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OCCASIONAL SURVEY:

Lysosomal enzymes and inflammation

with particular reference to rheumatoid diseases

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Origin of the concept of 'lysosomes'

For as long as cells have been studied by normal light microscopy cytologists have known that many cells contain small refractile granules, about 0·5–1 μ in diameter. In living fibroblasts grown in proliferative tissue culture, they occur predominantly around the nucleus. In leucocytes they are the characteristic leucocyte granules. In other cells their refractility has caused them to be called 'lipochondria' to distinguish them from granular mitochondria which they otherwise resemble superficially. Their function in cells was totally unknown.

Knowledge of the function of sub-cellular organelles was greatly advanced by two different types of investigation. The first, namely cytochemistry, came into being in 1939 when independently Gomori and Takamatsu demonstrated the principles which allowed the activity and localization of one enzyme, alkaline phosphatase, to be demonstrated in cells in tissue sections. However, it took over 20 years before cytochemistry became generally applicable (Gomori, 1952). The second type of investigation originated from the work of Claude (1946a, b), in which it was found that the cells of a tissue could be burst open; that the contents dispersed into the surrounding medium; and that intracellular structures could be collected, amassed into 'fractions' or collections of particles of equal density (or mass) by differential centrifugation. For example, the nuclei of all the cells of the particular tissue or organ studied could be sedimented together in a nuclear pellet by relatively low-speed centrifugation, and this allowed biochemists to obtain considerable information concerning the biochemistry of nuclei. Once the nuclei had been sedimented, further and higher-speed centrifugation sedimented the mitochondria into a fairly compact pellet, which contained morphologically recognizable mitochondria (Hogeboom, Schneider, and Pallade, 1948); it was soon found that this 'mitochondrial pellet' contained two distinct components, a light and a heavy fraction. With mitochondria and nuclei removed, it was possible to centrifuge the residual matter of the cytosol (ground cytoplasm) and, with very high centrifugal forces, it was found that this contained a vast array of small particles (Chantrenne, 1947) which were visible only with the electron microscope. These were first called 'the microsomes' (now known to be fragments of the endoplasmic reticulum and ribosomes).

Thus it was possible to remove selectively enough mitochondria (or any other cellular organelle) from all the cells of a large sample of tissue to allow biochemists to investigate their properties in great detail. For this reason biochemical 'cytology' (see Roodyn, 1967) became more popular than microscopic cytochemistry which still lacked the refined techniques available to biochemists. In fact, both types of study are necessary and complementary; the value of combining both is well demonstrated by the advances in knowledge concerning lysosomes (see Dingle and Fell, 1969).

The lysosome concept

The mitochondrial pellet from rat liver contained oxidative enzymes and hydrolytic enzymes. In a series of intricate studies, de Duve and his associates at Louvain showed that they could separate the oxidative granules from the lytic granules by careful centrifugation of the mitochondrial pellet. As they purified the heavier granules they increased the specific activity of the oxidative enzymes and reduced that of the lytic enzymes and *vice versa* in purifying the lighter granules. They were finally able to purify true mitochondria, which contained cytochrome oxidase as a specific marker (Hogeboom, Schneider, and Striebich, 1952), from the lighter granules of this fraction which contained acid phosphatase as a typical marker enzyme (Berthet and de Duve, 1951). A feature of especial interest was the fact that the hydrolytic enzymes in these lighter granules were latent, that is their full activity could not be measured until the granules had been treated in such ways as would damage a normal

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membrane. Finally, it became clear that the crude mitochondrial fraction contained completely characteristic particles which possessed all the hydrolytic enzymes of this fraction but held them in a latent condition behind a semi-permeable organelle membrane (see de Duve, 1959; 1969). To these particles de Duve gave the name 'lysosome', *i.e.* lytic body (de Duve, Pressman, Gianetto, Wattiaux, and Appelmans, 1955). As first conceived, they were 'membrane-bounded granules containing five acid hydrolases in latent form' (de Duve, 1969). As extracted from various tissues, they are now known to contain very many more lytic enzymes (Table). It will be obvious from the Table that lysosomes contain all the enzymes needed to digest cells and extracellular matrices. It is still not certain whether each lysosome in any one tissue contains all the hydrolytic enzymes found in that tissue, or whether each lysosome in any one cell may contain only part of the total complement of the hydrolases of that tissue (see de Duve, 1969, p. 16; de Reuck and Cameron, 1963).

Table *Enzymes present in lysosomes of various tissues (from Tappel,* 1969)

Class of enzyme	*Example*	*Numbers described*
Proteases and peptidases	Cathepsins A, B, C and D Collagenase Arylamidases	8
Nucleases	Acid deoxyribonuclease Acid ribonuclease	2
Phosphatases	Acid phosphatase	5
Enzymes hydrolysing the carbohydrate chains of glycoproteins and glycolipids	Sialidase	11
Enzymes degrading glycosaminoglycans	Hyaluronidase	5
Lipids	Esterase	6

Normal function of lysosomes: the vacuolar system

Biochemical studies were essential for defining 'the lysosome', but if its function was to be understood the organelle had to be examined inside the cell. This work was pioneered by Novikoff (1957; 1961). His great contributions were due to the fact that he made co-ordinated studies of lysosomes by biochemical procedures, by electron and light microscopy, and by cytochemistry (*e.g.* Novikoff, Beaufay, and de Duve, 1956; Novikoff, 1961). Co-ordinated studies were also made during this same period by Straus (1958), who used the ingenious device of injecting an enzyme for studying the fate of proteins taken up into cells. The cytochemical method of Holt (1959) for acid phosphatase, based on Gomori's earlier and somewhat capricious procedure, gave a reproducible technique for the light and electron cytochemistry of lysosomes (Holt and Hicks, 1961a, b; Essner and Novikoff, 1961). A vast amount of work has now been done by electron microscopists (*e.g.* Daems, Wisse, and Brederoo, 1969), whose criteria of a lysosome is any structure of the correct order of size, which is bounded by a single membrane and which gives a positive electron histochemical reaction for acid phosphatase. (For other criteria, see Daems and others, 1969.) It may be noted that latency, which is the critical property by which lysosomal hydrolases are characterized, cannot be demonstrated by electron histochemistry because of the fixation and other processes at present needed for electron microscopy. Moreover, there are grounds for doubting the absolute reliability of the acid phosphatase reaction as seen in electron micrographs (Daems and others, 1969). But from such studies has emerged the concept of a spectrum of lysosomal forms which together form the vacuolar system of cells (de Duve, 1966; 1969). The results of these investigations may be summarized briefly as follows:

Phagocytosis by macrophages and polymorphs is a well-known phenomenon and is akin to the way amoebae ingest food by means of pseudopodia, which are large outswellings and invaginations of the cell membrane. It is now known that most types of cells engulf matter from their external environment by means of 'drinking movements' or pinocytosis (Lewis, 1931; Holter, 1959). This process involves invaginations of the cell membrane which vary from those clearly seen by light microscopy down to microinvaginations of 600–1300 Å in depth. In all these cases the cell membrane is invaginated and the margins join to engulf the outside medium; in so doing, the membrane is interiorized as a vacuole which passes into the cytoplasm. This is often called 'endocytosis'; the vacuole is the phagosome (Straus, 1958; 1961) or the phagocytic or endocytotic vacuole. According to Cohn and Fedorko (1969), the primary lysosome is 'a somewhat ephemeral organelle' (Cohn and Fedorko, 1969), which is probably made from the Golgi vesicles (de Duve, 1969; Novikoff, Roheim, and Quintana, 1965; Novikoff, 1967) and which is probably only seen by light microscopy in leucocytes as the typical leucocyte granules (Cohn and Fedorko, 1969). Primary lysosomes fuse with endocytotic vacuoles to form the digestive organelle of the cell, which is the secondary lysosome. Cohn and Fedorko (1969) consider that it is the secondary lysosome which has the characteristic cytochemical activity and the characteristic density

when studied biochemically by differential centrifugation. On the other hand, other workers consider the primary lysosome to be the normal form of the lysosome and consequently to be the organelle which is studied by these cytochemical and biochemical procedures.

Secondary lysosomes can also be formed by autophagic processes, in which parts of the cytoplasm, including mitochondria, are taken up into a digestive vacuole which fuses with primary lysosomes. This process of autophagy (Novikoff, 1959; Ericsson, 1969) can account for the turn-over of parts of cells but has been reported mainly in cells which have suffered some sort of stress or damage. These autophagic vacuoles (de Duve, 1963) have been called cytolysomes by Novikoff (1963). They have been observed in plasma cells (Page Thomas, 1969) and in A type cells of the synovial lining in rheumatoid synovia (Barland, Novikoff, and Hamerman, 1964; Wyllie, Haust, and More, 1966).

Ultimately, the digestive vacuole, whether derived by heterophagy and pinocytosis (phagosome) or by autophagy (autophagic vacuole or cytolysome), may shrink apparently by virtue of digestive processes and absorption. If it contains indigestible matter it may remain as a 'residual body' or it may move to the surface of the cell and extrude its contents to the exterior by exocytosis.

One major feature underlies all these phenomena, namely the fusion of membranes. This fusion occurs between primary lysosomes and the endocytotic or the autophagic vacuole, or between the secondary lysosome and the cell surface in exocytosis. But all these changes of the lysosomal-vacuolar system occurs without the lysosomal enzymes being allowed to enter the interior of the cell. Dingle (1968a, b; 1969) has suggested that coalescence of lysosomal and cell membranes plays a major part in the secretion of lysosomal enzymes from the cells into the intercellular matrix. He considers that, in living connective tissue, these enzymes degrade the matrix in the immediate microenvironment of the cell from which the enzymes have been extruded, and that the cell then takes up the degraded material by endocytosis. This is the 'two-stage digestion of extracellular macromolecules' (Dingle, Fell, and Glauert, 1969). But, apart from one example, there is no definite evidence that the lysosomal enzymes in a cell can leak out of the lysosomes into the cytoplasm and digest the cell in which they normally reside. The one case in which they do seem to act in this way is that in which cells ingest silica particles which are taken up into lysosomes and then react with the lysosomal membrane. This results in the extrusion of intact silica particles out of disrupted lysosomes into the cytoplasm, assumably together with the lysosomal enzymes, and leads to the death of the cell. The effect of silica on lysosomes and the possible intracellular release and effect of lysosomal enzymes have been reviewed by Allison (1969).

It is also possible that certain photosensitizing substances may affect cells by disrupting their lysosomes. Slater and Riley (1966) showed loss of lysosomal enzymes when lysosomes took up phylloerythrin and were irradiated with light. Allison, Magnus, and Young (1966) found that anthracene and also porphyrins became concentrated into lysosomes in living cells and that the lysosomes became activated when these cells were exposed to light; the cells died some hours later suggesting a direct effect, *i.e.* intracellular release of lysosomal enzymes. Both these substances produce erythema when placed on human skin and both were found in the lysosomes of the endothelial cells of dermal capillaries. However, it is not certain that this effect was not due either to the altered lysosomes influencing the production of a mediator (see later) or to the extracellular release of lysosomal enzymes.

On the other hand, in a long series of careful studies, the group of workers at the Strangeways Laboratory, Cambridge, has been able to alter lysosomal membranes considerably without damaging the cells in which the lysosomes resided; they did show, biochemically, the considerable release of lysosomal enzymes into the medium in which the cells or tissue was growing. This work was reviewed by Dingle (1969). There is as yet no definite conclusion as to the extent to which intracellular release of lysosomal enzymes affects cells; limitations of all the methods used for studying lysosomes contribute to the fact that this question is still unresolved; the fact of extracellular secretion of lysosomal enzymes, however, seems to be firmly established.

SUMMARY: CONCEPT AND FUNCTION OF LYSOSOMES

It will be apparent from the above, and from many of the chapters of the book 'Lysosomes in Biology and Pathology' (Dingle and Fell, 1969), that what began as a simple concept of a granule which contained latent lytic enzymes has grown into a maze of vacuoles of various types meandering through the cell as a complex internal digestive system. 'The lysosome' has become 'the vacuolar system' and hence a spectrum of lysosomal forms. For all that, it is still reasonable to define 'the lysosome' as a granule, normally of about $0 \cdot 5$–$1 \cdot 0$ μ in diameter, which is bounded by a semipermeable membrane within which acidic lytic enzymes of the cell are sequestered in a latent form.

