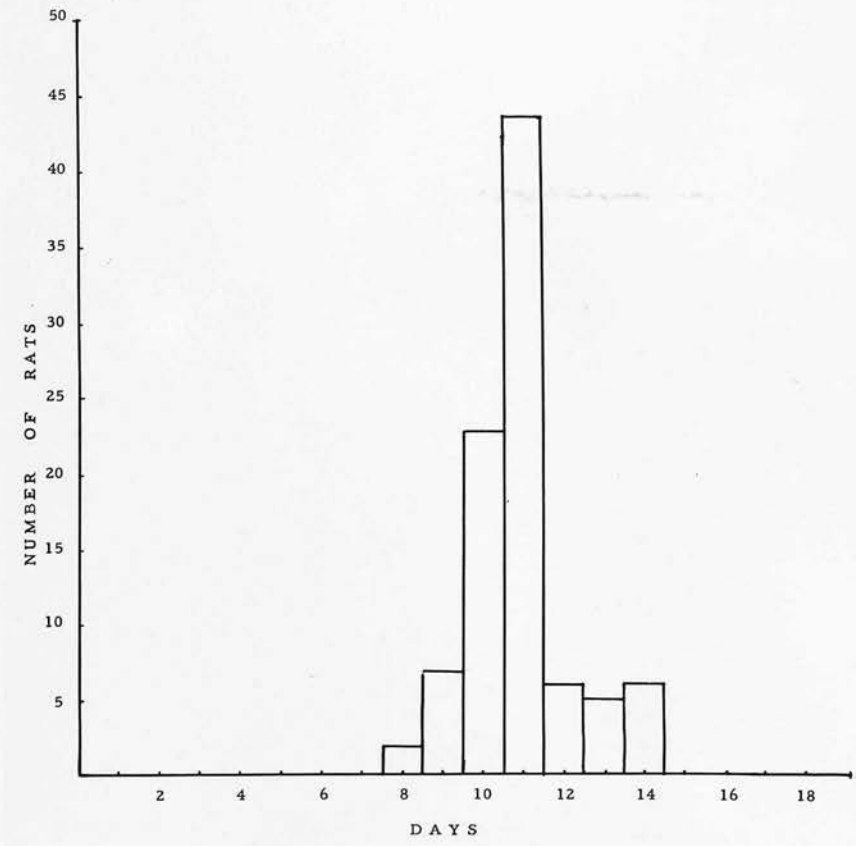
Type of plaster of Paris jacket used to protect the skin graft.

Figure 2



Skin homograft (top) and autograft (bottom) at twelve days. The homograft has been rejected.

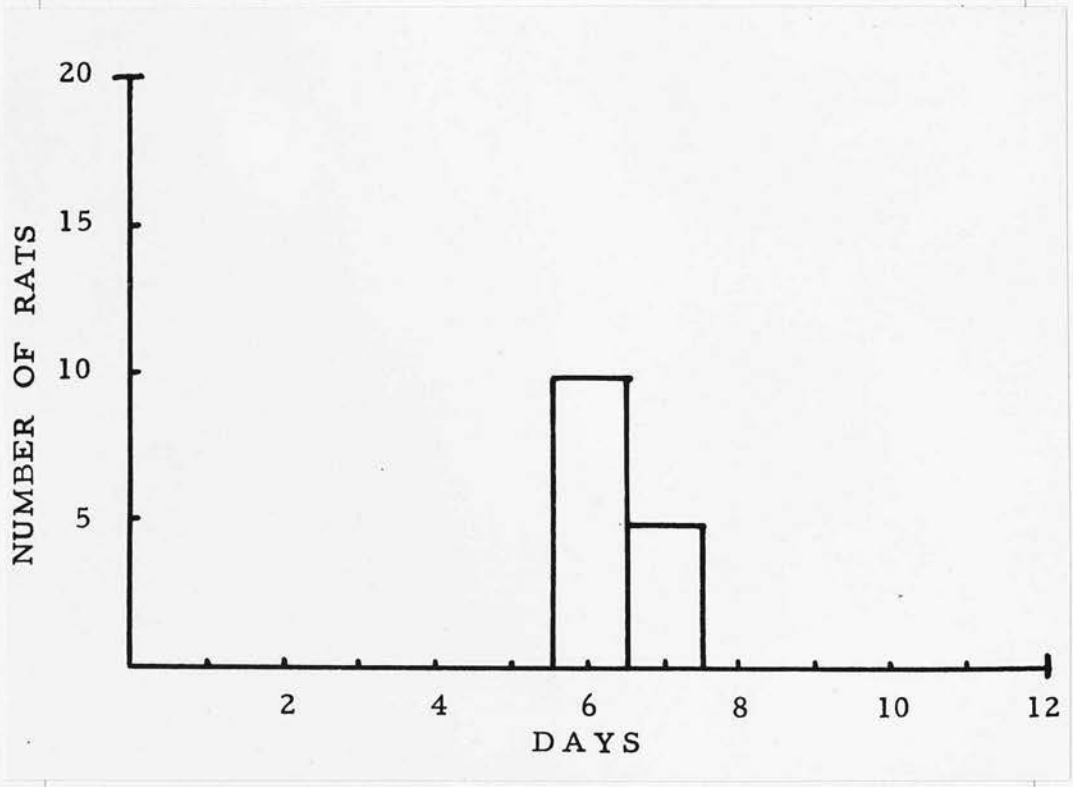
Figure 3



Rejection times of first set skin homografts in 93 rats.

Fifteen second set skin homografts to rats immunised 15 to 19 days before by skin grafts from the same donor had a mean survival time of 6.3 days (Fig.4).

Figure 4



Rejection times of second set skin homografts in 15 rats.

RESULTS

In assessing the histological preparations, particular attention was paid to the following points -

(1) evidence of cell survival in the graft;

(2) character of any new bone formation in the vicinity of the graft;

(3) the local reaction of the host to the graft.

Series I: Bone autograft (Table 2)

At two days most of the lacunae in the grafted bone appeared empty, or contained only pyknotic remnants of osteocytes indicating that the greater part of the bone had died. In some of the grafts, however, a few osteocytes retained their normal morphological character, suggesting a limited survival. Organisation of the haematoma at the graft site was complete in six to eight days.

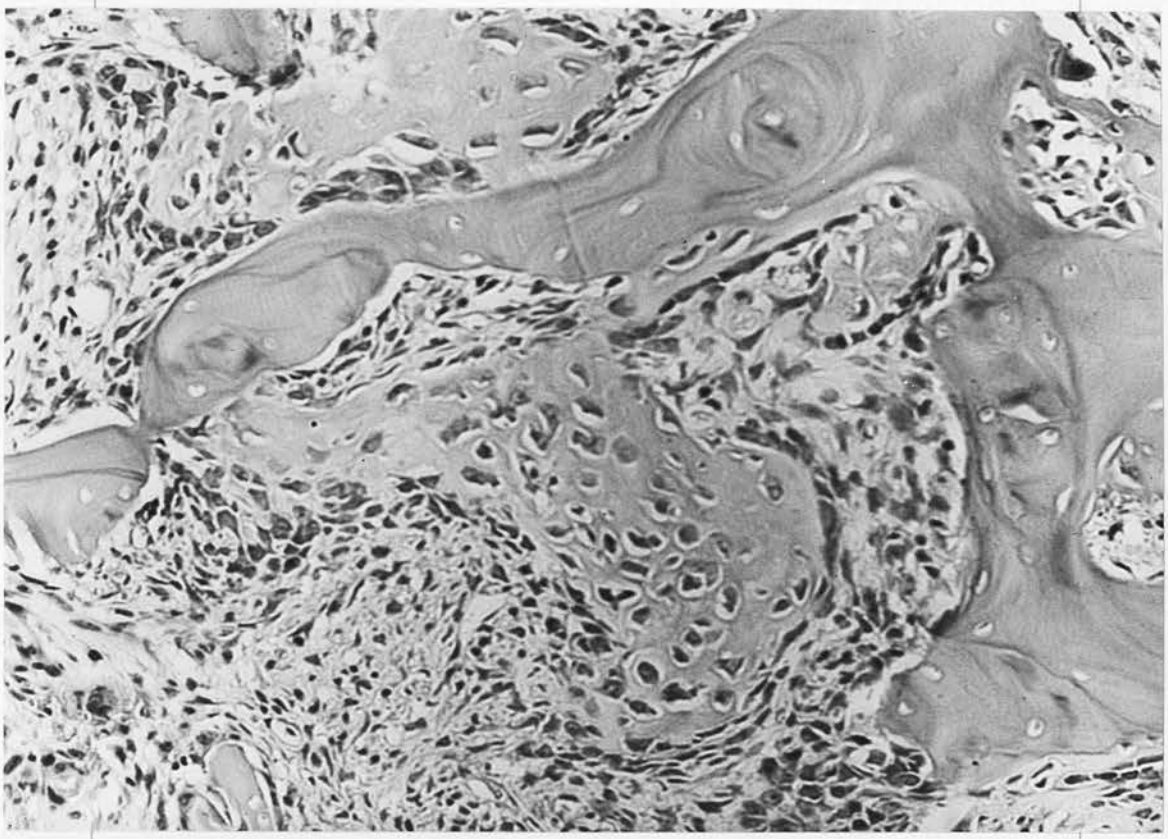
At four days new woven bone began to appear on the surface of the graft fragments. This new bone grew vigorously, linking the graft fragments and extending into the surrounding connective tissue (Figs. 5 - 7). At fourteen days lamellar bone was seen for the first time; at sixteen days the new bone which had hitherto been arranged in a haphazard manner began to show organisation in irregular spheres or ossicles containing haemopoietic marrow (Fig. 8). These ossicles became progressively more regular in outline, usually elongated in their long

axes corresponding with the direction of the adjacent muscle fibres (Figs. 9 and 10). Osteoclastic resorption proceeded from the fourth day, removing dead graft and new bone which did not contribute to the ossicle. After the mature ossicle had formed, little further osteoclast activity was seen. The dead bone of the original graft was not always completely removed, being occasionally found incorporated in the wall of the ossicle (Fig. 10). The reaction of the host site to the graft consisted initially of rapid invasion of the haematoma by granulation tissue and round cells. This settled by eight days to a less vascular fibrous tissue which finally became a thin fibrous periosteum to the forming ossicle.

TABLE 2

DURATION	RAT NO.	STRAIN	OSTEOCYTE SURVIVAL IN GRAFT	NEW BONE FORMATION	COMMENT
2 day	32	A	+	-	
	40	H	+	-	
4 day	184	A	+	+	First appearance of new bone.
	20	A	-	-	
	183	H	-	+	
	29	H	-	-	
6 day	45	A	+	+	
	7	A	+	-	
	19	H	+	+	
8 day	24	A	+	+	
	185	H	+	+	
10 day	33	A	-	+	
	30	H	+	+	
12 day	25	A	-	+	
	23	H	-	+	
14 day	22	A	-	+	New bone of lamellar character first seen.
	15	H	+	+	
16 day	17	A	-	+	
	113	H	-	+	
18 day	23	A	-	+	Tendency of new bone to grow in form of an ossicle.
	64	H	-	+	
4 week	35	A	-	+	
	12	H	-	+	
6 week	5	A	-	+	Ossicles now regular in outline and well formed.
	28	H	-	+	
8 week	34	A	-	+	
	16	H	-	+	
3 month	38	A	-	+	
	14	H	-	-	This graft was quite atypical showing no new bone and the presence of a marked inflammatory response. Possibly an infected graft.
4 month	192	A	-	+	
	11	H	-	+	
5 month	60	A	-	+	
	13	H	-	+	
6 month	9	A	-	+	
	10	H	-	+	

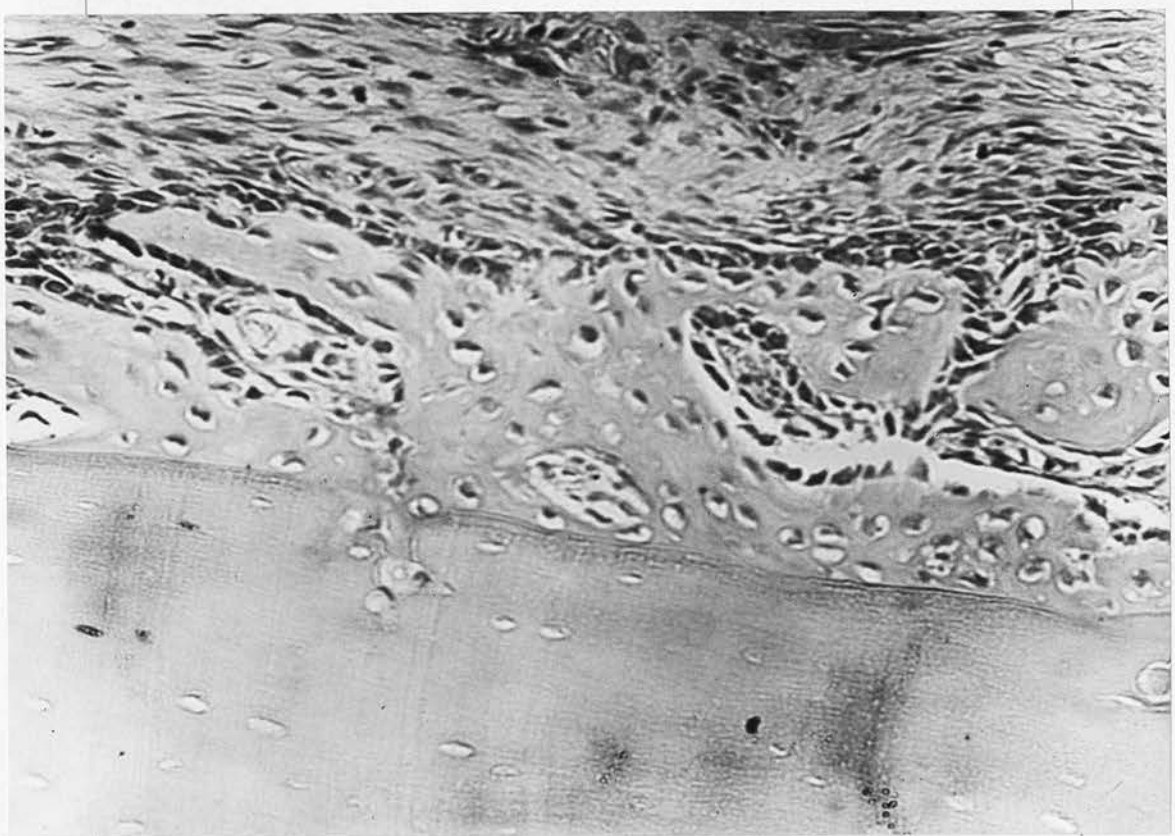
Figure 5



Autograft, six days (x 330)

The lacunae of the graft are empty or contain only shrunken remnants of osteocytes. New bone has begun to grow on the surface of the graft and is extending into the surrounding connective tissue.

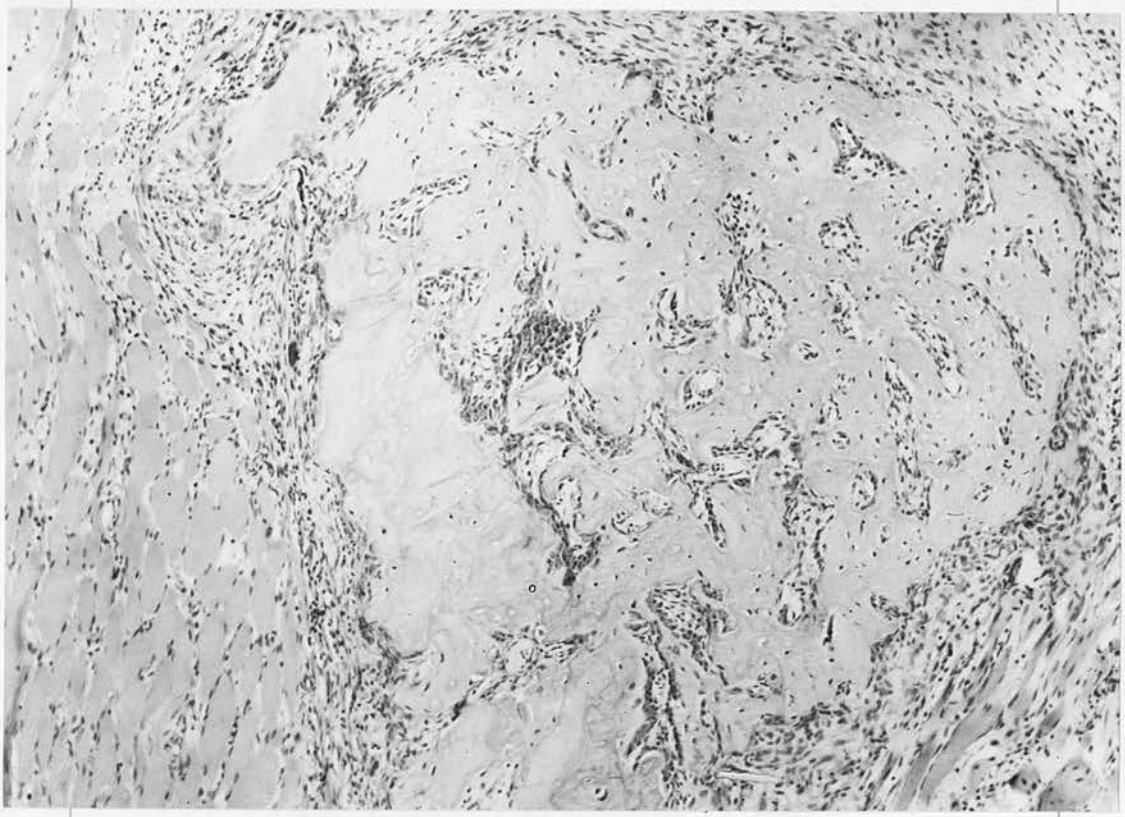
Figure 6



Autograft, ten days (x 330)

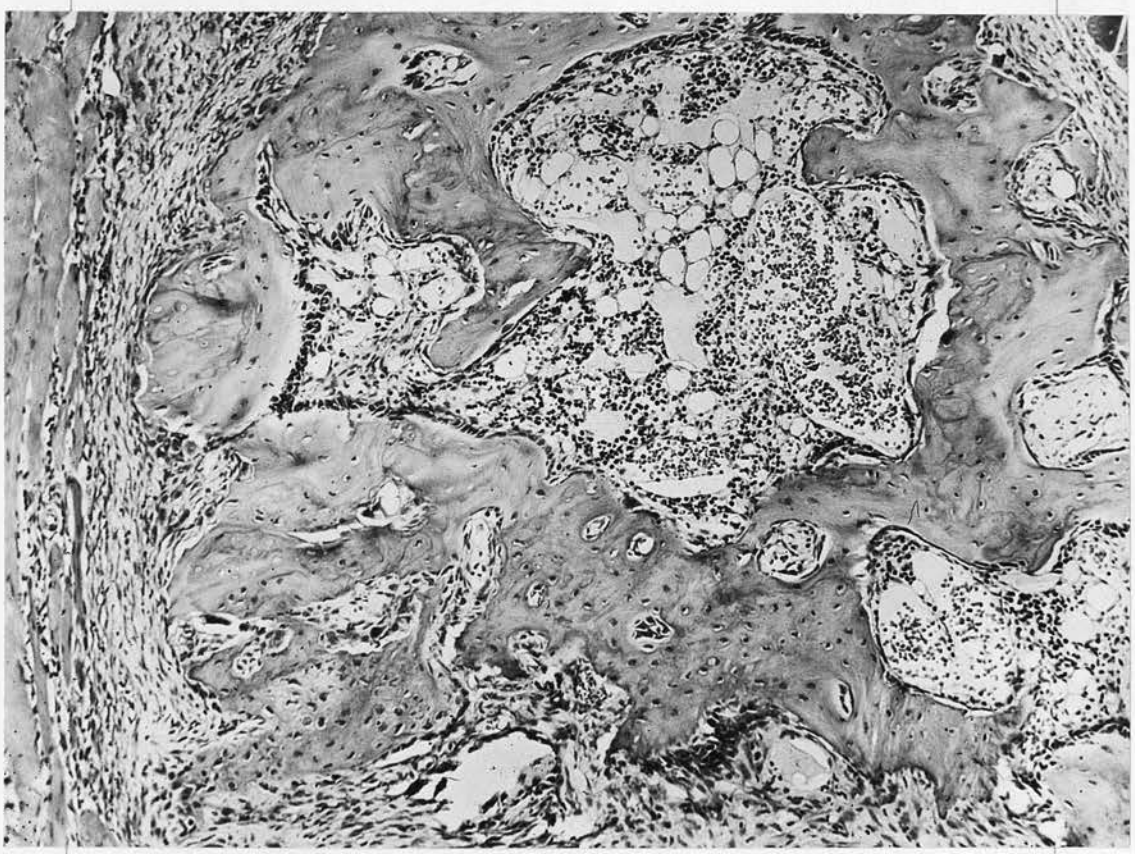
The original graft is largely acellular. There is a vigorous proliferation of new bone from the graft surface.

Figure 7



Autograft, fourteen days (x 110)
Growth of new bone continues in haphazard manner.

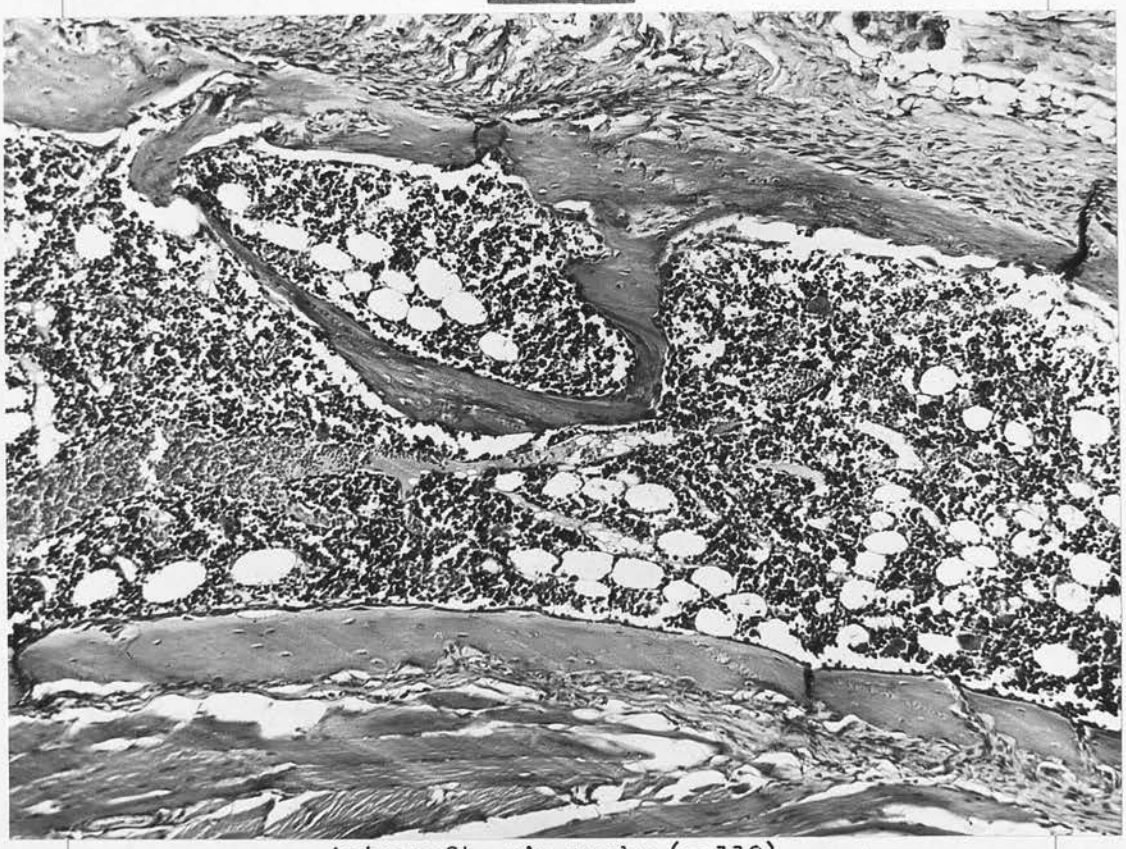
Figure 8



Autograft, eighteen days (x 110)

The bone has now grown into an irregular sphere containing haemopoietic marrow. Active remodelling is in progress as shown by the osteoblastic activity on some surfaces and osteoclastic on others.

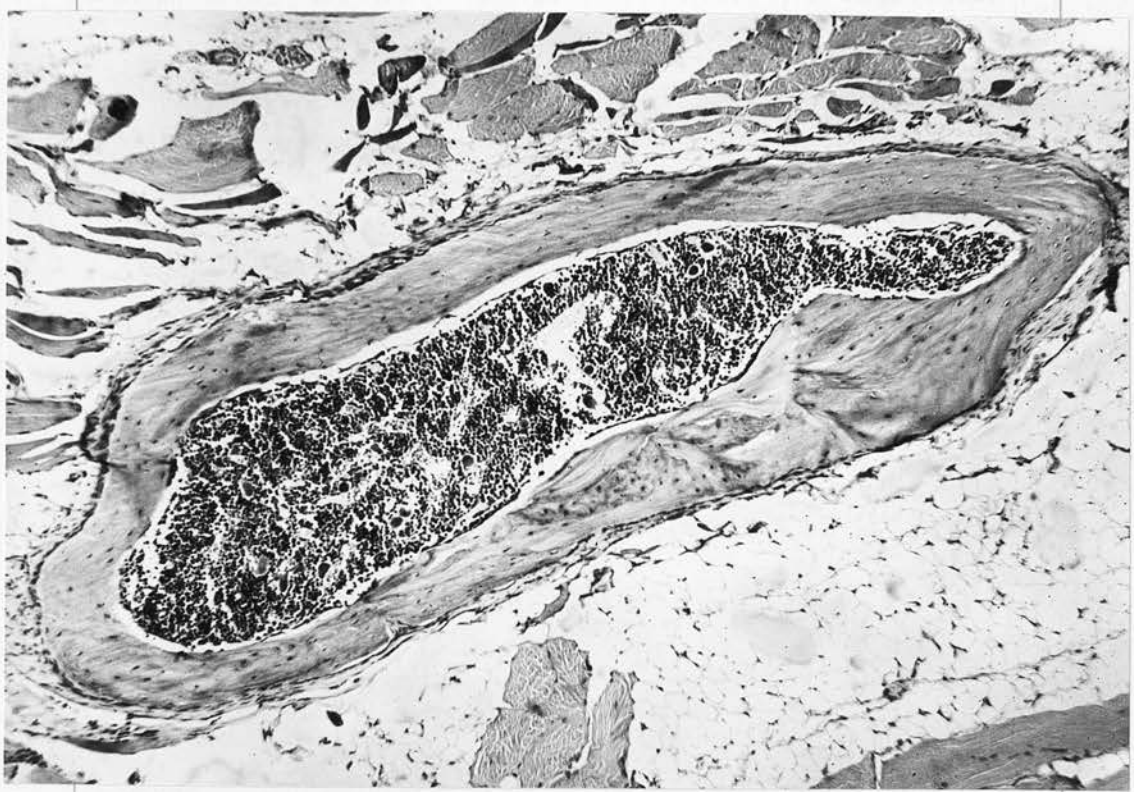
Figure 9



Autograft, six weeks (x 110)

The ossicle has become complete and more regular. It is elongated in the direction of the adjacent muscle fibres.

Figure 10



Autograft, six months (x 75)

A late ossicle. A few small fragments of original graft are incorporated in the cortex of the ossicle. There is a complete absence of reaction in the surrounding tissue.

Series II: Bone homografts in normal rats - (Table 3)

Initially these grafts behaved in a very similar manner to the autografts. Only very few osteocytes appeared to survive in the grafted bone. Granulation tissue rapidly extended through the graft area. New bone appeared on the graft surface at four to six days and grew until eight days (Fig. 11). In contrast to the autograft, this new bone formation, although variable in amount, was usually scanty: in four of twenty-two grafts of six to eighteen days' duration it could not be identified.

After eight days the progress was entirely different from that of the autograft. The surface layer of osteoblasts on the new bone became flattened and more basophilic and quickly disappeared (Figs. 12 and 13). The deeper osteocytes of the newly formed bone also became pyknotic and fragmented (Figs. 13 and 14) and in their turn, disappeared. In those grafts in which a large amount of new bone was produced, the dissolution of the bone cells was frequently delayed until sixteen or even eighteen days (Fig.15). Death of this new bone left a characteristic vacuolated bone matrix, the empty lacunae of the immature bone being large in comparison with the amount of matrix. This enabled the early phase of new bone formation to be identified even after the death of the cells concerned (Fig. 16). Resorption of this dead new bone proceeded rapidly, so that after four weeks it could rarely

be found.

A second phase of new bone formation sometimes occurred in homografts of four weeks and older. This phase consisted of slowly growing lamellar bone. It was never large in amount, and did not organise into an ossicle. It remained closely applied to the surface of the graft, a well marked cement line being present at the interface (Figs. 17 and 18). This type of new bone was seen in five of fourteen grafts of four weeks and older.

Table 4 lists the characteristic features of the autograft new bone and the early and late phases of homograft new bone.

Figure 19 is a graphic impression of the time relationship of these three types of bone and their relative quantity.

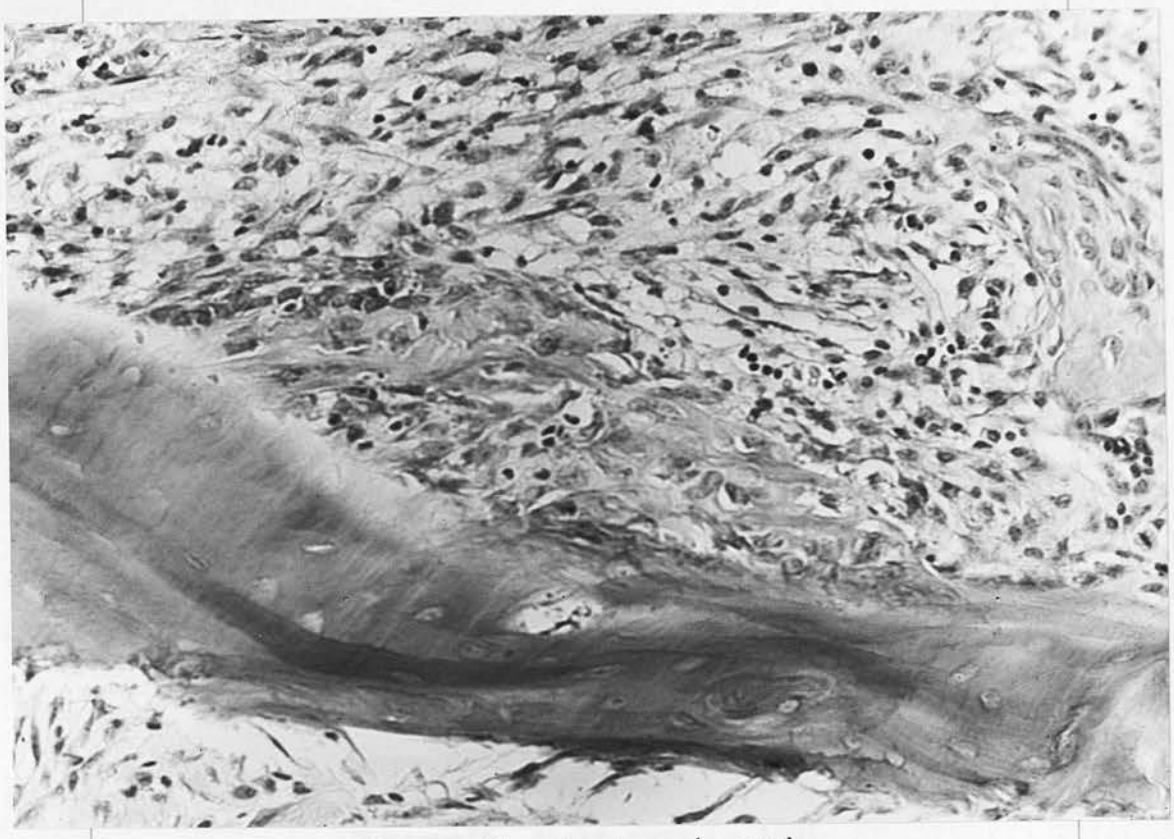
Osteoclasts were present from the fourth day, but resorption of the dead homograft proceeded more slowly than of the dead autograft. The local response of the host tissues resembled that to the autograft until the eighth day. Thereafter there was an increase in fibrous tissue and a heavy infiltration with lymphocytes and varying numbers of plasma cells, eosinophils and histiocytes. This inflammatory reaction persisted until four to five months.

TABLE 3

SERIES II - BONE HOMOGRAFTS

DURATION	RAT NO.	STRAIN	OSTEOCYTE SURVIVAL IN GRAFT	NEW BONE FORMATION E - Early phase L - Late phase	COMMENT
2 day	43	A	+	-	
	43	H	-	-	
4 day	196	A	+	-	
	41	A	-	-	
	196	H	+	-	
	41	H	+	E	New bone living.
6 day	194	A	+	-	
	44	A	+	E	New bone living.
	194	H	+	E	New bone living.
	44	H	+	E	New bone living.
8 day	45	A	+	E	New bone living.
	45	H	-	E	New bone living.
9 day	156	A	+	E	New bone dead.
10 day	24	A			Graft not found.
	195	H	-	E	New bone living.
	24	H	+	E	New bone living.
12 day	195	A	-	E	New bone dead.
	48	A	+	-	
	48	H	+	E	Most of new bone dead.
14 day	52	A	+	-	
	52	H	+	E	Most of new bone dead.
16 day	21	A	+	E	New bone dead.
	5	A	+	E	New bone dead.
	153	H	+	E	Most of new bone dead.
	21	H	+	E	New bone living.
18 day	6	A	+	E	New bone dead.
	23	A	+	-	
	23	H	+	E	Most of new bone dead.
	6	H	+	E	New bone dead.
4 week	8	A	+	L	
	8	H	+	L	New bone dead
6 week	22	A	-	-	
	22	H	+	E	New bone dead.
8 week	9	A	+	-	
	9	H	-	-	
3 month	200	A	+	L	
	10	A	-	-	
	10	H	-	E	New bone dead.
	200	H	+	E	New bone dead.
4 month	1	A	+	L & E	Early phase new bone dead.
	1	H	+	-	
5 month	7	A	+	L	
	7	H	+	L	
6 month	2	A	-	-	
	2	H	+	-	

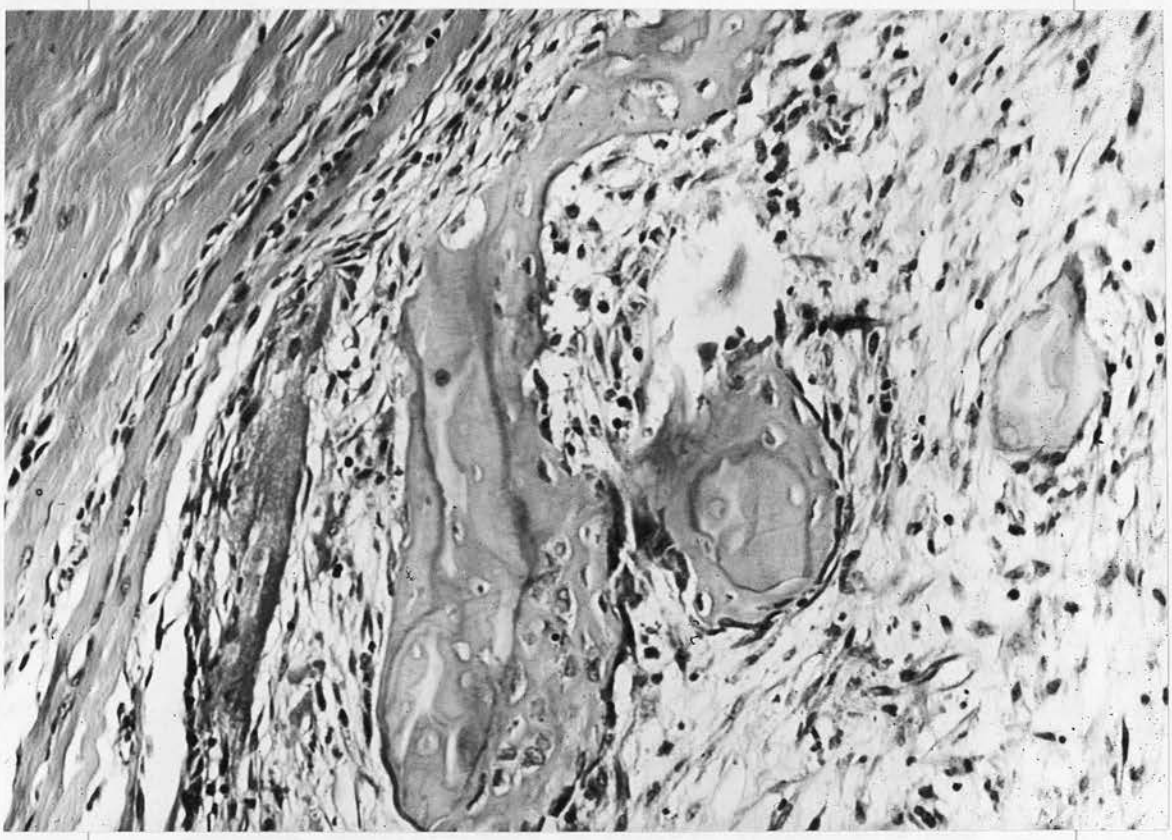
Figure 11



Homograft, six days (x 330)

Most of the original graft lacunae are empty. New bone is being laid down on the graft surface.

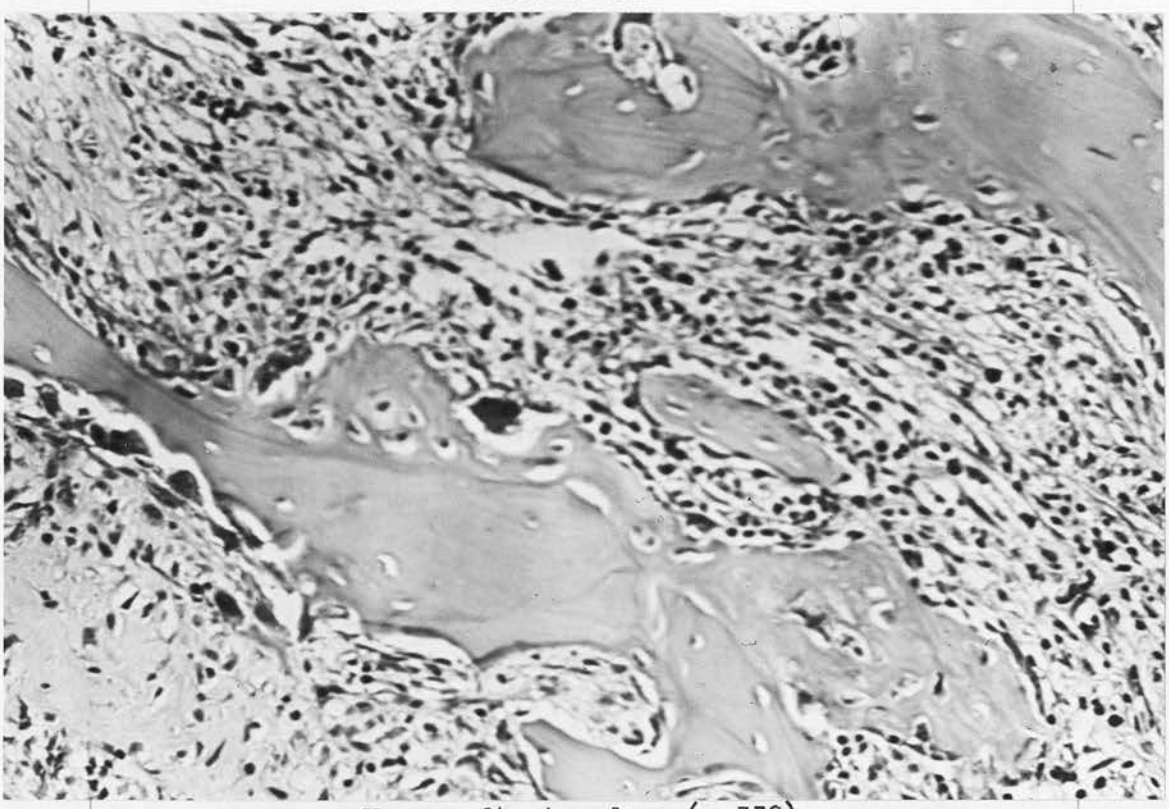
Figure 12



Homograft, eight days (x 330)

The surface layer of osteoblasts on the new bone are flattened and basophilic although most of the cells in the newly formed bone still appear normal. If this is compared with the autograft at ten days (Fig.6) the change in the appearance of the osteoblasts is well shown.

