PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 165, 170–177 (1980)

The Isolated Hepatocyte: A Cellular Model for Aging Studies (40954)

D. L. KNOOK

Institute for Experimental Gerontology TNO, Rijswijk, The Netherlands

Abstract. Based on the concept that the absence of cell division leads to senescence and that aging changes are most clearly manifest in long-lived postmitotic cells, isolated hepatocytes are proposed as a suitable model for the study of cellular aging. These cells, aged *in vivo* under normal physiological conditions, will be of the same age as the donor after isolation. Several possibilities and advantages of this system are discussed. Examples given of the use of isolated hepatocytes for aging studies include the investigation of the relation between age-related alterations in rat liver functions (drug clearance, albumin synthesis) and aging processes at the cellular level. The potential use of isolated hepatocytes for studies on basic cellular aspects of aging is illustrated by experiments on changes in the activities of lysosomal enzymes as well as their multiple forms. A new additional approach to the study of cell aging, particularly with regard to phenomena such as cell contacts, cell recognition, and mutual influences between cells of various types, is offered by the possibility of cocultivating hepatocytes and sinusoidal cells from young and old rat livers.

Aging and cellular division capacity. On the basis of proliferative activity, mammalian cells can be classified into three types: (a) continually dividing intermitotic cells (hemopoietic stem cells, fibroblasts); (b) postmitotic cells, formed early in life and not replaceable after their specialization (neurons, striated muscle cells); (c) reverting postmitotic cells with a potential dividing capacity triggered only after a special stimulus (hepatocytes, osteocytes).

As recently reviewed (1), there is growing evidence that long-lived postmitotic cells exhibit the most pronounced symptoms of cellular senescence and that organisms age more as a consequence of detrimental changes in their nondividing cells than as the result of a limited renewal capacity of intermitotic cells (1, 2). Thus, senescence is not caused by the loss of division capacity; on the contrary, the absence of cell division causes senescence (1). Accepting this concept, studies on cellular aging should primarily deal with age-related changes in postmitotic cells and reverting postmitotic cells, which together probably constitute over 90% of the total cell number of mammals (1).

Impairment of cell function. It is difficult to obtain conclusive evidence of intrinsic cellular causes for the senescence in postmitotic cells *in vivo*. Functions of cells in their natural surroundings are influenced by several extracellular factors, which may be primarily responsible for a decrease in cell functions with age. The physiological changes in blood circulation and distribution are good examples. As a result of aging changes in cardiovascular dynamics, a redistribution of the blood volume in several organs will occur (3). In general, the result will be a decreased blood supply to organs and the cells constituting these organs will be hampered in several of their functions.

Evidence for the impairment of functions of postmitotic cells with age has come mainly from microscopical observations of structural changes as well as from biochemical studies, e.g., with tissue homogenates. Both types of studies have inherent limitations. Regarding microscopical studies, the relation between structural and functional changes in most cellular organelles is far from clear. The main problems in the biochemical studies involve (a) the cellular heterogeneity of the starting material, resulting, for example, in preparations of cellular organelles derived from various cell classes; (b) harmful effects of the preparation procedure on the experimental material; and (c) the loss of cytoplasmic factors during homogenization and isolation.

Isolated long-lived cells as models for

0037-9727/80/100170-08$01.00/0
Copyright © 1980 by the Society for Experimental Biology and Medicine.
All rights reserved.

aging studies. Most of the theoretical and experimental problems concerned with the *in vivo* studies on cellular aging as well as with the biochemical studies can be solved by another approach to the study of cell aging which is based on the use of suspensions consisting of single postmitotic cells. The basic idea behind this approach is that postmitotic cells aged *in vivo* under normal physiological conditions and isolated from donors of several ages will be of the same age as the donor. In this way, old cells obtained from an old individual can be compared with young cells isolated from a young individual. This experimental model system has been used by our group for the study of aging in postmitotic cells [for reviews, see (4–6)], while on the basis of experimental evidence, Schneider and Mitsui (7) have proposed the system of isolating fibroblasts from young and old donors as an appropriate one for studying aging in intermitotic cells. Another general advantage of using isolated cells is the great potential for comparison of findings on isolated cells with observations or results obtained *in vivo*.

