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studied with isolated hepatocytes

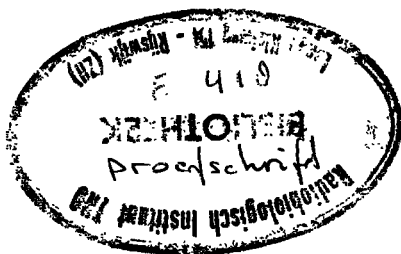
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CELLULAR BASIS OF LIVER AGING
STUDIED WITH ISOLATED HEPATOCYTES

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studied with isolated hepatocytes

C.F.A. van Bezooyen

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ABBREVIATIONS

BSP	bromsulphophthalein
BSP-GSH	glutathione-bromsulphophthalein
DEAE	diethylaminoethyl
DNP	2,4-dinitrophenol
EDTA	ethylenediamine tetraacetate
FCCP	carbonyl cyanide p-trifluoromethoxyphenylhydrazone
GA	golgi apparatus
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulphonate
RER	rough endoplasmic reticulum
SER	smooth endoplasmic reticulum
TCA	trichloroacetic acid

CHAPTER I

INTRODUCTION

The general objective of experimental gerontological research is to elucidate the mechanisms which underlie the aging processes of man. The clarification of these mechanisms might enable one to combat the infirmities of old age. Consequently, the quality of life in this stage of human life span would be improved. Therefore, the motto used for the first time by Lord Anulree and applied by most scientists working in the field of experimental gerontology runs as follows: "To add life to years, not years to life".

The elucidation of the mechanisms underlying aging processes of man may be partly realized by investigating the cellular basis of organ aging, which is the more specific objective of this study. Many organs, including brain, heart, kidney, liver and lung, show a decrease in their functional capacities with age, as reviewed by Shock (1961, 1968), Norris and Shock (1966), Comfort (1972), Riess (1972), Timiras (1972), and Platt (1976). The cellular basis of organ aging changes can be studied at different levels of biological organization; including the organ, cellular and subcellular levels.

CELLULAR BASIS OF ORGAN AGING STUDIED AT THE ORGAN LEVEL

The cellular basis of organ aging may be studied in the intact organism. However, several organs or organ systems may interfere with the functions of others, thereby complicating the interpretation of age-related changes observed in individual organs. For example, the aging of the nervous system may play a general role in the onset of organ aging because of its coordinating function (Frolkis, 1968; Hansche, 1975; Ordy et al., 1976, and Strehler, 1976). Another governing system is the endocrine system, which also has in certain aspects an interrelationship with the central nervous system. General discussions on endocrine factors in relation to aging have been provided by Bellamy (1970), Gusseck (1972), and Everitt and Burgess (1976). Besides, or in addition to alterations in the nervous and endocrine systems, changes in the blood distribution may also influence the functions of organs (Ries, 1972). A decreased functional capacity of the heart or changes in the blood vessels such as a thickening of the basal membrane of the capillaries and a decrease in the number of capillaries per unit organ volume may occur during aging. The changes in the blood distribution pattern, due to changes in heart function and in the vascular system may result in a decreased blood supply to the various organs. Consequently, these organs may be deficient in their oxygen and food supply.

From the data mentioned above, it can be concluded that the intact organism, in which many complicating factors influence the functional capacity of an organ, is not the appropriate system to determine the cellular basis of organ aging.

The cellular basis of organ aging might also be studied by transplanting organs from old donors to young recipients or by perfusing intact organs of different ages and determining the functional capacity of these transplanted or perfused organs. By transplanting organs from old donors to young recipients, a distinction can be made between reversible aging changes in an organ occurring under the influence of the intact aging organism and the irreversible aging changes in the organ itself. A limitation which must be accepted is that it is essential to restrict oneself to transplantations which are performed with inbred strains, so that the transplantations are not complicated by a rejection of the transplant. Orthotopic transplantations of whole organs, which means that the organs are transplanted on the correct anatomical location in the recipient, have been performed to study aging changes in ovarian (Krohn, 1963) and renal (Hollander, 1970, and Van Bezooijen et al., 1974) function. These latter studies were complicated by the occurrence of ischemia during transplantation. However, an advantage of these transplantation studies involving transplanting organs from old donors to young recipients is that the functional capacity of an organ can be studied beyond the normal life-span. The functional capacity of heterotopic organ transplants from old to young animals was investigated with adrenal (Geiringer, 1956) and prostate (Franks, 1973) transplants.