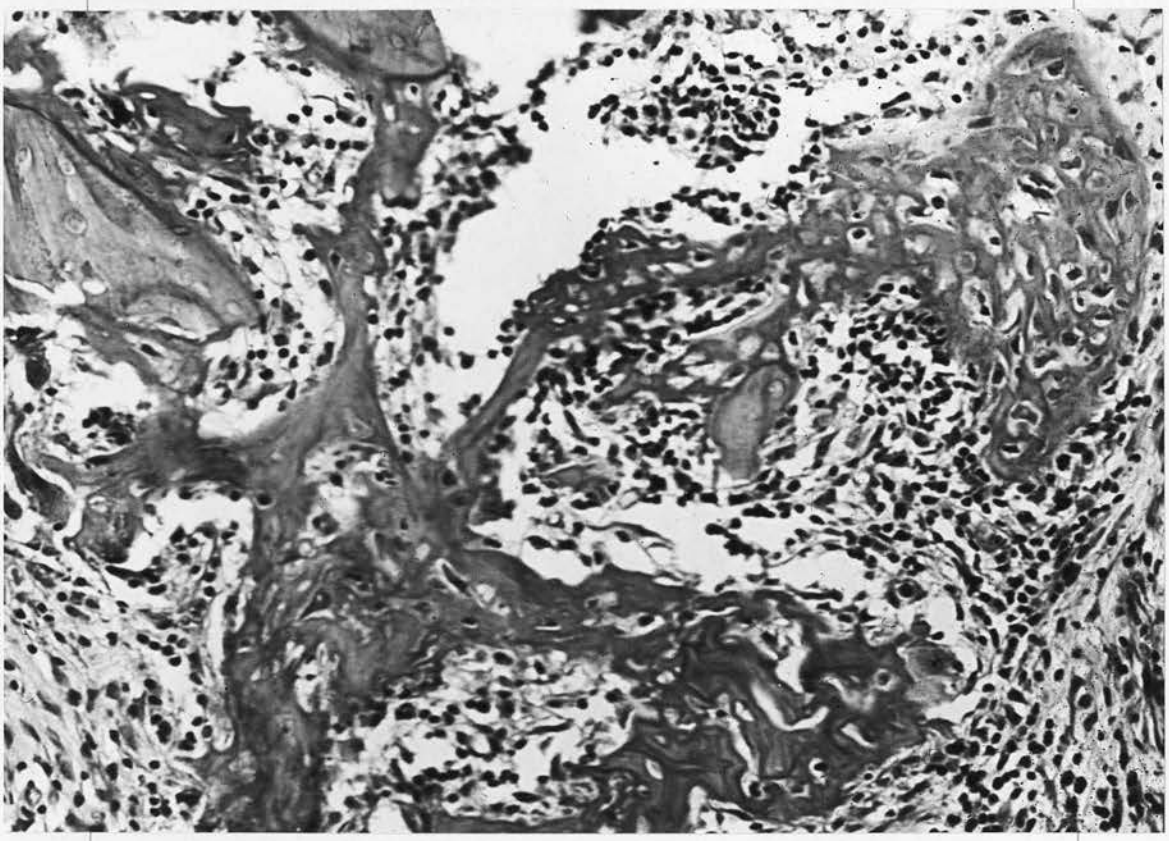
Figure 13



Homograft, ten days (x 330)

Showing disappearance of surface osteoblasts and pyknosis of the cells of the new bone. Osteoclastic resorption of the original graft and of the new bone is proceeding.

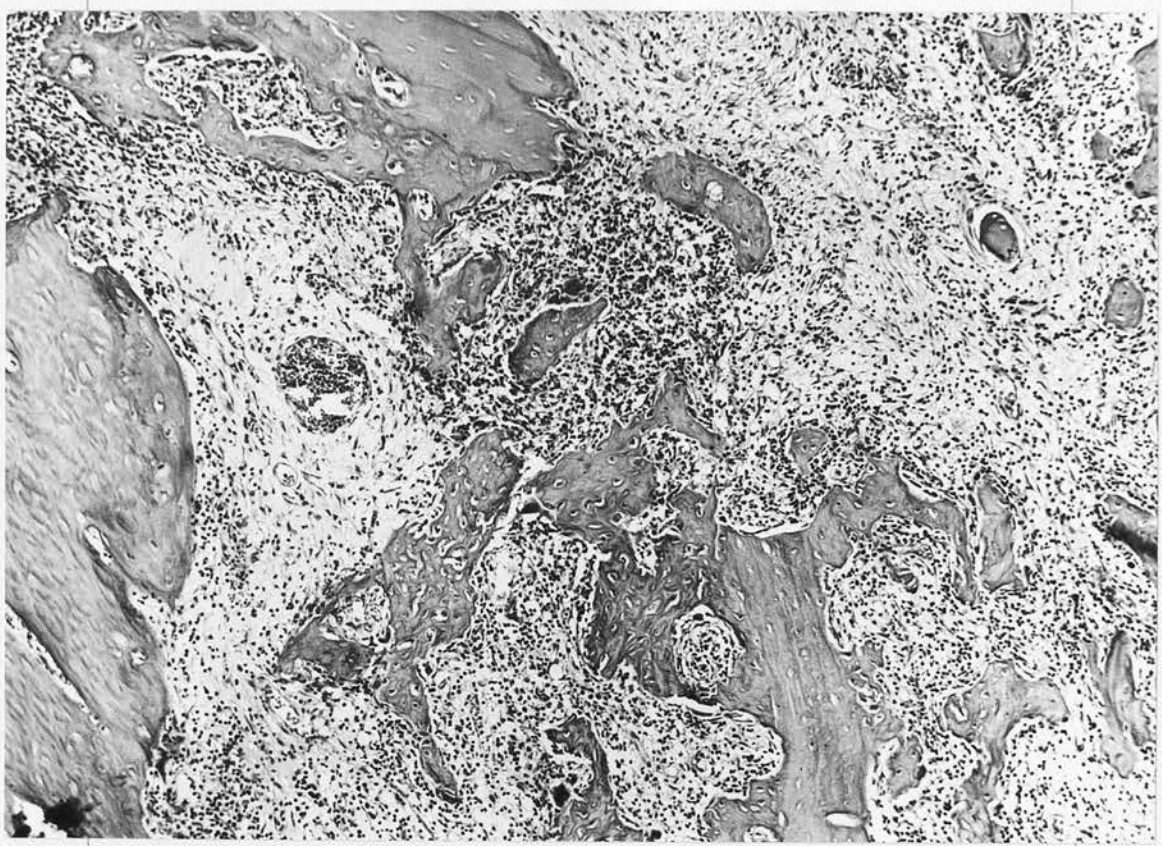
Figure 14



Homograft, twelve days (x 330)

Some of the lacunae of the new bone are now empty while the remaining cells show degenerative changes. The inflammatory reaction to the graft is well shown.

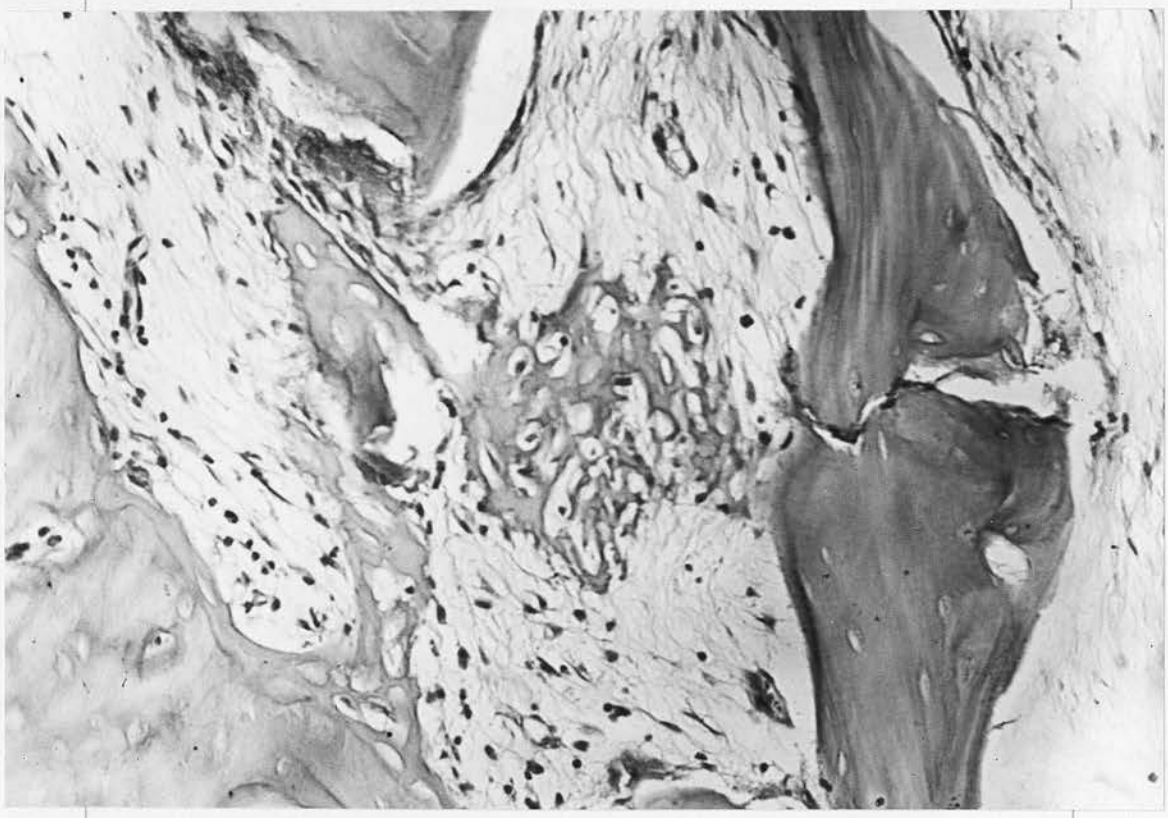
Figure 15.



Homograft, sixteen days (x 110)

In this homograft at sixteen days many of the osteocytes of the new bone persist although no further osteogenesis is taking place.

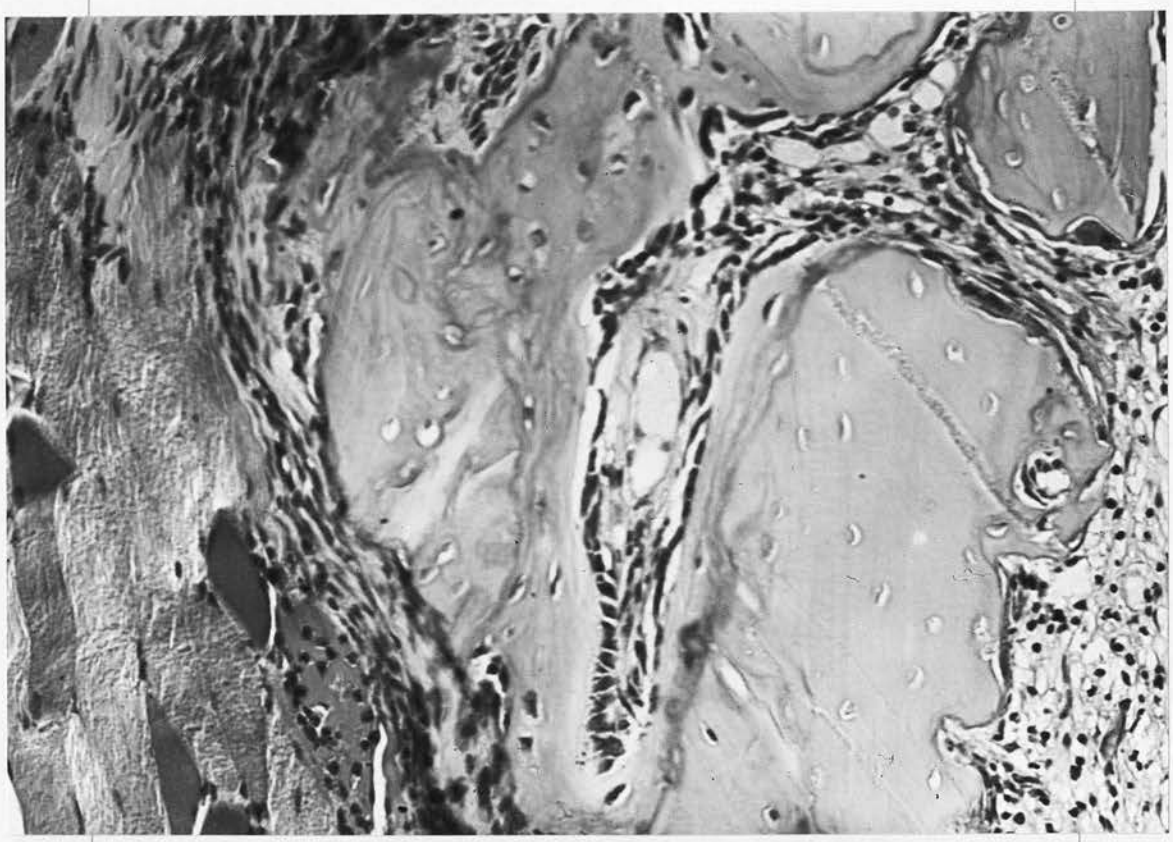
Figure 16.



Homograft, four weeks (x 330)

The new bone which has formed in relation to the bone homograft, although dead and acellular still is identifiable because of its characteristic open matrix.

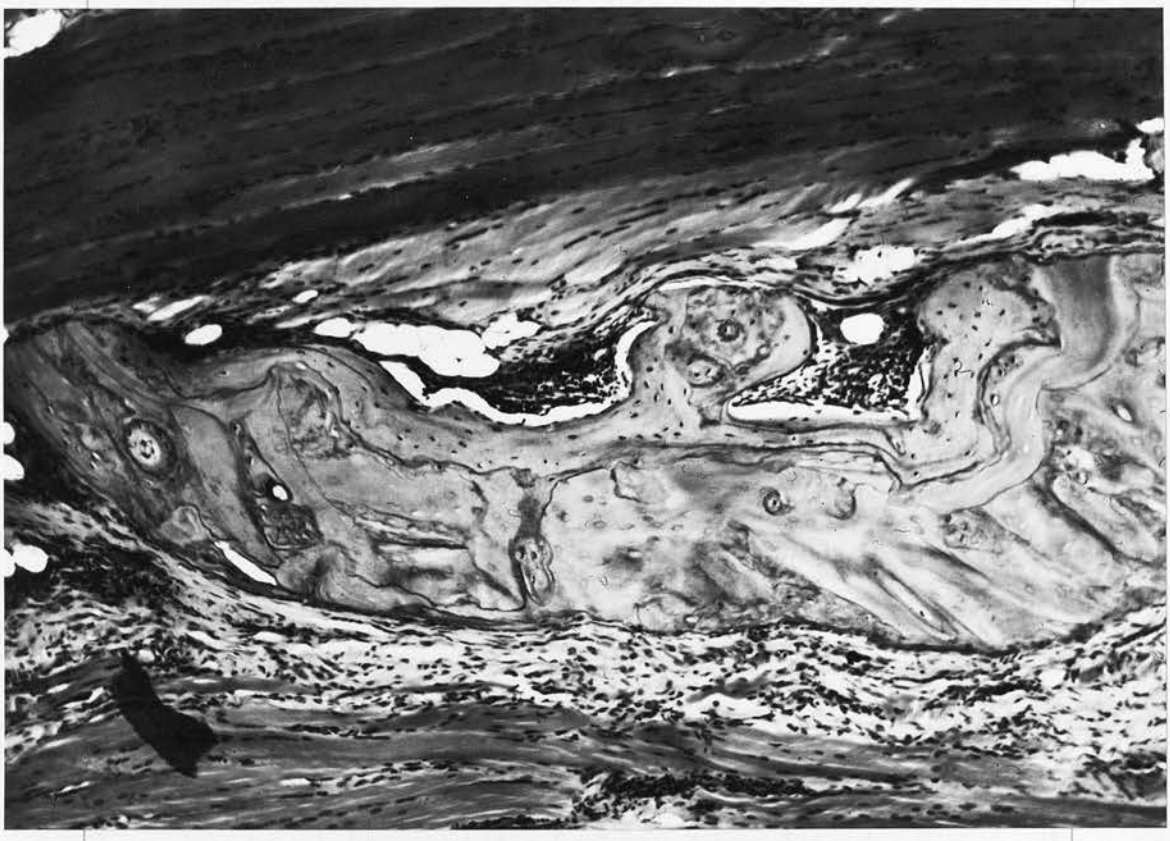
Figure 17



Homograft, five months (x 330)

Active new bone formation of the late phase has commenced on the graft surface. The inflammatory reaction to the homograft is still present.

Figure 18



Homograft, ten weeks (x 110)

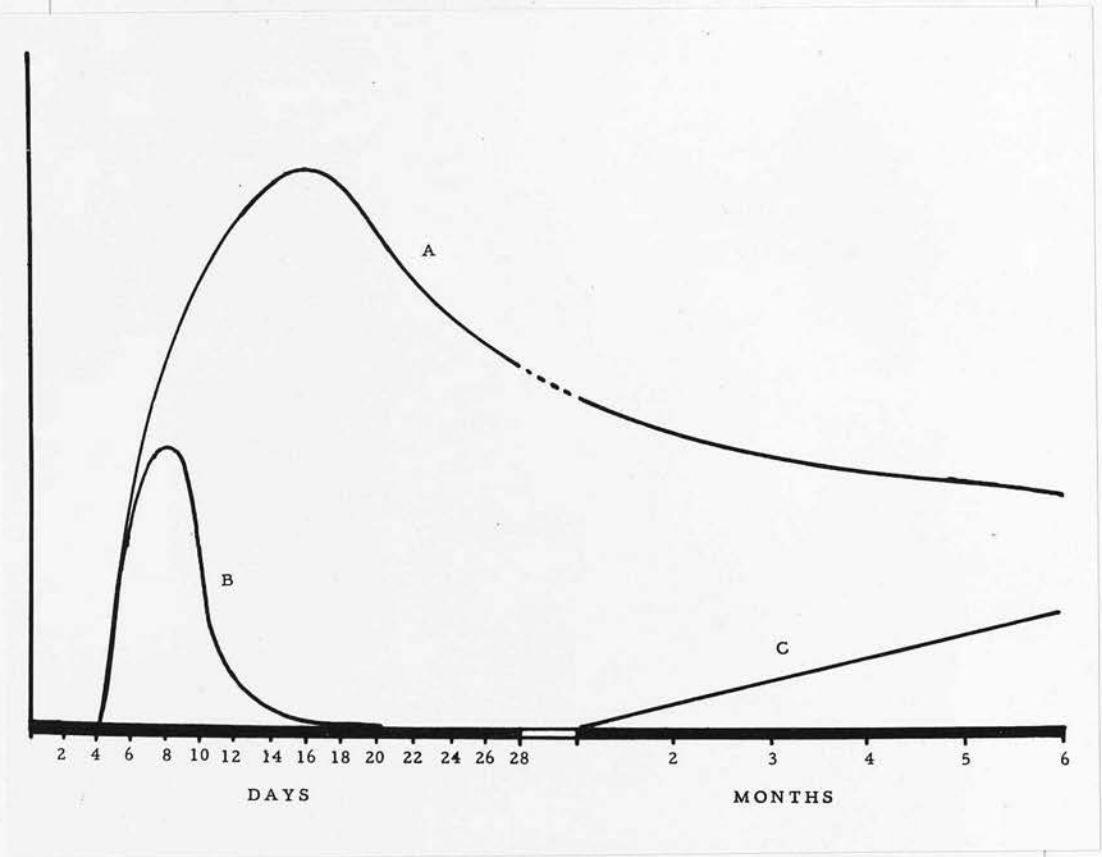
Another example of the late phase of homograft new bone. Its lamellar character and its close application to the original graft from which it is separated by a well marked cement line are quite characteristic of this phase of homograft new bone.

TABLE 4

A Comparison of Autograft New Bone and Early and Late Phases of Homograft New Bone

	Autograft	Homograft	
		Early phase	Late phase
Duration of growth and survival.	Four days to at least six months.	Four days to eighteen days.	Four weeks to at least six months
Type of bone.	Woven at first becoming lamellar at fourteen days.	Woven	Lamellar
Quantity	Profuse	Variable but usually scanty.	Scanty.
Incidence	100 per cent	80 per cent	30 per cent
Progress	Forms ossicle containing haemopoietic marrow.	Resorbed rapidly after death of the bone.	Forms plaque on surface of dead graft.

Figure 19



General curves indicating the amount and duration of new bone which forms in relation to autograft, (A), homograft early phase (B) and homograft late phase (C).

Series III: Freeze-dried bone homograft (Table 5)

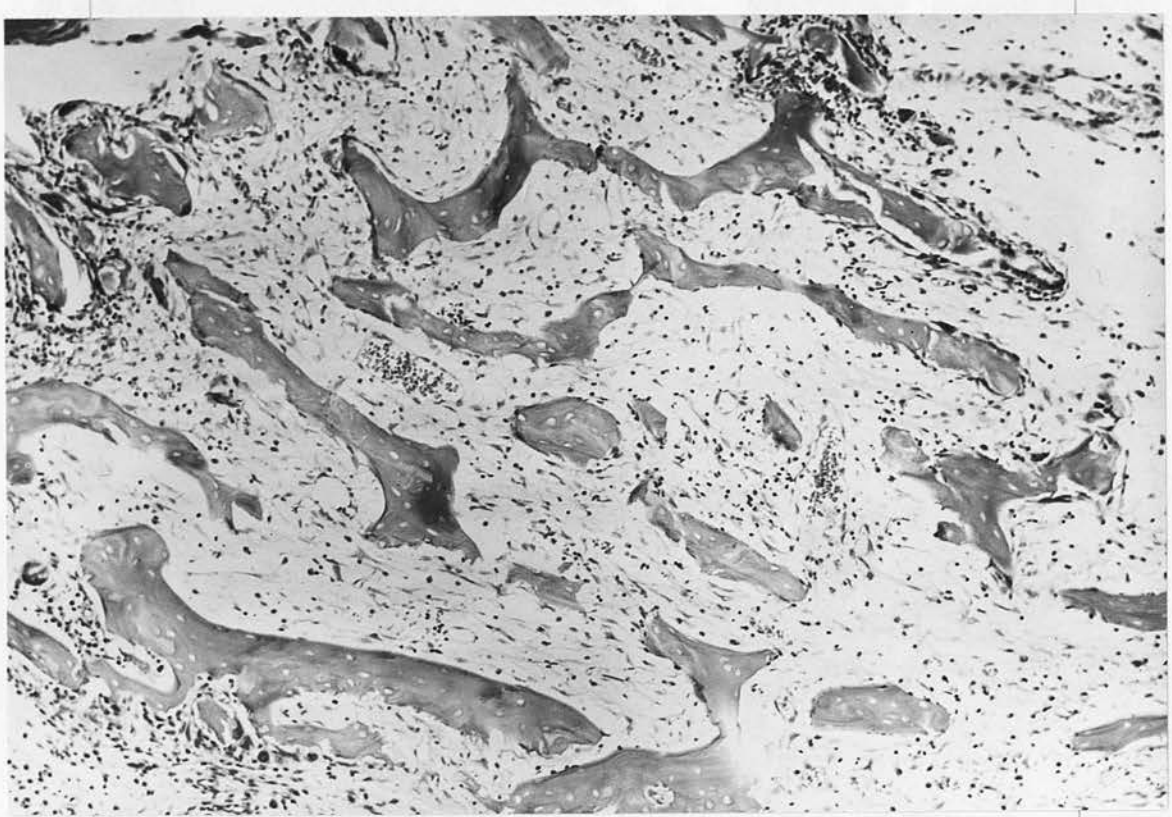
No survival of osteocytes was observed in this series, all lacunae in the grafted bone being empty. No new bone of the early phase occurred. Giant cells resembling osteoclasts were present from the sixth day and resorption proceeded slowly. The inflammatory reaction was less intense and later in onset than with the fresh homograft (Fig. 20). An additional number of grafts, of five to ten months' duration, was added to the original series. Of these, two of fourteen showed small amounts of new bone with the characteristics of late phase homograft new bone (Fig. 21).

TABLE 5

SERIES III - FREEZE-DRIED HOMOGRAFT

DURATION	RAT NO.	STRAIN	OSTEOCYTE SURVIVAL IN GRAFT	NEW BONE FORMATION	COMMENT
2 day	128	A	-	-	
	160	H	-	-	
4 day	130	A	-	-	
	161	H	-	-	
6 day	131	A	-	-	
	162	H	-	-	
8 day	133	A	-	-	
	163	A	-	-	
	163	H	-	-	
10 day	129	A	-	-	
	164	H	-	-	
12 day	165	A	-	-	
	134	A	-	-	
	165	H	-	-	
14 day	136	A	-	-	
	166	H	-	-	
16 day	111	A	-	-	
	109	H	-	-	
18 day	137	A	-	-	
	106	H	-	-	
4 week	105	A	-	-	
	105	H	-	-	
6 week	104	A	-	-	
	104	H	-	-	
	167	H	-	-	
8 week	107	A	-	-	
	107	H	-	-	
3 month	108	A	-	-	
	108	H	-	-	
4 month	110	A	-	-	
	110	H	-	-	
5 month	112	A	-	-	
	141	A	-	-	
	140	A	-	+	Lamellar bone typical of the late phase homograft bone.
	112	H			Infected.
	141	H	-	-	
6 month	113	A	-	-	
	139	A	-	-	
	143	A			Graft not found.
	125	A	-	-	
	127	A			Infected.
	113	H	-	-	
	139	H	-	-	
	143	H	-	+	Lamellar bone typical of the late phase homograft bone.
	126	H	-	-	
7 month	144	A	-	-	
	144	H	-	-	
10 month	139	H	-	-	

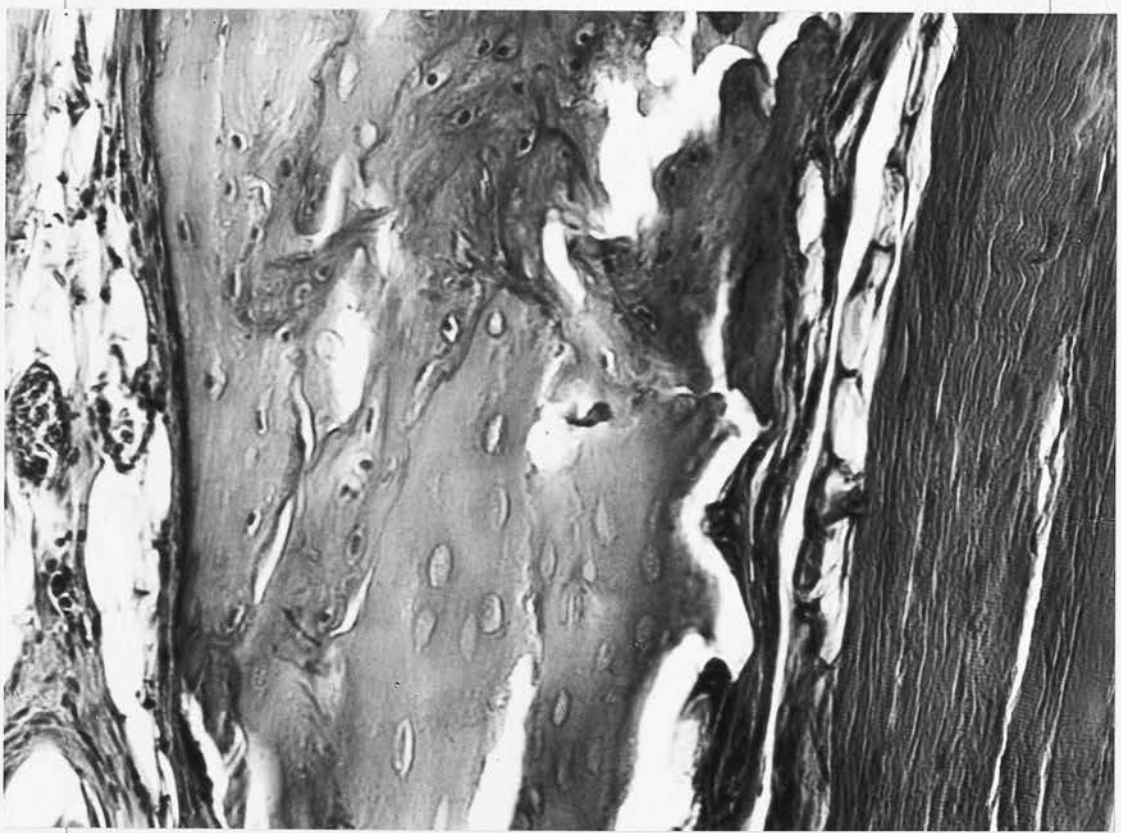
Figure 20



Freeze dried homograft twelve days (x 110)

The graft bone is entirely acellular. No new bone formation has occurred. The inflammatory reaction is less intense than that which develops around fresh homografts at this stage.
(Compare with Figs. 14 and 15).

Figure 21



Freeze dried homograft, six months (x 330)

Lamellar new bone with characteristics of the late phase of homograft new bone is growing on the surface of the dead graft.

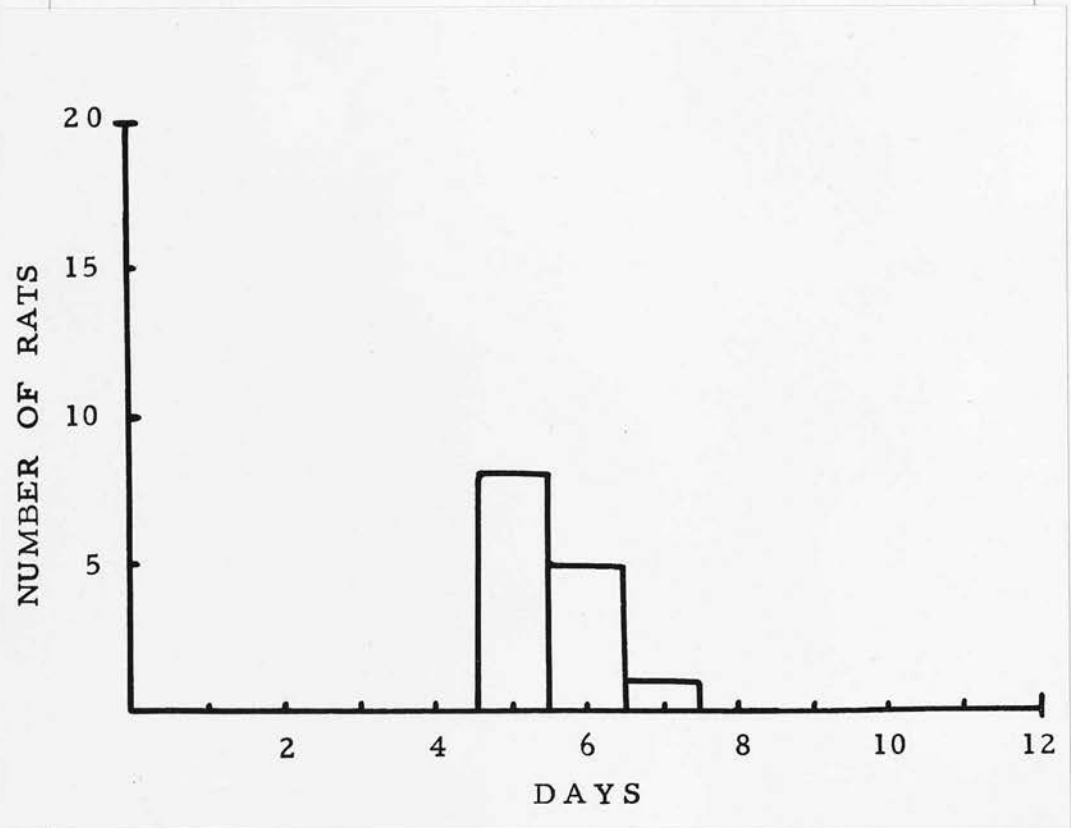
Series IV: Skin homograft three weeks after fresh bone homograft from the same donor

There were fourteen animals in this series. The skin grafts had a mean survival time of 5.6 days (Fig. 22).

Series V: Skin homografts three weeks after freeze-dried bone homografts from the same donor

There were fourteen rats in this series. The skin grafts had a mean survival time of 12.0 days (Fig. 23).

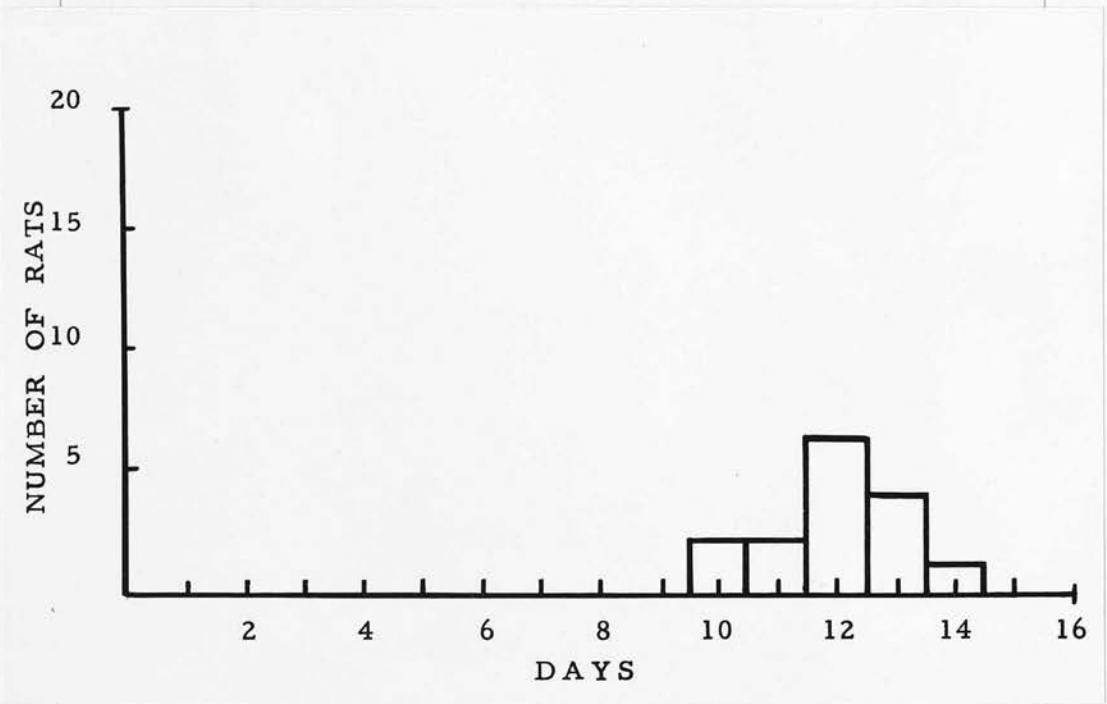
Figure 22



Rejection times of skin homografts on rats previously grafted with fresh bone homografts from the same donor.

(Compare with rejection times of second set skin homografts Fig.4)

Figure 23



Rejection times of skin homografts on rats previously grafted with freeze dried bone homografts from the same donor.

Series VIa: Bone homograft three weeks after skin homograft from the same donor. (Table 6)

These grafts differed from those in unprepared animals in the following respect.

No new bone developed in relation to any of the grafts of this series - otherwise the behaviour was similar. The inflammatory reaction of the host tissues to the graft appeared more intense in the early stages although this was an impression based on histological appearances rather than on precise measurement.

Series VIb: Bone homografts four months after a skin homograft from the same donor (Table 7)

There were six animals in this series. The bone grafts were examined at thirteen days as previous experience indicated that this was the optimal time for the recognition of homograft new bone. In each case new bone characteristic of the early phase of homograft new bone was present. There was an inflammatory reaction to the grafts as in Series 2.

TABLE 6

SERIES VIa - BONE HOMOGRAFT THREE WEEKS AFTER SKIN HOMOGRAFT

DURATION	RAT NO.	STRAIN	OSTEOCYTE SURVIVAL IN GRAFT	NEW BONE FORMATION	COMMENT
2 day	30	A	+	-	
	30	H	+	-	
4 day	59	A	-	-	
	59	H	+	-	
6 day	31	A	-	-	
	31	H	+	-	
8 day	57	A	+	-	
	57	H	+	-	
10 day	40	A	+	-	
	58	H	+	-	
14 day	33	A	+	-	
	33	H	+	-	
16 day	37	A			Graft not found.
	37	H	+	-	
18 day	60	A	+	-	
	60	H	+	-	
4 week	32	A			Graft not found.
	32	H	-	-	
6 week	35	A	-	-	
	35	H	-	-	
8 week	36	A	+	-	
	36	H	+	-	
3 month	26	A	+	-	
	26	H			Graft not found.
4 month	53	A	-	-	
	53	H	-	-	
5 month	39	A	-	-	
	39	H	-	-	
6 month	56	A			Graft not found.
	56	H	-	-	

TABLE 7

SERIES VIb - BONE HOMOGRAFTS TO RATS IMMUNISED BY SKIN FOUR MONTHS PREVIOUSLY

RAT NO.	STRAIN	DATE OF SKIN GRAFT	DATE OF BONE GRAFT	DURATION OF BONE GRAFT	NEW BONE FORMATION	COMMENT
203	H	6. 11. 56	5. 3. 57	13 days	+	In each case the new bone was typical early phase homograft new bone. Active osteogenesis had ceased and the formed bone was dead or dying.
203	A	6. 11. 56	5. 3. 57	13 days	+	
206	H	6. 11. 56	5. 3. 57	13 days	+	
206	A	6. 11. 56	5. 3. 57	13 days	+	
207	H	6. 11. 56	5. 3. 57	13 days	+	
207	A	6. 11. 56	5. 3. 57	13 days	+	

Series VII: Bone Homograft four weeks after bone homograft from the same donor (Table 8)

In this group there were two grafts to examine in each animal, the second grafts being of two days' to six months' duration whereas the first grafts were twenty-eight days older. Occasional osteocyte survival was observed in both. The second grafts gave four examples of early type new bone in the twenty eight grafts from six to eighteen days. Late phase new bone occurred in three of eighteen grafts of four weeks' duration and older. In the first grafts, the late phase new bone occurred in nine of forty eight grafts. The inflammatory reaction and slow rate of resorption of these grafts corresponded to Series II.

TABLE 8

SERIES VII - BONE HOMOGRAFT TO BONE IMMUNISED

2ND GRAFT					1ST GRAFT		
DURATION	STRAIN	RAT NO.	OSTEOCYTE SURVIVAL IN GRAFT	NEW BONE FORMATION E - Early phase L - Late phase	DURATION	OSTEOCYTE SURVIVAL IN GRAFT	NEW BONE FORMATION E - Early phase L - Late phase
2 day	H	146	+	-	29 day	+	-
4 day	A	208	+	-	32 day	+	L
	A	151	..... INFECTED			-	-
	H	208	+	-		-	-
	H	151	+	E		-	-
6 day	A	209	-	-	33 day	-	-
	A	148	+	-	33 day	-	-
	H	209	GRAFT NOT FOUND		35 day	+	-
	H	148	+	E	33 day	-	L
8 day	A	210	+	-	36 day	+	-
	A	150	-	-	37 day	+	-
	H	210	+	-	37 day	GRAFT NOT FOUND.	
	H	150	+	-	36 day	-	-
10 day	A	211	+	-	37 day	+	-
	A	149	+	-	37 day	+	-
	H	217	+	-	39 day	+	-
	H	149	+	-	37 day	+	E & L
12 day	A	212	+	E	39 day	-	E
	A	147	-	-	39 day	-	-
	H	212	-	E	41 day	-	-
	H	147	+	-	39 day	-	E
14 day	A	213	-	-	42 day	+	-
	A	152	+	E	42 day	+	-
	H	213	+	-	43 day	-	L
	H	152	+	-	42 day	+	-
16 day	A	216	+	-	42 day	-	-
	A	154	-	-	42 day	+	-
	H	216	-	-	45 day	-	E
	H	154	+	-	43 day	+	+
18 day	A	215	+	-	45 day	+	L
	A	155	+	-	45 day	+	-
	H	215	+	-	47 day	+	L
	H	155	+	-	45 day	+	-
4 week	A	82	+	-	56 day	-	-
	H	78	+	E		-	-
6 week	A	83	+	L	10 week	-	-
	H	83	+	-		-	L
8 week	A	79	+	L	12 week	-	-
	H	79	-	-		-	-
3 month	A	80	-	-	4 month	-	-
	H	80	-	-		-	-
4 month	A	76	-	E	5 month	-	-
	A	158	-	-		-	-
	H	74	-	L		+	L
	H	158	+	E		+	L

Table 8 continued overleaf.