Of great importance in the potential usefulness of the proposed model system is the choice of starting material. If possible, the following prerequisites should be fulfilled: (a) Preference should be given to an organ which consists primarily of only one type of postmitotic cell with a well-understood relationship between physiological and functional competence at the organ level and biochemical processes occurring in the distinct cell type. (b) With regard to aging, clear changes in the functional capacity of the organ of choice as well as of the cells constituting the organ should be described; ideally, there should be indications for a mutual relationship. (c) Practical prerequisites include the technical possibility of isolating sufficient cells in a viable state and with measurable functions.

Organs consisting primarily of postmitotic cells are brain, heart, and liver. The brain is perhaps the first organ to come to mind as a potential source for postmitotic cells. On second thought, it will be clear, however, that, apart from technical problems with regard to cell isolation and determination of functions in isolated cells, the gap in our knowledge of the relation between changes in brain functions and the underlying cellular mechanisms is still far too great for a good exploration of this system. In addition, although neurons exhibit characteristic aging phenomena such as cell death and the accumulation of lipofuscin, these aging changes are to a considerable extent restricted to certain specified areas of the brain and are most probably not common to all brain cells (8). Similar considerations hold for physiological and cellular aging changes in the heart (8). Fortunately, as will be outlined below, the hepatocytes in the mammalian liver appear to fulfill most of the requirements for a good model system for studies on cellular aging.

Hepatocytes and cellular aging. Isolated hepatocytes are particularly suitable as a model for the study of cellular aging for a number of reasons.

(a) The two main cell types in the mammalian liver are hepatocytes or parenchymal cells and sinusoidal lining cells. The hepatocytes occupy about 90% of the total liver volume. They can be considered as reverting postmitotic cells with a low mitotic incidence of about 0.01% under normal physiological conditions (9, 10). Consequently, nearly all parenchymal cells live for as long as the rat does after they have differentiated (11).

(b) There is well-documented evidence for an age-related decline in several functions of the liver in man, the mouse, and the rat. The drug-metabolizing capacity in the rat (12, 13) and the plasma clearance of drugs metabolized by the liver in man (14) show a decrease with advancing age. There are also indications for an age-dependent decline in cholesterol synthesis (15) and bile salt synthesis (16) as well as for a latency in several adaptive responses (17–19). Although in the hepatic response to some hormonal stimulations changes may be due to aging in extrahepatic systems (17, 18), a functional alteration of the aging liver is also involved in several cases (17, 19). With regard to the hepatocytes, stereological analyses of the livers of male rats (20) and

female rats (21, 22) of various age groups revealed definite age-related changes in the ultrastructure of these cells.

(c) A procedure which allows the isolation of hepatocytes from rats ranging in age from 2 weeks to 36 months has been developed (23, 24). Parenchymal cells with a purity of at least 95% are obtained. The yield is 35–40 × 10^6 cells per gram wet weight of liver and does not significantly change with the age of the donor rat. Cells from both young and old rats have a normal ultrastructural appearance. Recent studies have also demonstrated that the functional capacity of the hepatocytes isolated from young rats closely resembles that of hepatocytes in the perfused liver [for a review, see (6)]. The availability of intact hepatocytes in suspension provided the opportunity for further separation of different subpopulations according to the degree of cellular ploidy (25). It is also now possible to isolate pure suspensions of the two main types of sinus lining cells, viz., Kupffer cells and liver endothelial cells (26, 27). This offers the possibility of making interesting comparisons on cellular aging in long-lived hepatocytes and sinus lining cells which turn over rapidly (4, 5).

(d) The pure hepatocyte suspensions are suitable for the preparation of primary monolayer cultures [for a review, see (28)]. This is a useful system for studying the effects of complex extrahepatic factors which may influence the cellular aging process *in vivo*. The effects of hormones, toxins, drugs, and chemical carcinogens on the hepatocytes can now be studied *in vitro*. A new approach to the study of cellular aging is offered by the possibility of cocultivating hepatocytes and sinusoidal cells from young and old rat livers (29). In this way, the roles of cell contacts, cell recognition and aggregation, cellular polarity, and mutual influences can be ascertained. This system will be described in more detail later on.