Contents of lysosomes

Apart from enzymes which will degrade most proteins, lysosomes also contain hyaluronidase (Aronson and Davidson, 1965), collagenase (Mandl, 1961; Woods and Nichols, 1965; Lazarus, Brown,

Daniels, and Fulmer, 1968), and elastase (Janoff and Scherer, 1968). Consequently they are of special interest in any condition which involves loss of collagen or cartilage (other significant enzymes are listed in the Table and by Tappel, 1969). Lysosomes from polymorphs have been shown to contain phagocytin (Cohn and Hirsch, 1960); a cationic 'inflammatory' protein (Janoff and Zweifach, 1964); an activator of plasminogen (Lack and Ali, 1964); a protease which induces changes in capillary permeability (Movat, Uriuhara, Macmorine, and Burke, 1964; Uriuhara, Macmorine, and Franklin, 1965); and haemolysins (Desai and Tappel, 1965). Lysosomes have the ability to concentrate many substances which are fed to cells. This was shown dramatically by Allison and Young (1969), who found that they concentrated acridine orange; Fell and Dingle (1969) found they took up sucrose, which they were unable to digest; Persellin and Ziff (1966), Ennis, Granda, and Posner (1967), and Lorber, Cutler, and Chang (1968) showed that lysosomes of phagocytic cells concentrated gold salts which act as inhibitors of lysosomal hydrolases.

With particular relevance to erosion of extracellular matrix, enzymologists have recently been to some pains to characterize the precise nature of the proteases found in lysosomes from various tissues (Barrett, 1969). Ali (1964) demonstrated that cartilage (from the ear of the rabbit) contained a protease which he considered to resemble cathepsin B and which was inhibited by epsilon-aminohexanoic acid; chondrolysis was also inhibited by this compound. The evidence that this acid protease was cathepsin B was based on its activation by cysteine and its inhibition by chloroquine, iodoacetamide, mercury, and arginine analogues (Ali, Evans, Stainthorpe, and Lack, 1967). Dingle (*e.g.* 1969) and his colleagues have concentrated on another acid protease, present in cartilage lysosomes, which they characterized as cathepsin D. They then purified it in some bulk from rabbit, chicken, and man (Dingle, Barrett, and Weston, 1971), and produced a specific antiserum to this purified enzyme. The antiserum inhibited the action of purified cathepsin D derived from various organs and the isoenzymes of cathepsin D, but did not inhibit other enzymes (Dingle and others, 1971). They followed the degradation of chick limb-bone cartilage, as measured by the release of chondroitin sulphate, when the cartilage was treated with 'Triton X-100' which disrupted the lysosomes of the chondrocytes. They found that pre-treatment with the specific antiserum to cathepsin D gave 98 per cent. inhibition over the first 4 hrs, with less inhibition over prolonged periods (Weston, Barrett, and Dingle, 1969). The inhibition was dose-dependent and has been confirmed for the autolytic degradation of the cartilage (Dingle and others, 1971). It seems reasonably clear form these data that the autolytic degradation of chicklimb cartilage, is caused prodominantly by cathepsin D. Poole, Barrett, and Dingle (1971) have used this specific antiserum and immunofluorescence techniques to demonstrate that cathepsin D is localized in small particles, of the size of lysosomes, in cells of various tissues.

Cathepsins A, B, C, and D have been found in lysosomes from different tissues. Cathepsin D is defined by the fact that it will not act on most synthetic peptides but is active in degrading proteins, particularly haemoglobin which is used as its substrate in most assays of this enzyme. Cathepsins A, B, and C correspond in specificity to pepsin, trypsin, and chymotrypsin (see Barrett, 1969; Barrett and Dingle, 1971 for full review). There has been much discussion of which of these enzymes are present in the lysosomes of any particular tissue (*e.g.* see Barrett and Dingle, 1971); this is a difficult question at present and turns on the criteria used for the purity of the enzyme preparation. It is equally noteworthy that the intense purification required by enzymologists often results in their collecting only 10 per cent. of the proteolytic activity initially present in the sample (*e.g.* see Barrett, 1971). And there is considerable evidence that purified enzymes may be very different from the same enzymes when present in tissues (Siekevitz, 1962). The work of Dingle and his colleagues on cathepsin D, by the use of its specific antibody, is therefore a refreshing and decisive contribution to this tangled subject.

Intimately mixed up with the proteases of lysosomes are the enzymes best known as 'naphthylamidases' or 'arylamidases'. These enzymes hydrolyse leucyl-β-naphthylamide (leucine 2-naphthylamide) but are distinct from leucine aminopeptidase (Tappel, 1969). Mahadevan and Tappel (1967) demonstrated that arylamidase activity was high in purified lysosomes from various tissues and differed from microsomal arylamidases in the following features: they are inhibited by divalent metal ions and by p-chloromercuribenzoate but are activated by dithiothreitol. Snellman (1969) purified cathepsin B to give a specific activity over 200 times greater than was obtained in the crude preparation and found that this purified enzyme had strong arylamidase activity. The use of puromycin seems to be of great value in differentiating between these enzymes. It is a potent competitive inhibitor of arylamidases (Marks, Datta, and Lajtha, 1968) but does not appreciably inhibit cathepsin B (Barrett and Poole, 1969). Although its mode of action on those arylamidases which act on amino acid-naphthylamides has not been clarified, both puromycin and the puromycin aminonucleoside inhibit the lysosomal dipeptidyl arylamidase II (McDonald, Reilly, Zeitman, and Ellis, 1968) by virtue of their size and their cationic charge. The physiological function of the amino acid and the dipeptidyl

arylamidases is not known, but there has been a suggestion that they may de-activate regulatory peptides such as bradykinin (Behal, Little, and Klein, 1969). On the other hand, Hopsu-Havu, Mäkinen, and Glenner (1966) showed that one arylamidase can release the terminal lysine from the 10-peptide kallidin-10 and so form the 9-peptide bradykinin. Kallidin-10 is normally formed from the action of trypsin or of kallikrein on the serum α_2-globulin fraction.