TABLE 8 (continued)

SERIES VII - BONE HOMOGRAFT TO BONE IMMUNISED

2ND GRAFT					1ST GRAFT		
DURATION	STRAIN	RAT NO.	OSTEOCYTE SURVIVAL IN GRAFT	NEW BONE FORMATION E - Early phase L - Late phase	DURATION	OSTEOCYTE SURVIVAL IN GRAFT	NEW BONE FORMATION E - Early phase L - Late phase
5 month	A	81	-	-	6 month	-	-
	A	159	-	-		+	-
	H	81	+	-		+	-
	H	159	+	-		-	-
6 month	A	157	-	-	7 month	-	-
	H	157	-	-		+	-

Series VIII: Freeze-dried bone homograft four weeks after skin homograft from the same donor (Table 9)

This series behaved as Series III. No survival of osteocytes was seen. No new bone developed by six months. In one graft which was left for 10 months a small amount of late phase homograft new bone was seen. Resorption associated with the presence of osteoclast-like giant cells progressed slowly.

As no method of quantitating the inflammatory response could be evolved it was not possible to demonstrate any difference in the inflammatory reaction to the freeze-dried bone in unprepared and preimmunised hosts. The response appeared mild in both groups.

TABLE 9

SERIES VIII - FREEZE-DRIED BONE INTO SKIN IMMUNISED RATS

DURATION	RAT NO.	STRAIN	OSTEOCYTE SURVIVAL IN GRAFT	NEW BONE FORMATION	COMMENT
2 day	124	A	-	-	
	124	H	-	-	
4 day	122	A	-	-	
	122	H	-	-	
6 day	117	A	-	-	
	117	H	-	-	
8 day	115	A	-	-	
	115	H	-	-	
10 day	116	A	-	-	
	116	H	-	-	
12 day	120	A	-	-	
	120	H	-	-	
	119	H	-	-	
14 day	121	A	-	-	
	121	H	-	-	
16 day	114	A	-	-	
	114	H	-	-	
18 day	123	A	-	-	
	123	H	-	-	
4 week	85	A	-	-	
	85	H	-	-	
	88	H	-	-	
6 week	86	A	-	-	
	86	H	-	-	
8 week	87	A	-	-	
	87	H			Graft could not be found.
3 month	90	A	-	-	
	90	H	-	-	
4 month	91	A	-	-	
	91	H			Graft could not be found.
5 month	89	A	-	-	
	89	H	-	-	
6 month	84	A	-	-	
	84	H	-	-	
10 month	118	A	-	+	Small amount of lamellar bone typical of late phase.

DISCUSSION

Before evaluating the results of these experiments it is necessary to outline briefly some of the characteristics of the homograft immune reaction.

(1) The typical homograft is initially accepted by the host in a manner similar to an autograft. It acquires a blood supply, survives for a few days and may grow to a limited extent.

At the end of a period which is surprisingly constant for a wide variety of tissues in a wide range of vertebrate animals, the graft dies. This is accompanied by the development of an inflammatory reaction (characterised by an infiltration with round cells of varying type) and by the interruption of the blood supply to the graft.

(2) Once a host has produced this response to a homograft it remains in a state of heightened sensitivity so that a second graft from the same donor to the same host is rejected at an earlier time (the second set phenomenon). This state of heightened sensitivity slowly subsides over a period of several months, (Billingham, Brent and Medawar, 1954).

(3) Several conditions are necessary for the fulfilment of the reaction.

The antigens evoking the immune response which brings about the homografts ultimate rejection (the T antigens) have been shown to be contained within cell nuclei (Billingham, Brent and Medawar, 1956a). In general only living cells liberate sufficient T antigen to elicit the immune reaction.

The antibody which effects the destruction of the homograft is linked with the circulating lymphocytes of the host and is not present in the serum. Direct contact between these cells and the grafted tissue is a necessary prerequisite for rejection. Any barrier to this contact allows prolonged survival of a homograft. This barrier may be natural as in the case of the matrix of cartilage (Loeb, 1930; Peer, 1954), or it may be created experimentally by the diffusion chamber, (Algire, Weaver and Prehn, 1954; Woodruff, 1957).

Freely circulating serum antibodies do also occur but these are dependant on a different set of antigens than the T antigens which have been described above. These H antigens are more

stable than the T antigens and are present in tissues which have been killed by such means as freeze-drying.

Killed homografts may release H antigens but not T antigens. They do not arouse a typical homograft immune response in the host; indeed, a subsequent living homograft from the same donor may survive slightly longer than normal. This is called the "enhancement effect", which is thought to be due to the H antigen/antibody inter-action although the precise mechanism is the subject of controversy - (Billingham, Brent and Medawar, 1956b; Kaliss and Bryant, 1958).

(4) Tissue antibodies are individual specific - immunity aroused by one homograft prevails against further homografts only from the same donor or from identical twins or from other members of a pure inbred strain. There is considerable evidence that many and perhaps all the tissues of an individual have tissue antigens in common.

Against this background the results of the experiments outlined in this chapter demonstrate clearly that bone homografts participate in immune reactions in quite a typical manner.

The failure of the bulk of fresh bone grafts whether autogenous or homogenous to survive transplantion has made it difficult to demonstrate that homografts of bone can in fact remain viable for even a few days. However, although most of the osteocytes failed to persist there was evidence that the dormant osteoblasts which are present on all bone surfaces did in fact survive in both the fresh autograft and homograft. These as a result of the trauma of transplantation became active and commenced forming bone after a latent period of four days. In the case of the autograft, growth continued uninterruptedly, but in the homograft it was arrested by about the 8th day and the new bone which had formed during that limited period subsequently died.

As a result only a very limited amount of new bone was ever seen in relation to a homograft, which may account for the fact that this has not previously been recognised, although Danis (1956) described a similar phase of new bone formation with homografts of bone marrow in rabbits. As stated on page 6, Hutchison (1952) observed some new bone growth around homografts of bone, but held that this bone growth had arisen from the host tissues rather than from grafted cells. There is little doubt that bone grafts can stimulate the induction of bone formation in adjacent soft tissues (see page 59) and by examining a histological preparation one could not say with certainty that new bone had origin in graft or host cells. Even so, workers in

this field have expressed dogmatic and entirely opposing views on the basis of essentially similar evidence (Danis, 1956; Levander, 1945; Lacroix, 1951).

While direct proof is lacking, there is a great deal of indirect evidence to support the view that the early phase of new bone around the homograft is in fact a product of the graft cells. Firstly, its limited duration of growth and survival observed in Series II and its complete failure of development in Series VIa correspond exactly with first and second set homograft reactions of other tissues, and can only be explained if the bone is of homograft origin. Secondly, had the early phase new bone been 'induced' one might have expected its occurrence in relation to the freeze-dried homografts, (Series III), but it was not in fact observed in this series.

The demonstration of a second set response by skin following a sensitising graft of bone (Series IV), is further evidence that bone participates in a homograft reaction. It indicates also that bone and skin have antigens in common.

The limited duration of the homograft immunity is shown by Series VIb in which each of the bone homografts carried out four months after a skin homograft from the same donor produced early new bone. This is in keeping with the duration of immunity against other tissues in rats recorded by Lehrfeld, Taylor and Converse (1955) and in

mice by Billingham, Brent and Medawar (1954).

Freeze-drying appears to inactivate T antigens in bone homografts since subsequent skin grafts from the same donor did not undergo an early rejection but survived slightly longer than normal (Series VIII). This prolongation of survival time, although small, was statistically significant*, and may be an example of the enhancement effect. The modified antigenicity of freeze-dried bone homografts is reflected in the reduced inflammatory reaction at the host site. Pre-immunisation of the host did not obviously increase this reaction (Series VI) as it does with some other killed tissue homografts (Darcy, 1955).

While the work described in this chapter was being carried out, another study of bone homografts to normal and immunologically prepared animals was reported by Enneking, (1957).

This author studied the histological progress of orthotopically placed autografts and homografts of segments of femoral shaft in the rat. These transplants were secured in position by means of a Kirschner wire. Presensitisation was carried out either by the insertion of a similar homograft two to three weeks before a second

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*Mean survival time of first set skin homografts - 10.9 days. Standard error 0.13. Mean survival time of skin homografts following freeze-dried bone homografts - 12.0 days. Standard error 0.29. Difference of mean of the two groups - 1.1 days. Standard error of the difference 0.32. Application of the 't' test indicates that this difference is significant at the 0.01 level.

homograft of bone from the same donor or by exchanging segments of femur between rats previously joined in parabiotic union.

He observed some new bone formation around all the transplants at five days which ceased in the homografts by fifteen days although persisting in the autograft. He found no difference between homografts in the normal and presensitised hosts with respect to the amount and duration of this new bone formation.

Subsequently, many of the homografts in both normal and presensitised hosts became surrounded by fibrous tissue and were resorbed or sequestrated without replacement by host bone, whereas the autografts went on to sound union.

An inflammatory reaction developed around the homografts, which was most intense in relation to the grafts in presensitised hosts.

Considerable variability in homograft behaviour was observed in these experiments, some homografts behaving in a similar manner to autografts. This was attributed to "low order antigenic complexity" between host and donor.

In general, Enneking's findings are in accordance with those reported in this chapter. Such differences as do appear in the results of the two studies can be attributed to the use of different host sites, different methods of

sensitisation of the host and to the use by Enneking of a partially inbred colony of rats. The most important of these differences is the apparent development of some new bone around the bone homografts to immunologically sensitised hosts in Enneking's study and its almost complete suppression in the author's work. Enneking made use of an orthotopic graft site which makes it difficult to exclude the possibility that host osteoblasts were responsible for the new bone formation. Further he did not confirm that the techniques he used for sensitising the hosts did in fact achieve that end. By using parabiosis as a method he was automatically selecting pairs of low antigenetic diversity, for only these (35 per cent of the total) survived the procedure.

Returning to the consideration of the experimental results recorded in this chapter there are several observations which do not appear to accord with the classical pattern of the homograft immune response and require further comment.

First, there is the observation that a few osteocytes in both fresh auto- and homograft appeared to retain their normal morphology throughout the period of observation. Retention of normal staining and histological characteristics is generally held to indicate viability of these cells. The apparent paradox of surviving osteocytes in the homograft in the presence of an immune

reaction is, however, readily explained. These cells are surrounded by a continuous matrix of bone which prevents their contact with host cells which is necessary for the fulfilment of the immune response. They are in this respect similar to the chondrocytes of homo-transplanted cartilage. The matrix of the new bone of the early phase is so incomplete and ill-formed, (see Fig.16) that it is too ineffective a barrier to protect the osteocytes of this bone.

Secondly, and more difficult to account for, is the incomplete appearance of the 'second set' phenomenon in Series VII in which the initial immunising stimulus was a bone homograft from the same donor. In this series two out of fourteen grafts produced early phase new bone, while following an immunising stimulus of a skin homograft in Series VIa there was complete suppression of the early phase new bone.

A possible explanation for this inconsistency lies in the different sites of implantation of the two first set grafts. It is known that levels of immunity may vary with the site of implantation (Medawar, 1948; Billingham, Brent and Mitchison, 1957). Skin is known as a highly favourable site while the status of muscle in this respect is largely unknown, although Earle (1954) stated that tumour homografts survived longer in muscle than they did subcutaneously.

Bone induction

The histogenesis of the late phase of homograft new bone remains unsolved. As with the early phase both the graft itself or the adjacent connective tissue of the host are theoretically possible sources. The fact that homograft immunity has been established against the graft does not eliminate the possibility that graft cells may be responsible, for it has been suggested that homografted tissues which are kept alive beyond a certain critical period are no longer susceptible to the immune response (Woodruff, 1952). One could argue that the living osteocytes which were demonstrable in the fresh homografts might become liberated during the process of resorption and resume osteogenic function. Evidence in favour of the host origin of this bone is provided by the fact that it developed in relation to several of the freeze-dried bone grafts (Series V). Freeze-drying kills mammalian skin (Billingham and Medawar 1952) and mature bone (Ray et al. 1952; Pate 1954). In the present experiments there was no histological evidence of cell survival in any of the freeze-dried bone grafts used.

The fact that bone formation in non-skeletal tissues can be 'induced' has been established beyond doubt since the classic experiments of Huggins (1930) who used transplants of bladder mucosa as the inducing agent. Whether bone

itself can provide this inducing stimulus is the subject of more controversy. There are many reports of new bone developing around grafts of dead bone in soft tissues (Wilson 1951; Axhausen 1953; Urist and McLean, 1952; Curtiss and Wilson 1953; Maatz 1955; De Bruyn and Kabisch 1955), but others have failed to confirm this (Ray et al. 1952; Ham and Gordon 1952; Keith 1934; Campbell et al. 1953; Danis 1956). The experiments recorded in this paper suggest that dead bone can induce metaplastic bone formation in non-skeletal connective tissue, but that this occurs infrequently and only after a long period.

SUMMARY

Bone homografts exhibit the following features which indicate that bone behaves in a typical manner with respect to homograft immunity.

(1) A limited growth of bone arising from the homograft takes place from the fourth to eighth day following transplantation. This bone subsequently dies.

(2) A state of heightened activity can be demonstrated in the host against a second set skin homograft following a first set bone homograft from the same donor and conversely to a second set bone homograft following a skin homograft from the same donor.

(3) Killed bone homograft fails to evoke an immune reaction.

The experiments have also demonstrated that bone formation in non-skeletal tissues may be induced by the presence of a bone graft.

CHAPTER II

The Growth of Transplanted Foetal Bones in Different Immunological Environments

Substantial growth of immature bone homografts in various sites has been observed by many workers including Leopold (1881), Fischer (1882), Von Helferich (1899), Willis (1936 and 1939), Huggins and Blockson (1936), Hancox (1947), Ray *et al* (1957), Felts (1955, 1957a, 1957b).

Such growth is contrary to the behaviour of mature bone homografts which, as has been shown in Chapter I, are capable of only very limited growth, restricted to the first few days after implantation.

A closer examination of the work of the above authors suggests that the difference may be more apparent than real, and might in some instances be explained by the use of particular host sites, or by the use of partially inbred laboratory animals.

For example, Willis (1936) used brain as the host site and it is now known that homografts of adult tissues transplanted to the brain may enjoy prolonged survival (Medawar 1948). Willis (1939) had less success with the use of the subcutaneous site.

Leopold (1881) and Ray *et al* (1957) also made use of a privileged site, the anterior chamber of the eye, in which

prolonged homograft survival may take place (Woodruff and Woodruff, 1950). Felts (1955) demonstrated the growth of bones homografts exchanged between 5 day old litter mate rats. The purity of strain is not stated; therefore, it is possible that inbreeding had reduced the incompatability between hosts and donors. In a later paper, Felts (1957b) compared isografts and homografts of 2 day old mouse humeri placed subcutaneously, and found that the later grew to only a limited extent, resembling the behaviour of mature homografts.

Huggins and Blockson (1936) had a variable experience with homografts of tail bones from newborn rats transplanted intraperitoneally, some growing well whereas other showed limited cartilage growth only.

In view of the general uncertainty in this field a further investigation appeared desirable.

The following experiments were planned to show the influence of a range of different immunological environments on a foetal bone.

Method and Materials

To overcome the technical difficulties of a foetal autograft, pure strains of mice were used. Grafts exchanged between individuals of a pure strain behave as autografts and are known as isografts.

The grafts used in all experiments were femurs taken from 18 day old foetal Strong A mice. These were transplanted into the spleens of the following hosts of both sexes aged 6 - 7 weeks at the time of grafting -

Host	Status of Graft
Strong A mice	Isograft
C_3H mice	Homograft
C_3H mice previously immunised against Strong A tissues	Second set homograft
Long Evans Rats	Heterograft

Each series of experiments was arranged so that the progress of a pair of femurs from one foetus could be observed in differing hosts -

Series I Compared isograft and homograft.

Series II Compared first and second set homograft.

Series III Compared homograft and heterograft.

A total 180 transplants were carried out.

In Series I, a pair of animals was sacrificed at intervals from 2 days to 3 months but in Series II and III, the maximum duration was 8 weeks, as it was found that few changes occurred in the grafts after that time. The transplants were dissected free from soft tissues and were

weighed, photographed, and X-rayed under standard conditions so that measurements of total length and length of diaphysis could be made. Each graft was examined histologically. In a few additional mice of Series I and II the vascular pattern of the graft was visualised by injecting the host's left ventricle with an Indian ink solution at the time of death.

Technical procedures

The graft

The femurs were dissected from 18 day foetal strong A mice with the aid of a dissecting microscope. They were kept moist with sterile saline during the dissection and were stored in saline at 37°, until all the femurs from the litter had been obtained, a process usually taking about 2 hours.

The transplantations were carried out immediately thereafter.

The 18 day foetal femur is largely cartilaginous with a small diaphysis of true bone (Fig. 24). The cartilage consists of uniformly immature cells. The epiphyseal cartilage plate is not identifiable at this stage. The total length and the length of bony diaphysis were measured by means of a micrometer ocular, and their weights were recorded. The values obtained from 180 femurs were -

total length 4.1 (± .2)mm.; weight 2.8 (± .7)mgm.

Operative procedure

With the host anaesthetised with intraperitoneal nembutal, a small incision was made in the left flank and the spleen exteriorised. A stab wound was made in the spleen and the foetal femur inserted with its long axis parallel to the long axis of the spleen. The abdominal wall and skin were closed with a single suture. Excessive bleeding was not encountered.

Method of immunisation

The technique used for the initial immunisation in Series II was based on that described by Billingham, Brent and Mitchison (1957). Spleens were removed from 2 adult strong A mice immediately after killing with ether, cut into small pieces and placed in a thimble of 50 mesh tantalum gauze. Gentle pressure with a glass rod was used to work the spleen cell through the mesh into 10 cc. saline. Clumps of cells were allowed to settle and a cell count made of the supernatant suspension (usually about 30 X 10^6 nucleated cells/ml). Half of the C_3H host mice in the series were then given an intraperitoneal injection of about 6 million cells.

The foetal bone homograft was inserted three weeks later.

Histology

The grafts were fixed and decalcified in Bouin's solution, embedded in celloidin, sectioned on their long axes and stained with haematoxylin and eosin. In the specimens which were injected with Indian ink alternate thick (200 micra) and thin (6 micra), sections were made.

The data for the growth of the normal femur were obtained by sacrificing a pair of mice, usually one male and one female at each of the time intervals indicated in Table 10. Measurement of total length, diaphyseal bone length and weight were made by the same techniques as for the grafts.

RESULTS

SERIES I: Comparison of 18 day foetal femur isograft and homograft

There was very little interruption in growth in length and weight of both isograft and homograft following transplantation (Table 10 and Figs. 57 and 58). There was, however, a delay of 2 days before the diaphysis began to increase in size. Thereafter growth proceeded in an orderly manner and both isograft and homograft developed identically until the sixth day (Figs. 25 and 26). Histological preparations showed growth of bone and cartilage in both groups, with normal endochondral ossification (Figs. 37 and 38).

After the sixth day the progress of the two grafts differed. The isograft continued to grow uninterruptedly, secondary centres of ossification appeared in the correct sequence, although a day or two earlier than in the normal femur (Figs. 27 - 34). Maximum growth in length reached at about 8 weeks was 80% of that of the normal femur (Table 10 and Fig. 55). (This limitation of growth was not related to the age of the host for several pairs of femurs were implanted isogenously into 6 weeks and 8 months old mice, and no difference was observed in the length of the grafts). The articular cartilage of the isograft was thinner and more irregular than that of the normal femur.

After six days the homograft showed no further growth of bone, and the bone which had developed up to that time largely died - only a few osteocytes persisting -(Figs. 40 and 51). The cartilage, however, continued to grow so that the total weight of the femur kept pace with the isograft (Table 10 and Fig. 58). The epiphyseal cartilage plate differentiated normally, but the hypertrophic, calcified cartilage, which accumulated in large amounts both in the metaphyseal region and in the epiphyses, was unreplaced by bone -(Figs. 40 - 49). This hypertrophic cartilage was so fragile that fractures frequently occurred resulting in distortions which made measurements of length impossible.

The duration of growth of homograft cartilage corresponded closely to that of the isograft as judged by weight measurement and by the microscopic appearance of the epiphyseal cartilage plate. In both groups the plate became much narrower after the eighth week, corresponding to the reduction in the rate of growth (Figs. 47 and 48).

The hypertrophic cartilage of the homograft was invaded increasingly by blood vessels from the periphery after the third week, causing irregular areas of resorption in which accumulation of inflammatory cells occurred. These areas were examined carefully for evidence of new bone formation, but this was found in only one graft at 12 weeks in which some new bone had developed in the distal epiphysis (Fig. 52).

In homografts in which the vascular pattern had been visualised by Indian ink injection, a sudden shut-down in the vascular bed within the diaphysis was seen after the sixth day, coinciding with the arrest of ossification and death of the bulk of the previously formed bone. Thereafter only a few irregularly disposed vessels were seen within the diaphysis - (Figs. 53 and 54).

The medullary space of the homograft became occupied with fibrous tissue and infiltrated with inflammatory cells, chiefly plasma cells and lymphocytes, contrasting with the haemopoietic marrow of the isograft - (Figs. 50 and 51).

A most interesting difference was observed in the gross appearance of the isograft and homograft. In both, the growing cartilage began to lay down in some detail the secondary characteristics of a femur such as the lesser trochanter and intertrochanteric ridge, but in the isograft these secondary characteristics became submerged and disappeared as the cartilage was replaced by bone. In the homograft, on the other hand, these secondary characteristics persisted throughout the period of growth. Tubulation or remodelling of the "metaphysis" did not occur in the homograft (Figs. 35 and 36).

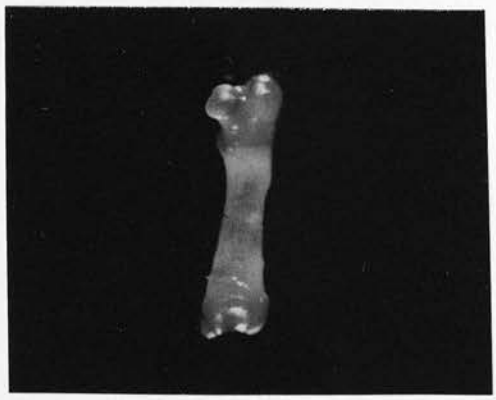
TABLE 10

SUMMARY OF BONE MEASUREMENTS

AGE OF NORMAL FEMUR		EQUIVALENT GRAFT AGE	TOTAL LENGTH (mm)					LENGTH OF DIAPHYSEAL BONE (mm)					WEIGHT (mg)				
			NORMAL	ISOGRAFT	HOMOGRAFT	HOMOGRAFT IN PREIMMUNIZED HOST	HETEROGRAFT	NORMAL	ISOGRAFT	HOMOGRAFT	HOMOGRAFT IN PREIMMUNIZED HOST	HETEROGRAFT	NORMAL	ISOGRAFT	HOMOGRAFT	HOMOGRAFT IN PREIMMUNIZED HOST	HETEROGRAFT
-2		0	4.1	4.4	4.4	4.5	4.2	1.6	1.5	1.5	1.5	1.3	2.8	3.5	3.3	3.0	4.1
0		2	4.8	5.3	5.2	5.0	4.5	1.9	2.2	2.2	1.5	1.4	3.7	5.3	5.7	5.1	8.7
2		4	5.3	5.5	5.3	5.4	5.1	2.4	3.2	3.0	1.6	1.4	5.5	6.8	6.6	6.0	
4		6	6.0	5.6	5.7	5.9	5.1	3.0	3.8	3.1	1.5		8.3	8.4	8.1	9.5	
6		8	6.7	6.7	6.6	6.5	5.3	3.4	4.7	3.2	1.7		10.6	11.1	10.9	10.6	
8		10	7.3	7.5	7.0	5.9	4.9	4.7	4.3	3.2	1.3		13.8	12.4	11.1	10.9	5.1
10		12	8.1	7.5	7.0	6.5	5.1	5.3	5.0	2.5	1.4		16.4	13.8	13.6	12.3	5.4
12		14	8.7	8.0			5.0	5.8	6.0	2.8	1.4		20.5	15.6	16.3	9.4	5.1
14		16	9.1	8.8			5.0	6.5	6.5	2.3	1.6	1.6	20.3	18.2	15.8	11.7	5.7
16		18	9.5	8.8			5.3	6.9	7.0	3.1	1.8		23.9	21.5	16.8	6.5	5.0
18		20	9.9	8.9				7.6	7.5				26.7				
	4		10.1					7.5					26.0				
	5		11.3	9.3	- TOO DISTORTED TO MEASURE -			8.5	7.1	3.2	1.6	1.3	30.8	15.3	18.5	17.1	6.9
	6		11.9	9.7				9.1	7.1	3.0	1.4	1.4	35.7	19.3	17.8	18.5	8.3
	7		12.1	10.7				9.6	8.0	2.7	1.5		35.8	20.8	22.6	21.5	7.8
	8		13.2	9.2				10.5	7.8	3.2	1.4		44.3	20.5	21.0	21.5	
	9		14.1	10.6				11.0	8.1	2.2	1.8		50.3	25.2	21.1	17.9	
	10		14.0	11.5				11.1	7.9	3.2	-		53.0	18.7	24.2	-	
	11		14.2	9.5				11.0	7.0	2.9			55.6	22.6	22.8		
	12		14.4	12.0				11.4	9.8	2.5			54.6	19.7	17.9		
				11.3				12.0		2.9			54.5	24.1			

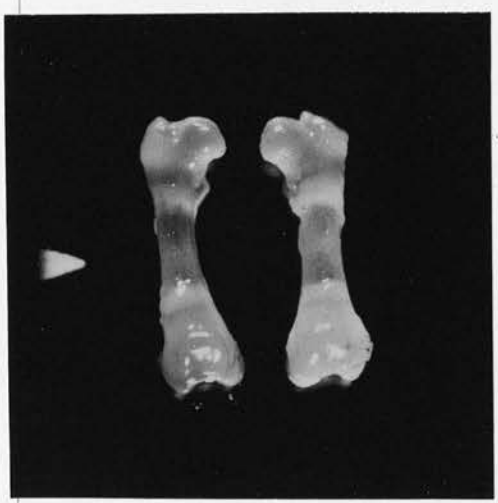
Mean values only are recorded whenever two or more pairs of hosts of the same series were sacrificed at the samt time interval.

Figure 24



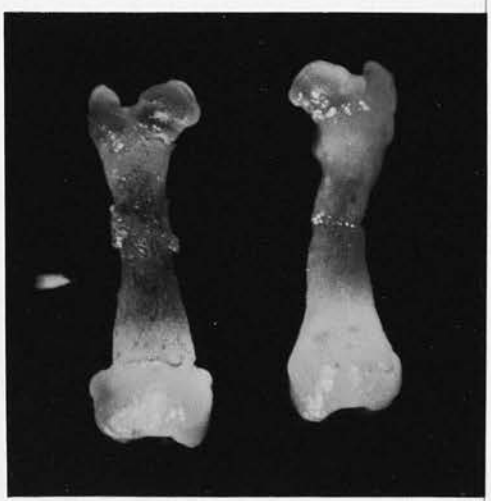
Femur of an 18-day old mouse foetus (x 9)

Figure 25



Isograft (L) and Homograft (R)
2 days (x 9)

Figure 26



Isograft (L) and Homograft (R)
6 days (x 9)

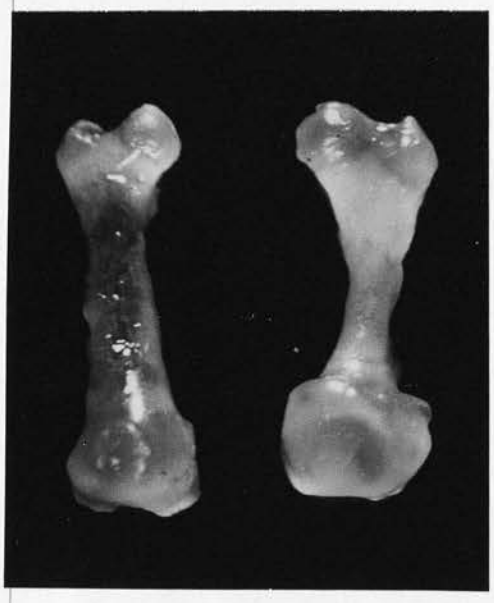
Up to six days there is a general similarity between isograft and homograft both with respect to total length and length of diaphysis (which corresponds to the red segments of the shaft).

Figures 27 - 30

These photographs illustrate different progress of the femur isografts and homografts after six days. While ossification proceeds normally in the isograft, the diaphysis of the homograft ceases to grow and becomes paler in colour. Cartilage growth continues in the homograft to the same extent as in the isograft, although fractures and distortions (Figure 30) may cause dissimilarity in length.

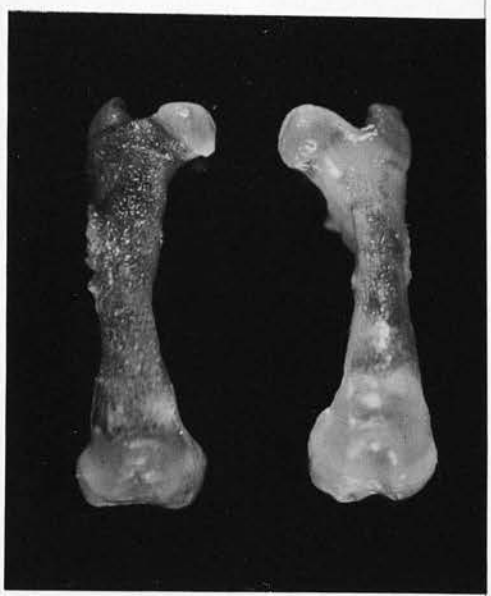
The increasing opacity in the cartilage in the homograft is due to calcification.

Figure 27



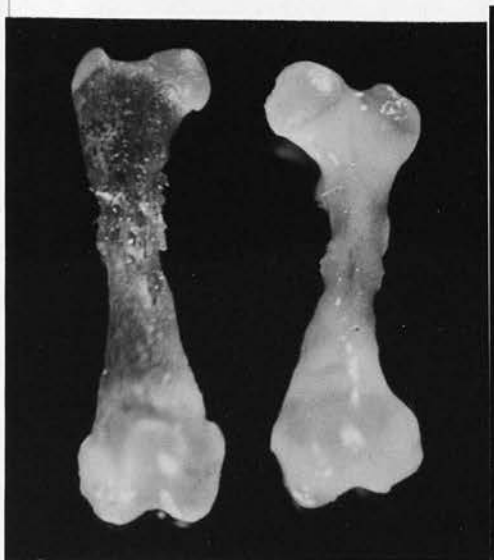
Isograft (L) and Homograft (R)
8 days (x 9)

Figure 28



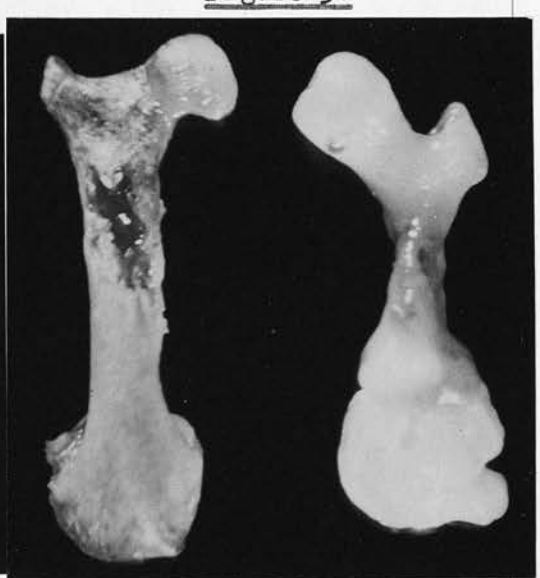
Isograft (L) and Homograft (R)
10 days (x 9)

Figure 29



Isograft (L) and Homograft (R)
12 days (x 9)

Figure 30



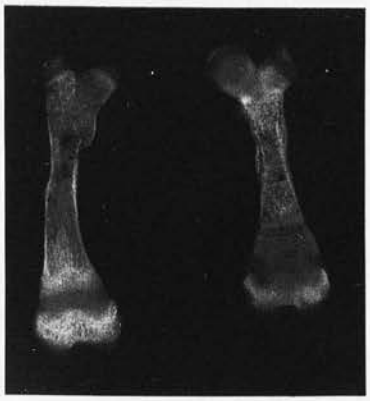
Isograft (L) and Homograft (R)
4 weeks (x 9)

Figures 31 - 34.

In these x-rays the bone can be distinguished from the calcified cartilage by virtue of its trabecular arrangement. In the homograft the length of the column of bone in the diaphysis remains approximately the same whereas in the isograft it increases steadily.

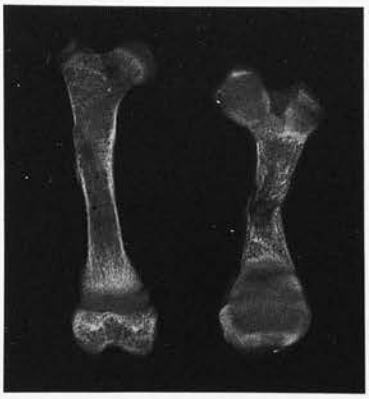
Note the progressive reduction in the width of the epiphyseal cartilage as the rate of growth diminishes.

Figure 31



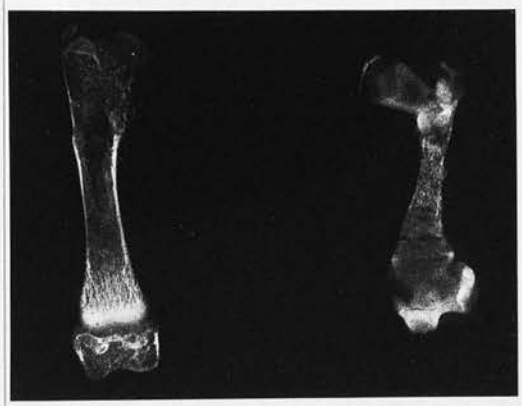
Isograft (L) and Homograft (R)
14 day
x-ray (x 6.5)

Figure 32



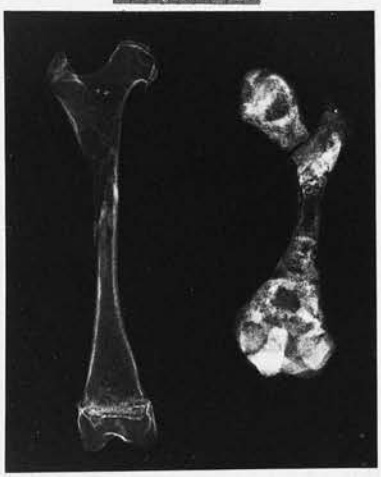
Isograft (L) and Homograft (R)
16 day
x-ray (x 6.5)

Figure 33



Isograft (L) and Homograft (R)
20 day
X-ray (x 6.5)

Figure 34



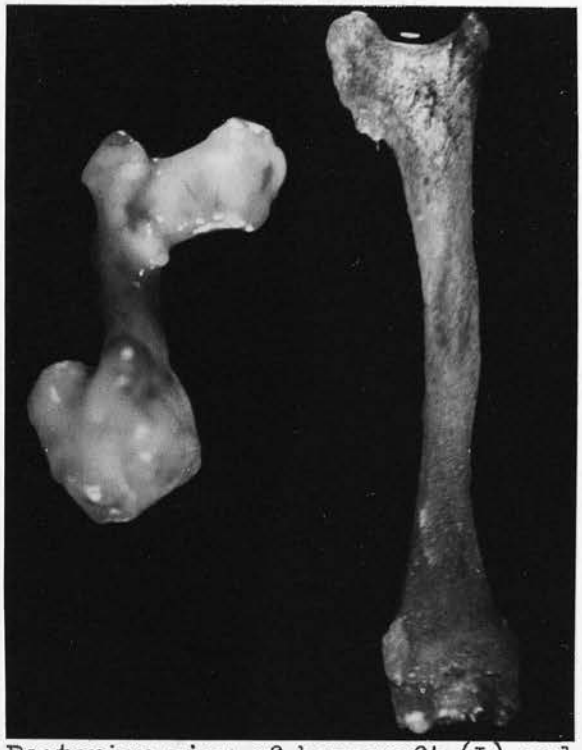
Isograft (L) and Homograft (R)
9 weeks
X-ray (x 6.5)

Figures 35 and 36

Note that while the homograft retains a well marked lesser trochanter and intertrochanteric ridge, these features are missing in the isograft.

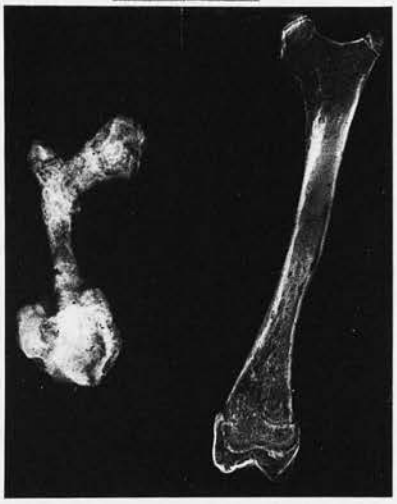
(These photographs were taken by the author with improvised equipment and do not demonstrate adequately these morphological differences between the isograft and homograft which were clearly apparent under the dissecting microscope).

Figure 35



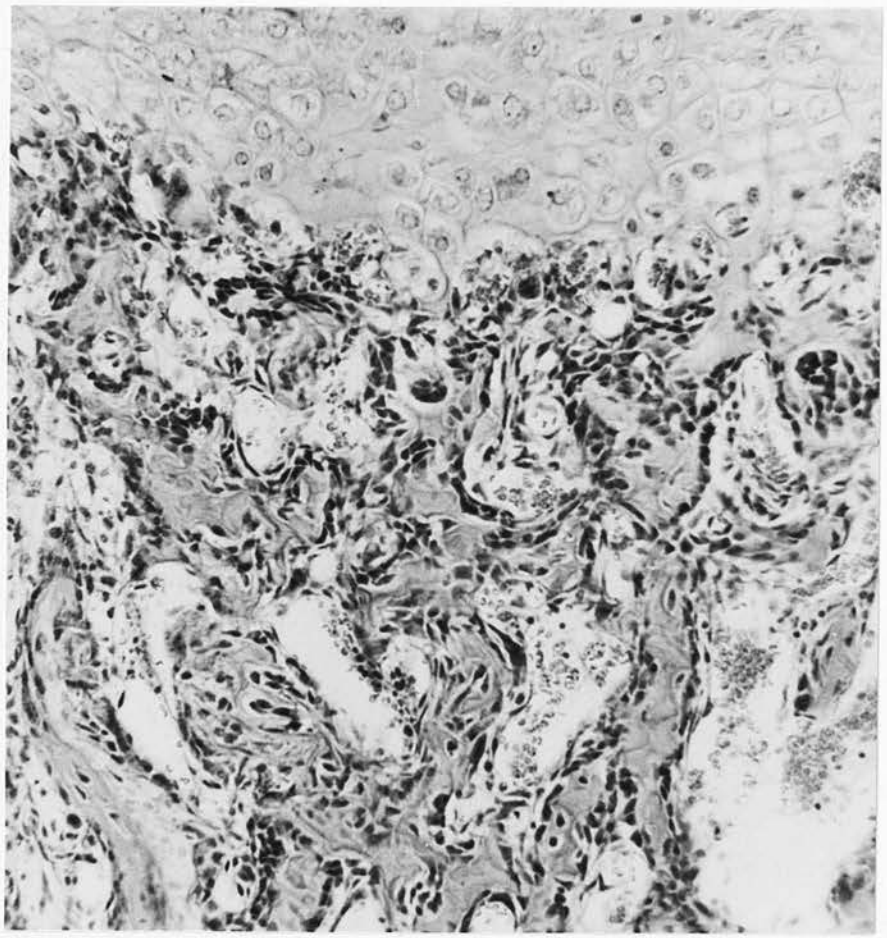
Posterior view of homograft (L) and isograft (R) at eleven weeks (x 9).

Figure 36



X-ray (x 6.5) of the homograft and isograft pair shown in Figure 35.

Figure 37

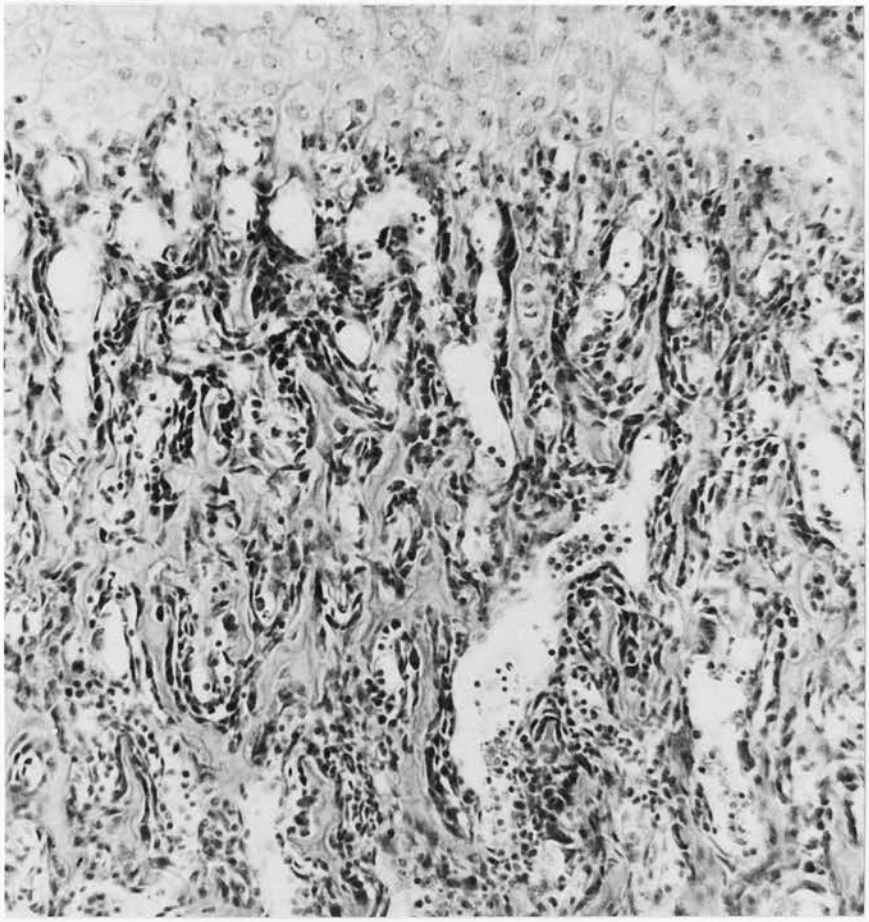


Isograft six days

Photomicrograph of junction of cartilage and bone. Normal endochondral ossification is proceeding.