(e) The functional, cytochemical, and biochemical techniques available for studying the liver are advanced and they can now be applied to isolated hepatocytes, various subpopulations of hepatocytes, and hepatocytes in maintenance culture. This makes possible correlated studies on the cellular basis of aging changes in liver functions.

Several examples of the use of isolated hepatocytes for studies on cellular aging are given below.

Cellular basis of liver aging studied with isolated hepatocytes. One of the major functions of the liver is the transfer of a number of organic anions, including steroids, bilirubin, and many drugs, into the parenchymal cells and finally into the bile. The capacity of the human and the rat liver to take up these components can be determined *in vivo* by the clinical bromsulfophthalein (BSP) retention test. As recently reviewed (6), several authors have reported an age-related decrease in functional liver capacity when this test was used in various groups of aged people. Also for female rats of two different strains, the BSP retention test showed a decrease with advancing age (6, 30).

As a first step in the study of BSP uptake by isolated hepatocytes, the mechanism of BSP transfer from the plasma into the cells was investigated. The rate of BSP uptake appeared not to be proportional to the BSP concentration in the medium outside the cell membrane, but approached a maximum level, indicating a carrier type of transport (31). Furthermore, it could be concluded from the observations that because free BSP was transported into the hepatocyte against a concentration gradient and BSP uptake was dependent on temperature and metabolic energy, BSP is transferred by an active transport system (31).

Hepatocytes from 3-month-old female WAG/Rij rats appeared to store 11.9 nmole BSP/10^6 cells or 8.1 nmole BSP/mg cellular protein (Fig. 1). A sharp decrease in the amount of BSP stored by the hepatocytes was observed between 3 and 12 months of age; this was followed by a less pronounced decline up to 36 months (Fig. 1). These data strongly suggest that the observed decrease in the capacity of the liver to remove BSP from the blood is at least partly due to an age-related decline in the BSP storage capacity of the individual hepatocytes.

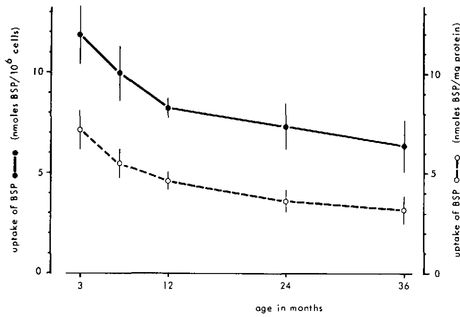

FIG. 1. BSP storage capacity of hepatocytes isolated from 3-, 7-, 12-, 24-, and 36-month-old female WAG/Rij rats. The number of different cell preparations used for the determinations in these age groups was 11, 4, 8, 7, and 6, respectively. Values represent the mean and standard error of the mean. For experimental details, see (32).

As a second liver-specific function, the synthesis of specific proteins can be mentioned. Results obtained for albumin synthesis in intact rats (33) indicated that the capacity to synthesize this protein could be used as an indication of the functional reserve capacity of the rat liver (8, 33). Possible age-related changes in the synthesis of albumin by isolated hepatocytes were investigated in a series of experiments. Hepatocytes isolated from 3-month-old female WAG/Rij rats synthesized 5.8 μg albumin/hr/10^6 cells (34), which nearly equals the amount synthesized in an *in vivo* situation (35). This finding implies that no dramatic loss of functions occurs during isolation of hepatocytes. The albumin synthesis by isolated hepatocytes showed a significant decrease between 3 and 24 months. Hepatocytes from 24-month-old rats synthesized only 2.3 μg albumin/hr/10^6 cells. In contrast, a sharp increase was found between 24 and 36 months (34). To some extent, the age-related pattern of the total protein synthesis by isolated liver parenchymal cells was comparable with that of the albumin synthesis. A decrease in total protein synthesis between 3 and 24 months followed by an increase between 24 and 36 months was observed (24). Similar age-related changes in protein synthesis by isolated hepatocytes were observed for male Fischer F 344 rats (36). The reason for the increase in albumin and total protein synthesis after 24 months of age is not yet clear. Several observations exclude the possible explanation based on senescent proteinuria (8).

