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ROLE OF BIOLOGICAL MEMBRANES IN SOME SKELETAL REACTIONS

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INTRODUCTION

The Cambridge Research Hospital, now the Strangeways Research Laboratory, was founded 56 years ago for the study of rheumatoid arthritis and related diseases. Originally the research was purely clinical and pathological, but our founder, T. S. P. Strangeways, gradually came to the conclusion that these diseases would never be properly understood until more was known about the physiology of the cells of the joints. So in 1923 he closed the wards, and for the last three years of his life devoted himself entirely to the study of living cells.

But it must be confessed that for many years there was little support for Strangeways's view that the investigation of living cells would shed light on the aetiology of rheumatic diseases. Then the situation changed, and during the past decade, spectacular advances in cell biology and microchemistry have made it possible to examine the functional activities of skeletal cells from new points of view. As Strangeways predicted, experiments on living tissue *in vitro* have played a prominent part in this research, and I shall hope to show you that results are now being obtained by this means that are indeed relevant to the physiology and pathology of the skeleton.

Most of the work that I am going to talk about today has been done on cartilage and bone in organ culture.

In an ordinary cell culture, the normal architecture of the tissue is deliberately destroyed by enzymic digestion of the intercellular material, and the resulting cell suspension is cultured in flasks or bottles for long periods in a state of abnormally active proliferation. Naturally such cultures seldom give the same response to biologically active agents as the differentiated tissues from which they were derived.

But in an organ culture the histological architecture of the tissue is jealously preserved, because the object of the technique is to maintain in culture, a differentiated, functionally active tissue which will behave *in vitro* in essentially the same way as it does *in vivo*. Fortunately, in general, intact tissues grown in this way respond to biologically active agents in basically the same manner as they do in the body.

I need hardly stress the enormous advantage of this to the investigator. Here we have a tissue giving its characteristic reactions to such substances as hormones and vitamins (for review see Fell, 1964a) while isolated in a closely controlled, closed system, where not only the tissue itself, but—equally important—its humoral environment also are available for detailed biochemical study.

One of the most important contributions of electron microscopy to cell biology has been the demonstration of the complex systems of cytoplasmic membranes with which the cell is endowed (Figs 1, 2, 3). What part do all these membranous structures play in the physiology and pathology of the skeleton? For some years my colleagues and I have been trying to obtain at least part of the answer to this intricate and fundamentally important question. Today I am going to talk about some of the results of this work, and their possible application to the behaviour of the skeleton in the body.

VITAMIN A

Our research on the role of membrane systems in skeletal reactions, stems from experiments made some 15 years ago by the late Sir Edward Mellanby and myself (Fell and Mellanby, 1952). I hope you will forgive me if I very briefly refer to this early work, because it was the starting point for all that we have done since.

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Fig. 1.—Diagram of cell, showing membrane systems.
Modified from Brachet (1961) by G. H. Haggis. Reprinted from Haggis (1966), by courtesy of the author and publishers.

When an animal is given a diet containing excess of vitamin A, there is extensive bone resorption sometimes accompanied by spontaneous fractures (Wolbach and Bessey, 1942). Cartilage also is affected, and at least in some species, its matrix loses its metachromasia (Thomas, McCluskey, Potter, and Weissmann, 1960). The object of our experiments was to discover whether the vitamin

had a direct action on the skeleton, as this was a source of controversy in 1951. The obvious way to settle the matter, was to grow bone and cartilage in organ culture, in medium containing an excess of the vitamin.

The result of this procedure was dramatic. In the presence of the vitamin, embryonic (chick) cartilage and bone seemed to melt away, and after

Fig. 2.—Electron micrograph of chondrocyte (embryonic chick cartilage) showing mitochondria (m), endoplasmic reticulum (e.r.), Golgi (G), nucleus (n), cell membrane (c.m.), intercellular material (i.m.). (Photograph by A. M. Glauert.)
Reprinted from Glauert, Fell, and Dingle (1969), by courtesy of the editors.

Fig. 3.—Electron micrograph of osteoblast (embryonic chick bone) showing membrane systems. Lettering as in Fig. 2. (Photograph by A. M. Glauert.)

8-10 days hardly any intercellular material remained in the explants (Fig. 4). We obtained similar results in experiments on explanted limb-bones from foetal mice near term (Fig. 5).