H. and E. x 135.

Figure 38



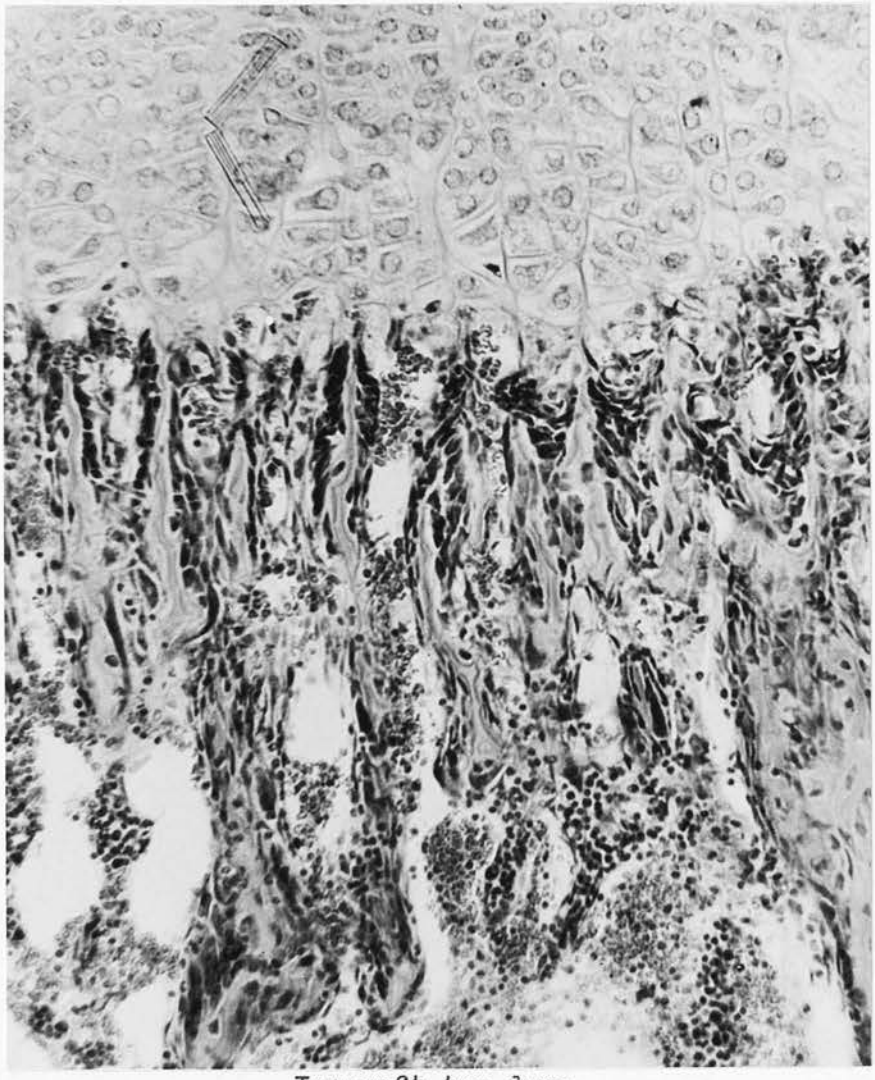
Homograft six days

A corresponding field to Fig. 37

Active endochondral ossification is proceeding. The resemblance to the isograft at this same stage is close

H. and E. x 135

Figure 39

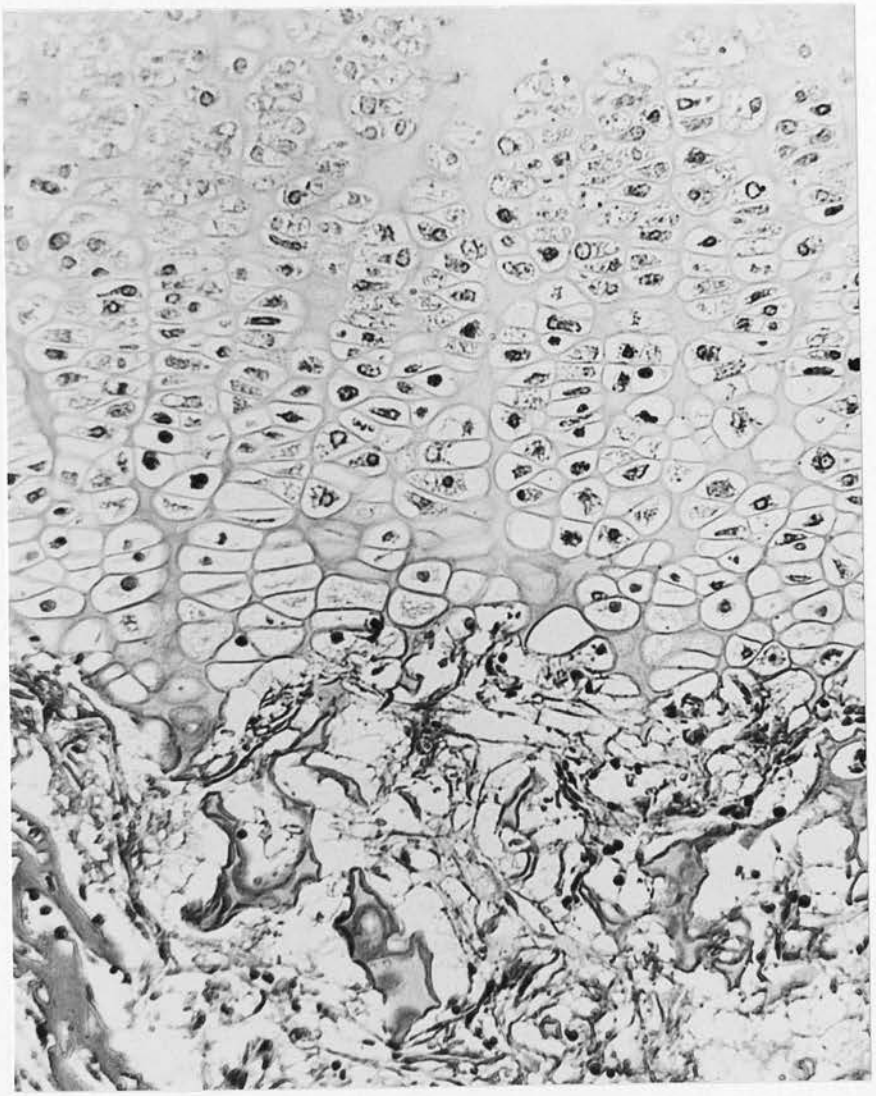


Isograft ten days.

Cartilage bone junction showing normal appearances of endochondral ossification.

H. and E. x 135.

Figure 40

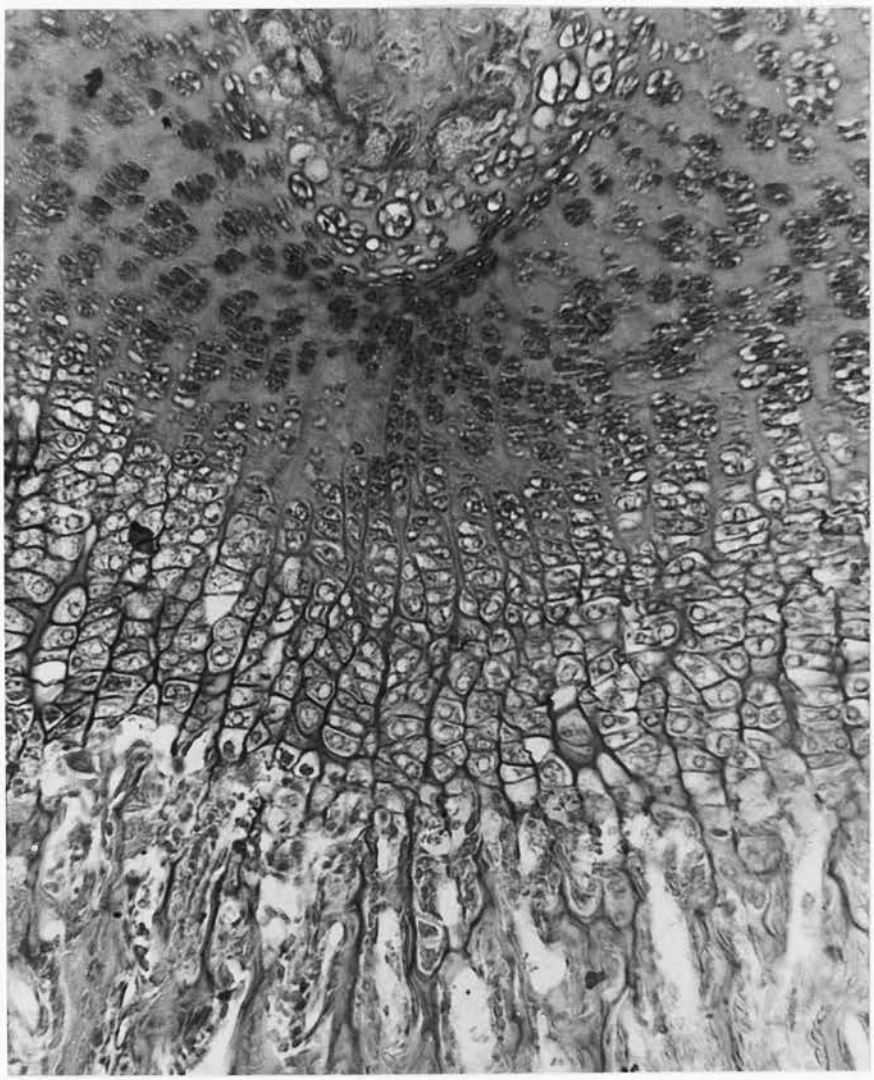


Homograft ten days.

A similar field to Figure 39. No osteogenis is occurring. The metaphyseal zone appears avascular.

H. and E. x 135.

Figure 41



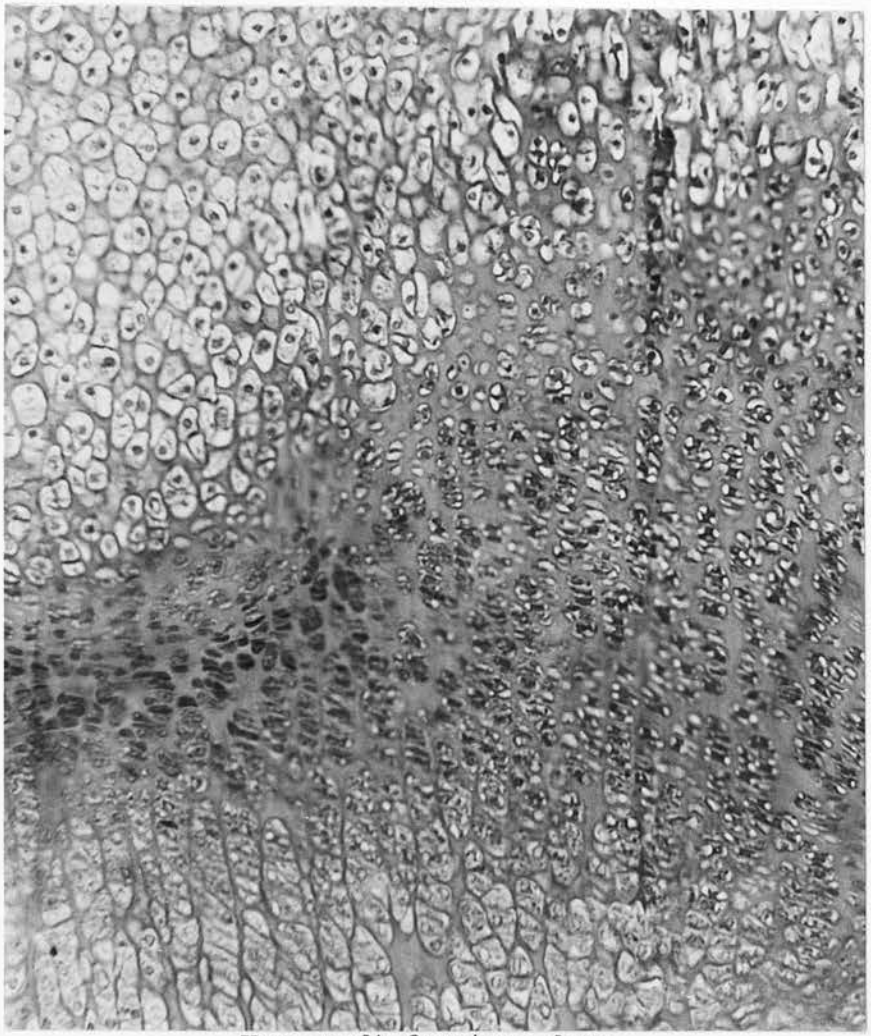
Isograft fourteen days.

A field of the epiphyseal plate.

The various stages of maturation of growing cartilage are well shown. Orderly replacement of mature cartilage by bone is taking place. A secondary centre of ossification has appeared in the epiphysis.

H. and E. x 135.

Figure 42



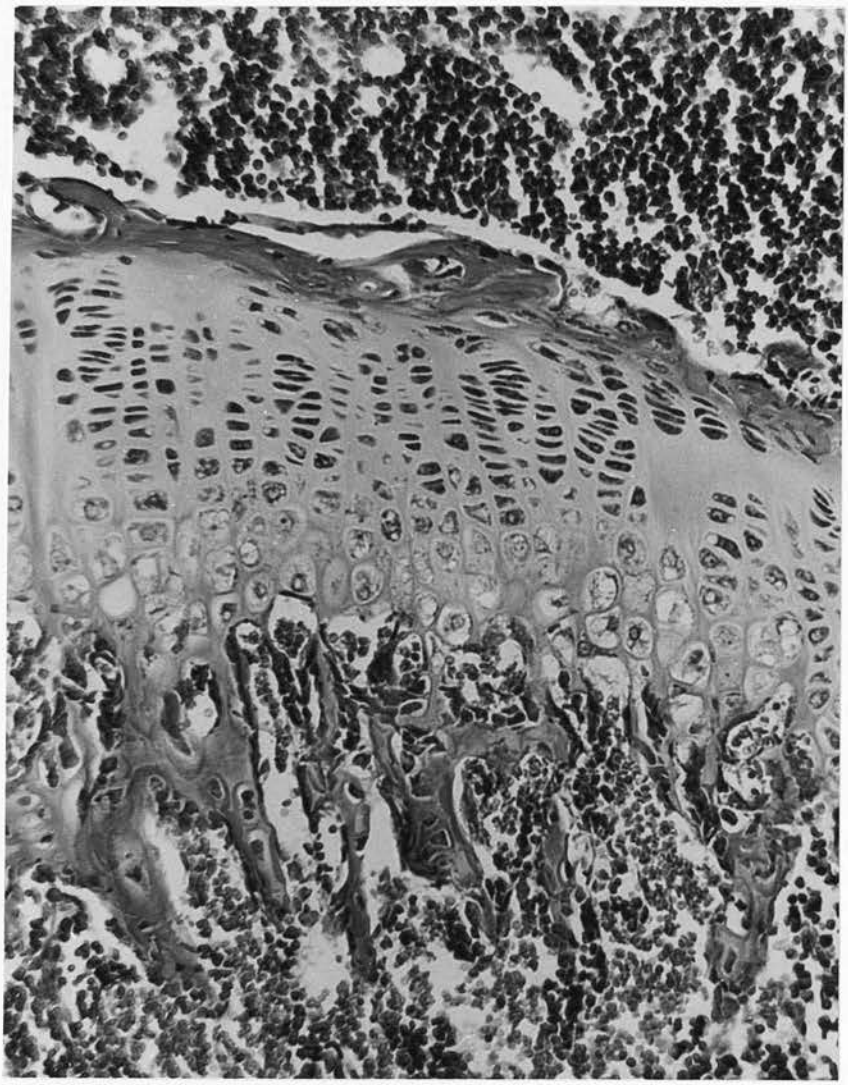
Homograft fourteen days.

A field corresponding to Fig. 41.

The epiphyseal cartilage plate is similar to that of the isograft but the hypertrophic cartilage is accumulating, unreplaced by bone.

H. and E. x 135.

Figure 43

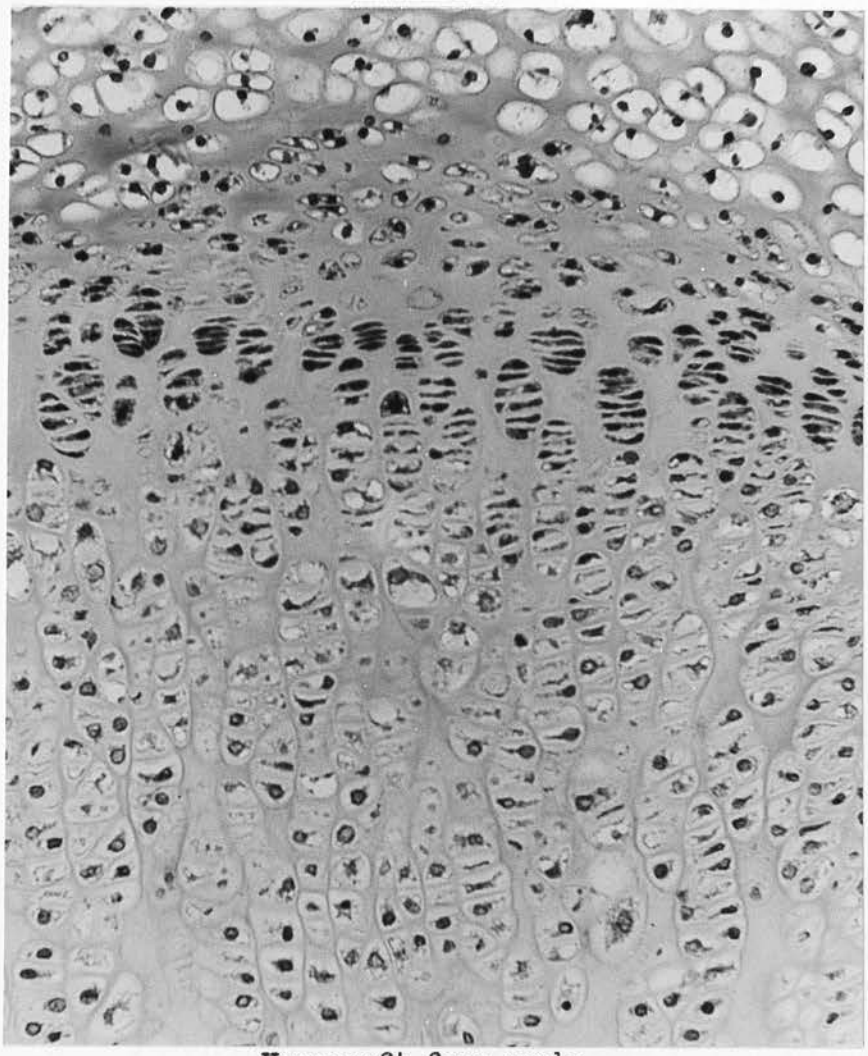


Isograft five weeks.

The epiphyseal cartilage plate presents an entirely normal appearance. The epiphyseal centre of ossification is now fully developed.

H. and E. x 135.

Figure 44



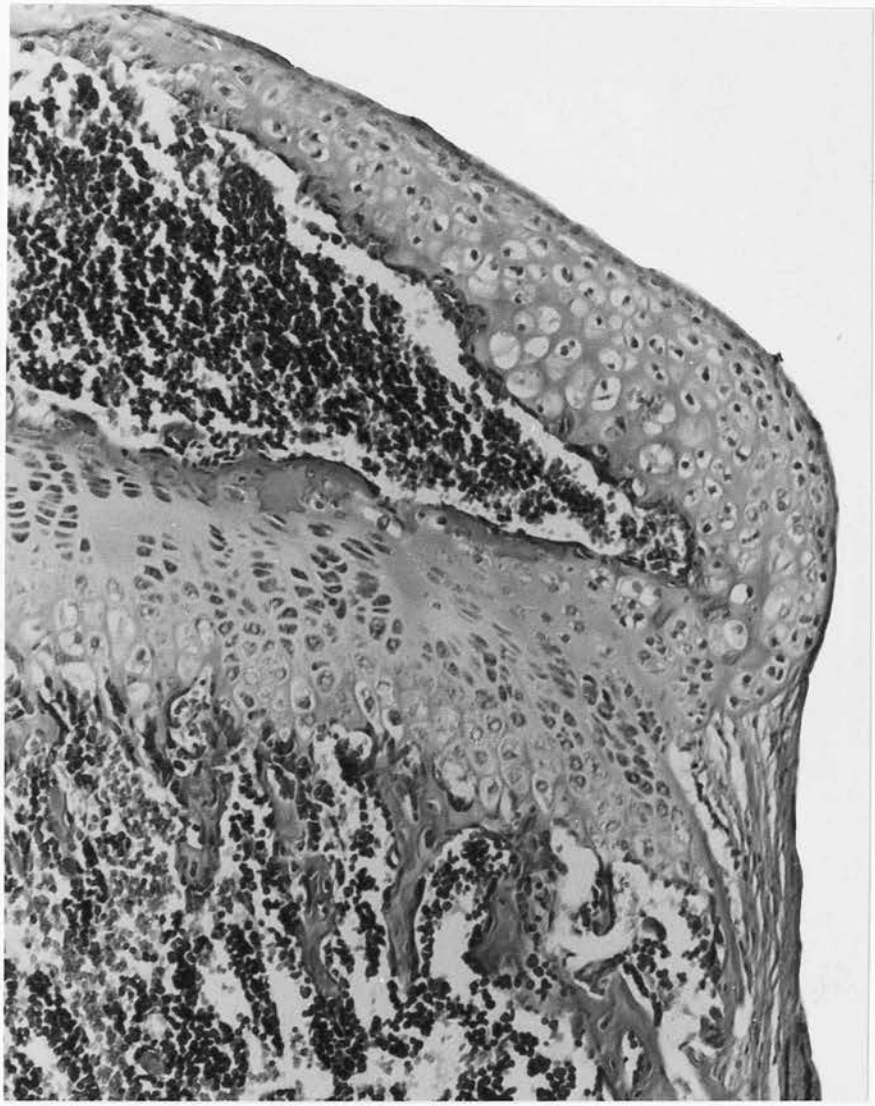
Homograft four weeks.

A similar field to Fig. 43

There is a general correspondence in the appearance of the growing cartilage, but no replacement of the hypertrophic cartilage by bone.

H. and E. x 135.

Figure 45

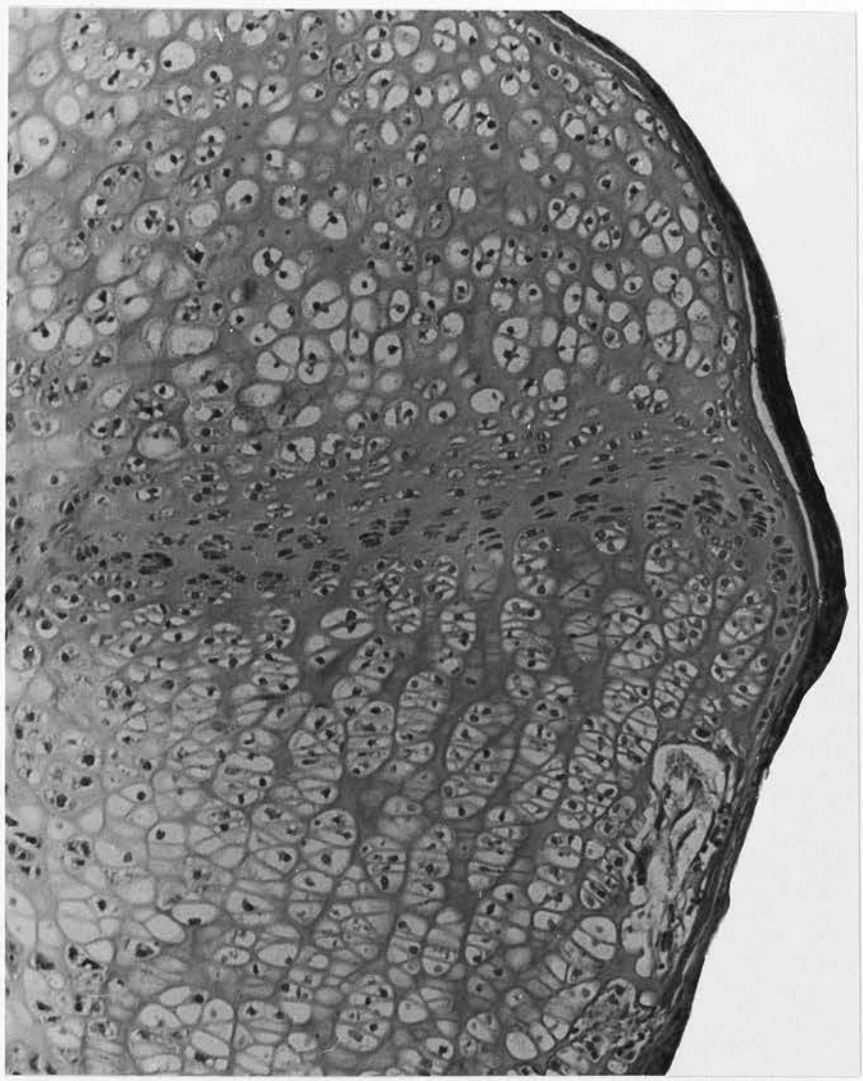


Isograft five weeks.

A lower magnification showing the general arrangement at the epiphysis. The appearance is similar to that of a normal bone.

H. and E. x 100.

Figure 46



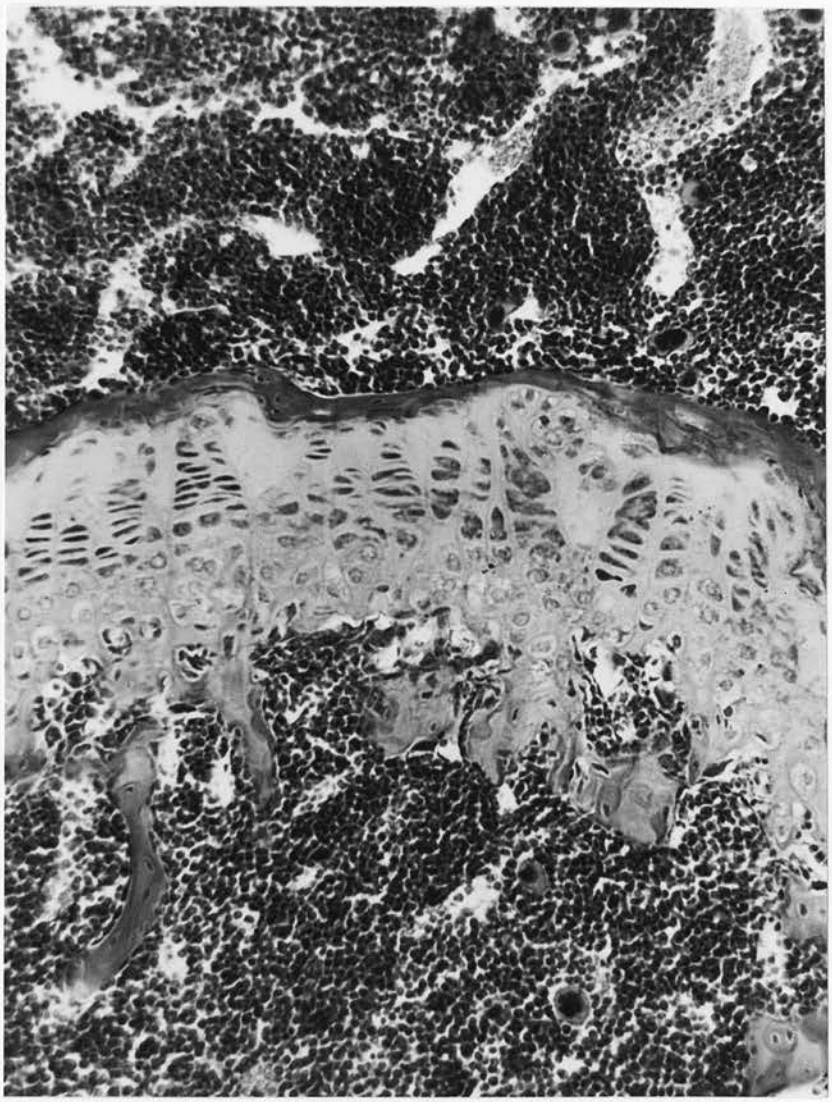
Homograft five weeks.

A corresponding field to Fig. 45.

The epiphyseal cartilage plate remains well differentiated despite the lack of bone replacement. Note an area of vascular erosion of the hypertrophic cartilage.

H. and E. x 100.

Figure 47

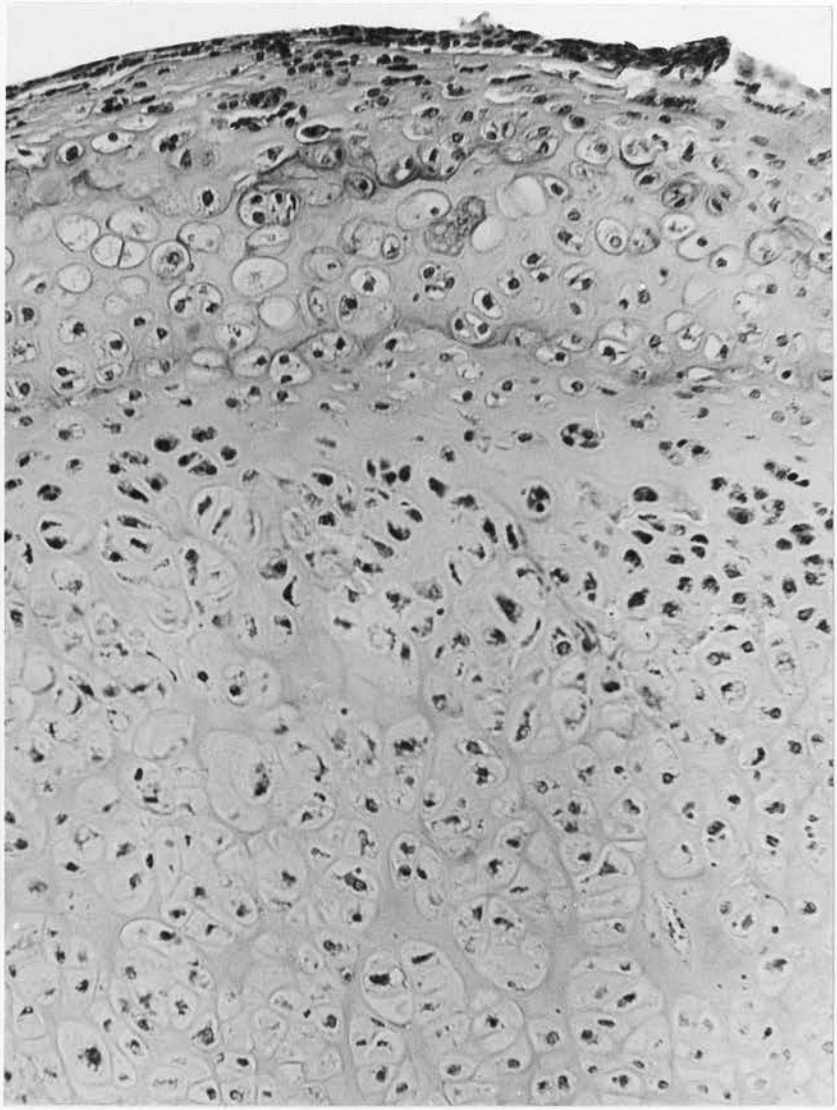


Isograft nine weeks.

The growth plate persists but is becoming narrower and endochondral replacement by bone is less obvious indicating a slowing down of growth.

H. and E. x 135.

Figure 48

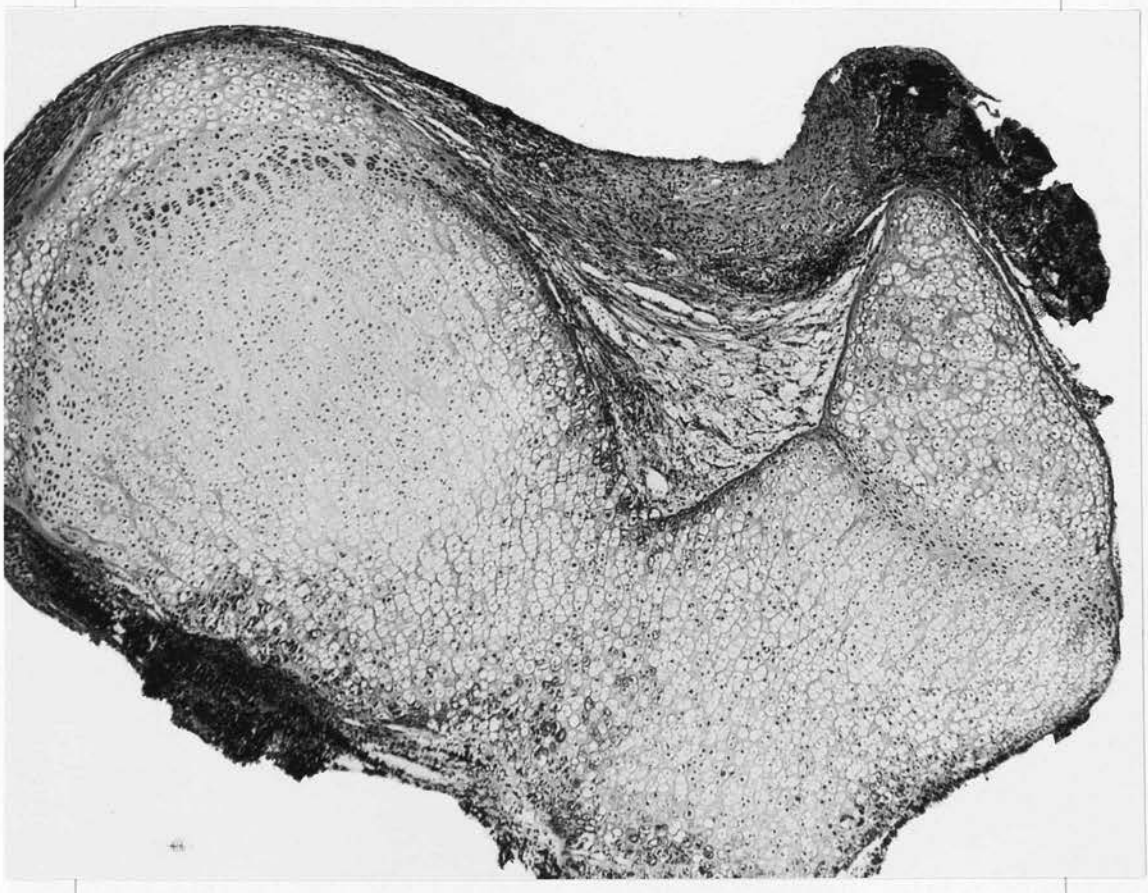


Homograft ten weeks.

The field of the growth plate likewise shows less activity and less clear differentiation than hitherto.

H. and E. x 135.

Figure 49.



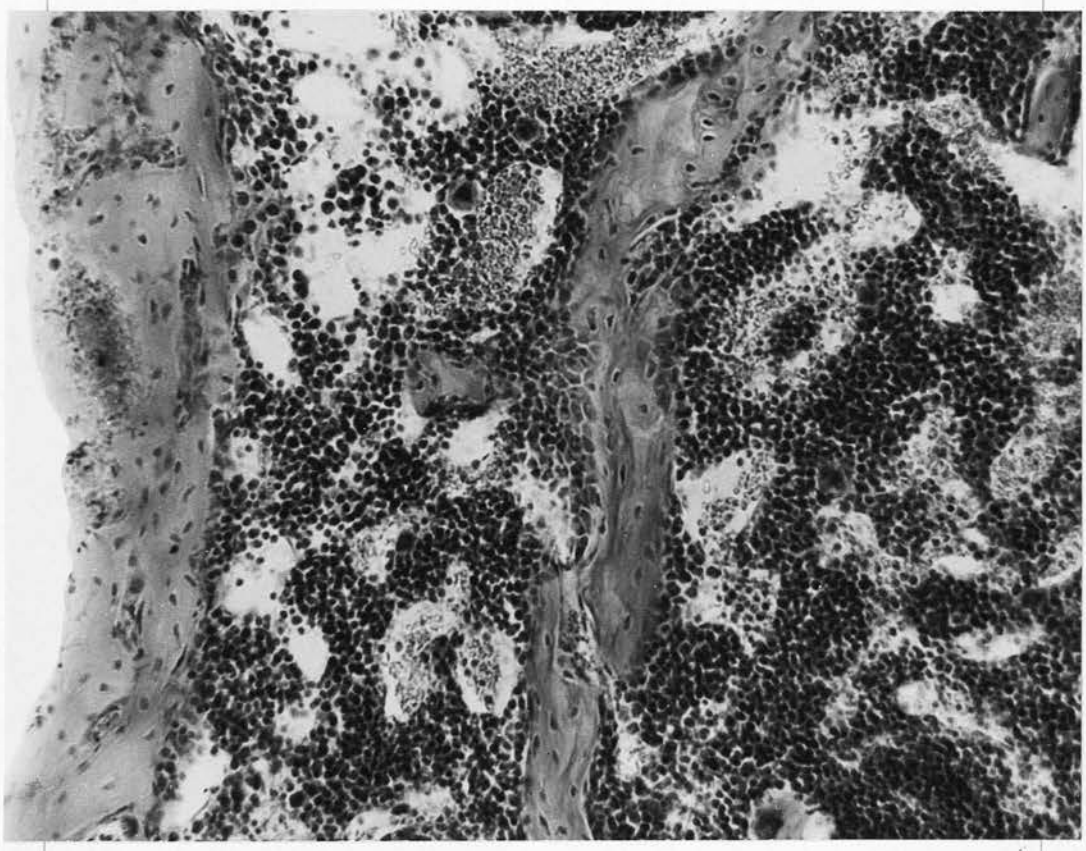
Homograft four weeks.

A low power view of the upper end of the femur.

The general shape is well maintained and the epiphyseal cartilage plates of the head and greater trochanter are normally sited. There is a vast accumulation of mature cartilage and complete absence of bone.

H. and E. x 30.

Figure 50

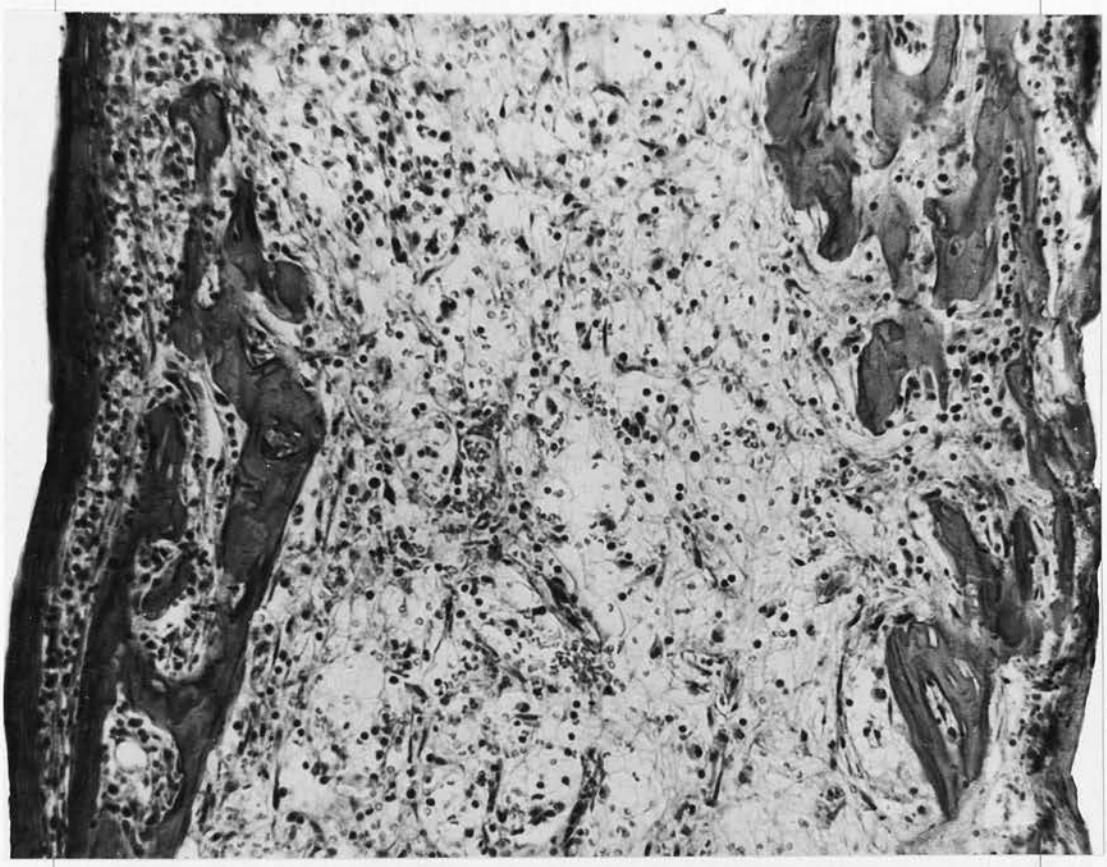


Isograft ten days.