The perfusion technique offers a possibility to exclude the disturbing factors mentioned above for the intact organism, such as changes in the nervous or endocrine system. Even if the influences of other organs or organ systems on the target organ are ruled out or kept to a minimum, interpretation of the results obtained with a whole organ still do not warrant solid conclusions about the cellular mechanisms of organ aging. For, an additional complicating factor is that organs consist of different cell types and each of these cell types may age in a different way. Furthermore, age-related changes in the capillaries may still influence the functional capacity of a perfused organ. Therefore, it might be difficult to trace back any age-related change in the functional capacity of an organ to changes in one or more functions of a special cell class. Moreover, aging changes in organ functioning may also be due to a loss of cells.

CELLULAR BASIS OF ORGAN AGING STUDIED AT THE SUBCELLULAR LEVEL

An alternative way to study the cellular basis of organ aging is represented by studies performed at the subcellular level. During the last ten years, many experimental data on subcellular aging changes have been obtained from studies performed with homogenates or suspensions of subcellular organelles. The literature on this subject has been extensively reviewed by Goldstein (1971), Kormendy and Bender (1971), Comfort (1974), and Hayflick (1975). A criticism on the use of homogenates or organelle suspensions is that they are derived from total organs; consequently, the homogenates and organelle suspensions originate from all different cell populations constituting an organ. Other disadvantages of using homogenates or organelle suspensions are the absence of the genetic control mechanisms located in the nucleus and the lack of several components present in the cytoplasm. An example of the consequences of these disadvantages is the observation that the capacity of isolated liver microsomes to synthesize protein was linear for only 5 minutes and had already reached a plateau after about 20 minutes of incubation (Mainwaring, 1969; Hrachovec, 1971; Buetow and Gandhi, 1973; Chen et al., 1973, and Layman et al., 1976). These periods are very short, since, for example, isolated liver parenchymal cells synthesize proteins in a linear way for at least 4 hours (this study). Furthermore, the rate of *in vitro* protein synthesis with a microsomal system is only about 4 percent of the *in vivo* rate of protein synthesis (Richardson et al., 1971).

With respect to the cellular basis of organ aging, it can be stated that it is very difficult to organize the subcellular results which have been obtained up to now into one governing hypothesis. Continued investigation of subcellular changes accompanying aging would be of great value if more attention would be paid to the reintegration of subcellular data into functioning physiological units. A testable physiological unit is that of the individual cell.

CELLULAR BASIS OF ORGAN AGING STUDIED AT THE CELLULAR LEVEL

In choosing a model system which might be appropriate to determine the cellular basis of organ aging, one must take into account that an organ is constituted of a variety of specialized cells. Cells can be characterized on the basis of their proliferative activity (Leblond, 1964; Bakerman, 1969; Kohn, 1971, and Cameron and Trasher, 1976). On

this basis, the following cell types can be distinguished:

- a. intermitotic cells, which continuously or periodically divide to compensate for cell loss. To this population belong for example the haemopoietic stem cells, fibroblasts and the basal cells of the epidermis.
- b. postmitotic cells, which do not divide after their differentiation. They can be subdivided into short-lived and long-lived fixed postmitotic cells (Kohn, 1971). Short-lived postmitotic cells are highly specialized. Some examples of this type of cells are red blood cells, plasma cells and granulocytic leucocytes. Long-lived fixed postmitotic cells are formed early in life, persist mostly as long as the animal and are also highly specialized. Cells in this category are neurons and striated muscle cells.
- c. reverting postmitotic cells, which normally do not divide or divide rarely, but can develop mitotic activity if they receive an appropriate stimulus. The cells are highly differentiated. Liver parenchymal cells and osteocytes belong to this group.