Effect of lysosomal contents

Weissmann, Spilberg, and Krakauer (1969) prepared lysosomes from rabbit leucocytes. When lysates from these lysosomes were injected into the skin and joints of rabbits, they produced acute inflammation at the site of injection. Repeated injection into joints gave hypertrophy and hyperplasia of the synovial lining cells; round cell infiltration of the synovium in the vicinity of blood vessels; pannus formation; and cartilage erosion. These results have been confirmed by Page Thomas (1969), who reported even more intriguing findings. He prepared lysosomes from rabbit liver and injected these into one joint, or one footpad, of litter mates. The injected joint showed the same 'proliferative synovitis' as was found by Weissmann and his colleagues, but there were also similar changes in the non-injected knee joint. When the injection was into the footpad, Page Thomas found changes in that ankle and also in the non-injected ankle.

Weissmann and Spilberg (1968) showed that purified lysosomes could degrade bovine nasal cartilage. When the lysosomes were obtained from leucocytes, they found that epsilon-aminocaproic acid inhibited most of this degradation but that this substance had no effect on the break-down of cartilage induced by lysosomes obtained from liver. These observations reiterate the following facts:

(*a*) That lysosomes contain various enzymes which can degrade cartilage (Table);

(*b*) That lysosomes from different tissues or cell-types may contain different enzymes or, at least characteristic proportions of the full complement of lysosomal enzymes (see Weissmann, 1965; Chayen, Bitensky, and Poulter, 1970a);

(*c*) That, in studying a disease process, there is no substitute for the actual diseased tissue; that is to say that model systems, while helping to define the problem, can differ in critically important detail from the human disease process or diseased tissue.

Weissmann, Becher, Wiedermann, and Bernheimer (1965) produced acute and chronic arthritis by injecting streptolysin S into the joints of rabbits; they found histochemical and electron microscopical changes in the synovial lining cells 5 hrs after injection. Streptolysin S is not antigenic (Bernheimer, 1954), and Weissmann, Keiser, and Bernheimer (1963) controlled their experiments by the use of a similar streptococcal material obtained from a mutant form which lacked streptolysin S. Moreover they showed that streptolysin S could act on living cells, disrupt their lysosomes, and cause their liquefaction and death (Hirsch, Bernheimer, and Weissmann, 1963). Page Thomas (1967) pointed out that streptolysin S, being a very powerful damaging agent of cell surfaces, could be acting primarily on the cell surface. Damage to the cell membrane could affect the lysosomes inside the cell (as shown by Dumonde, Bitensky, Cunningham, and Chayen, 1965; see also Bitensky, 1963a). Page Thomas (1967) suggested that, if this were the case, it would resemble the effect on the cell membrane in the arthritis produced experimentally by fibrin or by inflammatory exudate (Dumonde and Glynn, 1962).

Janoff and Zweifach (1964) produced inflammatory change in the microcirculation by injecting cationic proteins which they had extracted from isolated lysosomes. It disrupted mast cell granules. Movat and others (1964) described a protease from lysosomes which increased capillary permeability and which was inhibited by epsilon-aminocaproic acid.

Lysosomes and immune effects

Antigenic viruses and bacteria have been shown to be taken up into phagolysosomes; endocytosis of bacteria is facilitated if they are coated with antibody or with opsonins (Allison, 1968b). It also appears that antigens are taken up into lysosomes by endocytosis and are 'processed' by them (Allison, 1968b; Weissmann, 1967). The lysosomes of polymorphs seem to be more effective in totally degrading such macromolecules than are lysosomes of macrophages. Uhr and Weissmann (1965) have shown that some ingested macromolecules can remain inside lysosomes in macrophages in an immunogenic high molecular weight form. It seems to be a general rule that the more stable lysosomes (as in polymorphs) are capable of more complete degradation of such molecules and of bacteria than are the less stable lysosomes, as found in macrophages (see in Allison, 1968b). There is a strongly held view that lysosomes of macrophages, in particular, 'process' antigens and pass them on to the lymphoid, antibody-synthesizing cells (Weissmann, 1967; Allison, 1968b). Treatment which labilizes the lysosomal membranes prolongs the 19S antibody response (Weissmann, 1967). Since production of this antibody is dependent on the persistence of antigenic stimulation, this work implies that the antigen is still present and has not been degraded (Uhr, 1964). Weissmann (1964; 1966) has argued that this type of phenomenon could be

involved in autoimmunity. Antigens and other macromolecules such as fibrin (Ghadially and Roy, 1967) are taken up by endocytosis and are degraded in the phagolysosomes. Weissmann considered that autophagy (uptake of cytoplasm and organelles into lysosomal vacuoles of the same cell) would be induced by intense endocytotic activity. If such material was not fully degraded it might be extruded from the cell and passed on to the antibody-forming cells, so producing an immune response to the original cell. This hypothesis requires cells which show autophagy and endocytosis; they should also have labile lysosomal membranes if the general impression is correct, namely that incomplete degradation with retention of antigenicity is found when lysosomal membranes are labile. All these conditions are, in fact, present in rheumatoid synovial lining cells. A similar hypothesis has been advanced by Page Thomas (1969) with reference to the work of Lack (1969).