Part of the diaphysis showing living bone and haemopoietic marrow.

H. and E. x 135.

Figure 51



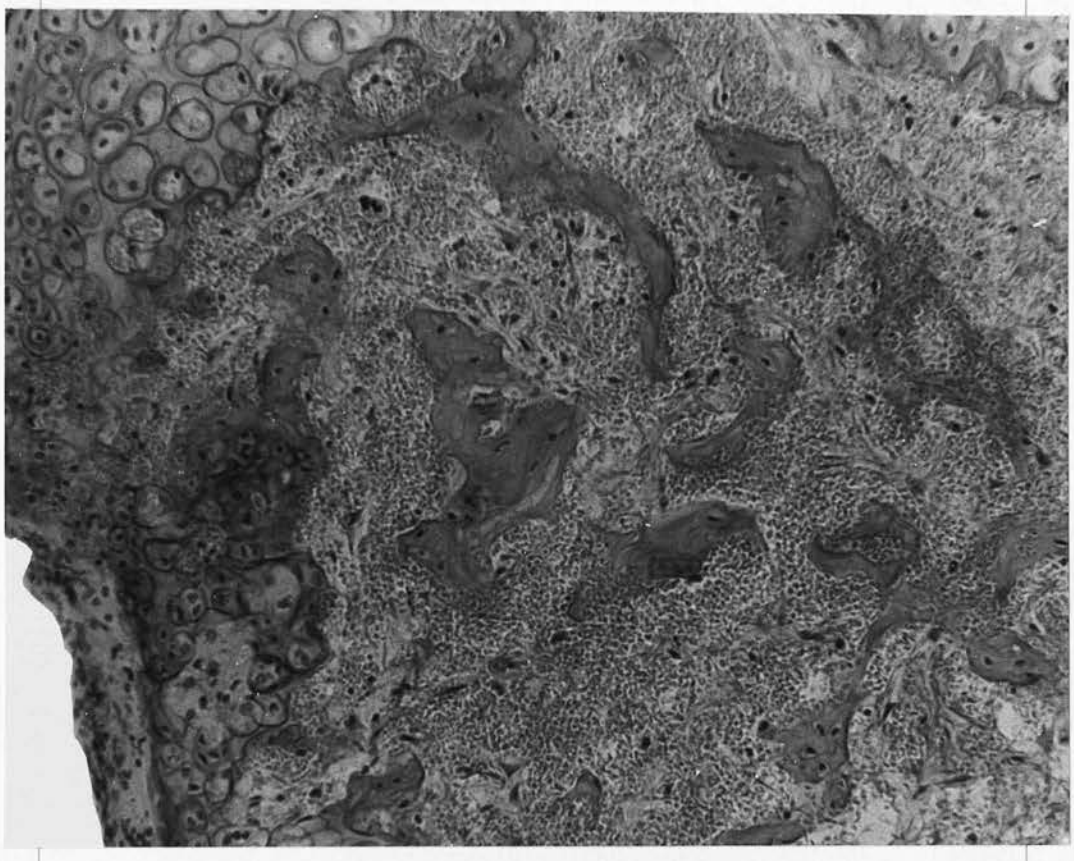
Homograft ten days.

Part of the diaphysis

The bone is mostly acellular and no new bone formation is occurring. The marrow space is infiltrated with inflammatory cells.

H. and E. x 135

Figure 52

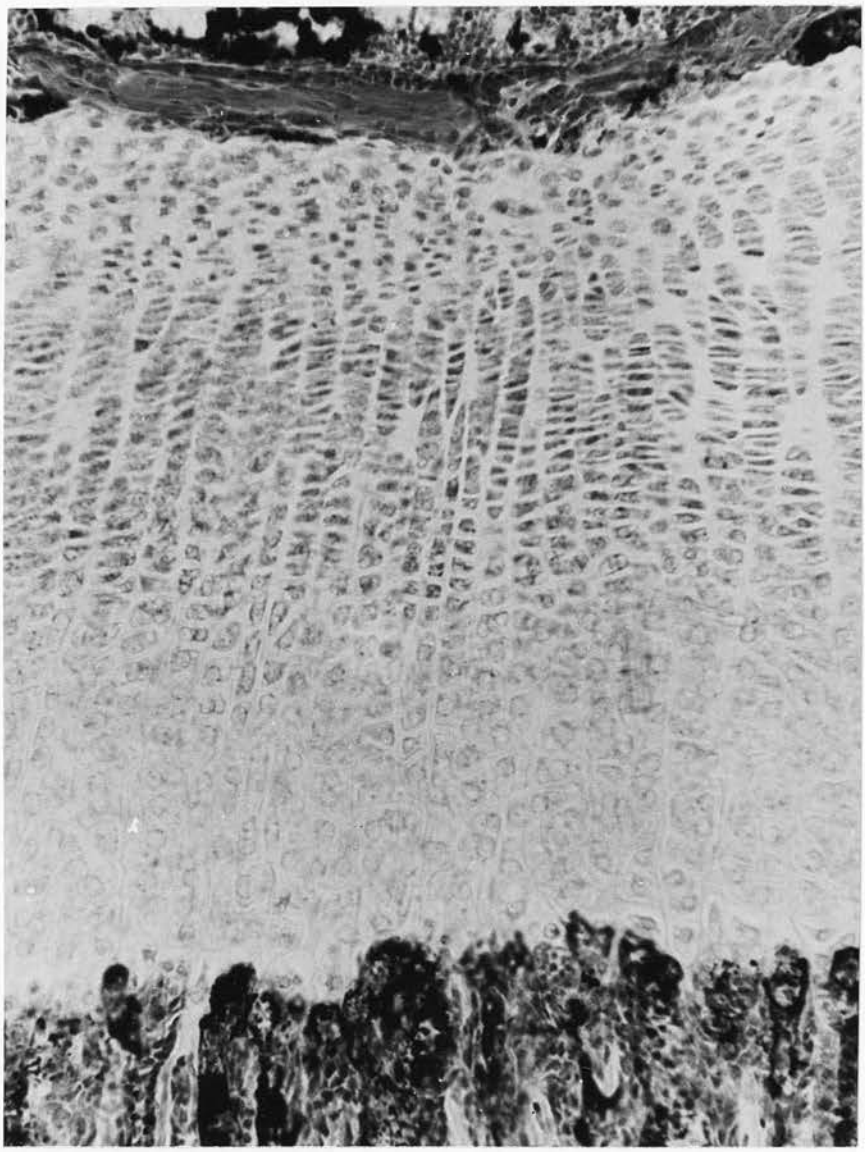


Homograft twelve weeks.

This field shows some living bone within the epiphysis of a homograft. It is surrounded by a highly vascular connective tissue but no haemopoiesis is in evidence. This is the only example of bone formation seen after the sixth day in the homograft femur.

H. and E. x 135.

Figure 53

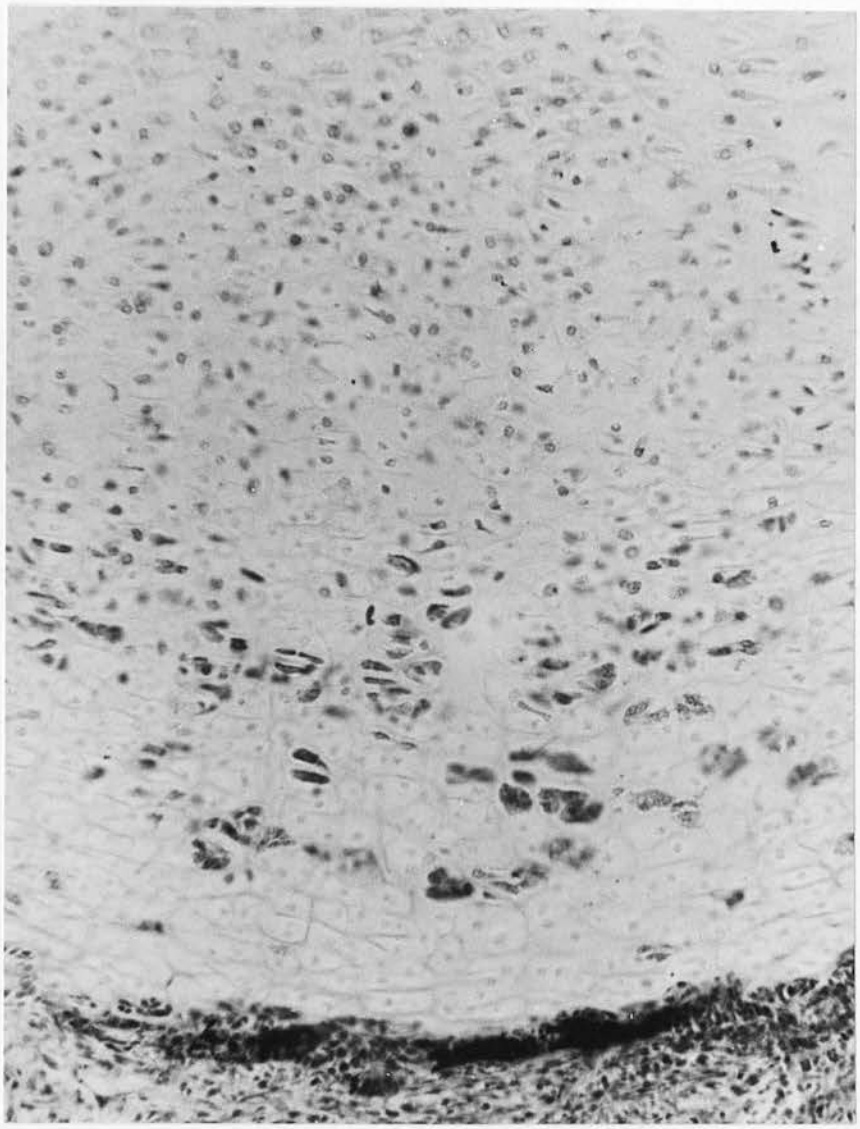


Isograft sixteen days.

This section is of the bone cartilage junction in an injected preparation. The normal pattern of capillary hair-pin loops at the zone of endochondral ossification is visualised by the Indian ink.

H. and E. x 135.

Figure 54



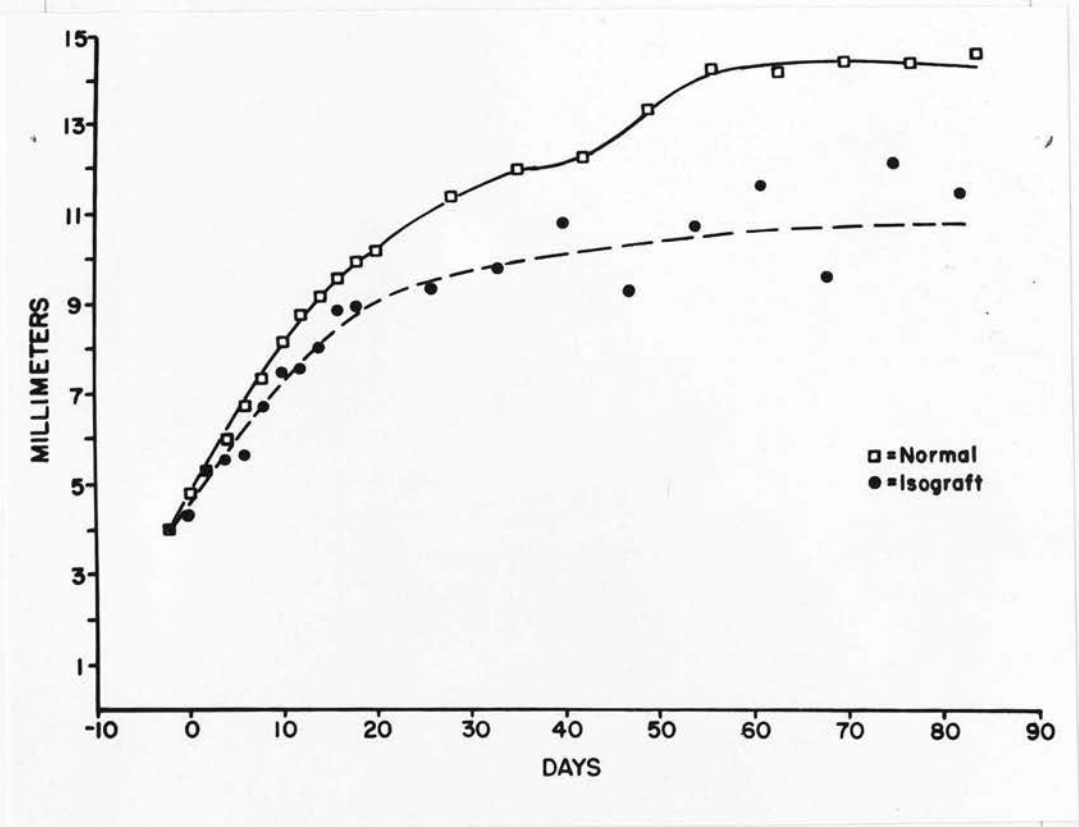
Homograft sixteen days.

A similar preparation to Fig.53.

The injected vessels are scanty and quite irregular in their distribution.

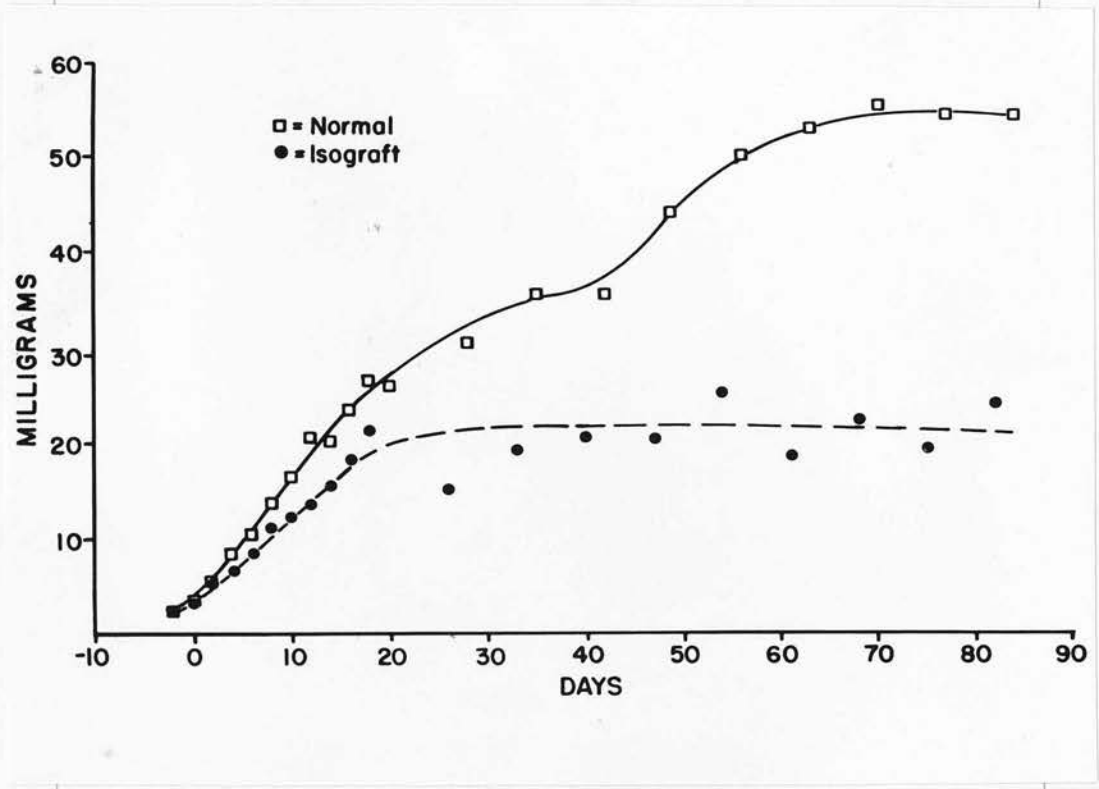
H. and E. x 135.

Figure 55



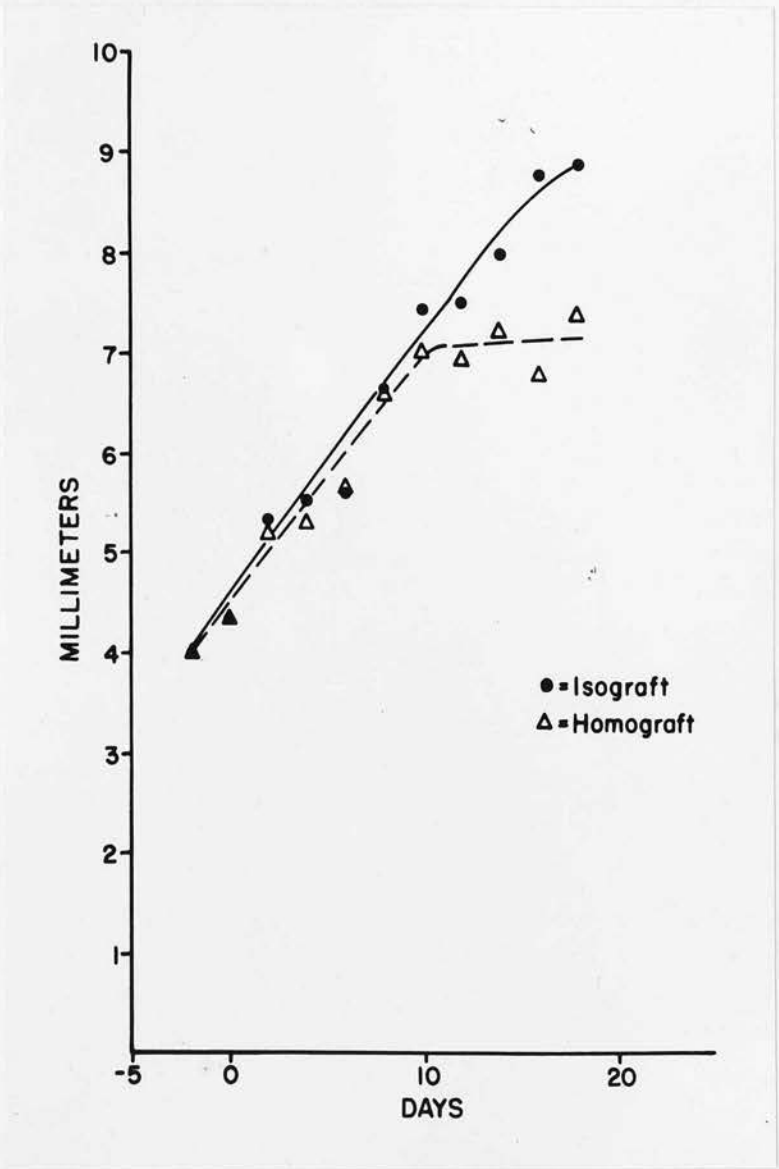
Increase in length of normal and isograft femurs.

Figure 56



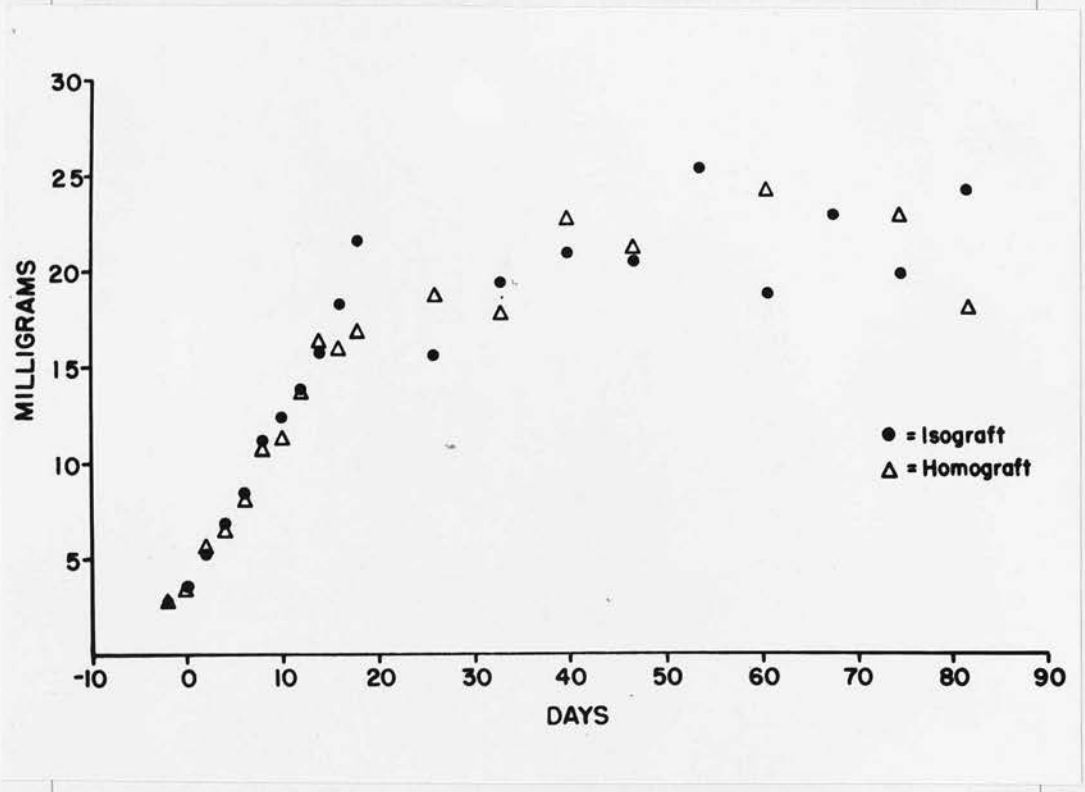
Increase in weight of normal and isograft femurs.

Figure 57



Increase in length of isograft and homograft femurs.

Figure 58



Increase in weight of isograft and homograft femurs.

SERIES II: Comparison of Homografts in Normal and Preimmunised Hosts

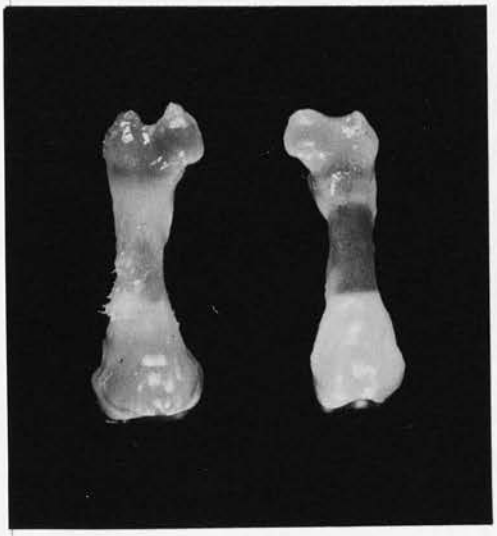
In the hosts which had been immunised against Strong A tissues 3 weeks prior to the foetal bone homograft, the grafts showed one clear and consistent difference from the similar homografts in the untreated hosts. The diaphyseal bone did not grow at all after implantation whereas growth during the first six days was seen in the normal homograft (Table 10, Figs. 59 - 64, 71 and 72). Growth and organisation of the cartilage was similar to that of normal homograft.

Unfortunately, the histological preparations from this group were unsatisfactory, but the material that was available failed to show any evidence of new bone formation in the grafts to preimmunised hosts - even during the initial six days.

Figures 59 - 62

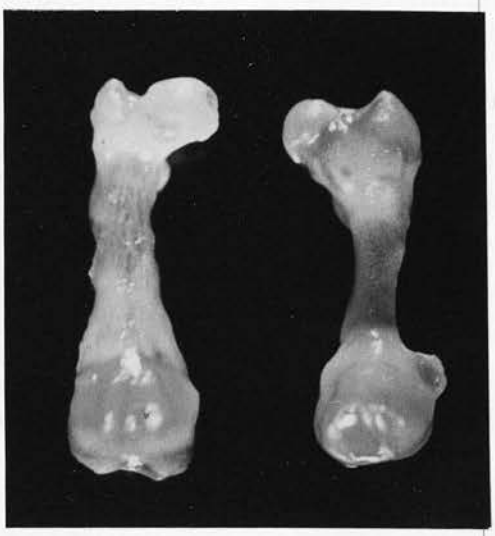
These photographs show the similarity in total growth of the femur homografts in normal and preimmunised hosts (series 2). The only difference between these grafts lay in the bony diaphysis, which in the preimmunised hosts did not increase in size after transplantation, while in the unprepared hosts the diaphysis increased in size during the first six days. The disparity between the diaphyseal sizes both with respect to total length and thickness is well shown in these photographs.

Figure 59



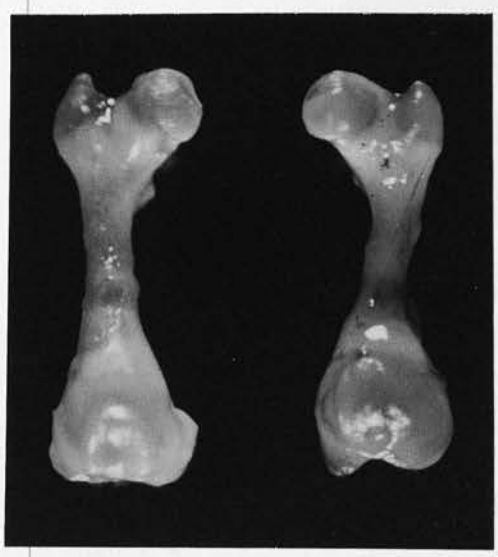
Homografts to (L) normal and (R) preimmunised hosts.
4 days (x 9)

Figure 60



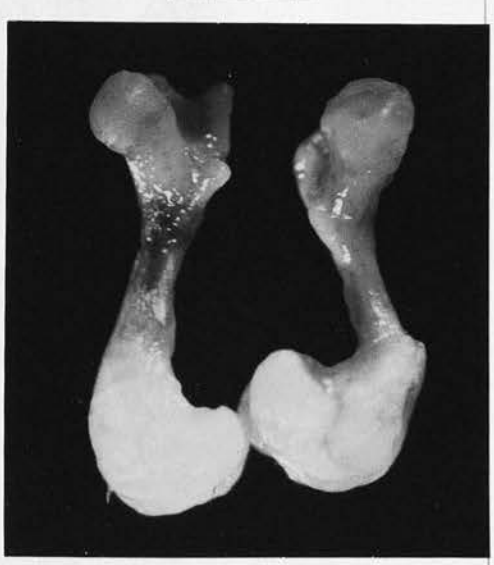
Homografts to left normal and (R) preimmunised hosts.
8 days (x 9)

Figure 61



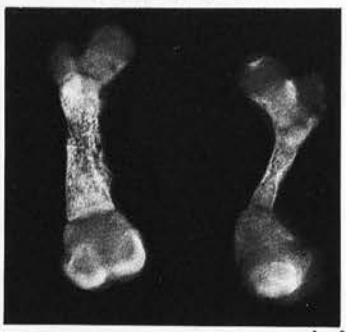
Homografts to (L) normal and (R) preimmunised hosts
10 days (x 9)

Figure 62



Homografts to left normal and (R) preimmunised hosts.
5 weeks (x 9)

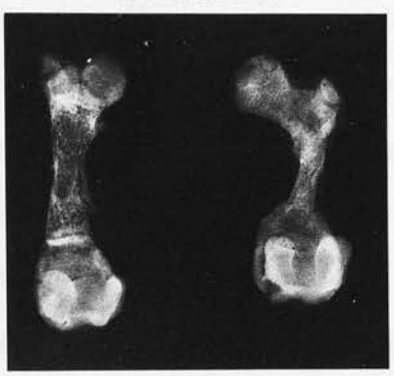
Figure 63



X-rays of homografts to (L) normal and (R) preimmunised hosts.

14 days (x 6.5)

Figure 64



X-rays of homografts to (L) normal and (R) preimmunised hosts.

5 weeks (x 6.5)

These two figures show very clearly the difference in diaphyseal size which was the striking feature of series 2. The length of the diaphysis in the homograft to the preimmunised host has not increased following transplantation, while the cartilage growth in the two grafts is comparable.

SERIES III: Comparison of Homograft and Heterograft

The heterograft differed from the homograft in the following respects. The diaphyseal bone did not increase in size after implantation (Figs. 71 and 72). The total length of the graft increased during the first six days by a maximum of 1.5 mm. and thereafter grew no more (Table 10). The weight increased during the same period by an average of 2 mgm. and subsequently showed no change (Table 10). Calcification of the epiphyses occurred at inconstant and variable times and in irregular patterns. After six days no changes took place in the gross appearance of the heterografts, so that at two months it remained as a miniature but well proportioned femur (Figs. 65 - 69). A dense fibrous capsule developed around the heterograft, which although not adherent to the graft, was so tough that it was extremely difficult to dissect without damaging the bone.

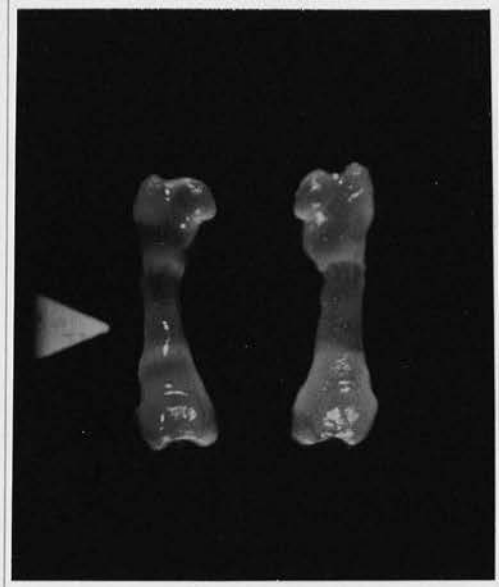
Histologically no evidence of surviving bone cells was found in the heterografts after transplantation. Much of the cartilage, particularly towards the centre of the mass, lost its cellular character and took on a confluent amorphous appearance which failed to stain with haematoxylin and eosin. However some of the cells, particularly at the periphery, retained their normal morphology for at least six weeks, and in the early period after transplantation an attempt at differentiation of an epiphyseal cartilage plate occurred (Fig. 70). The marrow space became the site of

an intense inflammatory reaction after fourteen days - up to that time it appeared avascular and largely acellular.

Figures 65 - 67

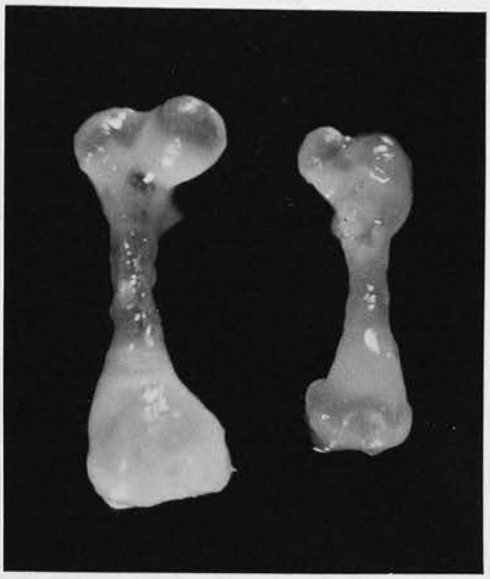
In these photographs of homograft and heterograft pairs from series 3, the homograft is shown to increase steadily in size, whereas the heterograft remains almost unaltered in size following transplantation.

Figure 65



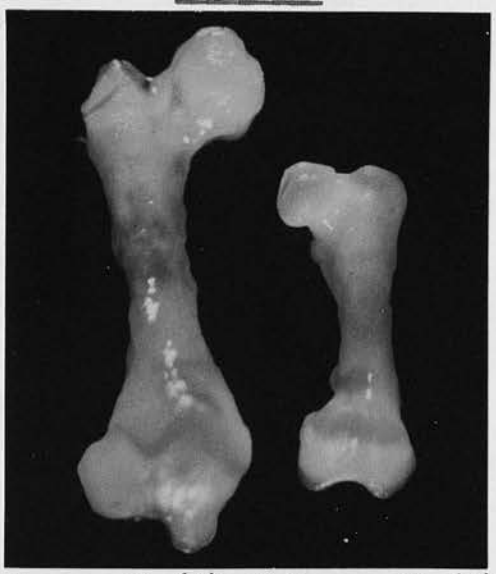
Homograft (L), Heterograft (R)
2 days (x 9)

Figure 66



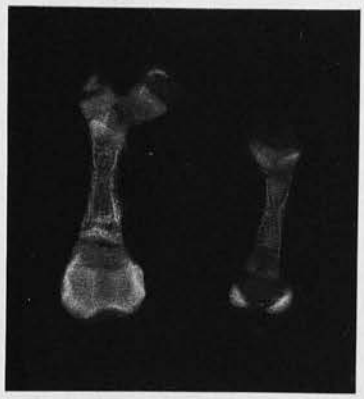
Homograft (L), Heterograft (R)
12 days (x 9)

Figure 67



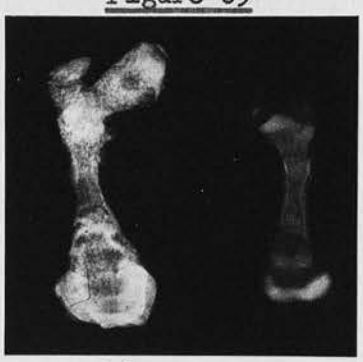
Homograft (L), Heterograft (R)
8 weeks (x 9).

Figure 68



Homograft (L), Heterograft (R)
20 days
X-ray (x 6.5)

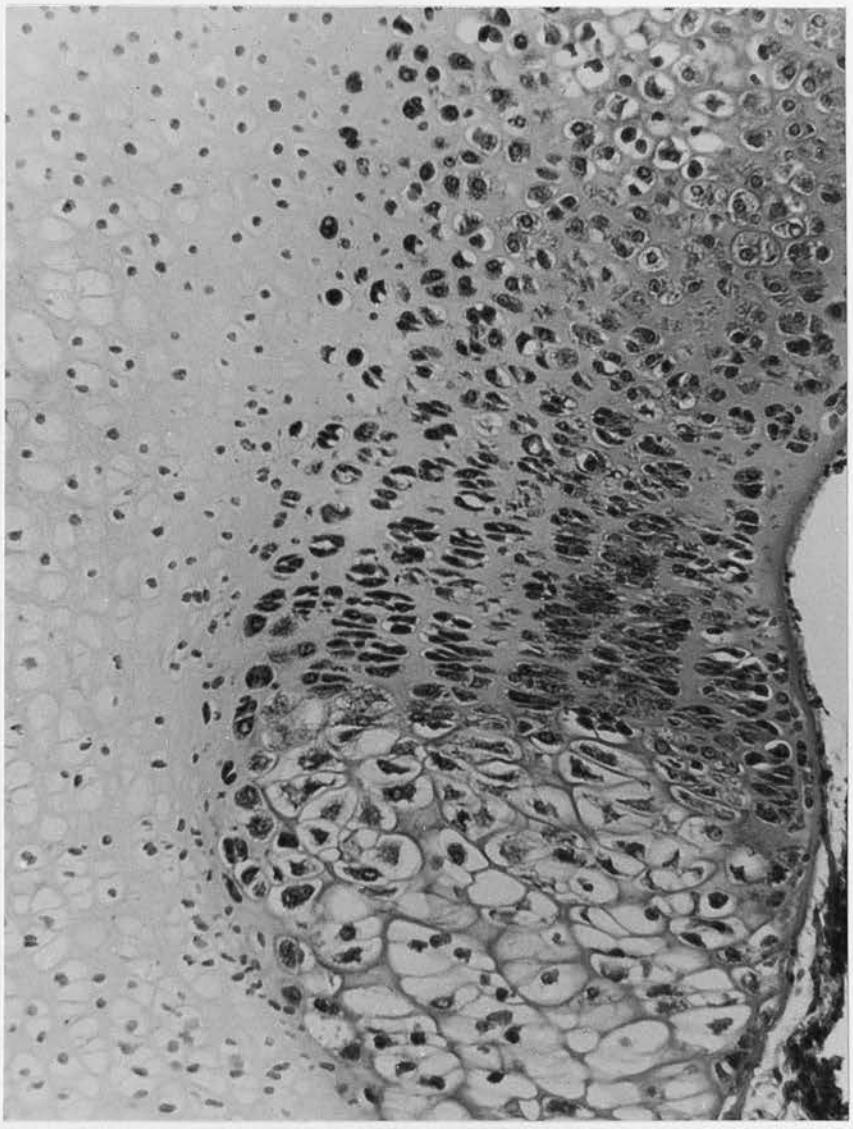
Figure 69



Homograft (L), Heterograft (R)
8 weeks
X-ray (x 6.5)

These two figures demonstrate the unchanging appearance of the heterograft. The diaphysis has not altered in size since transplantation.

Figure 70

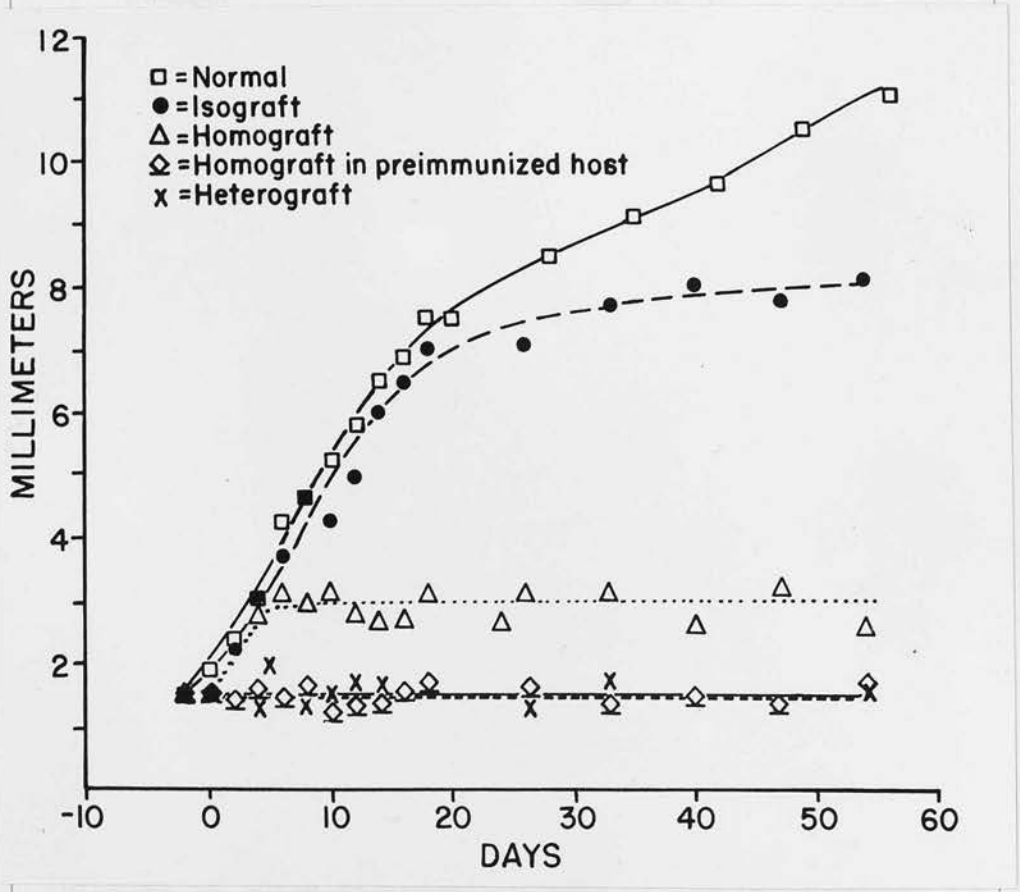


Heterograft seven days.