The study of aging changes in intermitotic cells has been approached experimentally by using cultures of one of this cell type, namely, the fibroblast. Hayflick (1965) showed that normal human diploid fibroblasts in culture underwent a limited number of population doublings (viz. about 50) and then died. He considered this finite lifetime of normal cultured cells as a manifestation of biological aging at the cellular level. However, there may be some important objections to the use of this model system. Criticism is especially leveled at the heterogeneity of the late passage cells, which are the cell cultures which have undergone about 50 population doublings. The heterogeneity might be due to an increase in the nondividing fraction of the population (Cristofalo and Sharf, 1973). However, this explanation has been contradicted by Macieira-Coelho (1974), who reached the conclusion that hardly any nondividing cells were present in late passages of human embryonic fibroblasts. It seems to be more likely that the heterogeneity of the cells in the late passage cultures might be attributed to a lengthening of the cell cycle in subpopulations of the cultured cells (Hill, 1977, and Macieira-Coelho, 1977). An additional problem is the correlation of the *in vitro* cellular aging with the *in vivo* human age. For example, Schneider and Mitsui (1976) observed that the changes in several characteristics such as the cell population replication rate, the *in vitro* life-span and the onset of the cell culture senescence of fibroblasts aged *in vitro* were qualitatively and quantitatively different from the changes observed with fibroblast aged *in vivo*. Moreover, the characteristics: modal cell volume and cellular macromolecular

contents were altered as a function of *in vitro* aging but were not altered *in vitro* as a function of donor age *in vivo*. Therefore, Schneider and Mitsui (1976) concluded that, for studying human cellular aging, the system of isolating fibroblasts from young and old human donors is more appropriate than the *in vitro* aging of normal cultured diploid human fibroblasts.

A more promising system to study the cellular basis of organ aging is offered by suspensions of cells isolated from organs of animals of different ages. The disadvantages mentioned above for the use of normal diploid cells in culture do not apply to the *in vitro* system of cells isolated from animals of different ages. In addition, if long-lived postmitotic cells are isolated from organs of different ages, the functional capacity of individual cells which have aged under normal physiological conditions can be determined. Furthermore, different functional characteristics can be determined from a cell suspension isolated from one animal. Finally, the functional capacity can be determined from cell suspensions isolated from organs of different ages of which the functional capacity has been previously determined *in vivo*. In this way, it can be investigated whether a change in the functional capacity of an organ with age might be attributed to a change in the functional capacity of the cells constituting that organ. From the gerontological point of view, organs consisting of long-lived postmitotic cells are supposed to play an important role in the aging process of the individual. A defective function will be particularly serious for cells that are not periodically renewed after they have differentiated. Therefore, degenerative changes in postmitotic cells might be an important cause of organ aging. To the organs that are largely made up of postmitotic cells belong brain and heart. However, isolating cells from brain or heart in an intact state and measuring the functional capacity of these isolated neurons or heart muscle cells is still difficult to achieve.

Another group of long-lived cells are the reverting postmitotic cells. Under normal circumstances these cells live as long as the individual. Liver parenchymal cells belong to the reverting postmitotic cells. The liver consists of a variety of specialized cells. In an adult rat, the liver contains two major cell populations, viz., the parenchymal cells and the sinusoidal lining cells. A minor group consists of blood vessel wall, bile duct and connective tissue cells. The parenchymal cells, also called hepatocytes, represent the main cell type in the liver of young, adult or old rats. The fraction of the total liver volume occupied by these cells is about 85 percent (Striebich et al., 1953; Weibel et al., 1969, and Greengard et al., 1972). About 60 percent of all liver cells are parenchymal cells (Stowell, 1952; Grant and Rees, 1957; Daoust and Cantero, 1959, and Fabrikant, 1968).