I want to emphasize one very important point. The cells in these vitamin A-treated explants are *not dead*. And what is more, we found that a very high toxic dose of the vitamin was much less effective than a lower dose. So to produce this remarkable dissolution of the intercellular material, what was needed were cells that were physiologically active but physiologically abnormal.

Sir Edward and I had achieved our main objective—from our results it seemed clear that vitamin A did indeed have a direct action on bone and cartilage.

But the biochemical basis of these changes was unknown, and this extension of the research had to wait until the techniques of both organ culture and microchemistry had developed further. Ten years later (unfortunately after Sir Edward's death), my biochemical colleagues, John Dingle and Jack Lucy, and I decided that we were in a position to make another attack on this problem. To cut a very long and complicated story short, we found that in cultures of cartilaginous limb-bone rudiments from

Fig. 5.—The effect of hypervitaminosis A on late foetal mouse bones in culture; sections of tibiae from the same foetus after 6 days' growth (a) in control medium and (b) in the presence of 10 i.u. vitamin A/ml. Note the almost complete disappearance of cartilage and bone matrix in response to the vitamin. (Stain: Delafield's haematoxylin and chromotrop).

Reprinted from article by H. B. Fell in Symposium ed. H. B. Frost (1964), by courtesy of the editor and publishers.

7-day embryonic chicks, vitamin A greatly increased both the total synthesis and the extracellular release of an acid protease (Fig. 6, opposite) (Fell and Dingle, 1963), and in addition we obtained evidence that after release this enzyme was largely responsible for the degradation of the cartilage matrix (Barrett, 1966; Weston, Barrett, and Dingle, 1969). Recently John Reynolds (1968), in our Laboratory, has shown that vitamin A also causes intense

Fig. 4.—Effect of hypervitaminosis A on embryonic cartilage in culture. Serial photographs of a pair of living femora from a 6-day embryonic chick, grown in organ culture for 8 days. Left: control explant. Right: explant grown in medium to which 10 i.u. vitamin A (retinol) per ml. had been added.

Fig. 6.—Curves showing effect of vitamin A on the release of lysosomal acid protease from the limb-bone rudiments of 7½-day chick embryos. During 6 days' cultivation the explants exposed to 10 i.u. vitamin A/ml. have released far more acid protease than their paired controls in normal medium.

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resorption in culture of calvariae explanted from late foetal rats and mice, and that this bone-resorption too is accompanied by an increased synthesis and release of a similar acid protease.

The acid protease that Dingle and his colleagues have demonstrated in skeletal tissue, has the characteristics of a cathepsin, and appears to be cathepsin D (Woessner, 1967; Weston and others, 1969). Now cathepsins are lysosomal enzymes. The lysosomes (Fig. 1) are membrane-bound cytoplasmic particles which were discovered in 1955 by de Duve and his co-workers (de Duve, Pressman, Gianetto, Wattiaux, and Appelmans, 1955) at the University of Louvain. These organelles have been found to contain some 25 hydrolytic enzymes in a bound form, one of which is cathepsin D. Later in my lecture, I shall consider the question of the probable mode of action of the cathepsin on intercellular material.

Naturally we wanted to know the mechanism whereby vitamin A liberated the bound lysosomal enzymes from the cell. We still have not got a complete answer to this question, but I think that we now know the vitamin's site of action. Dingle (Dingle, 1961; Dingle and Lucy, 1962) discovered that vitamin A alters the physico-chemical properties of biological membranes. In his original experiments (Dingle, 1961), he made a lysosome-rich preparation from rat liver by appropriate homogenization methods and then added vitamin A to the fraction; in the presence of the vitamin, the lysosomes appeared structurally intact, but they rapidly released their bound enzymes.

Further work by Dingle, Lucy, and their colleagues (for review see Dingle and Lucy, 1965) showed vitamin A to be a very versatile membrane-active

substance. It proved to be a potent haemolytic agent, it caused swelling of isolated mitochondria and, when it was added to cell cultures, electron micrographs showed that every membrane system in the cell was affected. It was interesting that these effects of vitamin A had a high degree of molecular specificity (Fell, Dingle, and Webb, 1962), and out of a large group of related compounds, only those with biological activity *in vivo* produced the changes that I have described.