There has been a partial differentiation of the epiphyseal cartilage plate at the periphery of the femur, while the central part of the cartilage mass had lost its staining character and the chondrocytes in this area are shrunken.

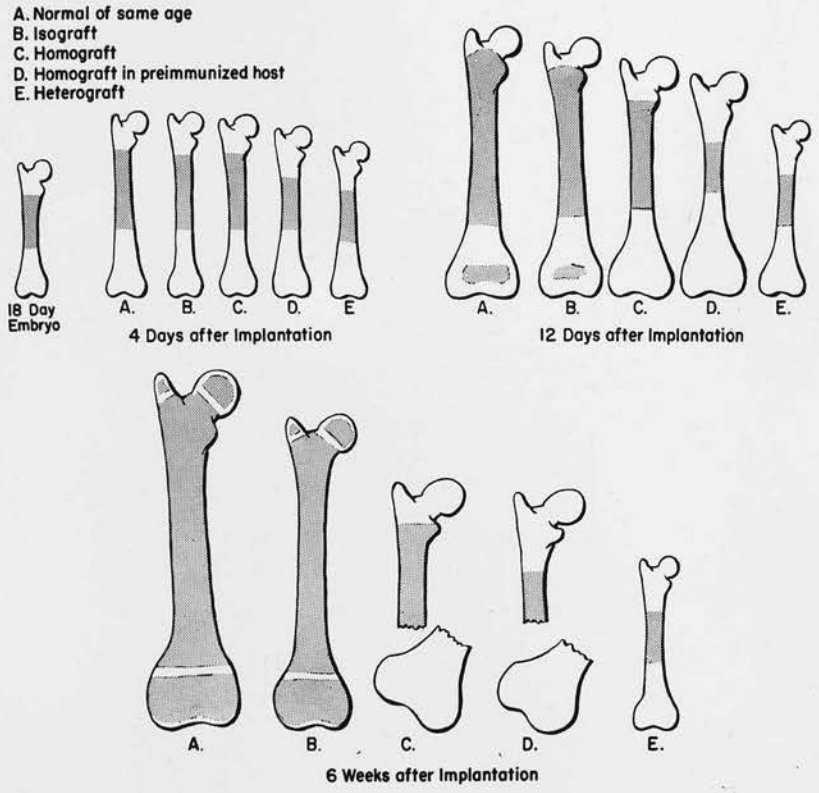
H. and E. x 135.

Figure 71



Increase in diaphyseal length of the foetal femur in the different circumstances described in the text.

Figure 72..



Drawing to scale illustrating some of the differences which were observed in growth and development of the foetal femur in the various experimental situations. The shaded areas represent bone.

DISCUSSION

In each of the experiments described above the foetal femur was observed to grow to a greater or lesser degree. However, the foetal femur is comprised of two principal tissues - cartilage and bone - which individually showed great differences in behaviour.

If each of these tissues is considered separately a clearer understanding of the different behaviour of the grafts is possible -

The Bone

In the isografts, the diaphyseal bone growth continued almost without interruption and replaced the maturing cartilage by the normal process of endochondral ossification until growth of the transplant ceased. In the homografts to normal hosts the bone grew for a period of only six days and thereafter largely died, while in the homografts to preimmunised hosts no bone growth was observed.

These findings agree so closely with the behaviour of the first and second set homografts of mature tissues in general and of mature bone in particular - see Chapter I - as to leave little doubt that from an immunological standpoint there is no difference in behaviour between 18 day foetal bone and adult bone homograft.

The failure of bone survival in the foetal heterografts

is entirely in accordance with the fate of heterografts in general.

The cartilage

The isograft cartilage grew and differentiated in a manner which differed from that of normal bone only in that the articular cartilage was thinner and more irregular.

The homograft cartilage also grew and differentiated in a very normal fashion and was responsible for the increase in size of the femur homograft. This growth appeared uninfluenced by the development of immunity or by prior immunisation, except insofar as the death of the true bone and reduction of circulation prevented the replacement of hypertrophic cartilage by bone, so that large masses of hypertrophic and calcified cartilage accumulated. This continued growth of the foetal homograft cartilage does not, however, indicate that foetal cartilage differs immunologically from adult cartilage because cartilage homografts in experimental animals and in man have been shown to survive and even grow when taken from more mature donors (Nunnemacher, 1939, Lacroix, 1951, Peer, 1954, Basich and Wyburn, 1947 and 1955).

The heterograft cartilage grew to a very limited extent and survived only in part. This agrees with the experience of others. Cartilage heterografts appear to be unique amongst tissues in showing even this limited

capacity for survival (Loeb and Harter, 1926). The explanation is probably the same as that which is believed responsible for cartilage homograft survival (see page 51).

The Organisation of Bone Growth

The different behaviour of cartilage and bone growth in these experiments has afforded an excellent opportunity of observing the relative importance of these two tissues in influencing the form and size of the developing femur. It has been known since the classic studies of Fell and her colleagues,(Fell and Robison, 1929, Fell and Canti, 1934, Fell, 1939), that the cartilage precursor of bone has the ability to develop recognisable characteristics of a particular bone even in the tissue culture chamber.

In the homograft transplants described above in which cartilage growth continued but not bone, it was apparent, that, not only was there an inherent growth pattern in immature cartilage which could produce the characteristic features of a particular bone in great detail, but that the duration of growth and ultimate size of the bone were also governed to a great extent by intrinsic factors within the young cartilage.

In the isograft in which the normal sequence of endochondral ossification took place, the cartilage continued to lay down the characteristics of a femur and to regulate its ultimate size, but as bone replaced the

cartilage many of the secondary characteristics of the femur became submerged in the course of the remodelling which took place at the metaphysis.

These results considered in conjunction with the behaviour of the autograft in Chapter I indicate the importance of the relative roles of bone and cartilage in shaping the skeleton.

Immature cartilage is endowed with a potential for growing into a specific shape of finite size independent of its normal anatomical relationships and even of its normal endocrinological environment.

Bone on the other hand appears to be more dependent on extrinsic influences for the maintenance of form. If guided by a cartilage model to assume the form of a specific bone, it quickly loses many of its secondary prominences to become a slender tubular structure in the absence of muscular attachments.

The limit of organised growth of bone by itself is the formation of a small lens shaped ossicle (see page 14 and Fig. 10, also, Lacroix, 1951 and Danis, 1956).

The induction of bone by cartilage

Transplants of growth cartilage have been found by other workers to be potent inducers of bone formation in surrounding soft tissues (Lacroix, 1956, Bridges and

Pritchard, 1958).

It was expected that evidence of such induced bone might be found in or around the cartilage of the homograft transplants, but although a particular search was made for this, in only one graft at 12 weeks was living bone found which might be accounted for in this way. It is possible that if the grafts had been observed over a longer period more evidence of bone induction might have been found for as suggested in Chapter I it may be a late development in relation to a graft.

Huggins, McCarroll and Blockson (1936) found the spleen to be a site in which bone induction by bladder mucosa transplants did not occur unless fascia was transplanted in conjunction. Perhaps the use of a site other than the spleen might have favoured induction in these experiments.

SUMMARY

Homografts of 18 day old foetal femurs showed no fundamental difference from the behaviour of grafts of more mature bone and cartilage.

Growth of bone was limited to a short period after transplantation and was abolished by prior immunisation. Cartilage growth alone was responsible for the increase in size of these transplants and did not appear to be influenced by the presence of immunity.

The very limited growth of cartilage and the total failure of bone survival in the heterografts indicate an immune reaction of a different order than that which develops against the homograft.

The different effect of the homograft immune reaction on cartilage and bone enabled certain conclusions to be drawn concerning the part played by these two tissues in determining the form of a bone. Cartilage growth and development is shown to be regulated in large part by intrinsic factors. Bone growth and form on the other hand is shown to be dependant largely upon extrinsic influences.

CHAPTER III

Comparative Study of Bone Graft Materials in the Dog

The clinical use of bone autografts is attended by several practical disadvantages. It necessitates an additional operative procedure. Complications may develop at the donor site such as a late fracture or infection. This latter, although uncommon, can be a major tragedy when it involves the one sound leg. Post operative chest complications in the elderly are greatly increased, as a result of pain on moving or coughing, when the iliac crest is used as a source of bone. Furthermore, the amount of autogenous bone available for grafting is limited, particularly in a child.

For these reasons many alternatives to autograft have been tried both clinically and experimentally, ranging from such obvious materials as homograft and heterograft of bone to some surprising choices such as cow horn, ivory, plaster-of-Paris and plastic sponges. Many of these materials have "worked" in that their use has not prevented a satisfactory outcome in clinical use, but of these materials only homografts (prepared in a variety of ways) are widely used at the present time.

Bone heterografts have enjoyed some popularity in the continent, but the published accounts of their use compare unfavourably with the reported experience of homografts both

clinically and experimentally (Kingma, 1960; Maatz, Lentz and Graf, 1954).

The two preceding chapters have demonstrated that there are marked differences in the behaviour of auto- and homografts of bone, these differences being due to the development of an immune reaction against the homograft. Nevertheless, an almost overwhelming volume of reports testify to the successful use of bone homografts. It is reasonable to assume therefore that the presence of an immune reaction need not prevent the graft from performing a useful function.

At the same time there is a general impression amongst orthopaedic surgeons that the behaviour of homografts is in general less satisfactory than that of autografts, although objective evidence for this belief is difficult to obtain, chiefly because clinical cases are not sufficiently comparable to enable a valid comparison of different bone grafting materials.

A number of experimental methods have been used to assess the value of different types of bone graft. A few workers have studied the pattern and rate of revascularisation of various bone grafts but the majority have relied on the gross or microscopic appearance of the grafts following transplantation. Most of the more readily available experimental animals have been used, and

many test situations devised ranging from small defects in rats' calvaria to massive transplants of half the femur in the dog. Among non-skeletal sites which have been used the anterior chamber of the eye and muscle have been most popular.

Vascular studies of bone grafts.

The rate of vascular penetration of bone grafts has been studied by Stringa (1957), who used an injection of a solution of Berlin blue and micropaque to visualise the circulation. Revascularisation of homografts was only slightly slower than that of autografts in the rabbit, while heterografts showed great delay.

Anderson, Schmidt and Lecocq (1959)using the anterior chamber of the rat's eye as a test site found that heterografts of small particle size were revascularised as well as cancellous autograft, whereas larger heterografts were poorly penetrated. In another paper, Anderson et al (1959) found deproteinized bone to be less well revascularised than fresh homograft.

Kiehn and Glover (1953) reported that the uptake of P^{32} was less in boiled and frozen homografts than in fresh autograft in dogs. They regarded this as a measure of the relative vascularity of these materials.

Histological Studies of Bone Grafts

Maatz 1955 using small defects in dogs' femurs (the "spongiosa" test) found deproteinized bone to be superior to fresh autograft, a view which was supported by Losee and Hurley (1956) using tibial inlay grafts in dogs, whereas Holmstrand (1957) in his excellent monograph found that this material was one of the least satisfactory of the graft materials which he studied in defects in rabbits' tibiae.

Using demineralized bone transplanted to small defects in rats' calvaria Ray and Holloway (1957) concluded that this material was the best alternative to autograft, being superior to frozen homograft. Losee and Hurley (1956), on the other hand, found this material to be unsatisfactory.

Herndon and Chase (1954) demonstrated that large fresh homografts in the dog healed much less rapidly than similar autografts, while Reynolds and Oliver (1950), studying transplanted segments of dogs' tibiae could find no difference in the ultimate rate of healing of these materials.

Campbell et al. (1953) using onlay grafts of split rib in dogs could demonstrate no difference in the progress of frozen autograft, frozen homograft and boiled bone. Reynolds and Oliver, (1950) and Gallie (1914) also were unable to demonstrate differences between fresh and boiled homograft in the dog. Holmstrand (1957), however, found that the resorption of boiled bone was much retarded.

These are but a few examples of the general uncertainty which prevails. The list could be extended almost indefinitely but without any further clarification.

Much of the confusion has arisen from the almost universal adoption of histological examination for the assessment of a graft's progress. This is a highly subjective method and incapable of precise measurement.

It is also apparent when reviewing this work that bone grafts behave very differently in different situations. It follows, therefore, that an experimental method or graft which has no resemblance to any surgical procedure cannot yield information which is relevant to clinical usage.

Having reviewed this work we felt that there was still no clear evidence to indicate which of the various alternatives to autograft was the most acceptable for clinical use. Where differences in behaviour were large, as for example between massive autograft and homografts, then relatively crude methods of assessment have given positive information - massive homografts being clearly inferior to similar autografts. Where small grafts have been used differences of behaviour are less apparent and cannot be measured by inspection of histological preparations alone.

Accordingly a further series of experiments was planned which fulfilled what we regarded as the two essential

requirements of a biological test of bone grafts.

Firstly, the test situation should be one which, while capable of standardisation, would also resemble a common clinical bone grafting procedure. Secondly, in order that different graft materials could be compared, the progress of the grafts must be capable of exact measurement.

The first of these requirements presented no problem, but the second was more difficult because of a lack of an accepted yardstick of a graft's function.

Possible Functions of a Bone Graft

Despite much research and more speculation there is still no generally accepted theory of how a bone graft works. In general, they are used clinically to fill defects or bridge gaps in or between bones; but how do they achieve this? Does a graft have just one function or can it do different things in different situations?

Several views have been held.

(1) That the graft is incorporated as a living unit in its new site.

Although this view was held by such an eminent authority as Albee, (1944), it is now known to be incorrect, because of the numerous observations that even grafts of fresh bone largely die, (see also chapter I), and because of the manifest success of grafts of killed bone.

(2) That the graft contributes osteogenic cells in areas where these are lacking or inactive

The work of chapters I and II shows that a permanent contribution of living osteoblasts can be provided by autografts alone. In view of the success of other types of grafting material, this is obviously not the prime function of a graft. Furthermore, it is reasonable to suppose that only those osteoblasts which are in close contact with the intact circulation at the host site, that is at the periphery of the graft, could survive. As in most grafting procedures there is an ample supply of host

osteoblasts at this situation, a further contribution appears redundant. However, this function of a graft cannot be dismissed altogether as unimportant. There are clinical situations in which a graft is used to bridge a very large gap, as in a Brittain's arthrodesis of hip or shoulder or in a plastic repair of the nose, in which it would appear to be advantageous to have foci of osteogenesis occurring along the length of the graft. Again in dealing with non-union of a fracture, where for some reason the local osteoblastic activity has become inadequate for repair, a further contribution of living osteoblasts might be desirable. It is in precisely such situations that the superiority of autografts is often most apparent.

(3) That the graft supplies "raw materials" which may be made use of by the new bone forming in the area

Two recent studies using radio-isotopes as tracers, have demonstrated that this does not occur. Using bone grafts labelled with Ca^{45}, Sr^{90} and Y^{91}, Urist, MacDonald and Jowsey (1958) found that the labelled constituents were carried into the systemic circulation and not localised to the reparative host tissue.

Cohen, Maletskos, Marshall and Williams (1957) obtained similar results using grafts labelled with Ca^{45}. There was no preferential transfer to host callus forming around the graft.

(4) That the graft provided a trellis or scaffolding which facilitates the extension of living bone from the host site.

This has been called the "osteoconductive" property of a graft and was first described by Barth (1908). There is much indirect evidence which substantiates this view of a graft's function. Histologically, it has been repeatedly demonstrated that living bone from the host site appears to spread over the surfaces provided by the graft, which in its turn is slowly resorbed enabling its gradual replacement by living bone. This process, which has been variously called creeping substitution (Phemister 1914) or appositional bone substitution (Kreuz et al. 1951), appears to take place with any acceptable bone graft material. This mechanism would explain why so many different graft materials both living and dead are able to perform a useful function.

Whether this process is dependent purely upon the physical structure of a graft or whether there is some further inducement such as a chemical stimulus from the graft is uncertain, but such evidence as is available, suggests that the mechanism is complex. Inert plastic materials with something like the physical structure of cancellous bone are not invaded by bone except at the very periphery when placed in a skeletal defect, (Struthers, 1955; Bryan, Janes and Grindlay, 1958).

On the other hand chemical extracts of bone have been

held to encourage the growth of new bone locally when placed adjacent to the calvaria of rats, (Moss 1958). In general, however, there have been more failures than successes with attempts to encourage new bone growth with chemical extracts.

Further confusion arose from the observations that bone from which the organic matrix has been extracted, (Ray and Holloway, 1957), and bone from which the inorganic matrix has been extracted (Losee and Hurley, 1956) both appear to have been used successfully as grafts, suggesting that a chemical stimulus must be of small importance, for in one or other of these materials it must surely be lacking.

In general, it may be held as established that the provision of an osteoconductive matrix is an important function of a bone graft but that the mechanism by which it performs this function is still unknown.

(5) That the graft provides a mechanical support

This function of a graft is so obvious as to require little comment. In many procedures such as onlay or inlay grafts for fractured long bones, or in certain techniques of arthrodesis such as the ischio-femoral arthrodesis of the hip or the subtalar strut graft a mechanical function is clearly required of a graft. However, other properties of a graft may also be valuable in such circumstances - notably the provision of living cells, and the provision of

osteoconductive matrix.

There is a current tendency in clinical practice to rely less and less on bone grafts for mechanical support now that reliable inert metal implants are available, for two reasons. Firstly, metal implants retain their strength while bone grafts become progressively weakened from the time of insertion and may break on this account at some later stage. Secondly, to provide mechanical support, cortical bone must be used and there is evidence that this is a less satisfactory osteoconductive matrix than cancellous bone (Editorial 1957). The combination of a metal plate plus cancellous strip grafts would appear to have advantages over a cortical inlay or onlay graft alone.

(6) That the graft induces the formation of bone in the surrounding tissue

In any skeletal site it would be quite impossible to state whether the new bone forming in and around the graft arose from pre-existing osteoblasts at the host site, or by induction. It seems unnecessary to presume that induction makes any important contribution, when in most clinical situations abundant supplies of fully differentiated osteoblasts are already available.

This question has been discussed more fully (page 59). From the evidence presented there it would appear that induction while remaining a theoretical possibility, is

unlikely to play an important part in the usefulness of a graft.

There are then three useful functions which a bone graft may perform -

1. provide living osteoblasts.
2. provide an osteoconductive matrix.
3. provide mechanical support.

In designing a biological test for comparison of bone grafts it was necessary to select one of these functions for measurement. The first could be eliminated as being applicable only to autograft. Of the remaining two it was decided to concentrate on the osteoconductive property as being the one most commonly required of a graft in clinical use.

The ultimate outcome of a successful bone grafting procedure is that the graft be replaced and supplemented by living bone which is strong enough to withstand the stresses applied to the part. This is effected by a process of remodelling in which the internal architecture of the bone becomes organised to give the greatest strength with the greatest economy of bone tissue. The graft once it has performed its function of osteoconduction, remains only as an embarassment to this process of remodelling.

The ideal bone graft, therefore, would appear to be one which while facilitating osteoconduction, was capable of

rapid resorption once that purpose had been fulfilled.

Both these processes - the rate at which bone formed within a graft area and the rate at which the graft itself was resorbed appeared capable of exact measurement, and were selected as the criteria for comparison of different grafting materials.

Method and Materials

The following materials were selected for this comparative study being the ones which seemed most likely to be of clinical value asfar as could be judged from published work -

Material	Preparation
Bone Autograft	Fresh
Bone Homograft	Fresh
	Frozen
	Freeze-dried irradiated
	Autoclaved
	Mineral matrix extracted
	Organic matrix extracted

Preparation of the Grafts

All the grafts were prepared from ribs which were chipped into fragments measuring less than 2 mm. in their largest diameter. Where possible the grafts for each series were prepared from the bone of one donor. Aseptic technique was observed during the procurement and subsequent handling of the grafts.

Frozen Homograft

The frozen graft was stored at -30°C for 1 - 2 months before use.

Autoclaved Homograft

These grafts were autoclaved at 15 pounds pressure for 20 minutes and subsequently kept at -30°C until used.

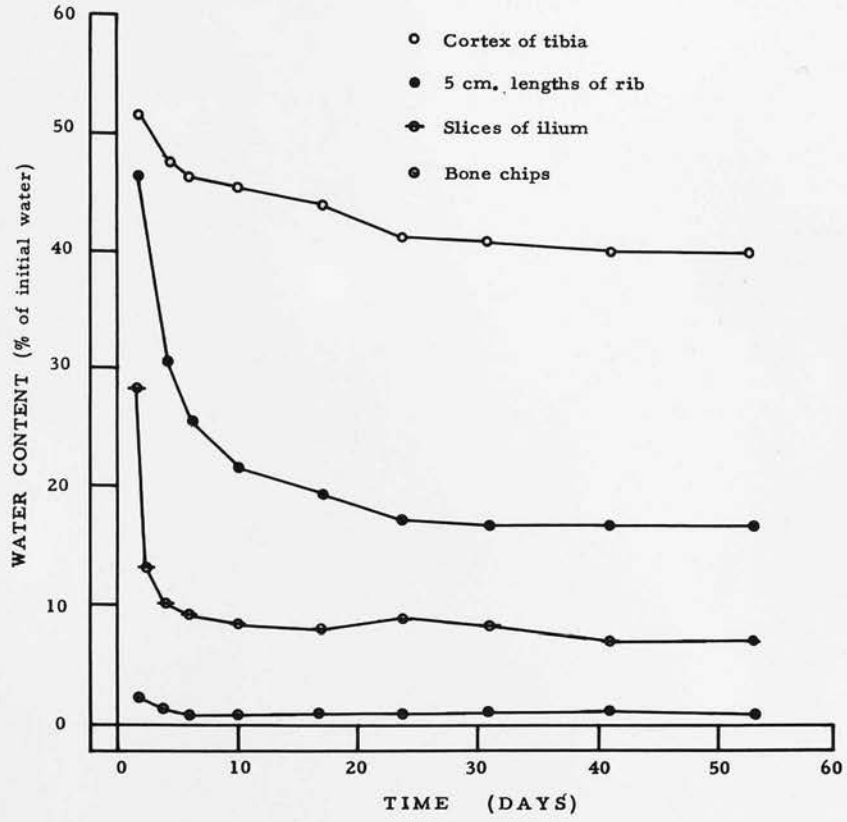
Freeze-dried Homograft

The apparatus used for freeze-drying was a modified Edwards bone and artery drier.

The bone was maintained *in vacuo* at -30° using P_2O_5 as a dessicant. The ease with which bone could be dried was found to vary considerably with the type of bone - cortical bone being particularly refractory. Drying curves for different types of bone are shown in Fig. 73.

It was decided to aim at a final water content of less than 1 per cent in the grafts used for these experiments and

Figure 73



DRYING CURVES FOR VARIOUS TYPES OF BONE TISSUE MAINTAINED IN VACUO AT -30°C.

the routine which was found satisfactory consisted of an initial drying at -30°C for two weeks followed by a further two weeks drying at room temperature.

The graft was subsequently stored at room temperature in vacuo in a sealed container. Several containers were tried some of which are illustrated in Fig. 74. Of these, the most reliable proved to be the sealed glass ampoule, occasional failures being experienced with all the demountable containers.

Freeze-dried irradiated homograft

The freeze-dried bone was prepared as above and then exposed to a cobalt 60 source receiving a dosage of 2.5 million roengten equivalent physical (r.e.p.)

Mineral extracted (demineralized) homograft

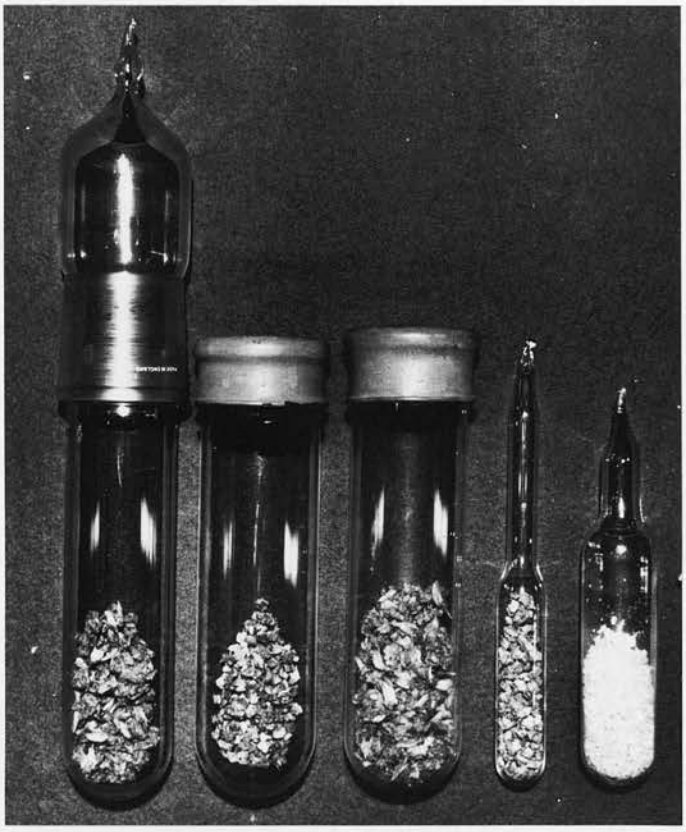
The chelating agent ethylene diamine tetra-acetic (E.D.T.A.) acid was used.

Bone was placed in a solution of E.D.T.A. prepared by dissolving 250 grammes in 1,750 millilitres of distilled water adjusting the pH to 7.0 by the addition of sodium hydroxide. X-rays were used to show when decalcification was complete. The graft was subsequently stored at -30°C after washing with saline.

Protein extracted (deproteinized) homograft

The method used was that described by Williams and

Figure 74



Some containers used for the storage of freeze-dried bone. Left to right:

Glass tube with vacuum-fitting silicone-greased cap;

Glass tube with rubber seal fixed with vacuum wax;

Glass tube with rubber seal;

Completely sealed, glass ampoules (2) - the most reliable of these containers.

Irvine (1954). Ethylene diamine was used as the solvent, the extraction being carried out in a soxhlet extractor for about 30 cycles. This reduced the nitrogen content of the bone to less than 0.1 per cent. A few further cycles of distilled water were used to remove the ethylene diamine and the material was subsequently stored at room temperature.

The experimental animal

Dogs were selected as being the only readily available experimental animal with bones large enough to provide a test site of a size which had clinical significance.

Young adult dogs weighing 16 - 20 kilograms were used.

As it was impossible to obtain dogs which were of the same breed and background it was felt that variation in results might occur which were due to differences in the host rather than differences in the graft. For this reason two grafts of different materials were implanted in each dog at the same time. When evaluating results comparison was made between the progress of the two grafts in the same animal.

The technique of grafting

The dogs were anaesthetised with intravenous nembutal. With aseptic technique the skin and deep fascia was incised over the lateral aspect of the distal femur. The

quadriceps muscle was reflected forward from the lateral intramuscular septum, exposing the femur.

A trephine hole 4 mm. in diameter was made in the lateral aspect of the femur at the level of the proximal margin of the femoral condyle. Through this hole a cavity measuring about 15 mm. in diameter was curetted out of the cancellous bone of the distal femur. Into this cavity was packed the graft material under test. The opposite leg was dealt with in the same way. The cortical plugs removed by the trephine were replaced and the wound closed in layers.

Each series, except the control Series I, consisted of nine dogs. Three dogs in each series were sacrificed at one month, three months and six months.

The femurs were removed and fixed in 10 per cent formal saline. A sagittal slab about 3 mm. in width was cut from the graft area, and these were decalcified in neutral E.D.T.A. (see footnote, page 9).

The tissue was then embedded in celloidin, sectioned and stained with haematoxylin and eosin. Various techniques for the measurement of new bone formation and graft resorption were explored and eventually the following procedure was evolved.

Technique of measurement of new bone and residual graft within the graft area

By means of projection microscopy enlarged drawings (X55) were made of sections of the whole graft area. On these drawings the living bone of the host was differentiated from the dead bone of the graft (Figs. 84 - 90). Photostat copies of these drawings were made onto paper of uniform thickness. From these photostats the pieces of paper corresponding to the different types of bone were cut out and weighed.

Knowing the weights of paper corresponding to the living bone and the dead bone and the weight of the paper corresponding to the entire graft area, it was possible to calculate the amount of the two types of bone as a percentage of the graft area occupied by each, and thus to give mathematical expression to the behaviour of different graft materials by which they could be compared one with another.

In order to test the reliability of the method, a preliminary series was carried out in which both femurs of each dog received the same type of graft - fresh autograft.

TABLE 11

SERIES OF EXPERIMENTS

Series I	Control series in which each leg received fresh autograft.
Series II	Comparison of fresh autograft and fresh homograft.
Series III	Comparison of frozen homograft and autoclaved homograft.
Series IV	Comparison of frozen homograft and freeze-dried homograft.
Series V	Comparison of frozen homograft and deproteinized homograft.
Series VI	Comparison of frozen homograft and demineralized homograft.
Series VII	Comparison of freeze-dried homograft and freeze-dried irradiated homograft.

RESULTS

Whatever graft material was used, the same general pattern of behaviour was observed. Differences in progress of the various grafts were of degree rather than kind and the same general description is applicable to each.

The haematoma which initially occupied the spaces between the graft fragments was rapidly invaded by granulation tissue from the periphery. Following closely in its wake new bone grew into the graft area, covering the surfaces of the graft fragments and extending from one to another until the entire defect was spanned by living bone. This process was completed by one month in each series.

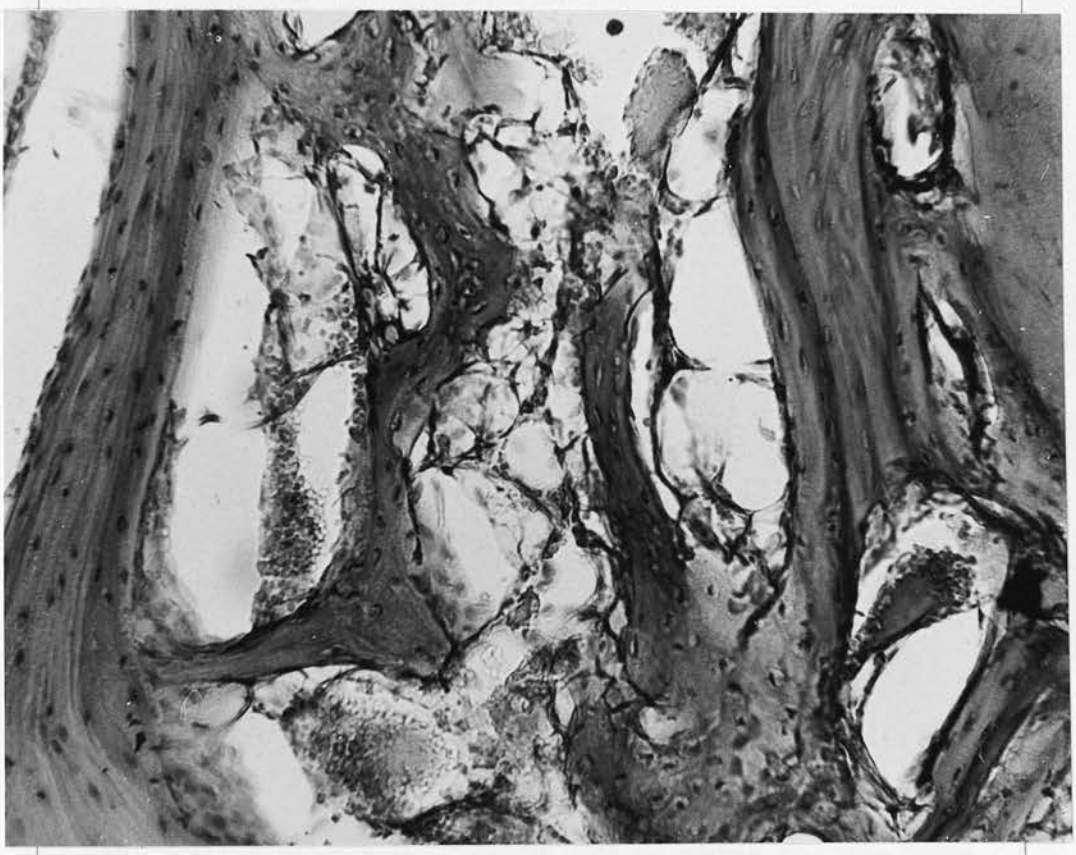
Progressive removal of the graft bone and remodelling of the newly formed host bone took place, eventually resulting in a normal pattern of cancellous arrangement with little residual graft (Figs. 75 - 80, 84 - 87 and 91).

In similar but ungrafted defects the repair process was much slower than in any of the grafted series. Even at three months such ungrafted cavities were incompletely bridged by bone (Fig.88). Cavities filled with plastic scaffolds such as nylon mesh or polythene granules likewise showed delayed and incomplete "healing" (Fig. 83).

With some of the graft materials tested differences in rates of resorption were so marked as to be apparent by

simple inspection of the histological preparations or from the outline drawings (Figs. 82 and 89) but for the most part differences between the various materials could only be demonstrated by actual measurements of the rates of new bone formation and of resorption of the graft.

Figure 75

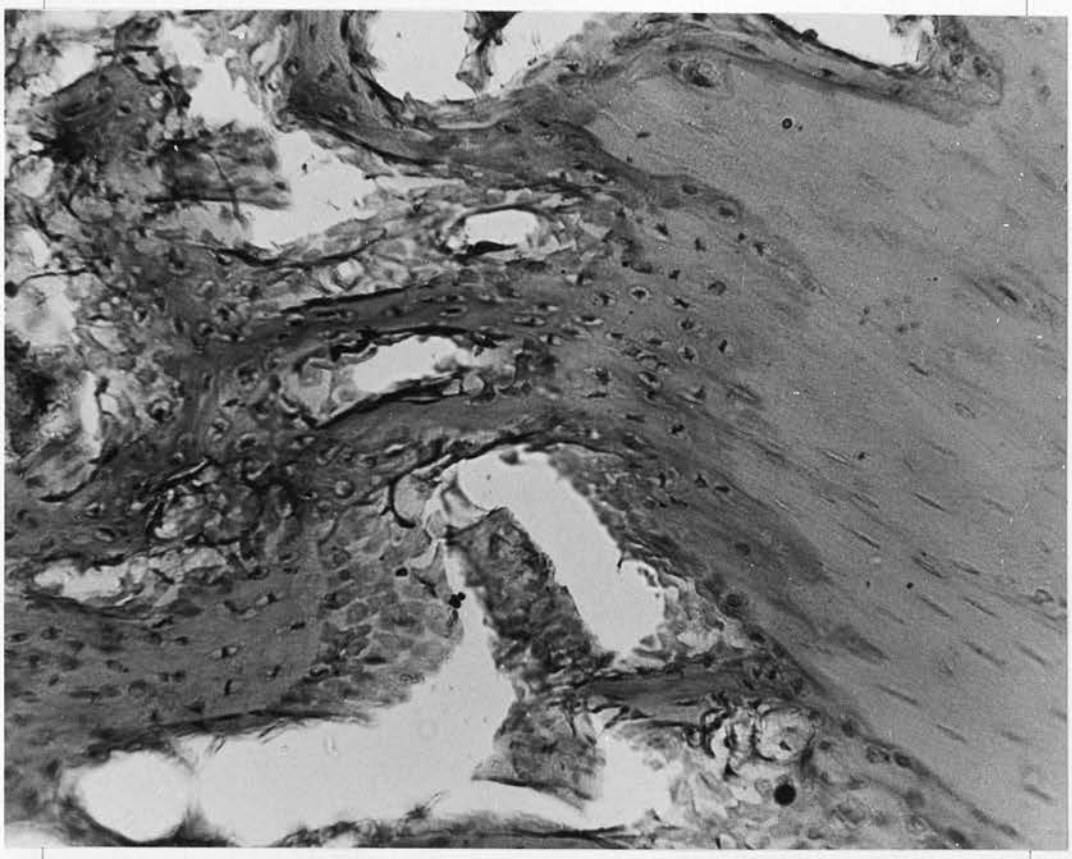


Autograft two weeks.

New bone is extending from a living host trabecula (L) towards the grafted bone (H) and is being laid down on the surface of the graft.

H. and E. x 150.

Figure 76

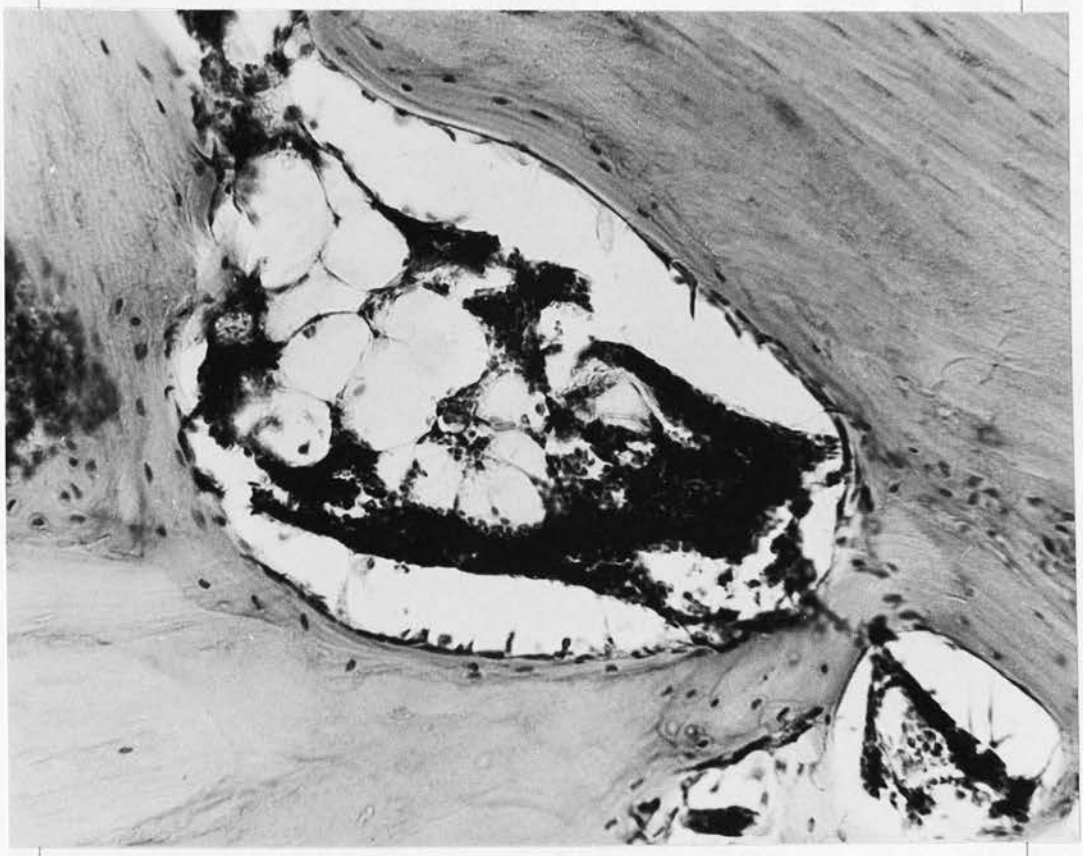


Autograft two weeks.

Another field from the periphery of the graft area showing the proliferation of new bone extending from the margin of the defect (L) and appearing to be guided toward the acellular graft fragment (R) to which the new bone has become united.

H. and E. x 150.

Figure 77

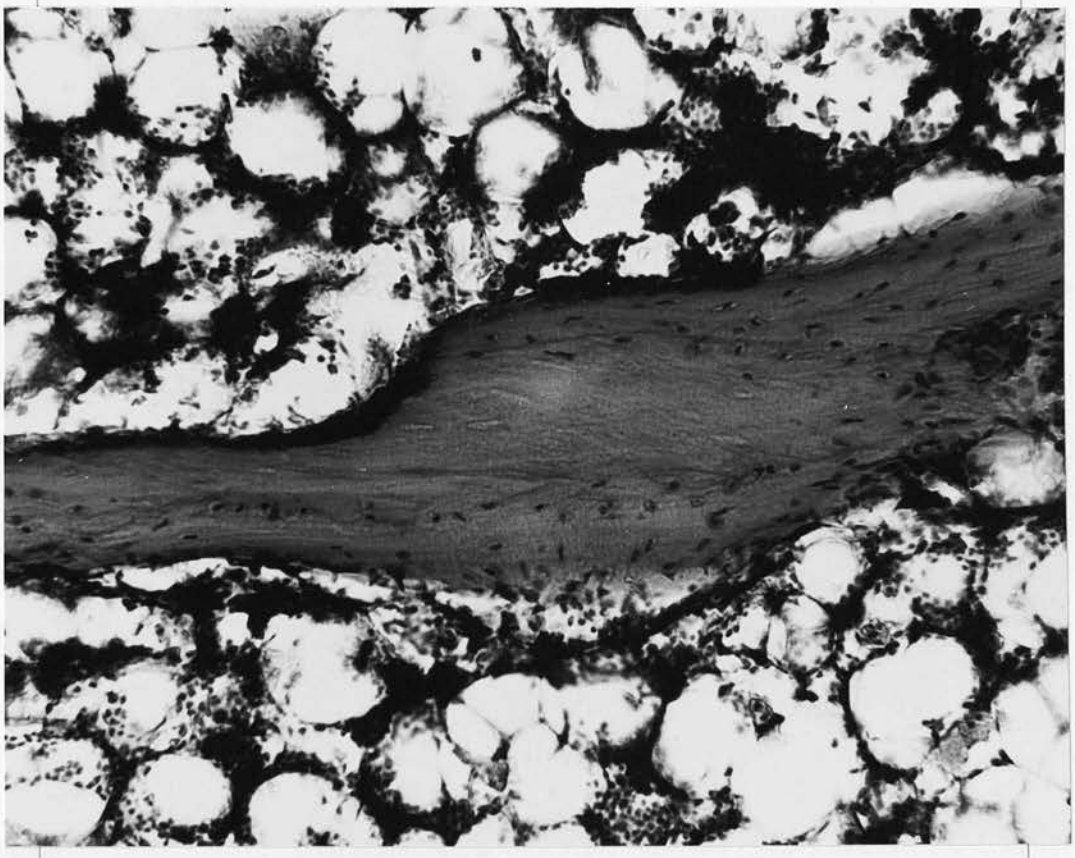


Autograft three months

Living bone which is now lamellar in character is linking together three pieces of graft bone and is covering their surfaces. The acellularity of the graft distinguishes it from the new bone.