Under normal physiological conditions, liver parenchymal cells show a very low mitotic incidence of about 0.01 percent (Abercrombie and Harkness, 1951; Stöcker et al., 1964; Nadal and Zajdela, 1966, and Greengard et al., 1972). Consequently, almost all rat liver parenchymal cells live as long as the rat after they have differentiated (Post and Hoffman, 1964). The proliferation is predominantly located in the periportal area of the rat liver lobule (LeBouton and Marchand, 1970, and Müller, 1970) and is mainly confined to the diploid hepatocytes (Post and Hoffman, 1965).

An important reason for the choice of the liver parenchymal cells as a model system to study the cellular basis of organ aging was that isolation of these cells seemed to be possible at the time this study was begun. About that time, many reports (Howard and Pesch, 1968; Berry and Friend, 1969; Capuzzi et al., 1971; Gallai-Hatchard and Gray, 1971; Lentz and Luzio, 1971; Weigand et al., 1971, and Seglen, 1972) appeared which described methods to isolate viable liver parenchymal cells. All of these investigators made use of the enzymes collagenase and hyaluronidase. These enzymes take care of the digestion of the framework of connective tissue. A calcium-free preperfusion of the liver was necessary for the cleaving of the desmosomes of the adjacent cells (Berry and Friend, 1969, and Berry, 1976a). For loosening the tight and gap junctions between the hepatocytes, mild mechanical treatment was required (Berry and Friend, 1969, and Wanson, 1976). Considering these aspects, a general method to isolate liver parenchymal cells requires a preperfusion of the liver with a calcium-free medium. Thereafter, the liver was perfused with the enzymes collagenase and hyaluronidase. Finally, pieces of liver tissue were incubated with the same enzymes with constant shaking. It is now possible to isolate viable liver parenchymal cells from rat and mouse as has been described in many reports, which have been reviewed by Schreiber and Schreiber (1973). Other species from which viable hepatocytes can be isolated are man (Bojar et al., 1976), sheep (Clark et al., 1976, and Elliott et al., 1976), monkeys (Capuzzi et al., 1975a, and Glinioer et al., 1976), chickens (Goodridge, 1973; Capuzzi et al., 1975b, and Anderson et al., 1976), hamsters (Rognstad et al., 1975) and guinea pigs (Elliott and Pogson, 1977).

For a realization of the objective of this study, it was necessary that the functional capacities of the isolated cells could be quantified and that they were nearly equal to the values reported for the cells in the perfused liver. In this way, it would be possible to investigate whether changes in the functional capacity of individual hepatocytes with age played an important role in aging changes of the

whole liver. The fulfilment of these prerequisites seemed to be possible at about the start of this study if the model system of isolated rat liver parenchymal cells was used. Recent studies also showed that the functional capacity of the isolated hepatocytes resembled that of hepatocytes in the perfused liver. The gluconeogenesis of isolated hepatocytes, for example, appeared to take place at the same rate as in the perfused liver. The amount of glucose formed by liver parenchymal cells isolated from starved rats (Ingebretson and Wagle, 1972; Williamson and Ellington, 1975, and Katz and Jungmann, 1976) was comparable to the amount of glucose formed by the perfused liver of starved rats (Ross et al., 1967; Chan and Freedland, 1972; Ingebretsen and Wagle, 1972, and Zehner et al., 1973). The rate of gluconeogenesis for the perfused liver after addition of lactate to the perfusion medium (Ross et al., 1967, and Chan and Freedland, 1972) was in the same range as that observed for the isolated hepatocytes after addition of lactate to the incubation medium (Ingebretsen and Wagle, 1972; Cornell et al., 1973; Garrison and Haynes, 1973; Williamson and Ellington, 1975; Katz and Jungermann, 1976, and Story et al., 1976). Furthermore, liver parenchymal cells isolated from starved rats synthesized glucose from alanine at rates comparable to those observed in the perfused liver (Johnson et al., 1972, and Ingebretsen and Wagle, 1972). The enzyme activities of glycogen metabolising enzymes such as glycogen synthase, phosphorylase and glucose-6-phosphatase were also similar in isolated hepatocytes and perfused liver (Walli and Schimassek, 1976).