A third, more general aspect of liver metabolism concerns cellular respiration and oxidative phosphorylation. A decline in these functions may account for the decline in the tolerance of older animals to physical stress. Ultrastructural changes in mitochondria of hepatocytes of mice, rats, and humans are indicative of a possible decline in energy of these cells with age (37). Hepatocytes isolated from 3-month-old female WAG/Rij rats have an endogenous respiration of 2.7 μmole oxygen per minute per gram wet weight (37), which is comparable with the respiration rate of 2.2 μmole per minute per gram weight observed for the perfused liver (38). This indicates that the isolated hepatocytes are intact with respect to respiratory capacity. No significant differences were observed in the endogenous respiration and in the ratio between the uncoupled and the blocked respiration of cells isolated from 3-, 12-, and 36-month-old rats (37). These data suggest that hepatocytes in the livers of old rats have an unimpaired energy supply mechanism.

Studies on lysosomal enzymes in isolated hepatocytes. In long-lived cells such as hepatocytes, most intracellular constituents are continuously renewed. Lysosomes play the major role in the controlled breakdown of cellular components, including lipids, proteins, and nucleic acids. In morphometric studies on hepatocytes of young and old rats of various strains, clear alterations in the lysosomal system were observed. The cytoplasmic volume occupied by lysosomal structures increased in hepatocytes of old rats (20–22) and the lysosomes became progressively loaded with indigestible material with age (39). Thus, in contrast to most other cellular components which are continuously renewed, lysosomes can be considered to actually age (40). In this respect, the accumulation of defective enzyme molecules demonstrated for several aging systems (41, 42) does not necessarily have to reflect cellular aging processes such as misincorporation of amino acids or post-trans-

lational modifications, but may also be due to alterations in the removal and breakdown of these defective molecules by the lysosomal system. This last possible explanation made it of interest to perform a detailed study of the hydrolytic enzymes in the aging lysosomes of the long-lived hepatocytes.

Although a considerable amount of information concerning aging changes in lysosomal enzyme activities with age was available, these changes showed several discrepancies. During our studies on lysosomal enzyme activities in suspensions of various liver cell classes, it emerged that the patterns of aging changes often differed considerably for liver homogenates, hepatocytes, Kupffer cells, and endothelial cells. For 3-month-old female BN/BiRij rats, the distribution of various lysosomal enzymes over the three classes of isolated liver cells showed remarkable differences (39, 43). The activities of nearly all investigated enzymes when expressed on a protein basis are much higher in Kupffer and endothelial cells than in parenchymal cells. In view of the great cellular differences in the specific activities, it is not surprising that the aging patterns reported for enzyme activities in liver homogenates often do not reflect the real alterations occurring with aging in the specific liver cell classes (39). Aging changes in lysosomal enzymes in one of these cell classes, the hepatocytes, are shown in Table I. Cathepsin D is the only enzyme which shows a clear age-related increase in activity on both a cellular basis and a protein basis. Since no decrease in specific activities was observed, the results do not indicate changed functions of the lysosomal enzymes in old hepatocytes. However, autophagy and hydrolysis are complex processes and specific activities of lysosomal enzymes represent only one aspect of the functioning of lysosomes. Another complicating factor is the presence of several isoenzymes or multiple forms of one enzyme, such as acid phosphatase (44) and cathepsin D (45), even within the one specific cell class of the hepatocytes. As a