H. and E. x 150.

Figure 78



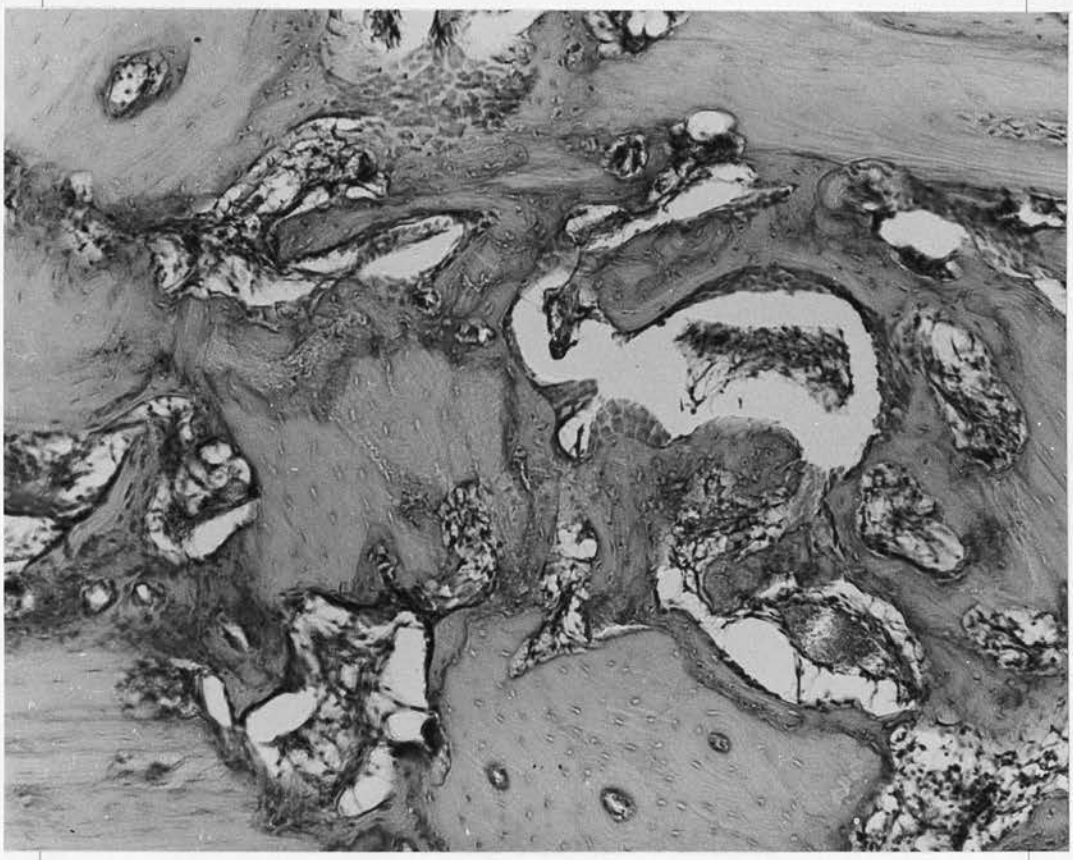
Autograft six months.

Field from the centre of the graft defect.

Only a small fragment of graft is left lying in a trabecula of living bone. At this stage remodelling within the graft area has restored the internal architecture of the bone to near normal arrangement.

H. and E. x 150.

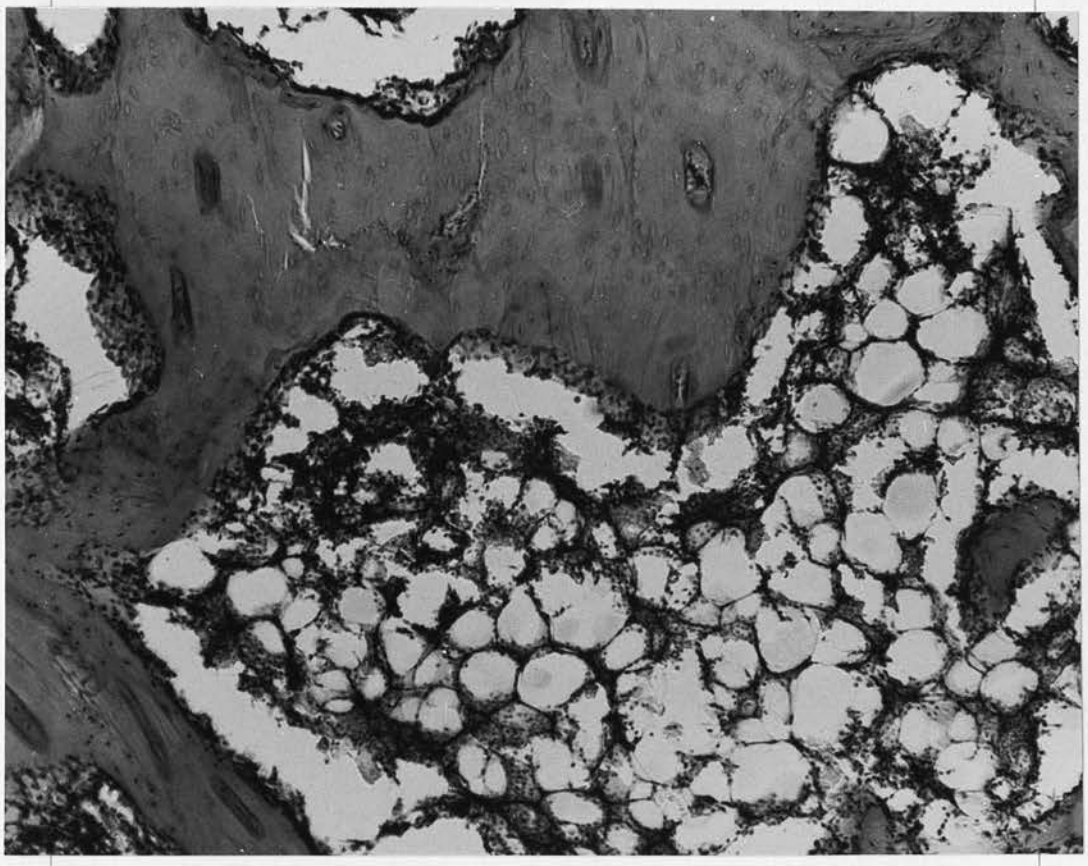
Figure 79



Frozen homograft two weeks.

Field from margin of graft area. New bone is linking the acellular graft fragments in a manner which is indistinguishable from an autograft at the same stage.

Figure 80

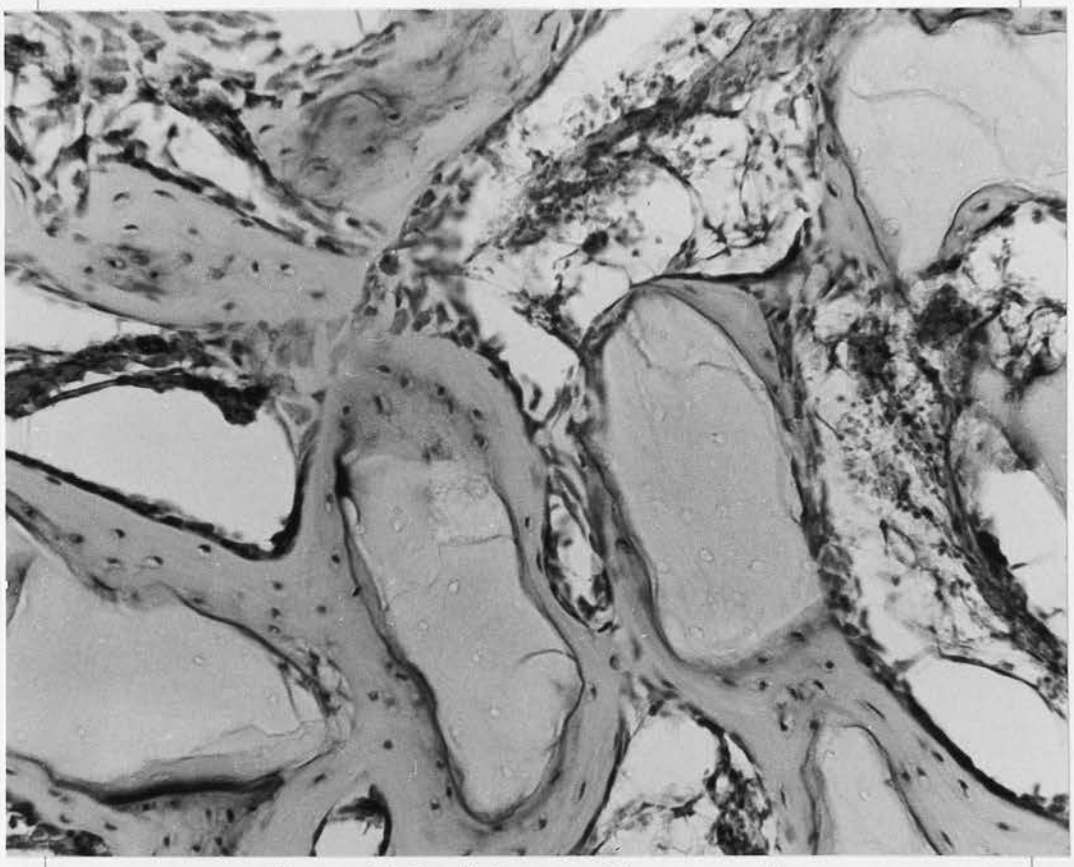


Frozen homograft three months.

Progressive resorption has removed much of the graft but the remaining fragments are linked and partly covered by new bone. Histological appearance shows no difference from an autograft at the same stage.

H. and E. x 150.

Figure 81

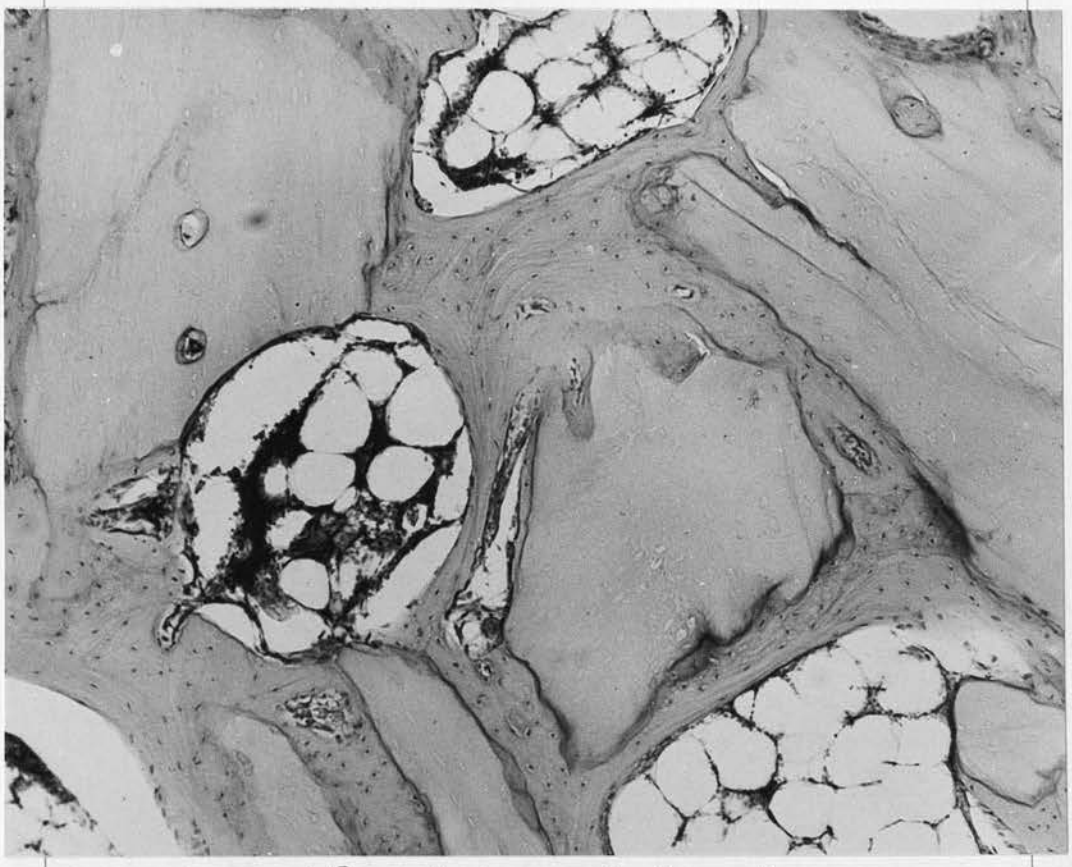


Deproteinized homograft one month.

Note the general similarity with the frozen homograft at the same stage (Figure 79). The guiding influence of the graft on the arrangement of the new bone is well shown.

H. and E. x 135.

Figure 82

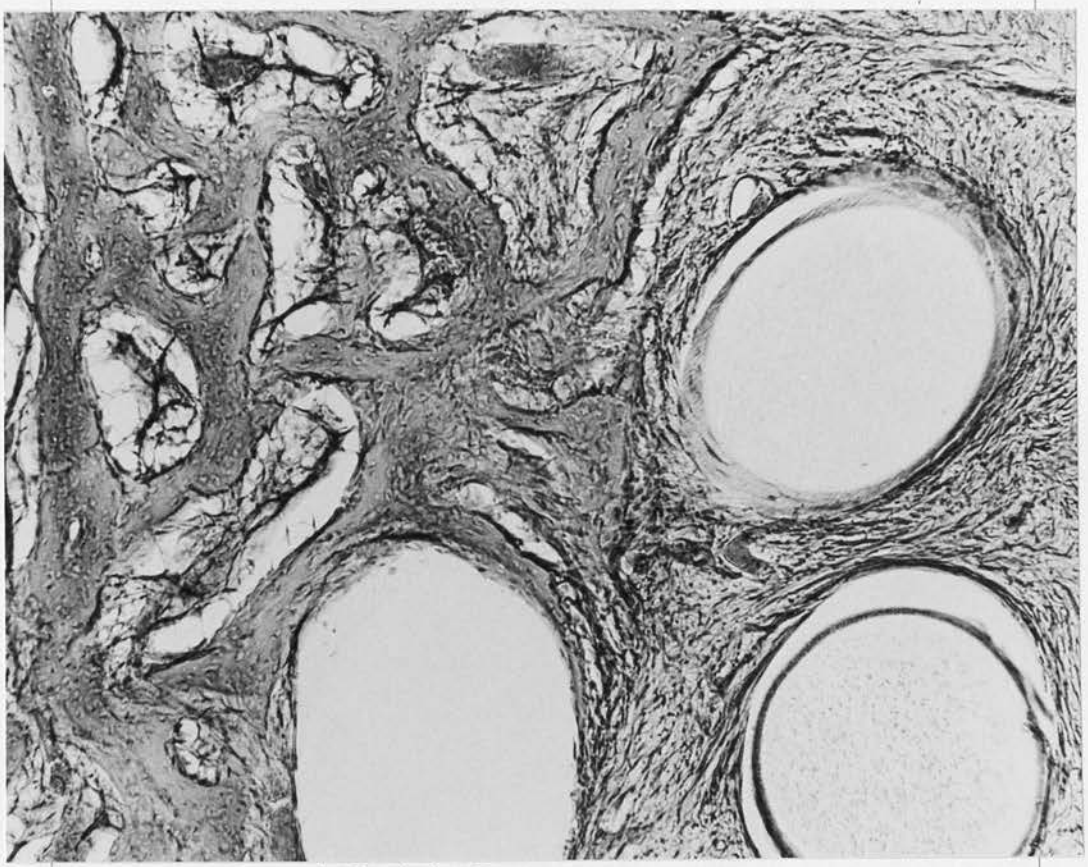


An organic homograft six months.

The amount of residual graft is much greater than for example in the autograft at the same stage (Figure 78). The presence of the graft prevents the remodelling of the new bone and so delays the final healing of the defect.

H. and E. x 70.

Figure 83

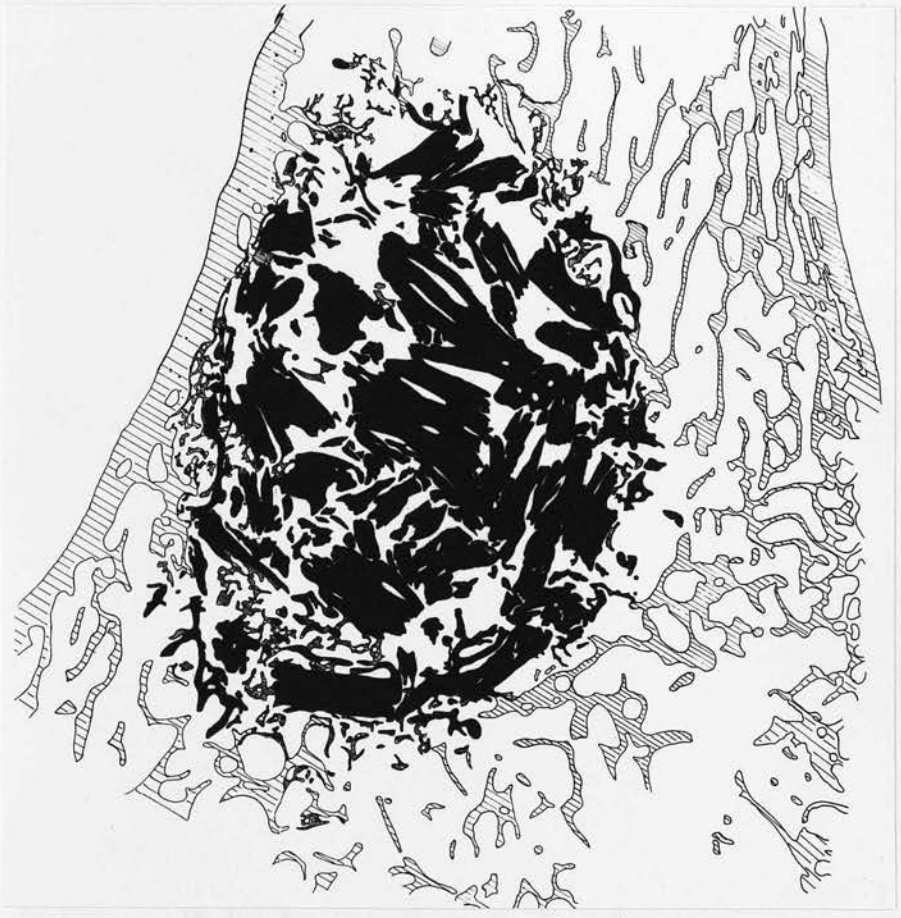


Bone defect implanted with nylon mesh.
Two weeks.

This field from the margin of the defect demonstrates the complete lack of osteoconductive influence in the nylon. The new bone shows no orientation toward it. (Compare Figure 76).

H. and E. x 70.

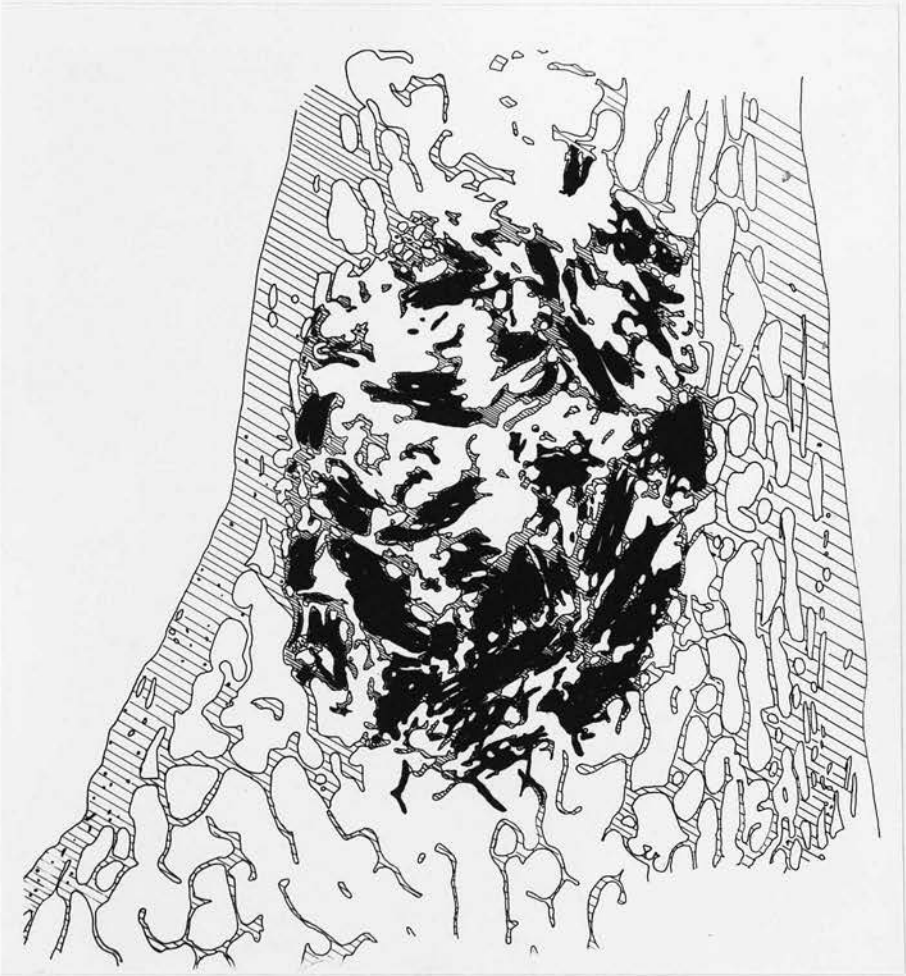
Figure 84



Autograft, one week (x 6)

Drawing of section through graft area. The graft is shown in black, the living host in cross hatching. At this stage new bone ingrowth is limited to the periphery of the defect.

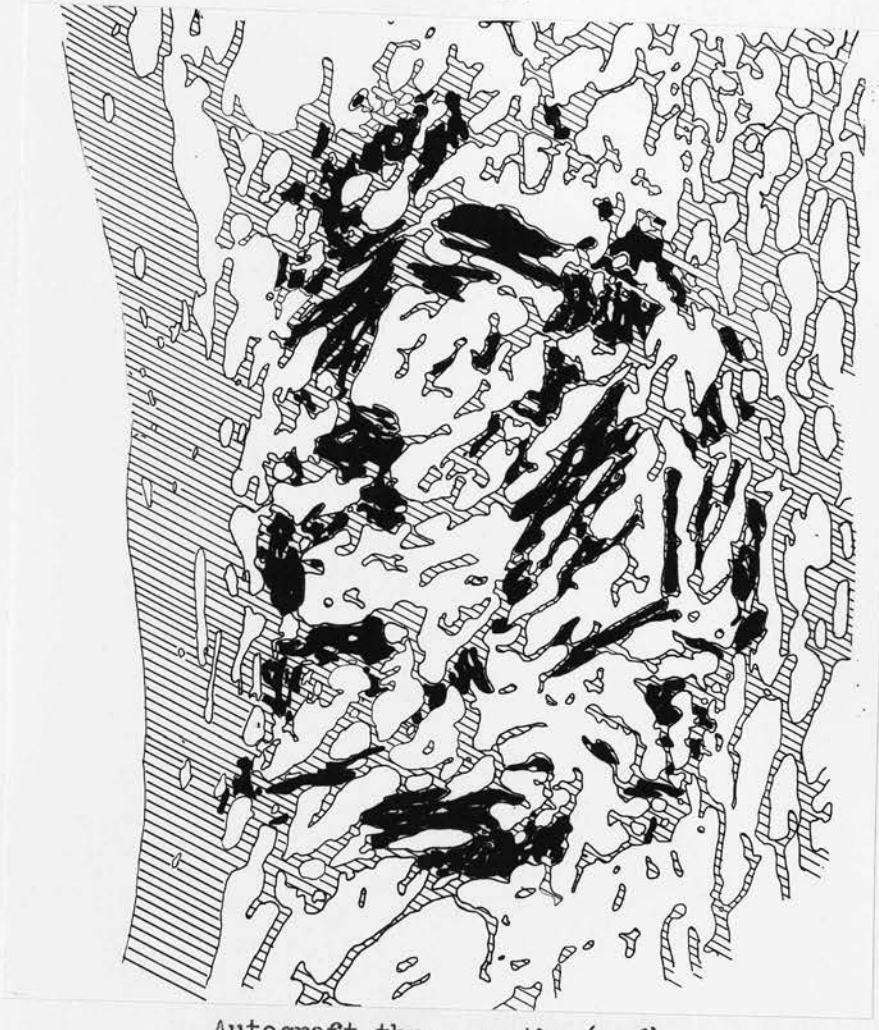
Figure 85



Autograft, one month (x 6)

New bone spans the graft area extending from one graft fragment to another. There is already a reduction in the amount of graft bone.

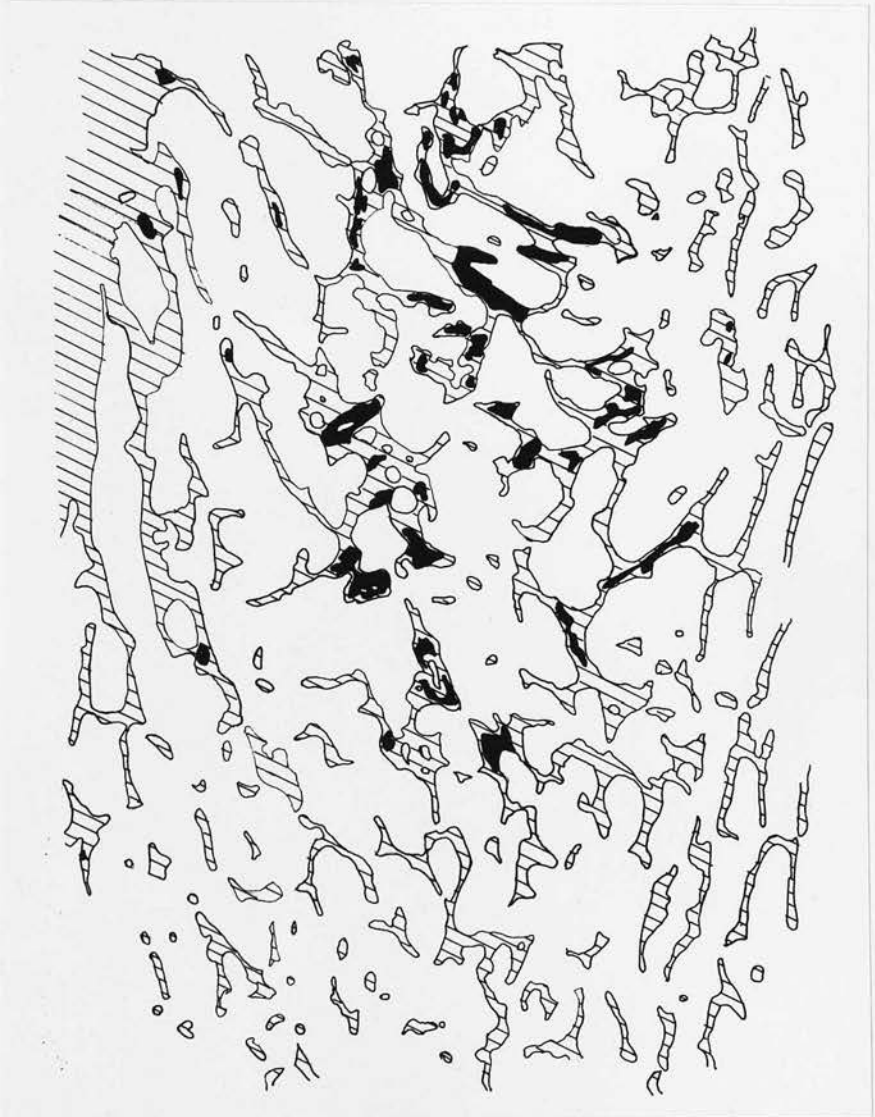
Figure 86



Autograft three months (x 6)

Further resorption of new bone has taken place and the new trabeculae are beginning to show a more ordered arrangement.

Figure 87



Autograft, six months (x 6)

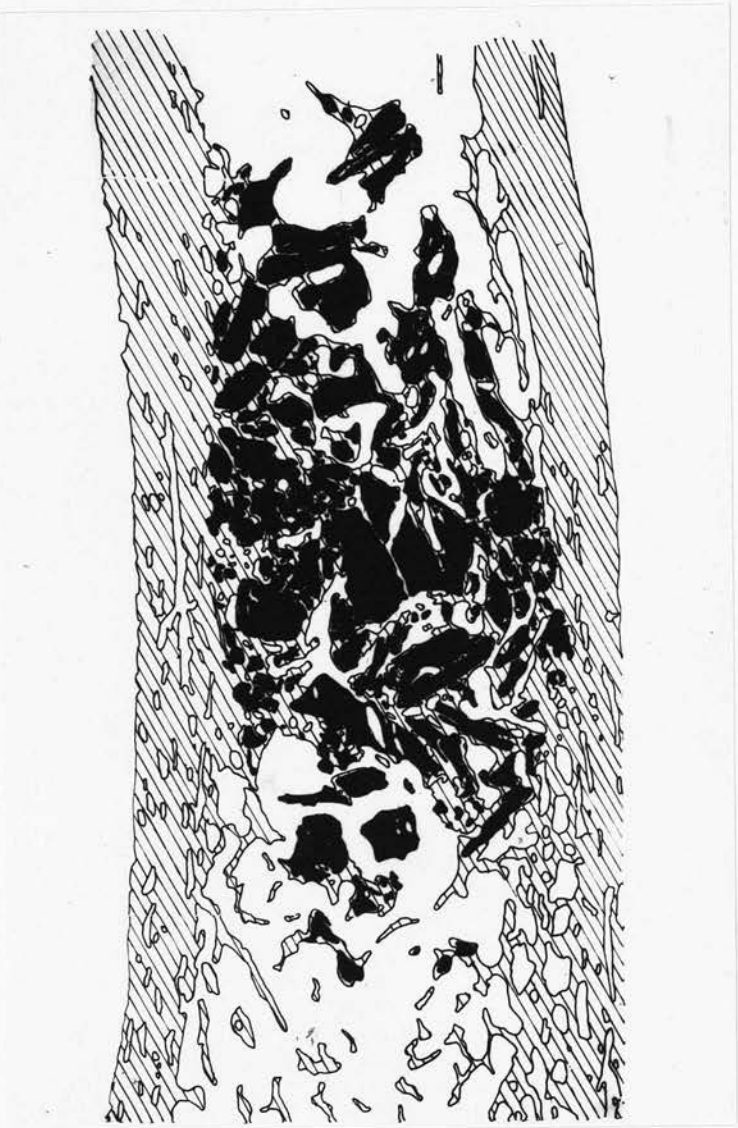
Only small fragments of residual graft remain. The arrangement of trabeculae is similar to the normal pattern in this area.

Figure 88

Drawing of ungrafted defect at three months (x 6).

The cavity which was the same size as those of the grafted series is incompletely bridged by bone, whereas in all the bone grafted cavities, new bone had extended throughout by this stage.
(Compare Figures 86, 89 and 90).

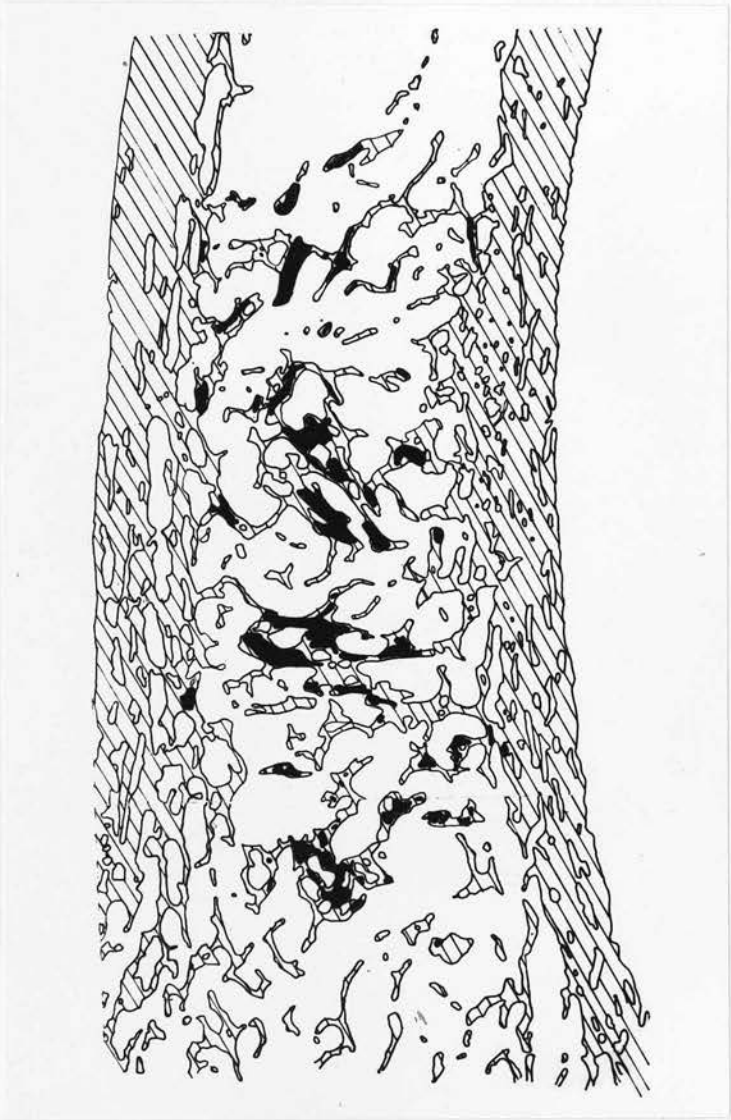
Figure 89



Deproteinized homograft, three months (x 6)

Compare with the frozen homograft which was grafted to the other leg of the same dog (Fig. 90). The amount of residual graft is much greater.

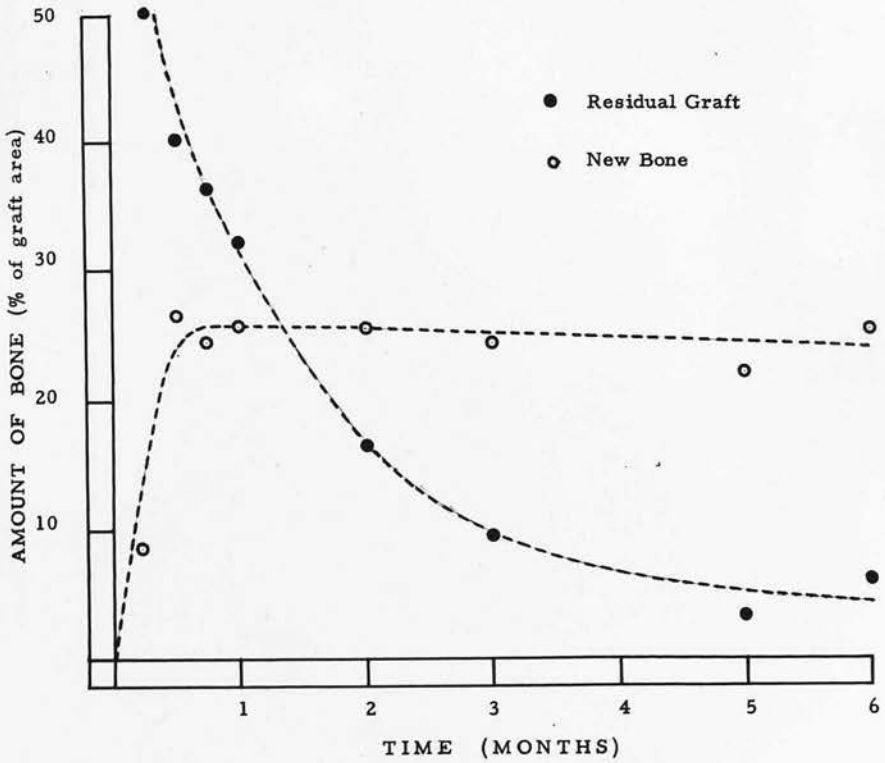
Figure 90



Frozen homograft, three months (x 6)

Although the extent to which the defect is filled by new bone corresponds to Figure 89, bone resorption is more advanced.

Figure 91



PROGRESS OF FRESH AUTOGRAFT

(Each point represents the mean value from grafts from 1 - 5 dogs)

Series I: Control Series comparing fresh autografts in both femurs

The results are presented in Tables 12 and 13.

Statistical analysis of these results fails to demonstrate any difference in the rate of graft resorption and new bone formation when the same graft is placed in both legs.

This established that the experimental method is reliable and valid for the comparison of different grafts' materials, in that if differences in behaviour are found with these materials the differences are likely to represent real variations in graft behaviour rather than any variability in the host site.

TABLE 12

SERIES I

Percentage of new bone in defect

NEW BONE IN GRAFT AREA				
Time	Dog	Left Leg Autograft	Right Leg Autograft	Difference
1 week	37	8.3	9.0	- 0.7
2 weeks	34	24.5	28.9	- 4.4
3 weeks	32	23.0	25.4	- 2.4
1 month	31	21.5	30.4	- 8.9
	22	32.4	23.7	+ 8.7
2 months	18	26.3	29.2	- 2.9
	17	24.5	23.0	+ 1.5
3 months	16	23.1	25.6	- 2.5
	15	36.9	28.8	+ 8.1
4 months	14	26.1	27.1	- 1.0
5 months	13	21.1	20.1	+ 1.0
	11	23.2	24.6	- 1.4
6 months	10	15.2	21.8	- 6.6
	9	32.0	32.0	0

When the series of differences is evaluated statistically from one month to six months, it is found that:-

Mean difference = 0.8%

Standard error of mean difference = 1.6%

Using the "t" test, t = 0.5

P = 0.6 (V = 10)

which means that the difference in the amount of new bone in the two legs is <u>not</u> significant.

TABLE 13

SERIES I

Comparison of fresh autografts in both legs

RESIDUAL GRAFT BONE				
Time	Dog	Left Leg Autograft	Right Leg Autograft	Difference
1 week	37	50.8	49.4	+ 1.4
2 weeks	34	41.1	39.3	+ 1.8
3 weeks	32	34.4	39.6	- 5.2
1 month	31	43.8	30.7	+ 13.1
	22	31.5	32.1	- 0.6
2 months	18	8.7	8.2	+ 0.5
	17	15.3	33.5	- 18.2
3 months	16	7.1	6.7	+ 0.4
	15	17.7	24.8	- 7.1
4 months	14	12.4	15.4	- 3.0
5 months	13	2.9	5.0	- 2.1
	11	3.0	1.9	+ 1.1
6 months	10	4.0	0.6	+ 3.4
	9	18.7	21.9	- 3.2

When the series of differences is evaluated statistically from one month to six months it is found that:-

Mean difference = - 1.4%

Standard error of mean difference = 2.3%

Using the " t" test, t = 0.61

P = 0.6 (V = 10)

which means that the difference in the amount of residual grafts in the two legs is <u>not</u> significant.

Series II: Comparison of fresh autograft and fresh homograft (see Tables 14, 15 and Fig. 92).

Statistical evaluation of these results failed to demonstrate any difference in the rate of removal of these two materials, but the amount of new bone formation was slightly greater in relation to the fresh autograft than that which formed in relation to the fresh homograft.