So these experiments on hypervitaminosis A gave us our first indication of the physiological importance of biological membranes in the response of skeletal tissue to environmental factors.

ANTISERUM AND COMPLEMENT

But very similar changes can be produced in cartilage and bone by an entirely different membrane-active agent, *viz.* a suitable complement-sufficient antiserum. We decided to investigate the effect of an antigen:antibody reaction on our skeletal explants, because certain skeletal diseases, for example rheumatoid arthritis and recurrent poly-chondritis, are often thought to be due to an auto-allergic reaction.

The first experiments on the effects of antiserum on skeletal tissue in culture were made in collaboration with Leonard Weiss (Fell and Weiss, 1965) on the explanted limb-bones from foetal mice near term. We obtained some very striking results, but these foetal mouse bones were too small for biochemical study; the more recent work that I want to talk about today was done on the much larger rudiments from 7 to 13-day embryonic chicks (Fell, Coombs, and Dingle, 1966; Dingle, Fell, and Coombs, 1967). Professor Robin Coombs took care of the immunological side of this research and John Dingle was responsible for the biochemistry.

For many of our experiments we used the humeri, tibiae and femora of 7 to 8½-day embryos. They consist largely of a continuous cartilaginous rod, but the shaft is covered by a layer of periosteal bone. These bone rudiments were grown in a chemically defined medium to which was added 5 per cent. of heated antiserum, prepared in rabbits, and 10 per cent. of rabbit serum complement; for the control cultures the serum complement was heat-inactivated. We used two different antisera: anti-Forssman, which is active against a lipo-polysaccharide component of the cell membrane, and an antiserum against washed fowl erythrocytes. Both antisera produced the same type of change in the explants.

In control medium, the rudiments enlarged to several times their original size and bone continued to form. But in the presence of antiserum and active serum complement, the explants ceased to

enlarge after a few days, and became at first flexible, then limp and gelatinous; eventually the cartilage matrix broke down altogether and the periosteal bone was largely resorbed.

The histological picture (Fell and others, 1966) was very remarkable. The cartilage matrix first lost its metachromasia (Fig. 7). The cellular partitions became very narrow and eventually they broke down altogether (Figs 8, 10a). The chondrocytes lost their characteristic appearance and assumed a fibroblastic form (Fig. 8).

Fig. 7.—Effect of complement-sufficient antiserum on embryonic cartilage. Femora from a $7\frac{1}{2}$ -day embryonic chick, cultivated for 6 days: (a) in chemically defined medium +5 per cent. (heated) antiserum against fowl erythrocytes +20 per cent. heat-inactivated serum complement; the cartilage matrix is intensely metachromatic; (b) in the same medium as (a) but with active (unheated) serum complement; note loss of metachromatic material. (Stain: toluidine blue.)

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Fig. 8.—Tibia from a $7\frac{1}{2}$ -day embryo grown for 4 days in the presence of complement-sufficient anti-fowl erythrocyte serum. The matrix of the distal hypertrophic cartilage has disappeared in the region shown at the top of the picture, and the released chondrocytes have assumed a fibroblastic form. (Stain: Azan.)

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The periosteal bone also was severely affected (Fig. 9). Osteogenesis ceased, the matrix of the existing bone was resorbed, partly by osteoclasia and partly by osteolysis, and like the chondrocytes, the osteoblasts and osteocytes became fibroblastic in appearance. Similar changes were produced in the more highly developed bone from older embryos (Fig. 11a, opposite).

Fig. 9.—Effect of complement-sufficient antiserum on embryonic bone. Tibiae from an $8\frac{1}{2}$ -day embryonic chick grown in culture for 6 days. (a) Control treated with anti-fowl erythrocyte serum and heat-inactivated serum complement. Note typical osteoblasts, new bone formation, and hypertrophic cartilage with basophilic matrix. (b) Opposite tibia grown in the presence of anti-fowl erythrocyte serum and active serum complement. The bone is largely resorbed, the osteoblasts have assumed a fibroblastic form, and the cartilage matrix has lost its basophilia; note the thin layer of necrotic tissue (n.t.) on the surface of the periosteum. (Stain: Delafield's haematoxylin and chromotop.)

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That the cells of these drastically affected explants were in fact viable was shown in the following way

(Fell and others, 1966; Fell, Coombs, and Lachmann, 1968).