TABLE I. EFFECT OF AGE ON LYSOSOMAL ENZYME ACTIVITIES IN PARENCHYMAL CELLS ISOLATED FROM FEMALE BN RATS OF VARIOUS AGES

Enzyme	Age (months)	Activity[a]	
		Per 10^6 cells	Per mg protein
Cathepsin D[b]	3	11.9 ± 0.6 (6)	6.9 ± 0.5 (6)
	12	20.1 ± 6.4 (3)	6.3 ± 1.7 (3)
	24	32.7 ± 3.1 (4)*	10.0 ± 1.0 (4)*
	30–35	30.7 ± 5.9 (8)*	19.1 ± 2.8 (8)*
Acid phosphatase[c]	3	63.5 ± 4.9 (14)	34.2 ± 3.7 (14)
	12	67.7 ± 15.8 (3)	22.4 ± 3.3 (3)
	24	118.8 ± 9.7 (4)*	36.1 ± 2.0 (4)
	30–35	78.1 ± 8.6 (10)	32.1 ± 3.0 (10)
Aryl sulfatase B[d]	3	13.4 ± 0.5 (4)	6.9 ± 0.2 (4)
	12	21.6 ± 4.8 (3)	6.8 ± 1.2 (3)
	24	33.5 ± 1.1 (4)*	10.3 ± 0.8 (4)*
	30–35	16.9 ± 3.4 (4)	8.8 ± 1.9 (4)
β-Galactosidase[c]	3	6.4 ± 0.5 (4)	3.3 ± 0.2 (4)
	12	5.1 ± 1.1 (3)	1.6 ± 0.3 (3)*
	24	10.1 ± 0.4 (4)*	3.1 ± 0.2 (4)
	30–35	7.0 ± 1.3 (4)	3.4 ± 0.3 (4)

[a] Each value represents the mean ± SEM, with the number of different cell preparations used for the enzyme assays in parentheses [for experimental details, see (39)].
[b] Activity expressed as nmole tyrosine/min/mg protein.
[c] Activity expressed as nmole 4-methylumbelliferone/min/mg protein.
[d] Activity expressed as nmole nitrocatechol/min/mg protein.
* Significant difference ($P < 0.05$) between indicated values and those of 3-month-old rats.

consequence, age-related changes in total enzyme activities may be the result of changes in activities of various enzyme forms and do not necessarily reflect the actual changes occurring in each specific form separately.

To investigate age-related alterations in the different multiple forms of acid phosphatase in isolated hepatocytes, biochemical characteristics such as affinities for substrates and physical characteristics such as isoelectric points were studied (46). The activities of the multiple forms of acid phosphatase in hepatocytes from young and old rats are given in Table II. As reported previously (39), the specific activity with 4-methylumbelliferyl dihydrogen phosphate as substrate does not increase significantly with aging; the specific activities of the two other multiple forms increase significantly. Isoelectric focusing experiments with isolated hepatocytes revealed an increase in heterogeneity in acid phosphatase enzymes with different isoelectric points in cells isolated from 33- to 34-month-old rats (46). These results indicate that modifications or substitutions of amino acids occur, resulting in charge differences in the multiple enzyme forms. Misincorporation of amino acids or post-translation modifications may be the primary causes for the alterations in the enzyme molecules. On the basis of other experiments (46), it was suggested that post-translational modifications may be primarily responsible for the charge alterations in the multiple forms of acid phosphatases in hepatocytes from old rats. This experimental approach clearly illustrates the potential for the use of isolated hepatocytes in studies on the cellular causes of aging.

TABLE II. ACID PHOSPHATASE ACTIVITIES ON THREE SUBSTRATES IN PARENCHYMAL CELLS ISOLATED FROM YOUNG AND OLD RATS[a]

Age (months)	Activity per mg protein[b]		
	4-MUP[c]	1-NP[d]	p-NPP[e]
3	27.67 ± 1.30	36.31 ± 1.33	43.60 ± 1.93
34–35	31.67 ± 3.04	45.18* ± 1.79	52.97* ± 1.02

[a] Female BN/BiRij rats, 3 months of age and 34–35 months of age, respectively.
[b] Expressed in nmole 4-methylumbelliferone, 1-naphthol, or p-nitrophenol released per minute at 37°C. Mean ± SEM of three determinations. For experimental details, see (44).
[c] 2.1 mM 4-methylumbelliferyl dihydrogenphosphate.
[d] 48 mM sodium 1-naphthylphosphate.
[e] 20 mM disodium p-nitrophenylphosphate.
* Value differs significantly ($0.025 > P > 0.01$) from young value.