TABLE 14

SERIES II

Comparison of Fresh Autograft and Fresh Homograft

NEW BONE IN GRAFT AREA						
Time	Dog	Autograft		Homograft		Difference
1 month	29	19.1	24.8	24.9	20.6	+ 5.8
	30	33.4		20.5		- 12.9
	35	21.9		16.3		- 5.6
3 months	26	22.8	21.9	20.9	19.2	- 1.9
	27	20.8		19.5		- 1.3
	28	22.1		17.2		- 4.9
6 months	20	31.6	26.2	23.2	18.4	- 8.4
	21	21.6		13.5		- 8.1
	24	25.4		18.6		- 6.8

(All figures are given as % of entire graft area)

When the series of differences is evaluated statistically, it is found that:-

Mean difference = - 4.9

Standard error of mean difference = 1.8

Using the "t" test, t = 2.7

P = 0.025 (V = 8)

which means that the differences are significant at the 1 in 40 level - i.e. the experiment shows that the amount of new bone formed in relation to a fresh homograft is less than that formed in relation to a fresh autograft.

TABLE 15

SERIES II

Comparison of Fresh Autograft and Fresh Homograft

RESIDUAL GRAFT BONE						
Time	Dog	Autograft		Homograft		Difference
1 month	29	40.1	31.1	35.3	32.9	- 4.8
	30	15.5		30.8		+ 15.3
	35	37.8		32.6		- 5.2
3 months	26	1.1	6.6	2.4	10.4	+ 1.3
	27	1.6		4.8		+ 3.2
	28	17.1		23.9		+ 6.8
6 months	20	5.7	2.8	13.1	6.1	+ 7.4
	21	0.8		5.1		+ 4.3
	24	1.9		0.2		- 1.7

(All figures are given as % of entire graft area)

When the series of differences is evaluated statistically, it is found that :-

Mean difference = + 3.0

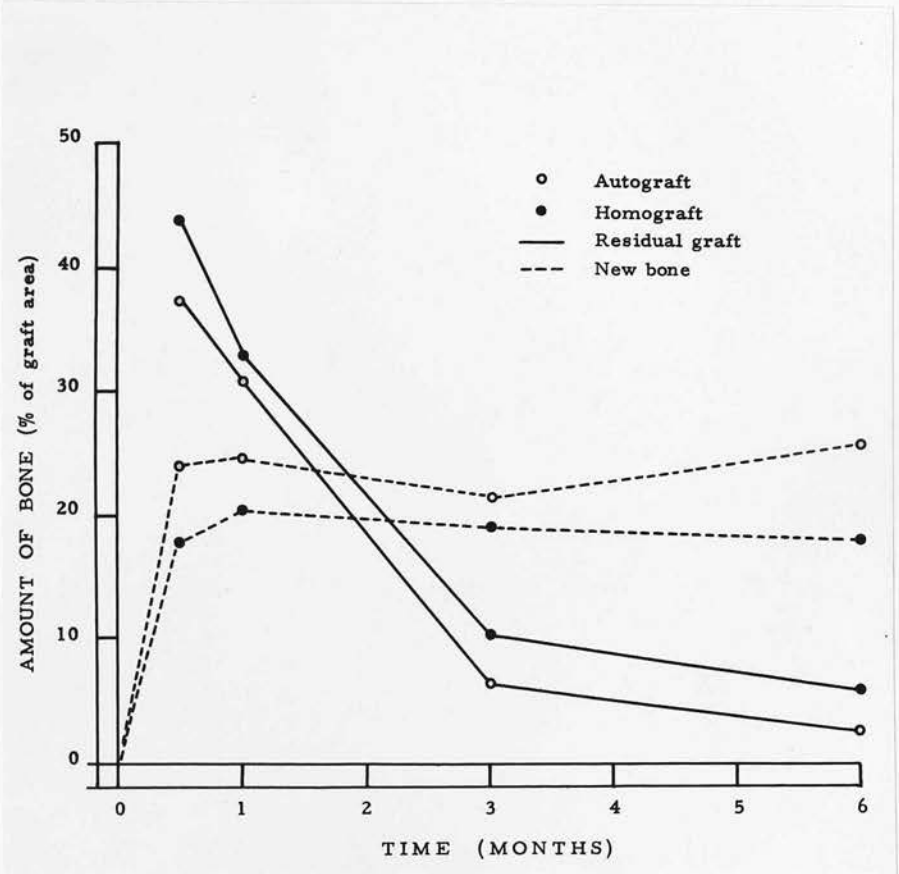
Standard error of mean difference = 2.2

Using the "t" test, t = 1.36

P = 0.22 (V = 8)

which means that the difference is <u>not</u> significant - i.e. the experiment does <u>not</u> show any significant difference in the rate of removal of autograft and homograft bone.

Figure 92



Comparison of fresh autograft
and fresh homograft (Series II)

Series III: Comparison of frozen homograft and autoclaved homograft. (see Tables 16,17 and Fig. 93).

Statistical evaluation of these results showed that these autoclaved homografts were less rapidly removed than frozen homograft, but that the amount of new bone forming in relation to these materials was the same.

TABLE 16

SERIES III

Comparison of Frozen Homograft and Autoclaved Homograft

NEW BONE IN GRAFT AREA						
Time	Dog	Frozen Homograft		Autoclaved Homograft		Difference
1 month	51	20.8	16.7	12.3	14.5	- 8.5
	52	9.7		15.0		+ 5.3
	53	19.7		16.2		- 3.5
3 months	44	10.1	14.2	20.1	18.9	+ 10.0
	47	18.0		16.0		- 2.0
	50	14.6		20.7		+ 6.1
6 months	43	16.7	14.4	16.2	17.1	- 0.5
	45	12.6		15.0		+ 2.4
	46	13.9		20.0		+ 6.1

(All figures are given as % of entire graft area)

When the series of differences is evaluated statistically, it is found that :-

Mean difference = + 1.7

Standard error of difference = 1.9

Using the "t" test, t = 2.7

P = 0.4 (V = 8)

which means that the differences are <u>not</u> significant - i.e. the experiment does <u>not</u> show any significant difference in the amount of new bone formed in relation to autoclaved homografts and frozen homografts.

TABLE 17

SERIES III

Comparison of Frozen Homograft and Autoclaved Homograft

RESIDUAL GRAFT BONE						
Time	Dog	Frozen Homograft		Autoclaved Homograft		Difference
1 month	51	27.5	24.5	32.2	30.2	+ 4.7
	52	21.9		30.7		+ 8.8
	53	24.0		27.8		+ 3.8
3 months	44	0.4	4.9	2.1	7.8	+ 1.7
	47	10.7		18.2		+ 7.5
	50	3.6		3.1		- 0.5
6 months	43	0.5	2.1	2.4	7.4	+ 1.9
	45	1.7		7.9		+ 6.2
	46	4.1		12.0		+ 7.9

(All figures are given as % of entire graft area)

When the series of differences is evaluated statistically, it is found that:-

Mean difference = + 4.8

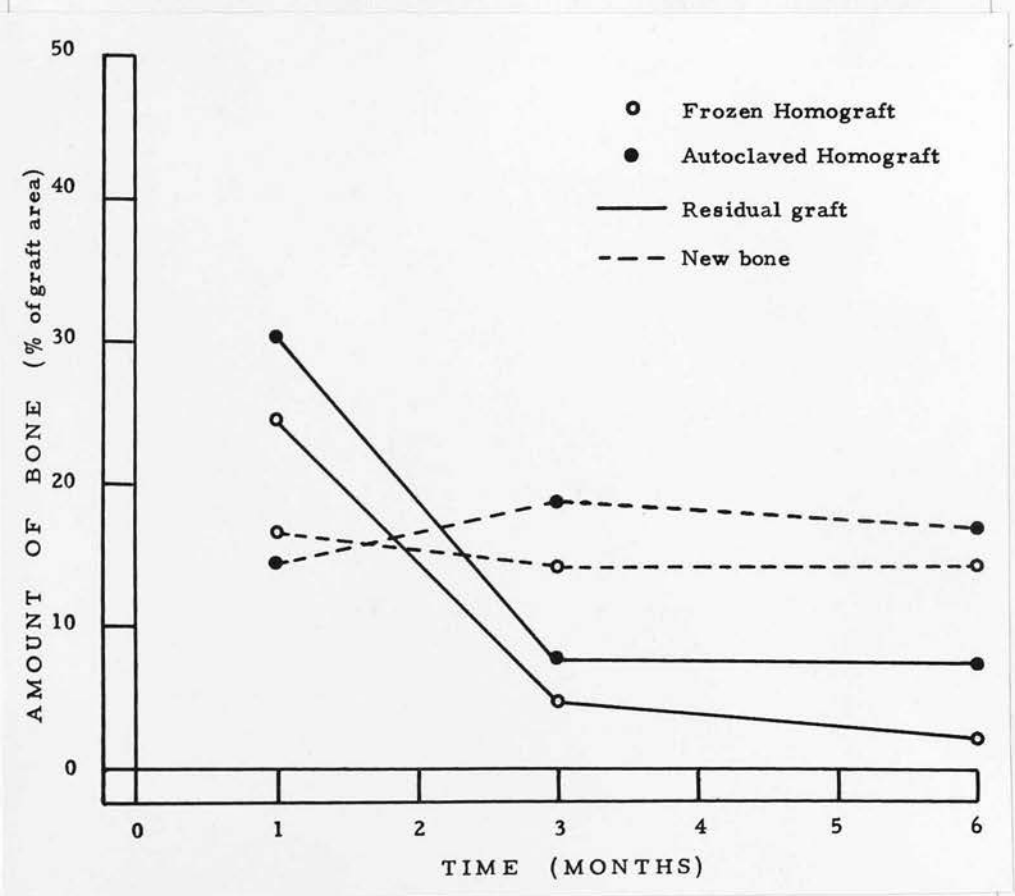
Standard error of difference = 1.0

Using the "t" test, t = 4.8

P = 0.002 (V = 8)

which means that the difference is significant at the 1 in 500 level - i.e. the experiment shows that autoclaved homograft bone is less rapidly removed than frozen homograft bone.

Figure 93



Comparison of frozen homograft and autoclaved homograft. (Series III)

Series IV: Comparison of frozen homograft and freeze-dried homograft (see Tables 18,19 and Fig. 94)

Statistical evaluation of these results showed that frozen homograft was slightly less rapidly removed than freeze-dried homograft, but that the amount of new bone forming in relation to these materials was the same.

TABLE 18

SERIES IV

Comparison of Frozen Homograft and Freeze-dried Homograft

NEW BONE IN GRAFT AREA						
Time	Dog	Frozen		Freeze-dried		Difference
1 month	56	15.3	17.7	13.1	13.9	- 2.2
	73	24.0		20.3		- 3.7
	129	13.9		8.3		- 5.6
3 months	57	23.5	17.2	24.3	20.4	+ 0.8
	58	12.3		19.8		+ 7.5
	60	15.8		17.2		+ 1.4
6 months	54	14.3	18.6	16.0	17.4	+ 1.7
	55	22.9		23.6		+ 0.7
	150	18.6		12.7		- 5.9

(All figures are given as % of entire graft area)

When the series of differences is evaluated statistically, it is found that:-

Mean difference = - 0.6

Standard error of mean difference = 1.4

Using the "t" test, t = 0.43

P = 0.68 (V= 8)

which means that the difference is <u>not</u> significant - i.e. the experiment does <u>not</u> show any significant difference in the amount of new bone formed in relation to frozen and freeze-dried homografts.

TABLE 19

SERIES IV

Comparison of Frozen Homograft and Freeze-dried Homograft

RESIDUAL GRAFT BONE						
Time	Dog	Frozen		Freeze-dried		Difference
1 month	56	34.2	26.9	26.6	26.7	- 7.6
	73	21.6		29.3		+ 7.7
	129	25.0		24.1		- 0.9
3 months	57	16.5	20.0	1.0	7.3	- 15.5
	58	21.9		10.7		- 11.2
	60	21.5		10.1		- 11.4
6 months	54	2.9	4.8	0.6	1.3	- 2.3
	55	7.1		2.4		- 4.7
	150	4.5		0.9		- 3.6

(All figures are given as % of entire graft area)

When the series of differences is evaluated statistically, it is found that :-

Mean difference = - 5.5

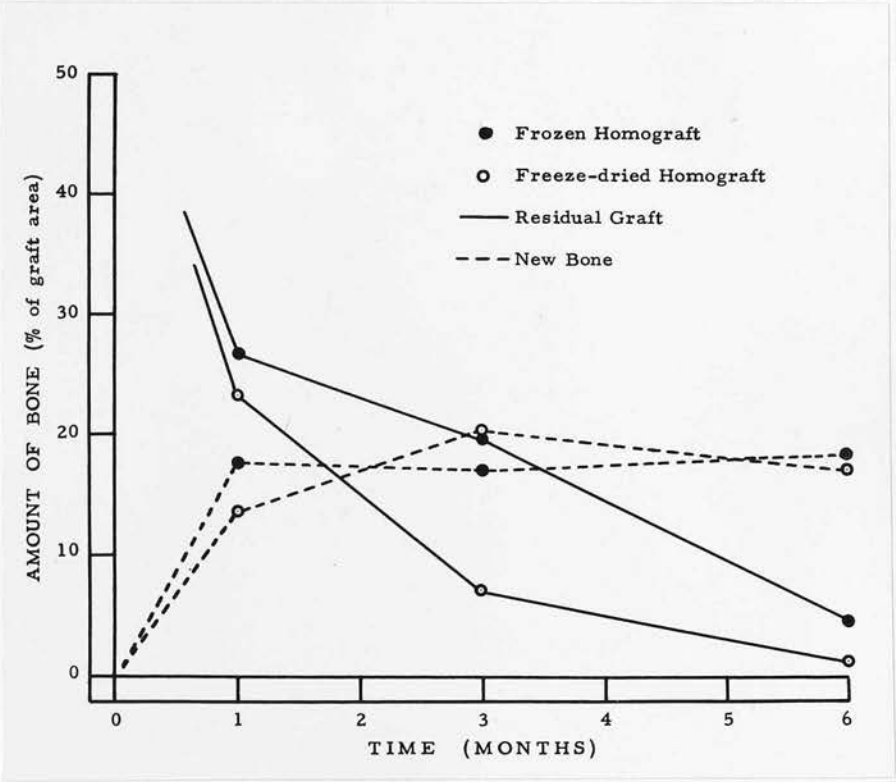
Standard error of mean difference = 2.3%

Using the "t" test, t = 2.39

P = 0.05 (V = 8)

which means that the difference is just significant as the results would be expected to occur 1 in 20 by chance - i.e. the experiment shows that frozen homograft bone is less rapidly removed than freeze-dried bone.

Figure 94



Comparison of frozen homograft and freeze dried homograft (Series IV)

Series V: Comparison of frozen homograft and deproteinized homograft
(see Tables 20, 21 and Fig. 95)

Statistical evaluation of these results showed that the deproteinized homograft was less rapidly removed than the frozen homograft, but that the amount of new bone forming in relation to these materials was the same.

TABLE 20

SERIES V

Comparison of Frozen Homograft and Deproteinized Homograft

NEW BONE IN GRAFT AREA						
Time	Dog	Frozen Homograft		Deproteinized Homograft		Difference
1 month	107	23.4	16.5	16.4	15.5	- 7.0
	147	9.2		11.3		+ 2.1
	148	15.9		18.8		+ 2.9
3 months	104	18.5	18.2	19.4	21.9	+ 0.9
	105	14.1		21.8		+ 7.7
	106	22.0		24.5		+ 2.5
6 months	100	24.9	17.2	28.4	20.1	+ 3.5
	101	15.6		19.8		+ 4.2
	102	11.1		12.1		+ 1.0

(All figures are given as % of entire graft area)

When the series of differences is evaluated statistically, it is found that:-

Mean difference = + 2.0

Standard error of mean difference = 1.3

Using the "t" test, t = 1.5

P = 0.2 (V = 8)

which means that the differences are <u>not</u> significant - i.e the experiment does not show any significant difference in the amount of new bone formed in relation to deproteinized homograft and frozen homograft.

TABLE 21

SERIES V

Comparison of Frozen Homograft and Deproteinized Homograft

RESIDUAL GRAFT BONE						
Time	Dog	Frozen Homograft		Deproteinized Homograft		Difference
1 month	107	8.0	23.0	29.0	28.7	+ 21.0
	147	27.8		30.9		+ 3.1
	148	33.2		27.1		- 6.1
3 months	104	14.6	10.9	35.5	33.9	+ 20.9
	105	12.1		31.0		+ 18.9
	106	6.0		35.2		+ 29.2
6 months	100	16.8	8.5	33.1	20.3	+ 16.3
	101	8.6		25.8		+ 17.2
	102	0.0		1.9		+ 1.9

(All figures are given as % of entire graft area)

When the series of differences is evaluated statistically, it is found that :-

Mean difference = + 13.6

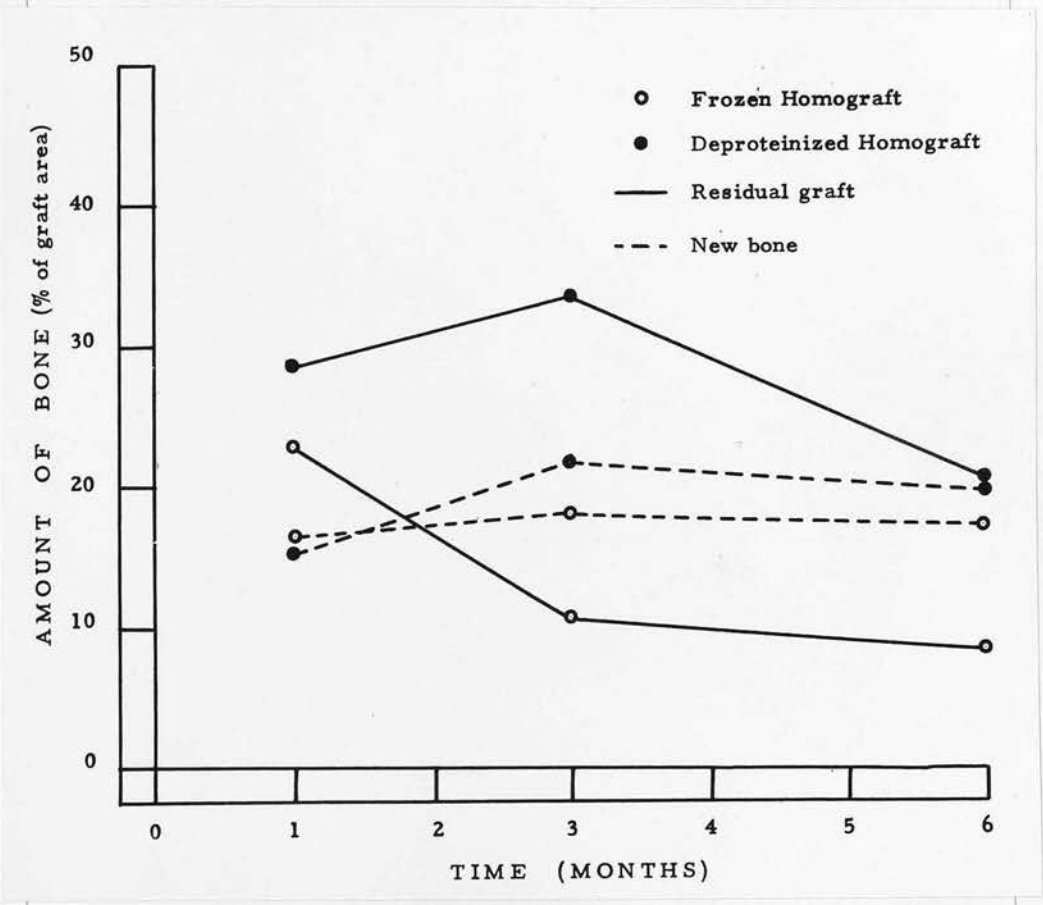
Standard error of mean difference = 3.8

Using the "t" test, t = 3.6

P = 0.007 (V = 8)

which means that the differences are significant at the 1 in 100 level - i.e. the experiment shows that deproteinized homograft bone is less rapidly removed than frozen homograft bone.

Figure 95



Comparison of frozen homograft and deproteinized homograft (Series V)

Series VI: Comparison of frozen homograft and demineralized homograft (see Tables 22, 23 and Fig. 96)

Statistical evaluation of these results showed that demineralized homograft was less rapidly removed than frozen homograft, but that the amount of new bone forming in relation to these materials was the same.

TABLE 22

SERIES VI

Comparison of Frozen Homograft and Demineralised Homograft

NEW BONE IN GRAFT AREA						
Time	Dog	Frozen		Demineralised		Difference
1 month	117	11.6	12.9	5.9	5.9	- 5.7
	122	7.6		7.4		- 0.2
	123	19.4		4.5		- 14.9
3 months	116	22.3	15.5	18.3	14.5	- 4.0
	154	13.4		13.7		+ 0.3
	155	10.7		11.5		+ 0.8
6 months	113	9.1	11.1	9.0	12.5	- 0.1
	114	10.5		13.6		+ 3.1
	115	13.7		14.8		+ 1.1

(All figures are given as % of entire graft area)

When the series of differences is evaluated statistically, it is found that :-

Mean difference = - 2.2

Standard error of mean difference = 1.8

Using the "t" test, t = 1.2

P = 0.26 (V = 8)

which means that the difference is <u>not</u> significant - i.e. the experiment does <u>not</u> show any significant difference in the amount of new bone formed in relation to demineralised homografts and frozen homografts.

TABLE 23

SERIES VI

Comparison of Frozen Homograft and Demineralised Homograft

RESIDUAL GRAFT BONE						
Time	Dog	Frozen		Demineralised		Difference
1 month	117	23.2	24.4	43.7	48.5	+ 20.5
	122	28.7		52.2		+ 23.5
	123	21.2		49.7		+ 28.5
3 months	116	0.2	5.1	25.6	25.4	+ 25.4
	154	6.3		19.1		+ 12.8
	155	8.8		31.4		+ 22.6
6 months	113	0.0	0.0	27.9	12.5	+ 27.9
	114	0.0		9.2		+ 9.2
	115	0.0		0.5		+ 0.5

(All figures are given as % of entire graft area)

When the series of differences is evaluated statistically, it is found that :-

Mean difference = + 19.0

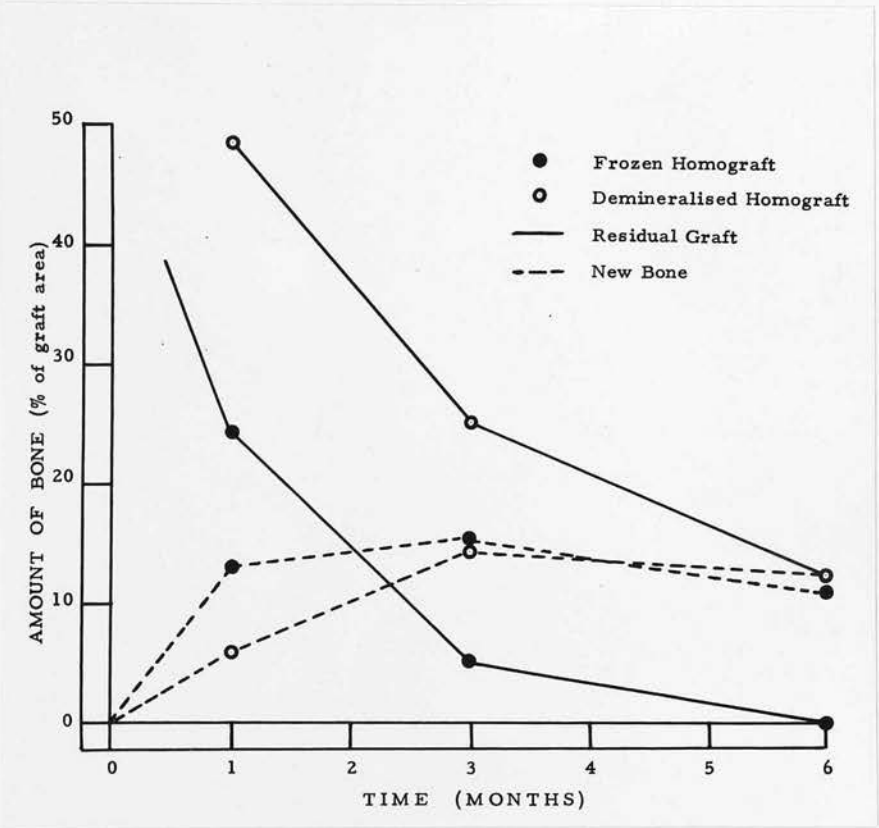
Standard error of mean difference = 2.0

Using the "t" test, t = 8.5

$P < .001$ (V = 8)

which means that the differences are significant at greater than the 1 in 1000 level - i.e. the experiment shows that the removal of demineralised bone is slower than that of frozen homograft.

Figure 96



Comparison of frozen homograft and demineralised homograft (Series VI)

Series VIII: Comparison of freeze-dried homograft and freeze-dried irradiated homograft (see Tables 24, 25 and Fig. 97)

Statistical evaluation of these results showed that the removal of freeze-dried irradiated homograft was slightly more rapid than freeze-dried homograft but that the amount of new bone forming in relation to these materials was the same.

TABLE 24

SERIES VII

Comparison of Freeze-dried Homograft and Freeze-dried Irradiated Homograft

NEW BONE IN GRAFT AREA						
Time	Dog	Freeze-dried		Freeze-dried Irradiated		Difference
1 month	142	25.2	20.7	20.2	22.3	- 5.0
	145	19.0		23.0		+ 4.0
	133	20.3		21.0		+ 0.7
	159	18.2		25.0		+ 6.8
3 months	134	22.4	15.1	17.8	10.7	- 4.6
	135	9. 9		10.8		+ 0.9
	132	13.0		3.5		- 9.5
6 months	136	10.0	12.8	10.9	14.8	+ 0.9
	138	15.5		17.8		+ 2.3
	144	13.0		15.7		+ 2.7

(All figures are given as % of entire graft area)

When the series of differences is evaluated statistically, it is found that :-

Mean difference = - 0.08

Standard error of mean difference = 1.5

Using the "t" test, t = 0.05

$P < 0.9$ $(V = 9)$

which means that the difference is <u>not</u> significant - i.e. the experiment shows that there is no difference in the amount of new bone formed in relation to freeze-dried and freeze-dried irradiated homografts.

TABLE 25

SERIES VII

Comparison of Freeze-dried Homograft and Freeze-dried Irradiated Homograft

RESIDUAL GRAFT BONE						
Time	Dog	Freeze-dried		Freeze-dried Irradiated		Difference
1 month	142	19.7	24.9	22.6	21.1	+ 2.9
	145	37.8		27.3		- 10.5
	133	22.2		21.6		- 0.6
	159	20.0		12.8		- 7.2
3 months	134	13.2	6.3	11.7	5.2	- 1.5
	135	4.6		3.7		- 0.9
	132	1.1		0.3		- 0.8
6 months	136	6.8	7.1	1.5	3.5	- 5.3
	138	2.7		1.8		- 0.9
	144	11.7		7.3		- 4.4

(All figures are given as % of entire graft area)

When the series of differences is evaluated statistically, it is found that :-

Mean difference = - 2.9

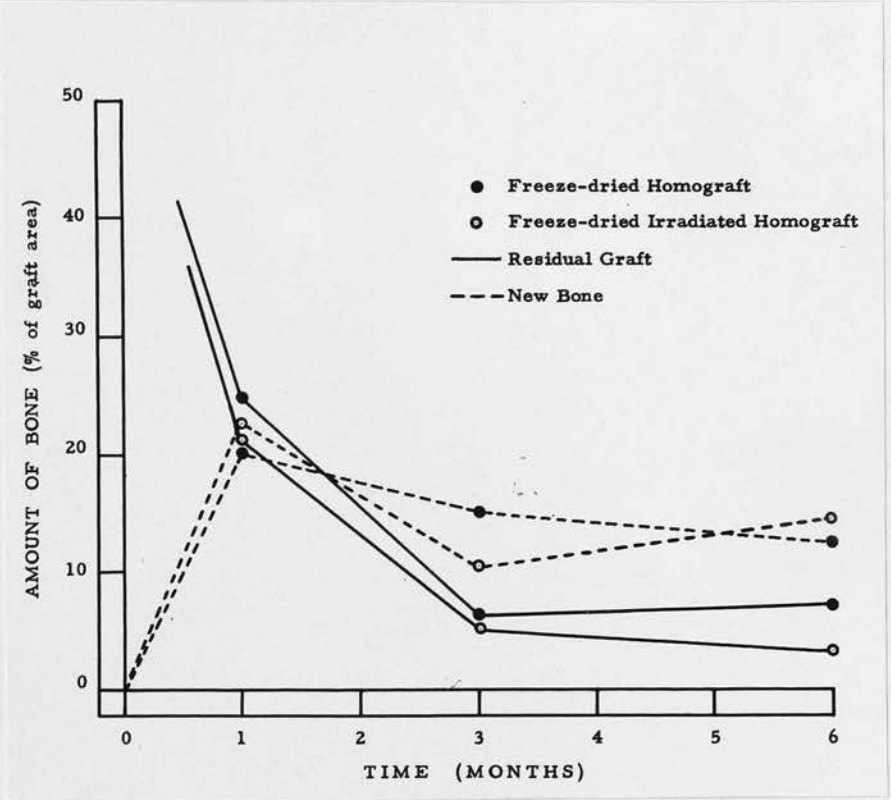
Standard error of mean difference = 1.2

Using the "t" test, t = 2.42

P = 0.04 (V = 9

which means that the differences are significant at the 1 in 25 level - i.e. the experiment shows that the removal of freeze-dried irradiated homograft is more rapid than that of the freeze-dried homograft.

Figure 97



Comparison of freeze-dried homograft and freeze-dried irradiated homograft (Series VII).

DISCUSSION

All the materials investigated in this study followed the same general pattern of behaviour. All seemed to perform a useful function in that the grafted cavities were all bridged with the new bone earlier and more completely than were similar ungrafted defects.

There was remarkable uniformity in the rate of this new bone production. In all grafted series it occupied about 15 - 20 per cent of the defect at one month, and continued at that level until the final remodelling was complete. (The amount of bone in the site used for the graft is normally 20 per cent.) A significant difference was found in the rate of new bone formation in Series II only and this difference was of such a low order that it is probably of little clinical importance. We may consider, therefore, that all the graft materials examined possessed an osteoconductive function to an approximately equivalent degree.

The rate at which the graft was removed showed much greater variation, freeze-dried irradiated, freeze-dried and frozen homografts in that order being more rapidly resorbed than autoclaved, demineralized and deproteinized homografts.

As rapidity of removal is likely to be a desirable feature of a bone graft (see page 130), it follows that of the materials tested, frozen, freeze-dried and freeze-dried

irradiated homograft are the most acceptable alternatives to autografts.

Such differences as did exist between these are small and selection of a material for clinical use will be influenced by convenience and practicability rather than by small biological advantages.

The most important practical consideration is that the graft can be stored to form a bone bank. (On this account, fresh bone homografts, which in other respects appear to be satisfactory, are not convenient for clinical use).

In the establishment of a tissue bank there are two basic requirements. Firstly, the biological properties for which the graft is used must be preserved. Secondly, the graft must be sterile.

Preservation of Bone

Among the different techniques which have been used for the preservation of bone, the earliest was refrigeration at 2 - 5°C (Inclan, 1942). Although apparently satisfactory, bone preserved by this method had the disadvantage of a short storage life.

An obvious extension of this method was the use of lower temperatures. Deep freezing -10 to -20°C permitted storage for at least 1 year (Wilson, 1951). The optimal temperature for the storage of bone has yet to be established.

It has been suggested that biological materials suffer least damage if frozen to very low temperatures (Meryman 1957). Hyatt (1960) recommends -60°C for the storage of bone, but in practice no disadvantage appears to have resulted from the use of temperatures in the -10° to -30°C range. Furthermore, the rate of cooling which is so important in the preservation of many materials does not appear to be critical in the case of bone.

The technique of freeze-drying which was applied originally to the preservation of bacteria and sera, (Flosdorf 1949), was more generally applied during the Second World War to the preservation of plasma and other biological preparations and food stuffs. Freeze-drying has proved to be a most satisfactory method of preservation, solutes remain evenly dispersed and no bacterial or enzyme change takes place (Flosdorf and Hyatt, 1952) during process and storage. Viability, however, is lost if drying to below 25 per cent of the original water content is achieved (Billingham and Medawar 1952).

Freeze-drying techniques were first applied to the preservation of bone by Kreuz et al.(1951) and subsequent reports (Hyatt and Butler 1957; Berkin et al. 1957) have confirmed the clinical success of this material. Freeze-drying has the disadvantage of reducing the strength of bone and therefore makes it less suitable for clinical use when mechanical fixation is required of the graft. Even

after reconstitution in saline for one hour its breaking stress is only 50 per cent of that of fresh bone (Chalmers, unpublished).

Various chemicals have been used for the preservation of bone - merthiolate (Reynolds et al. 1951), formaldehyde and acetone (Sabenas et al. 1955 a and b) and alcohol (Kleinberg, 1956). Of these, only the merthiolate bank has been given extensive clinical trial. The reported results have not justified its continued use (Frantz, Reynolds and Lipscombe, 1953).

Bone sterilization

The second problem of a tissue bank, that of ensuring sterility of the graft, has been tackled in various ways; by maintaining an aseptic technique during preparation and handling, or by subjecting the bone to an assortment of physical and chemical sterilizing agents, or by a combination of such methods.

The application of an aseptic technique has many limitations. Firstly, it requires that the source of bone is initially sterile. Such supplies are limited to small pieces of bone removed during the course of operations on bone, or to limbs which have been amputated for non-infective conditions. Facilities for obtaining sterile tissues from a cadaver have been developed at the U.S. Naval

Medical Centre in Bethesda (Hyatt and Butler 1957), but the cost of the necessary equipment and skilled personnel is so great that it has not been adopted elsewhere.

Even when bone from a presumed sterile source is available, a truly aseptic technique for the subsequent handling is difficult to achieve. In practice, a standard careful surgical technique has proved reasonably safe and the elaborate precautions described by Duthie(1953) have not been proved to be superior.

It is customary to carry out a bacteriological check of the prepared specimen by subjecting a sample or washings obtained from the specimen to culture. However, organisms deeply placed within the specimen may escape detection by such methods, and the cultural technique used may not reveal such organisms as the tubercle bacillus and the virus of infective hepatitis - both of which have been transmitted by bone homografts (James, 1958; Shutkin, 1954).

Some workers have added penicillin and streptomycin to the solution in which the graft is stored (Augustine et al. 1953; Fischer and Clayton, 1955) but these antibiotics will be effective against only a limited range of bacteria.

Several chemical sterilizing agents have been used. Some of these, which are also preservatives, have been mentioned already (page 189) and none has found favour in clinical use.

Beta propriolactone appears to hold more promise. Its use for sterilizing bone grafts in clinic surgery has been reported by Lo Grippo et al. (1957) and Berkin et al. (1957). This chemical antiseptic, however, suffers from the serious drawback of sterilizing only the surface of a tissue. Cancellous bone thicker than 6 millimetres and solid tissue thicker than 3 millemetres cannot be sterilized by this means (Lo Grippo et al. 1957).

Physical sterilizing agents have been more widely adopted.

Heat treatment by either boiling or autoclaving has been used for many years (Gallie and Robertson, 1918; Smith 1937; Lloyd-Roberts, 1952). The slower resorption of heat treated grafts may be attributable to its coagulating effect on the graft proteins rendering them more impenetrable to the host tissues. Heat also increases the size of bone crystals which may make the mineral less resorbable by reducing the surface area at which chemical activity can take place (Holmstrand, 1957). This could also account for the slower resorption of the deproteinized bone, Series V, for the preparation of this material involved heating to 118° C during extraction.

In recent years increasing use has been made of ionising irradiation as a sterilizing agent for many surgical

and biological products including tissue grafts (Meeker and Gross, 1951).

Several recent papers report the experimental and clinical usage of irradiated bone grafts. Ionizing irradiation if from a sufficiently powerful source can penetrate the entire thickness of a bone graft and its container, and thus is likely to be more effective than antiseptics which are able to sterilize surfaces.

Turner et al. (1956) using high voltage cathode irradiation found that a dose of 1.o million roengten equivalent physical (r.e.p.) would sterilize both frozen and freeze-dried bone which had been contaminated with Staphylococcus aureus. In experimental bone grafts to dogs they considered that a dosage above 2 million r.e.p. slightly impaired the healing of freeze-dried grafts while it caused slight acceleration of healing in frozen grafts. Their assessment of the grafts' progress, however, was based on inspection of histological sections, which is an unreliable method of demonstrating small differences in grafts' behaviour.

Many microorganisms are less sensitive to irradiation than Staphylococcus aureus. For example, the lethal dose for certain of the sporing bacteria such as Bacillus subtilis and Clostridium sporogenes is about 2 million r.e.p. This dose is recommended for the sterilization of foodstuffs.

(Department of Scientific and Industrial Research, 1955). Cohen (1955) using high voltage cathode irradiation found that a dosage of 2 million r.e.p. sterilized deliberately contaminated frozen bone grafts. In experimental transplants in cats he found no difference in the rate of healing or irradiated and non-irradiated grafts.

De Vries, Badgley and Hartmen (1958) using cobalt ^{60} source of gamma irradiation also found that 2 million r.e.p. sterilized bone contaminated with Clostridium botulinum and Bacillus tetanus. They reported the use of freeze-dried bone homografts irradiated at this level in 104 surgical procedures. There were 5 infections which were attributed to causes other than the graft. Otherwise the clinical results were satisfactory.

Basset et al. (1956) have also reported satisfactory clinical results using frozen homograft irradiated at 2 million r.e.p. Before irradiation, the bone which had been obtained from cadavers was contaminated with a variety of organisms. No positive cultures were obtained following irradiation. In one hundred patients, two wound infections occurred.

The dosage of 2.5 million r.e.p. used in the author's experiments was selected as giving a wide margin of safety over the generally accepted sterilizing doze of 2 million r.e.p.

Tables 26 and 27 summarise the various methods of preparation and sterilization of bone grafts which have been outlined above and indicate some of the advantages and disadvantages of each.

There is no single material in these lists which is entirely superior to the others and the clinician must weigh up the advantages and disadvantages of each before selecting which of these alternatives to autografts to use.

Of the methods of preservation, freezing, and freeze-drying are both satisfactory. As freeze-dried bone requires no special storage temperature it is the bank bone of choice if the distribution area of the bank is large, but it has the disadvantage of reduced strength which might render it unsuitable for a few surgical procedures. The frozen bone bank is satisfactory for limited local use.

Two methods of ensuring sterility are acceptable in that they do not have an adverse effect on the graft's function - adherence to an aseptic technique and exposure to ionizing irradiation. The former is restricted in its usefulness by the limitations in supply of sterile bone, and because it can never be 100% reliable. The latter has the advantages of absolute reliability thus making almost limitless supplies of unsterile bone available to the bone bank. It may be used with either of the preferred

methods of preservation and is to be regarded as the method of choice.

On the basis of these conclusions a freeze-dried irradiated bone bank has been established at the Royal National Orthopaedic Hospital in London and plans are in hand for the development of one based upon the Edinburgh Royal Infirmary.

TABLE 26

METHOD OF PREPARATION OF BONE HOMOGRAFTS	ADVANTAGES	DISADVANTAGES
Cooling at 2°C.	No special equipment required.	Limited storage life.
Refrigeration at -10°C or below.	Functions well as a graft.	Limited transportability.
Freeze-drying.	Functions well as a graft. Easily transported. May be stored at room temperature. Long storage life.	Reduced breaking stress.
In Merthiolate solution.	No special equipment required. Sterilises as well as preserves.	Has proved unsatisfactory in clinical use.
Preserved in alcohol, formaldehyde or acetone.	No special equipment required. Sterilises as well as preserves.	Have not been adequately evaluated experimentally and are therefore unsuitable for clinical trial at present.
Protein extracted.	Requires no special conditions of storage.	Chalk-like consistency. Unfavourable progress as graft.
Mineral extracted.	None.	Jelly-like consistency. Unfavourable progress as graft.

TABLE 27

METHOD OF STERILISATION	ADVANTAGES	DISADVANTAGES
Maintenance of aseptic technique.	Cannot interfere with the biological properties of the graft.	Limited supply of sterile bone. Absolute aseptic technique is an unobtainable ideal.
Application of heat.	Reliable.	Delays graft healing.
Antibiotics.	Do not interfere with biological properties of the graft.	Limited in their anti-bacterial spectra.
Beta propriolactone.	Simple to use.	A surface sterilising agent. Unsuitable for a thick graft.
Ionizing irradiation.	Reliable. If anything, enhances the acceptability of a graft. Can be used with any method of preservation. Enables unsterile sources of bone to be used.	None.

SUMMARY

Many bone graft materials have been used clinically or experimentally with apparent success as alternatives to autografts.

Six of these materials which appeared to hold most promise were subjected to a comparative experimental study.

Frozen, freeze-dried and freeze-dried irradiated homografts were found to behave more favourably than autoclaved, demineralized and deproteinized homograft.

The type of bone bank recommended for clinical use depends on the needs of the area, a frozen bone bank being satisfactory for local use, while a freeze-dried bone bank is preferable where wider distribution is required.

Ionizing irradiation is the most satisfactory method of sterilizing bone and may be used with either method of preservation.

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