Paired limb-bones from the same embryo were laid side by side in the same dish, and grown for various periods in complement-sufficient antiserum. One explant was then fixed and sectioned and the other was transferred to normal medium for a few days. The explants showed a surprising capacity for recovery. The limp, gelatinous rudiments soon became stiff and hard. When they were examined histologically, it was found that the fibroblast-like chondrocytes had resumed their normal appearance and were actively forming matrix (Fig. 10). Even

when the cartilage matrix had almost completely gone, regeneration took place. Bone also was regenerated (Fig. 11).

Fig. 10.—Recovery of cartilage from effects of antiserum. Tibiae from a 7½-day embryonic chick grown in complement-sufficient anti-fowl erythrocyte serum for 6 days. (a) was then fixed and sectioned; note the complete breakdown of cartilage matrix at either end of the shaft and severe loss of metachromasia. (b) was transferred to control medium for 8 days; the gross anatomy is very distorted, but the cartilage has regenerated (Stain: toluidine blue).

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Fig. 12.—Curves showing the effect of complement-sufficient antiserum on the release of lysosomal acid protease from 7-day limb-bone rudiments in culture. Explants exposed to anti-fowl erythrocyte serum and active serum-complement released much more acid protease than paired controls with heat-inactivated complement.

Fig. 11.—Recovery of bone from effects of antiserum. Mandibular rami from a 12-day embryonic chick exposed to complement-sufficient antiserum for 6 days. (a) was then fixed and sectioned; note atrophic periosteum, lack of osteogenesis, and osteoclasts (oc: osteoclast), (b) was transferred to control medium for 8 days; bone has regenerated. (Stain: Azan.)

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As we expected, complement-sufficient antiserum, like vitamin A, increases both the release (Fig. 12) and the total synthesis of the lysosomal acid protease and acid phosphatase (Dingle and others, 1967).

The changes produced by the antigen : antibody reaction closely resembled those caused by vitamin A, but we found that the mechanisms of action of the two agents were not identical. As I have already mentioned, vitamin A affects all the membrane systems of the cell. We have seen that it can release the bound enzymes from isolated liver lysosomes *in vitro*. But Weiss and Dingle (1964) found that complement-sufficient antibody, even when prepared against a lysosome-rich liver fraction, has no effect on a lysosomal preparation isolated *in vitro*, but it will release the enzymes from a liver slice. These results suggest that, as one might expect, the antibody and complement act on the cell membrane and not directly on the lysosomes.

PARATHORMONE

It is perhaps not surprising that parathyroid hormone which, as Gaillard (for review see Gaillard, 1961) has shown, causes rapid bone-resorption in culture, should also release lysosomal enzymes from bone explants. This was demonstrated by Gilbert Vaes (1965)—a pupil of de Duve, working in Gaillard's laboratory in Leiden.

SUCROSE

The lysosomal system of connective tissue cells can be activated in a completely different way from those I have just described, and that is by allowing the cells to ingest a non-toxic substance which they cannot digest.

de Duve's group found that, when they injected substances like dextran, polyvinyl pyrrolidone or the detergent Triton into animals (for review see Jacques, 1966), these compounds were taken up by the liver cells and accumulated in the lysosomal system. The lysosomes became very swollen and abnormally fragile. The injection of sucrose (Wattiaux, Wattiaux-de Coninck, Rutgeerts, and Tulkens, 1964) had a similar effect; normally sucrose is, of course, digested by invertase in the gut, and it is only slightly metabolized by the liver.

Quite by chance, John Dingle and I stumbled on another aspect of this sucrose effect. For reasons that I will not go into, we had to make some control experiments to find out whether the synthesis and release of lysosomal enzymes in our cultures of embryonic chick limb-bones would be inhibited by growth in a hypertonic medium. To investigate this point, we made the medium hypertonic in two ways: either by raising the concentration of salts or by adding sucrose. The bone rudiments grew quite well in both these hypertonic media, though they were rather smaller than their controls.

When Dingle investigated the synthesis and release of the lysosomal protease, a surprising fact came to

light. Fluid medium rendered hypertonic with NaCl or balanced salts had no effect on the production and secretion of the enzyme, but we were astounded to find that medium made hypertonic with sucrose, far from inhibiting the synthesis and release of the acid protease, had exactly the reverse effect and enormously increased both the production and secretion of the protease (Dingle, Fell, and Glauert, 1969) (Fig. 13).