Hollander and his associates have suggested an immunological-lysosomal mechanism for rheumatoid arthritis which has many similarities with these ideas of Weissmann (Hollander, McCarty, Astorga, and Castro-Murillo, 1965; Rawson, Abelson, and Hollander, 1965; Restifo, Lussier, Rawson, Rockey, and Hollander, 1965). They were attracted to the cytoplasmic inclusion bodies found in leucocytes in the synovial fluids of nearly all their rheumatoid patients. These inclusions apparently contained rheumatoid factor, which these workers released from the cells by sonication. They considered that aggregates of small (7S) altered gamma globulin 'might assume the role of an antigen in susceptible individuals'. Such aggregation and alteration could result from contact with antigens (Hollander and others, 1965); conceivably they might be formed by autophagic vacuoles, as in Weissmann's hypothesis. In Hollander's scheme the aggregated and altered gamma globulin would induce the formation of a 19S macroglobulin, namely rheumatoid factor, which acts as a secondary antibody. The rheumatoid factor reacts reversibly with the aggregated small gamma globulin to produce a 22S complex. This complex is then phagocytosed into phagolysosomes which release lysosomal contents which produce inflammation. In this context, he pointed out that phagocytosis of particulate antigen-antibody complexes by polymorphs was claimed to be essential in producing the inflammatory component of the Arthus reaction in rabbits (Cochrane, Weigle, and Dixon, 1959). Restifo and others (1965) demonstrated that injection of autologous and altered 7S gamma globulin into the uninvolved knees of serum-positive rheumatoid arthritics elicited an acute severe arthritis. One out of the six patients tested gave a negative serum latex-fixation test and he did not respond.

Lysosomes and the effect of anti-inflammatory drugs

As it has been shown above, lysosomal contents are capable of inducing inflammation. It is significant that, conversely, the substances which stabilize lysosomes include many which are well known for their anti-inflammatory effects. The only substances which have definitely been shown to stabilize lysosomes in life are cortisone and cortisol (see Allison, 1968b; Weissmann, 1969). Among the substances which stabilize isolated lysosomes are prednisone, beta-methasone, chloroquine, and various antihistaminic drugs such as chlorpromazine. This subject has been well reviewed by Allison (1968b) and by Weissmann (1968; 1969). There is some controversy whether aspirin stabilizes isolated lysosomes. Weissmann (1969) commented on the lack of apparent stabilizing effect of many non-steroidal anti-inflammatory drugs, such as salicylates, indomethacin, flufenemic acid, and colchicine, on isolated rabbit liver lysosomes. He did not exclude the possibility that they could act as stabilizing agents when administered to whole cells or tissues, by an 'indirect effect'. This is a generalized phenomenon; Fell (1969) has emphasized that even isolated cells do not respond to hormones and similar substances in the way that the cells of an organ culture will react.

For this reason the present authors have preferred to test the effect of anti-inflammatory drugs on human skin maintained in non-proliferative organ culture and to measure the effect of these drugs, and of inflammatory stimuli, on the lysosomes inside the cells of the organ culture (see Chayen, Bitensky, Butcher, Poulter, and Ubhi, 1970b; the methods used in such studies are considered later in the discussion of rheumatoid arthritis). The basic observations are as follows:

When mouse skin is painted with crotonaldehyde, a typical inflammatory response is seen. The lysosomes of the skin have been shown to become more permeable. Similar changes in subcellular membranes, including those of the lysosomes, have been reported in mouse skin injected subcutaneously with histamine, and in human skin maintained *in vitro* and subjected to histamine (10^{-6} to 10^{-4}M for 2–5 min.). Various anti-inflammatory agents, including aspirin, indomethacin, phenylbutazone, and topical steroids, have been shown to have quantitatively distinguishable effects in protecting the lysosomal membranes of such cultured skin from the damaging effect of histamine. This system has been used towards a quantitative assessment of anti-inflammatory potency (Chayen and others, 1970b).

CRYSTAL SYNOVITIS

A relation between deposits of crystals and the

symptoms of gout was suspected by Garrod (1876). Riehl (1897) reported the presence of crystals in regions which showed gouty necrosis and described phagocytosis of crystals by polymorphs and by mononuclear leucocytes. Freudweiler (1899) injected microcrystals of various urates, of xanthine, hypoxanthine, calcium carbonate, and creatinine subcutaneously into a number of animal species and found that all gave acute inflammatory responses which included phagocytosis of crystals by mononuclear and polymorphonuclear leucocytes. These effects were rediscovered by Seegmiller, Howell, and Malawista (1962) and by McCarty and his colleagues (McCarty, 1968). They led Seegmiller to suggest the vicious-cycle phenomenon in gout (Seegmiller and Howell, 1962): urate crystals precipitate in the tissue or synovial fluid and are phagocytosed by leucocytes. This results in a local production of lactic acid and consequently a drop in pH which causes further precipitation of urate from the exudate which, being in equilibrium with hyperuricaemic blood, is already supersaturated with urate. (It is likely that this hypothesis is an over-simplification, because urate crystals have been found, *e.g.* by Watts, Scott, Chalmers, Bitensky, and Chayen (1971), in tissues of patients whose blood levels of urates were well below the saturation level.)

McCarty (1968), in particular, has stressed that inflammation induced by crystals (urate in gout or pyrophosphate in 'pseudogout') is dependent on the presence of polymorphs (see also Phelps and McCarty, 1966). There is much evidence to support this contention, even though McCarty (1968) and the present authors have observed synovial fluids which contained very many crystals but few polymorphs, none of which had ingested crystals. The significance of these findings is unknown.

The crystals are phagocytosed by polymorphs into an endocytic vacuole and the cells degranulate (Weissmann, 1966). This process appears to involve the fusion of the granules, namely the lysosomes, with the endocytic vacuole to form a phagolysosome (Zucker-Franklin and Hirsch, 1964; Zucker-Franklin, 1966). There is some evidence (*e.g.* Allison, 1968a) that when lysosomes take up indigestible particles they may leak their enzymes into the cell (but see discussion of this subject, above); certainly Dingle and others (1969) found that, when cartilage and bone cells, in organ culture, took the indigestible sugar sucrose into their lysosomes, the lysosomes became swollen and there was synthesis of lysosomal enzymes which were found free in the culture medium. These results would indicate that lysosomal enzymes might be lost selectively from the polymorphs which have ingested crystals. On the other hand, studies like those of Jasani, Katori, and Lewis (1967) have shown that, in rheumatoid synovial fluid at least, the concentration of cytoplasmic enzymes such as lactate dehydrogenase was as greatly increased as was that of lysosomal enzymes. These results indicate that the free enzymes in the synovial fluid probably come from the disintegration of cells, particularly polymorphs, rather than by selective release. Of particular relevance to the fate of urate crystals is the fact that, in polymorphs, myeloperoxidase is apparently present in the lysosomes (Howell and Seegmiller, 1962; Woodin, French, and Marchesi, 1963; Cohn and Fedorko, 1969; Beaufay, 1969) and that slow uricolysis can be brought about by such peroxidases (Canellakis, Tuttle, and Cohen, 1955). As much as 5 per cent. of the whole dry weight of human polymorphs is myeloperoxidase (Schultz and Kaminker, 1962).