Studies on cellular aging performed with primary cultures of hepatocytes and with cocultures of several liver cell types. Maintenance cultures of isolated cells from various sources have become important tools in cell biology. The use of primary cultures of rat liver hepatocytes for the study of the formation of specific intercellular junctions and bile canaliculi along with their response to pharmacological agents shows some of the possibilities of this system (28), which can also be employed for the study of cellular aging (47). Isolated rat liver sinusoidal cells and purified Kupffer cells can also be kept in maintenance culture (48). A new approach to study the effect of aging on phenomena such as cell contacts, cell recognition, and the mutual influences of several cell types is offered by the recent possibility of cocultivating isolated rat hepatocytes with isolated endothelial cells, Kupffer cells, and other sinusoidal cells (29). Cocultures of hepatocytes and sinusoidal cells prepared from aged Sprague–Dawley rats have already been shown to increase the survival time of the cultured hepatocytes. The mixed cultures are devoid of cell debris and damaged cells for more than 7 days, possibly due to the strong phagocytic activity displayed by the cultured Kupffer cells. It appears that the improvement of the hepatocyte quality in the cocultures and their prolonged survival time due to the beneficial cooperation of hepatocytes and sinusoidal cells are not adversely affected by aging (47). These cocultures are also very useful for studies on metabolic functions in which several liver cell types are involved, such as hemoglobin degradation. Further studies are needed to analyze the reciprocal influences of the cell types as well as the influence of the aging process on these kinds of "multicellular" processes.

I would like to thank Elisabeth Sleyster, Dr. A. Brouwer, and Dr. C. F. A. van Bezooijen for their integral roles in the investigations performed in our group.