Fig. 13.—Curves showing effect of sucrose (0.08M) on the release of lysosomal acid protease from 7½-day embryonic (chick) limb-bones during 8 days in culture. Reprinted from Dingle, Fell, and Glauert (1969), by courtesy of the editors.

When we came to examine the histological structure of the explants (Fell and Dingle, 1969), we found an extraordinary picture. The cells of the perichondrial tissue, including the osteoblasts, were distended with a mass of clear vacuoles giving the cytoplasm a honeycomb structure (Fig. 14, p. 222). This is strikingly seen in electron micrographs (Glauert, Fell, and Dingle, 1969) (Fig. 15). The periosteal bone was much affected by the sucrose (Fell and Dingle, 1969). In the controls, periosteal ossification continued quite actively, and the bone had a dense, compact structure like that of the normal embryonic skeleton (Fig. 14). In the presence of sucrose, both osteocytes and osteoblasts became intensely vacuolated, and the osteoid matrix had a rather odd appearance. It was much less dense and formed a mat of discrete fibres; it gave the impression that the interfibrillar material was missing or much reduced. Electron micrographs (Glauert and others, 1969) showed alterations in both matrix and cells (Fig. 15, opposite).

In the cartilage, however, only the chondrocytes near the articular surfaces showed this extraordinary vacuolation, and even after 8 days the deeper cartilage cells were only slightly affected. Only in the articular region did the matrix become depleted; it lost its metachromasia and diminished in amount.

Fig. 15.—Electron micrograph of osteocytes in explants of tibial bone from 11-day embryonic chicks after 8 days in culture. (a) Osteocyte in control, surrounded by a zone of collagen fibres beyond which is mineralized matrix. (b) Osteocyte in explant exposed to 0.08M sucrose; note intense vacuolation of the cell which is surrounded by degraded material in which the collagen fibres appear to be disintegrating. (Photographs by A. M. Glauert.)

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Fig. 14.—Effect of sucrose on femora from a 7½-day embryonic chick, grown in culture for 6 days. (a) Control in chemically defined medium showing periosteum, dense bone, and hypertrophic cartilage. (b) Opposite femur grown in medium containing 0.08M sucrose; note intense vacuolation and distension of fibroblasts, osteoblasts, and osteocytes, and diffuse structure of the bone matrix. (Stain: Azan.)

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From these morphological studies it seemed clear that the greatly increased amount of lysosomal protease synthesized and released from the sucrose-treated rudiments must be produced mainly by the perichondrial tissue.

When transferred to normal medium, the explants rapidly lost both their sucrose (Dingle and others, 1969) and their vacuolation (Fell and Dingle, 1969), and at the same time the synthesis and release of lysosomal acid protease returned to normal. There was no evidence that the rudiments could metabolise sucrose; when we explanted them in medium containing 0.08M sucrose but no glucose, they ceased both to take up sucrose and to secrete acid protease, and soon degenerated.

We tested the action of a number of other sugars in this system (Fell and Dingle, 1969; Dingle and others, 1969). Glucose in a 0.08M concentration had no effect (Fig. 16). Maltose and mannose,

Fig. 16.—Curves comparing the release of lysosomal protease from 7½-day limb-bone rudiments cultivated in the presence of 0.08M sucrose and 0.08M glucose. Glucose which, unlike sucrose, is metabolized by the explants, has had no effect on the secretion of enzyme.

which can be metabolized, also were ineffective, but stachyose and melezitose were even more active than sucrose.

Naturally we wanted to know how the cytoplasmic vacuoles were formed. In order to find out, my colleague Ross Munro made a ciné film of the effect of sucrose on an ordinary monolayer culture of fibroblasts. Many years ago, Warren Lewis (1931) had shown that cells actively ingest globules of their surrounding medium, which stream into the cell as clear vacuoles, pass to the interior of the cytoplasm, and then rapidly disappear. Lewis called this process pinocytosis or drinking by cells. Munro (1968) found that, when sucrose is present in a culture of fibroblasts, pinocytosis takes place as usual, but the pinocytotic vacuoles do not disappear in the normal way when they reach the interior of the cell; instead they persist, often fusing with one another until the cell is stuffed with globules of fluid. Oddly enough the cells multiply quite happily in this condition, and their growth rate is only a little less than that of the controls.