In addition to the carbohydrate metabolism, the lipid metabolism of the isolated hepatocytes determined from the cholesterol production (Nilsson et al., 1973), the ketone body formation (Krebs et al., 1974) and the rate of fatty acid synthesis (Windmueller and Spaeth, 1966, and Thieden et al., 1976) also closely resembled that of the perfused liver.

With respect to the protein metabolism, Krebs et al. (1974) observed that the rate of urea synthesis by isolated hepatocytes was in the same range as that of the perfused liver.

With respect to the hormone responsiveness, it was found that the concentration of glucagon necessary to stimulate gluconeogenesis was exactly the same for the perfused liver (Exton and Park, 1968; Williamson et al., 1969, and Parilla et al., 1975) as for the isolated liver parenchymal cells (Oliver and Wagle, 1975, and Flockhart et al., 1976).

All data mentioned above indicate that, in many respects, the functional capacity of isolated liver parenchymal cells is similar to that of the cells in the perfused liver.

For a good comparison between *in vitro* and *in vivo* data, it is of importance to know whether the liver parenchymal cells, which are programmed *in vivo* with respect to their functional status, retain their program after isolation. This question was answered by Craig and Porter (1973), who prepared hepatocytes from rats fed a normal diet containing 4 percent fat and from rats fed with a fat-free diet. They observed that the nutritional state of the animal from which the parenchymal cells were isolated strongly correlated with the amount of radioactive leucine incorporated by the isolated hepatocytes into the lipogenic enzyme, fatty acid synthetase. In agreement with this finding, hepatocytes isolated from rats fed diets varying in protein content synthesized amounts of urea which were dependent on the amount of protein in the diet (Mendes-Mourao et al., 1976). Another indication that the hepatocytes are triggered *in vivo*, and that this triggering remains preserved during the isolation, was provided by Walker (1977). He isolated hepatocytes from rats conditioned to a controlled feeding schedule and observed that the isolated liver parenchymal cells synthesized amounts of glycogen which correlated with the time of the day the cells were prepared.

OBJECTIVE OF THE STUDY

From the above-mentioned data it can be concluded that isolated hepatocytes are in a functional state quite close to that of the *in vivo* situation. Measuring functional capacities of isolated liver parenchymal cells will provide much information on the physiological competence of these cells in the intact liver. Therefore, the isolated liver parenchymal cells are suitable as a model system to investigate the main objective of this study, viz. to determine whether changes in the functional capacity of the total liver with age are caused by age-related changes in the function of the individual hepatocytes.

Functions performed by liver parenchymal cells can be classified into three main groups:

- excretory functions: The liver excretes bilirubin and other bile pigments as well as exogenous substances such as bromsulphophthalein (BSP) which are removed from the blood into the bile. Age-related changes in the BSP metabolism will be extensively discussed in Chapter IV.
- detoxification: The liver is capable of converting toxic foreign compounds into relatively harmless substances which are subse-

quently excreted into the urine. The best known and most frequently used test of the protective functions of the liver is based on a conjugation reaction: the detoxification of benzoic acid with glycine to form hippuric acid, which is excreted into the urine. Little is known about the effect of age on the detoxification function of the liver. Stern et al. (1946), and Rafsky and Newman (1949) noted impaired hippuric acid synthesis in 70 to 80 percent of elderly human subjects.

metabolic activities: The liver plays an important role in carbohydrate, protein and lipid metabolism. With respect to changes in the protein synthesizing capacity of the liver and that of isolated microsomes with age, many controversial data can be found in the literature, as will be described in Chapter VI.