1. Evans, C. H., Med. Hypotheses **5**, 53 (1979).
2. Rockstein, M., Chesky, J., and Sussman, M., *in* "Handbook of the Biology of Aging" (C. E. Finch and L. Hayflick, eds.), p. 3. Van Nostrand Reinhold, New York (1977).
3. Kohn, R. R., *in* "Handbook of the Biology of Aging" (C. E. Finch and L. Hayflick, eds.), p. 281. Van Nostrand Reinhold, New York. (1977).
4. Knook, D. L., Akt. Gerontol. **7**, 1 (1977).
5. Knook, D. L., *in* "Genetic Effects on Aging" (D. Bergsma, D. E. Harrison, eds.), p. 171. Alan R. Liss, New York (1978).
6. Van Bezooijen, C. F. A., "Cellular Basis of Liver Aging Studied with Isolated Hepatocytes." Ph.D. thesis, Utrecht (1978).
7. Schneider, E. L., and Mitsui, Y., Proc. Nat. Acad. Sci. USA **73**, 3584 (1976).
8. Knook, D. L., *in* "Lectures on Gerontology" (A. Viidik, ed.). Academic Press, New York/London, in press.
9. Nadal, C., and Zajdela, F., Exp. Cell Res. **42**, 99 (1966).
10. Greengard, O., Federman, M., and Knox, W. E., J. Cell Biol. **52**, 261 (1972).
11. Post, J., and Hoffman, J., Exp. Cell Res. **36**, 111 (1964).
12. Kato, R., Biochem. Pharmacol. **13**, 1037 (1964).
13. Kato, R., *in* "Liver and Aging" (K. Kitani, ed.), p. 287. Elsevier/North-Holland, Amsterdam (1978).
14. Klotz, U., and Wilkinson, G. R., *in* "Liver and Aging" (K. Kitani, ed.), p. 367. Elsevier/North-Holland, Amsterdam (1978).
15. Lutton, C., and Chevalier, F., Biochim. Biophys. Acta **280**, 116 (1972).
16. Story, J. A., and Kitchevsky, D., Experientia **30**, 242 (1974).
17. Adelman, R. C., Fed. Proc. **34**, 179 (1975).
18. Adelman, R. C., Klug, T. L., Obenrader, M. F., and Kitahara, A., *in* "Liver and Aging" (K. Kitani, ed.), p. 277. Elsevier/North-Holland, Amsterdam (1978).
19. Kato, R., and Takanaka, A., Japan. J. Pharmacol. **18**, 389 (1968).
20. Schmucker, D. L., Mooney, J. S., and Jones, A. L., Science **197**, 1005 (1977).
21. Knook, D. L., Sleyster, E. Ch., and Van Noord, M. J., *in* "Cell Impairment in Aging and Development" (V. J. Cristofalo and E. Holecková, eds.), p. 155. Plenum, New York (1975).
22. Meihuizen, S. P., and Blansjaar, N., Mech. Ageing Develop. **13**, 111 (1980).
23. Van Bezooijen, C. F. A., Van Noord, M. J., and Knook, D. L., Mech. Ageing Develop. **3**, 105 (1974).
24. Van Bezooijen, C. F. A., Grell, T., and Knook, D. L., Mech. Ageing Develop. **6**, 293 (1977).
25. Bernaert, D., Wanson, J.-C., Mosselmans, R., De Paermentier, F., and Drochmans, P., Arch. Intern. Physiol. Biochim. **86**, 408 (1978).
26. Knook, D. L., Blansjaar, N., and Sleyster, E. Ch., Exp. Cell Res. **109**, 317 (1977).
27. Knook, D. L., and Sleyster, E. Ch., *in* "Kupffer Cells and Other Liver Sinusoidal Cells" (E. Wisse and D. L. Knook, eds.), p. 273, Elsevier/North-Holland, Amsterdam (1977).
28. Wanson, J.-C., Bernaert, D., and May, C., *in* "Progress in Liver Diseases" (H. Popper and F. Schaffner, eds.), Vol. VI, p. 1. Grune & Stratton, New York (1979).
29. Wanson, J.-C., Mosselmans, R., Brouwer, A., and Knook, D. L., Biol. Cell. **36**, 7 (1979).
30. De Leeuw-Israel, F. R., "Aging Changes in the Rat Liver." Ph.D. thesis, Leiden (1971).
31. Van Bezooijen, C. F. A., Grell, T., and Knook, D. L., Biochem. Biophys. Res. Commun. **69**, 354 (1976).
32. Van Bezooijen, C. F. A., and Knook, D. L., *in* "Liver and Aging" (K. Kitani, ed.), p. 131, Elsevier/North-Holland, Amsterdam (1978).
33. Chen, J. C., Ove, P., and Lansing, A. J., Biochim. Biophys. Acta **312**, 598 (1973).
34. Van Bezooijen, C. F. A., Grell, T., and Knook, D. L., Biochem. Biophys. Res. Commun. **71**, 513 (1976).
35. Jeejeebhoy, K. N., Bruce-Robertson, A., Ho, J., and Sodtke, U., Biochem. J. **130**, 533 (1972).
36. Coniglio, J. J., Lin, D. S. H., and Richardson, A., Mech. Ageing Develop. **11**, 77 (1979).
37. Brouwer, A., Van Bezooijen, C. F. A., and Knook, D. L., Mech. Ageing Develop. **6**, 265 (1977).
38. Krebs, H. A., Cornell, N. W., Lund, P., and Hems, R., *in* "Alfred Benzon Symposium 6th: Regulation of Hepatic Metabolism" (F. Lundquist and N. Tygstrup, eds.), p. 726. Munksgaard, Copenhagen (1974).
39. Knook, D. L., and Sleyster, E. Ch., Mech. Ageing Develop. **5**, 389 (1976).
40. De Duve, C., *in* "Aging into the 21st Century" (L. F. Jarrik, ed.), p. 25. Gardner Press, New York (1978).
41. Gershon, D., and Gershon, H., Gerontology **22**, 212 (1976).
42. Rothstein, M., Mech. Ageing Develop. **9**, 197 (1979).
43. Wisse, E., and Knook, D. L., *in* "Progress in Liver Disease" (H. Popper and F. Schaffner, eds.), Vol. VI, p. 153, Grune & Stratton, New York (1979).
44. Sleyster, E. Ch., and Knook, D. L., Arch. Biochem. Biophys. **190**, 756 (1978).

45. Sleyster, E. Ch., and Knook, D. L., in preparation.
46. Sleyster, E. Ch., and Knook, D. L., Mech. Ageing Develop. (1980), in press.
47. Mosselmans, R., Wanson, J.-C., Brouwer, A., and Knook, D. L., Abstract European Congress on Electron Microscopy, Den Haag (1980).
48. Brouwer, A., and Knook, D. L., *in* "Kupffer Cells and Other Liver Sinusoidal Cells" (E. Wisse and D. L. Knook, eds.), p. 343. Elsevier/North-Holland, Amsterdam (1977).

Received June 15, 1980. P.S.E.B.M. 1980, Vol. 165.