So it seems reasonable to suppose that the cytoplasmic vacuolation in our organ cultures was due to an abnormal persistence of the pinocytotic vacuoles. We have good evidence (Dingle and others, 1969) that sucrose does not increase the intake of fluid by the cells.

That there is a close connexion between pinocytosis and the lysosomal system has been clearly shown by a number of European and American workers. The functional relationship between the two processes is illustrated by the diagram (Fig. 17, opposite) drawn by Dr P. Jacques (1966), a member of Dr de Duve's laboratory.

Fig. 17.—Diagram of endocytosis. Material (solid or fluid) is engulfed by invagination of the cell membrane; the membrane-bound vesicles (phagosomes) thus produced pass into the interior of the cell where they fuse with enzyme-containing primary lysosomes to form secondary lysosomes. The ingested material is then digested, and the lysosome and its contents become a residual body, the ultimate fate of which is not entirely clear.

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It is very interesting that a dyspeptic cell that has stuffed itself with material that it cannot digest (there is no invertase in the lysosomes) should respond to the situation by this great overproduction of lysosomal enzymes. We are wondering whether, under certain pathological conditions, the ingestion of large quantities of naturally occurring substances, such as proteins, mucopolysaccharides, or their breakdown products, can evoke the same response by connective tissue cells as sucrose does. We share the view expressed by de Duve (1963) who wrote that "lysosomal enlargement with consequent evidence of apparent release [of enzymes] . . . also takes place as a result of various kinds of "over-feeding", especially with non-consumable diets, and may represent another cause of pathological changes".

It is interesting that vitamin A, antibody with complement, parathormone and sucrose, all fail to increase the release of a *non*-lysosomal enzyme, lactic acid dehydrogenase, so that the activation of the lysosomal system is not due merely to a general leakiness of the cell.

CORTISOL

Certain substances have the reverse effect to that of vitamin A or antiserum, and stabilize biological membranes. One of these is cortisol; as might be expected, it antagonizes some of the effects that I have described. A dose of as little as 0.1 μ g. cortisol per ml. of medium is effective where complement-sufficient antibody is concerned (Fell and Weiss, 1965; Fell and others, 1966) (Fig. 18).

Fig. 18.—Protective action of cortisol. Rats from a late foetal mouse, both grown in culture for 6 days in complement-sufficient anti-mouse-tissue serum. (a) Without cortisol; (b) in the presence of 0.1 μ g. cortisol/ml. medium. (Stain: Delafield's haematoxylin and chromotrop.)

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EXTRA- AND INTRACELLULAR DIGESTION

I want now to consider very briefly the mechanism that is responsible for the breakdown of the organic matrix in cartilage and bone. In this connexion I can offer a few facts stuck together with rather a lot of hypothesis.

My colleagues and I think that the breakdown is a two-stage process. Our results indicate that in the first stage the enzymes released from the cells digest certain components of the matrix. We suspect that in the second stage the breakdown products are then taken into the cells by pinocytosis or phagocytosis and that digestion is completed inside the lysosomal system; our evidence for this postulated second stage is mainly derived from electron microscopy.

It would seem that, in cartilage, extracellular digestion is more important in the degradation of the matrix than intracellular digestion, but that in bone and fibrous tissue it is probably the other way round, and that intracellular digestion plays the major role.

Cartilage.—My biochemical colleagues (Lucy, Dingle, and Fell, 1961; Barrett, 1966) have shown that the lysosomal acid protease that is secreted so actively under certain experimental conditions digests the protein moiety of the mucoprotein complex of which the matrix is largely composed; polymeric chondroitin sulphate, with some attached amino-acid residues, is thus released and the matrix disintegrates. Although the protease has an acid pH optimum, it nevertheless acts, though more slowly, at a neutral bulk pH (Woessner, 1967; Weston and others, 1969). Other lysosomal en-

zymes may also be involved in the degradation of cartilage matrix.