The choice of the functional capacities to be measured using hepatocytes isolated from rats of different ages was based mainly on the following reasons. Research on aging changes in the excretory capacity of the whole liver has already been investigated in detail by De Leeuw-Israel (1971). She measured the clearance of bromsulfophthalein (BSP) by means of the BSP retention test and adapted this widely used diagnostic test for liver function to the rat. De Leeuw-Israel (1971) found a decreased liver function with age in female RU rats. The most pronounced changes occurred between 3 and 12 months of age.

Besides determining the excretory capacity of the liver from the BSP clearance, the metabolic activity of the liver was studied indirectly by assessing the albumin level of the rat serum (De Leeuw-Israel, 1971). Age-related changes in albumin synthesis might be expressed by alterations in the serum albumin content. In man, the serum albumin content could be determined by its ability to bind specifically 2-(4'-hydroxybenzene azo) benzoic acid (HBABA) (Rutstein et al., 1954). The adaptation of this method for measuring rat serum albumin was achieved by scaling this method down to the microlevel (De Leeuw-Israel et al., 1967). De Leeuw-Israel observed that the concentration of albumin in rat serum did not change with age.

Considering the above-mentioned data, the functional capacity of the hepatocytes isolated from rats of different ages was determined on the basis of their competence to store BSP and to synthesize albumin. Furthermore, to determine whether possible changes in the albumin synthesis with age reflect overall changes in the protein synthesis of the hepatocytes or a change in the capacity to synthesize this liver-specific protein, the protein synthesizing capacity was also measured.

In the interpretation of aging data accumulated with isolated hepatocytes, one must always take into account that some morphological cellular aging changes might have influenced the results obtained. An example of such an age-related morphological phenomenon is the shift of the hepatocytes to a higher ploidy. Detailed information on changes in the ploidy state of the parenchymal cells with age will be given in Chapter IV.

In the human liver, a shift of the parenchymal cells to a higher ploidy with age was also observed. However, the shifts are less spectacular than those observed with rats, since about 70 percent of the cells are still mononuclear diploid in late age (Swartz, 1956; Altmann et al., 1966; Meek and Harbison, 1967, and Denkhaus, 1970).

To take into account the complicating factor of the shift of parenchymal cells to a higher ploidy state, the measured functional capacities were expressed not only on a cellular basis but also on a protein basis, so that the influence of an increase in cell volume (which means an increase in protein content) on the data obtained can be excluded.

Another morphological feature of the liver parenchymal cells which might also complicate the possible data obtained with age is the accumulation of lipofuscin (age pigment). In human liver, lipofuscin increased during senescence (Bachmann, 1953, and Findor et al., 1973). According to Findor et al. (1973), lipofuscin preferentially accumulated in the parenchymal cells of the centrolobular area. Tygstrup et al. (1965) compared the lipofuscin content of human livers from younger and older patients, using the age of 50 as the border line. However, they did not observe significant differences. In the rat liver an increase in lysosomal structures, including residual bodies like lipofuscin, has been reported by Knook et al. (1975), and Schmucker (1976). As in the human situation, Knook et al. (1975) observed that the lysosomal structures are preferably located in the central part of the lobule. If lipofuscin influences the functional capacity of a cell, it might be a complicating factor. Nevertheless, it is interesting to note that lipofuscin also accumulates with age in neurons and heart muscle cells. In this respect, hepatocytes also resemble the long-lived post-mitotic cells.

Summarizing, it can be concluded that the measurement of the functional capacity of hepatocytes isolated from rats of different ages on the basis of their competence to store BSP and to synthesize albumin and protein might be a suitable model system to study whether a change in the functional capacity of an organ with age might be attributed to a change in the functional capacity of the cells constituting that organ.