Rajan (1966) studied the behaviour of human polymorphs while they were presented with microcrystals of urate. Degranulation occurred within a few minutes once the cells had taken up a crystal; the cells remained viable as observed with phase-contrast microscopy or by vital staining with acridine orange. When the cells had been treated with colchicine, Rajan found that some cells failed to phagocytose, while others, which ingested crystals, failed to degranulate. Although McCarty (1968) found it technically difficult to confirm these findings and those of Howell and Seegmiller (1962), he conceded that the isotopic-labelling experiments of the latter authors provided convincing evidence that colchicine may act in this way in spite of other conflicting evidence. However, he considered it unlikely that this was the whole explanation which might also involve inhibition of polymorph motility and other metabolic effects.

Kinins at concentrations of 1,000–5,000 ng/ml. (equivalent to 10^{-7}M bradykinin) cause migration of leucocytes (Eisen, 1969). Eisen and Keele (1966) incubated cell-free synovial exudate with microcrystals of sodium urate and found that the crystals produced $1\text{–}5 \cdot 10^{-7}$M bradykinin in the exudate. Apparently urates and other negatively charged particles, including pyrophosphate and glass (Kellermeyer and Breckenridge, 1965), activate factor XII (Hageman factor) which is present in normal human and canine joint fluids (Kellermeyer and Breckenridge, 1966) and which converts kallikreininogen to kallikrein; this changes kininogens into active kinins (McCarty, 1968; Eisen, 1969). Goldfinger, Melmon, Webster, Sjoerdsma, and Seegmiller (1964) reported elevated levels of kinins in gouty joints and in other inflammatory arthritides, and Melmon, Webster, Goldfinger, and Seegmiller (1967) have shown that the joint effusions contained sufficient kinins to dilate blood vessels and possibly to excite pain receptors. It should be mentioned that some other evidence of McCarty (1968) concerning the effect of carboxypeptidase, which in-

activates bradykinin by converting it to the inactive octapeptide, casts a little doubt on the critical role of kinins in mediating the inflammatory reaction to crystals.

As discussed by Eisen (1969), Hageman factor is also activated by proteolytic enzymes liberated from phagocytosing leucocytes. Similarly, the digestion of cartilage by lysosomal enzymes liberates chondroitin sulphate which also activates this factor. Other lysosomal enzymes appear to act directly on kininogen to release kinins. The released kinins could then attract more leucocytes and so build up the inflammatory process.

RHEUMATOID ARTHRITIS

Nearly 10 years ago, Dingle (1962) suggested that the destruction of articular cartilage in arthritis was due to the local release of lysosomal enzymes from cells which were closely applied to the cartilage (also see Dingle, 1969). Weissmann (1964) put forward a similar hypothesis, emphasizing the possible function of lysosomes in autoimmune disease generally and rheumatoid arthritis specifically. The great bulk of knowledge which now exists on the role of lysosomes in the breakdown of cartilage and in the experimental induction of arthritis has come from the outstanding experiments of Dr. Weissmann and his colleagues in New York, and those of the group of workers at the Strangeways Laboratory in Cambridge under Dame Honor Fell and Dr. Dingle.

The inner surface of the joint capsule and all intra-articular structures of the diarthrodial joint, except that of the articular cartilage, are lined by what used to be called 'the synovial membrane'. The name is a misnomer because this tissue is derived from embryonic mesenchyme and the synovial lining cells appear to be modified mesothelial cells which lack a true basement membrane (Ghadially and Roy, 1969). It is therefore probably best to refer to synovial tissue, which is the intimal layer of synovial lining cells, one to four cells in depth in the normal tissue, and subsynovial tissue which can have various forms (Castor, 1960). The synovial lining cells abut close to the articular cartilage junction. Two cell types, designated Type A and B, have been distinguished by electron microscopy (Barland, Novikoff, and Hamerman, 1962): Type A cells characteristically have prominent Golgi complexes, many smooth-walled vacuoles, and little rough endoplasmic reticulum, while Type B cells have much rough endoplasmic reticulum (suggesting that they are involved in protein synthesis) but only scanty Golgi complexes or smooth endoplasmic reticulum. In human normal synovia there are more Type A than Type B cells. Intermediate forms are often seen, so that Ghadially and Roy (1969) have suggested that there is only one basic cell type which varies according to its functional activity.

Rheumatoid arthritis involves inflammatory changes in the synovial tissues and erosion of the articular cartilage. We have seen (above) that injection of the contents of lysosomes into joints produces both these changes. Moreover, substances which are found to be of value in inflammatory diseases of joints, such as corticosteroids, chloroquine, gold salts, and colchicine, all influence lysosomes and lysosomal enzymes (Weissmann, 1969). There is, therefore, experimental and circumstantial evidence that lysosomal enzymes may be involved in rheumatoid arthritis. The question was to decide from which cells these enzymes were derived.

The presence of lysosomal enzymes in the synovial fluid in rheumatoid joints is well documented (Jacox and Feldmahn, 1955; Lehman, Kream, and Brogna, 1964). Caygill and Pitkeathly (1966) found elevated levels of lysosomal enzymes but not of alkaline phosphatase; increased concentration of the latter would be expected if the enzymes of the synovial fluid were derived from leucocytes. This led Ghadially and Roy (1969) to suggest that the lysosomal enzymes of the synovial fluid were derived from the excessive numbers of lysosomes seen in the Type A cells of the rheumatoid synovial tissue (Barland and others, 1964; Wyllie and others, 1966; Norton and Ziff, 1966; Ghadially and Roy, 1967). On the other hand, Jasani and others (1967) found that the concentration of other cytoplasmic enzymes in rheumatoid synovial fluid was as greatly elevated as was that of lysosomal enzymes.