It is interesting that this continuous breakdown of the intercellular material in explants exposed to vitamin A (Dingle, Fell, and Lucy, 1966) or complement-sufficient antiserum (Dingle and others, 1967) does not seriously interfere with the synthesis of two important components of the matrix: hexosamine and hydroxyproline; but instead of being incorporated into new intercellular material as in the paired controls, in the treated explants, the greater proportion of the newly synthesized components is shed into the culture medium.

Electronmicrographs of the sucrose-treated rudiments (Glauert and others, 1969) give some indication that, in the articular cartilage, the chondrocytes ingest the partly degraded matrix around them, because the pinocytotic vacuoles contain material that closely resembles the surrounding matrix.

Bone and Fibrous Tissue.—The degradation of matrix in bone appears to be a far more complex process than in cartilage; in bone cultures both the

synthesis and release of lysosomal enzymes are much increased when the explants are exposed to vitamin A, complement-sufficient antiserum, parathormone, or sucrose, but what part these extracellular hydrolases play in the breakdown of the intercellular material is not yet clear. Moreover, the work of Gross suggests that a non-lysosomal collagenase may also be concerned in bone resorption (Gross, Lapiere, and Tanzer, 1963).

As I have already said, there is reason to think that intracellular digestion may be of crucial significance in the breakdown of both bone and fibrous tissue. Electron micrographs of sucrose-treated bone and fibrous tissue suggest that extracellular material has been ingested by the cells (Fig. 19).

CONCLUSION

I want to end my lecture by returning once more to the membrane systems of the cell, and indicating what we think is going on there under the various experimental conditions that I have described (Fig. 20, opposite).

The work of Novikoff and others (for review see

Fig. 19.—Electron micrograph of periosteal fibroblast in an explant of 11-day embryonic (chick) bone, grown for 8 days in the presence of 0.08M sucrose. A group of typical collagen fibres is enclosed in one vacuole, suggesting endocytosis of extracellular material. (Photograph by A. M. Glauert.)

Reprinted from Glauert, Fell, and Dingle (1969), by courtesy of the editors.

Fig. 20.—Diagram illustrating hypothesis of two-stage digestion of intercellular material. Lysosomal hydrolyases synthesized on the endoplasmic reticulum (ER) pass into the Golgi vesicles (GV); some Golgi vesicles move to the cell surface, fuse with the cell membrane (at F), and thus release the lysosomal enzymes into the environment where, possibly in conjunction with non-lysosomal enzymes, they partially digest the intercellular substance (C: collagen; CMP chondromucoprotein). It is suggested that the incompletely degraded material is taken into the cell by endocytosis, that the endocytotic vesicles (EV) fuse (F) with Golgi vesicles in the usual way to form a digestive vacuole (DV) where breakdown of the intercellular material is completed. AV: autophagic vacuole with mitochondrion in course of destruction; N: nucleus. Reprinted from Dingle (1969), by courtesy of the author and publishers.

Straus, 1967) has shown that the lysosomal enzymes, presumably synthesized on the polysomes, enter the endoplasmic reticulum and then pass into the Golgi system; there they are packaged into small vesicles—the primary lysosomes. According to our working hypothesis, some of the primary lysosomes pass to the surface of the cell, fuse with the cell membrane, and spill their enzymes into the intercellular material which is thus partially digested. We suggest that the cell ingests this partially degraded material by pinocytosis or phagocytosis, that primary lysosomes then fuse with the pinocytotic vesicles in the usual way, and that breakdown of the intercellular material is completed within the vesicles; there is reason to believe that the pH inside the vesicles is lower than outside the cell and therefore nearer the optimum for the lysosomal enzymes.

Why do membrane-active agents like vitamin A and an antigen: antibody reaction increase the secretion of lysosomal enzymes? At present we

can offer only a very tentative suggestion. We think that anything that reduces the stability of the plasma membrane is likely to promote fusion between the membrane and primary lysosomes that come in contact with it (see Fig. 20). Conversely, an agent like cortisol, which stabilizes biological membranes, might be expected to inhibit fusion between the primary lysosomes and the cell membrane, and so diminish secretion.

My talk today, on the role of biological membranes in skeletal reactions, rather reminds me of the map of Greenland in the atlas of my childhood. I remember that just within the coast-line of Greenland there was a narrow coloured border indicating the territory that had been explored; inside this border there was only a featureless white blank about which nothing whatever was known—I often used to wonder what there was in the blank area!

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