The stronger indication that the enzymes which erode cartilage come from the cells of synovial tissue is the finding of Ball (1968) that erosion of cartilage in rheumatoid joints proceeds from the outer edges inwards, *i.e.* from the junction of the cartilage with synovial tissue. Had it been caused by the enzymes in the synovial fluid, erosion would occur simultaneously or patchily over the cartilage surface; had the enzymes been released from the chondrocytes the erosion would begin inside the cartilage and proceed outwards. Hence it seems that the erosion of cartilage in rheumatoid arthritis may represent a particular example of the concept of Dingle (1968b; 1969) of the transport of lysosomal enzymes by exocytosis, possibly in small packages, as used normally for the extracellular digestion of intercellular matrix.

Electron microscopy of rheumatoid synovial lining cells

The ultrastructural changes seen in rheumatoid synovial tissue have been fully reviewed and illustrated by Ghadially and Roy (1969). The polymorphs and mononuclear cells in joint effusions show increased phagocytotic activity with many micro-

pinocytotic vacuoles. Phagocytosis also seems to be increased in the synovial lining cells, apparently aided by increase in length and number of the filopodia of these cells. Cellular debris and fibrinoid matter, including what seems to be true fibrin, have been detected in the phagosomes of these cells. Golgi complexes are reduced in size, a phenomenon which does not accord simply with the increased number of lysosomes found in the synovial cells if, in fact, new synthesis of lysosomes takes place in the Golgi apparatus. The lysosomes in rheumatoid cells do not seem to be more variable in structure than are those of nonrheumatoid synovial tissue. Cytolysomes (autophagic vacuoles) also appear frequently.

Cytochemistry

Ultrastructural studies have demonstrated the presence of what seem to be lysosomes in the synovial lining cells of rheumatoid and of non-rheumatoid joints (as discussed above). There are more of these structures in the former than in the latter. Otherwise no difference between these structures has been detected in the diseased and normal tissue. The presence of more lysosomes per cell does not imply that more lysosomal enzymes leak out of these cells. It is noteworthy that the electron micrographs show apparently normal single membranes bounding these structures in both normal and rheumatoid synovial cells. To determine whether the lysosomes in rheumatoid synovial cells are *functionally* different, it is necessary to test the function of the lysosomal membranes in such cells. Some assessment of this is achieved in conventional homogenate biochemistry by measuring the free : bound ratio of activity, but this, in fact, assesses how well the membranes resist their destruction by the homogenizing process (Allison, 1968b). A more direct test of the permeability of the lysosomal membranes, and of the latency of the lysosomal enzymes, is to measure the activity of lysosomal enzymes retained inside the lysosomes before and after experimental alteration of the permeability of the lysosomal membranes. This can be achieved by cytochemistry (see Bitensky, 1963b; see de Duve's agreement with this approach: de Duve, 1963). In Bitensky's fragility test (Bitensky, 1963b; Allison and Malucci, 1965; Allison, 1968b; Page Thomas, 1967), sections are exposed to β-glycerophosphate in an acidic acetate buffer. This substrate penetrates only slowly through normal lysosome membranes so that the normal lysosomes show little activity over short periods of incubation. With prolonged incubation in this buffer, the lysosomal membranes become more permeable; this treatment was used by de Duve and others (1955) to disclose the otherwise latent lysosomal enzymes. Consequently, in cytochemical studies, more enzyme activity becomes apparent because more substrate can now pass through the lysosomal membrane. In agreement with the results of Page Thomas (1967) who used this procedure, Bitensky and Butcher (unpublished data) found that it required 20-60 min. of incubation to disclose discrete, particulate acid phosphatase activity in nonrheumatoid synovial lining cells, but only 2-5 min. to disclose the same amount of activity in rheumatoid synovial lining cells. It is possible to use kinetic studies to extend such investigations to obtain more precise information concerning the function of the lysosomal membrane (*e.g.* Chayen, 1968).

Apart from its use in assessing the function of lysosomal membranes, cytochemistry is important for demonstrating which are the cells which are active enzymatically. Although Luscombe (1963), Hendry and Carr (1963), and Barrett and Poole (1969) could show that rheumatoid synovial tissue contained cathepsins, acid phosphatase, and β-N-acetylglucosaminidase, they could not demonstrate which cells were the most active for these enzymes. In contrast, cytochemical methods demonstrated that two different cell types of rheumatoid synovial tissue each contained their own elevated pattern of lysosomal enzymes (Chayen and others, 1970a; Chayen, Bitensky, Butcher, and Cashman, 1971). These methods also disclosed in the rheumatoid synovium that, in the cells adjacent to the articular cartilage and at the point where the cartilage had lost its metachromasia, the lysosomal membranes were virtually functionless as permeability barriers to enzyme substrates (Chayen, Bitensky, and Butcher, unpublished data).

Microdensitometry

The contributions which cytochemistry can make to the analysis of lysosomal function in rheumatoid arthritis have been enhanced by the development of scanning and integrating microdensitometry for the quantitative measurement of enzyme activity in each selected cell. (The principles of such microdensitometry have been discussed by Chayen and Denby, 1968; these techniques, and their application to the study of lysosomes have been reviewed by Chayen and Bitensky, 1968; Chayen, Bitensky, Butcher, and Poulter, 1969a; Chayen and others, 1970b; see also Bitensky, Butcher, and Chayen, in the press.) These methods have been used in simple determinations and in kinetic studies of lysosomal enzyme activity and, more significantly, of lysosomal membrane permeability. They demonstrated that, in the synovial lining cells of nonrheumatoid synovium lysosomal membrane restraint imposed almost 50 per cent. latency of intralysosomal enzyme, *i.e.* twice as much activity could be demonstrated in unit time if the membranes were made fully permeable. In synovia

from recently traumatized joints, the lysosomal membranes in the synovial lining cells were slightly more permeable, imposing only about 30 per cent. latency to the selected substrate. But, in synovial lining cells from rheumatoid synovia, the lysosomal membranes were virtually functionless. They imposed no latency on the lysosomal enzyme; treatment with acetate buffer at pH 5·0 produced progressive loss of enzyme from the lysosomes in contrast to the increased intralysosomal activity found in all nonrheumatoid synovial lining cells (Chayen, Bitensky, Butcher, and Poulter, 1969b; Chayen and others, 1971). It seems clear, therefore, not only that there are more lysosomes in each synovial lining cell in rheumatoid synovia (Ghadially and Roy, 1969) but that the lysosomal membranes are strikingly altered as regards their function as impermeable membranes.

Redox and lysosomes

Further microdensitometric studies demonstrated that the reduction-oxidation (redox) balance in rheumatoid synovial lining cells was altered towards a more reductive (hydrogen excess) state (Chayen and others, 1969b). Confirmation for this conclusion came when it was found possible to maintain rheumatoid synovial tissue in non-proliferative maintenance culture (adult organ culture) only if the pH was exceptionally acidic for this type of culture, namely at pH 7–7·2; in contrast, nonrheumatoid synovial tissue had to be maintained at about pH 7·8 for full viability (Poulter, Bitensky, Cashman, and Chayen, 1970). Measurement of tissue pH, by the use of pH indicator dyes, although obviously imprecise, also showed that the rheumatoid tissue was more acidic than the nonrheumatoid synovium (Poulter and others, 1970).

These results led Chayen and others (1969b) to test the effect of hydrogen donors and acceptors on synovial tissue maintained *in vitro*. They found that hydrogen-acceptors, like 2-methyl-1, 4-naphthoquinone or dehydroascorbate, removed the hydrogen-excess from rheumatoid synovial lining cells, so restoring the redox balance; concomitantly they restored normal function to the lysosomal membranes. Equally hydrogen donors, such as ascorbate, produced a more reductive redox balance in nonrheumatoid synovial lining cells and also decreased the latency of their lysosomal enzymes. In agreement with these experiments, Lucy and Lichti (1969) suggested that the well-known effect of vitamin A in labilizing lysosomal membranes could be due to the vitamin acting as an electron (hydrogen) donor.

Thus it would seem that the abnormality of lysosomes in rheumatoid synovial lining cells may be related to a fundamental redox imbalance in these cells. It is not yet known whether this effect is specific to the rheumatoid condition. In the range of nondiseased tissue, however, it does not occur in synovial tissue from recently traumatized joints.

Recently, Butcher and Chayen (1971) have searched for a possible cause of this redox imbalance. The reduction-oxidation state of cells is dependent, at least in part, on the rate of production of the reduced co-enzyme NADPH (in the oxidation of sugar-phosphates such as glucose-6-phosphate) and its rate of oxidation. That is to say, the redox balance of the cytoplasm depends on the ratio of NADPH : NADP. If there is a great excess of NADPH which cannot be oxidized by the tissue, the hydrogen from the NADPH can be available to reduce other cytoplasmic systems and so render the redox balance of the cytoplasm more reductive. They have found that rheumatoid synovial lining cells can produce NADPH four times as fast as nonrheumatoid synovial lining cells, although they oxidize it at only the same rate. It is of special interest that certain steroids have marked effects on the production and disposition of NADPH (Altman and Chayen, 1970; Butcher and Chayen, 1970).

SUMMARY: POSSIBLE ROLES OF LYSOSOMES IN RHEUMATOID ARTHRITIS

It is unlikely that rheumatoid arthritis is caused primarily by a lysosomal defect. But, as Weissmann (1966) indicated, joint diseases of various aetiologies may well be mediated by a 'final common pathway' in which lysosomes play a major role. They can act in the following ways:

Firstly, all theories require that lysosomes become 'activated' and that their membrane alters so that they either become so permeable that their contents leak out or so that they actively excrete their products by exocytosis. Alteration in the nature of the lysosomal membranes would conceivably make them more able to fuse with the cell membrane and to extrude their contents (Dingle, 1968b; 1969). The fact that the lysosomal membranes have become altered in rheumatoid synovial lining cells has been demonstrated (Chayen and others, 1969b; 1971). The contents of lysosomes can induce inflammation and all the changes associated with rheumatoid arthritis (Weissmann and others, 1969). They can facilitate the production of kinins and so attract leucocytes. Equally, the extruded lysosomal enzymes can digest cartilage; the chondroitin sulphate so liberated can also induce the production of kinins (Eisen, 1969). Extracellular digestion of cartilage, and of other extracellular matter, produces degraded proteins which are phagocytosed into phagolysosomes. These lysosomes 'process antigenic material' and could give rise to antigenic material and so set up an autoimmune response (Weissmann, 1964; 1966). Moreover increased pinocytosis may give rise

to increased autophagy in which the cells break down their own components and release them as 'new antigens' for autoimmune reaction. Increased pinocytosis and autophagy have been reported in synovial lining cells (Ghadially and Roy, 1969).

Thus lysosomal contents can erode cartilage; produce acute and chronic inflammation in joints; produce the effects of autoimmune disease; and induce kinin formation and leucotaxis. It is noteworthy that many of the drugs, including hydrocortisone, which have been found to be valuable in alleviating the symptoms of rheumatoid arthritis, have also been shown to be stabilizers of lysosomal membranes (Weissmann, 1968, 1969; Chayen and others, 1970b).

The aetiological factor which starts this whole process in rheumatoid arthritis is not known. Hollander and his associates (above) have suggested that the process begins with the formation of a peculiar immunological complex which becomes ingested into phagolysosomes. Ghadially and Roy (1969) consider that it could be initiated by phagocytosis of bacteria or viruses. Excessive phagocytosis of fibrin, possibly due to inefficient fibrinolysis, might trigger-off this chain of events (Page Thomas, 1969, quoting Lack; see also Lack, 1969). Abnormal amounts of fibrinoid matter in phagolysosomes in synovial lining cells have been reported (Ghadially and Roy, 1969). Chayen and others (1969b) have implicated a redox imbalance which might be induced by physiological hormonal imbalance or which could be the response of the cells to some other unknown stimulus. In fact, it would not be surprising if the release of lysosomal contents from abnormally permeable lysosomes in the cells in the vicinity of the articular cartilage might produce the condition of rheumatoid arthritis in response to many different stimuli.

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