CHAPTER II

THE ISOLATION OF LIVER PARENCHYMAL CELLS FROM RATS OF DIFFERENT AGES

<i>Contents:</i>	2.1	Introduction
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	2.3.2	Ultrastructural appearance
	2.3.3	Metabolic integrity
	2.4	Discussion

2.1 INTRODUCTION

In this chapter, a method to isolate liver parenchymal cells from rats of different ages will be described. To achieve the objective of this study, it was necessary that these isolated hepatocytes were morphologically and metabolically intact. The morphological integrity of the isolated hepatocytes was tested by means of light and electron microscopy. The metabolic integrity of the isolated cells was checked by means of their oxygen consumption.

2.2 MATERIALS AND METHODS

2.2.1 ANIMALS

Inbred virgin female and male WAG/Rij rats (a Wistar-derived strain) and female BN/Bi rats were used. The 90%, 50% and 10% survival ages of these rat strains are shown in Table 2.1 (Burek and Hollander, in press). The male WAG/Rij rat had a significantly shorter life-span than did the female WAG/Rij rat, while the survival ages of the female BN/Bi rat were comparable to those of the female WAG/Rij rat (Table 2.1). Burek (1978) studied the spontaneous lesions in female and male WAG/Rij rats and in female BN/Bi rats with age. He supposed that the difference in life-span between the female WAG/Rij and BN/Bi rats on

TABLE 2.1

THE 90, 50 AND 10 PERCENT SURVIVAL AGES OF FEMALE AND MALE WAG/Rij
AND FEMALE BN/Bi RATS

Strain	Sex	Number	Survival age (months)		
			90%	50%	10%
WAG/Rij	female	124	24	32	37
	male	78	13	24	31
BN/Bi	female	109	25	33	38

Data from: Burek and Hollander (in press)

the one hand and the male WAG/Rij rats on the other hand, might be explained by an earlier onset of all age-associated lesions in the male WAG/Rij rats. With respect to the liver, Burek (1978) reported the following age-related lesions pertinent to this study, viz., foci or areas of cellular alteration of hepatocytes, neoplastic nodules and bile duct cysts. The histological classification of these lesions was made according to the criteria decided upon during a workshop on classification of specific hepatocellular lesions in rats which was held in Silver Spring, Md., USA, in 1974 (Squire and Levitt, 1975). The incidence of foci or areas of cellular alterations was lower in male WAG/Rij rats and in female BN/Bi rats than in female WAG/Rij rats. In contrast, female BN/Bi rats showed a higher incidence of bile duct cysts than did female and male WAG/Rij rats. A relatively low incidence of the neoplastic nodules was found in the male WAG/Rij and in the female BN/Bi rat as compared with the female WAG/Rij rat. The spontaneous lesions of the livers of the female BN/Bi and the female and male WAG/Rij rat have been described in detail by Burek (1978). Although foci or areas of cellular alteration were the most frequently encountered liver lesions in female WAG/Rij rats, it can be mentioned that, during the isolation and washing procedures, cells with characteristics such as found in foci, areas or neoplastic nodules were probably selectively removed; such cells were not observed after isolation.

Detailed information on the conditions under which the animals were kept has been given by Hollander (1976). Briefly described, the animals were acquired from a specific-pathogen-free colony at the age of 3 months. Thereafter, the animals were maintained under conventional conditions. The rats were kept in autoclaved makrolon cages containing a maximum of 4 animals. They were housed in rooms with controlled relative humidity (60-65 percent) and temperature ($21 \pm 1^{\circ}\text{C}$). Nonautoclaved commercial pelleted rodent food (Diet AM II, Hope Farms, Holland) and tap water were available *ad libitum*. Pre-sterilized sawdust used as bedding material was changed twice a week. Cages and drinking bottles were also sterilized twice a week.

2.2.2 MEDIA

The standard medium employed in the parenchymal cell isolation procedure consisted of 0.1 mM L-aspartic acid, 0.2 mM L-threonine, 0.3 mM L-serine, 0.5 mM glycine, 0.6 mM L-alanine, 0.9 mM L-glutamic acid, 0.9 mM L-glutamine, 3 mM KCl, 0.5 mM NaH_2PO_4 , 0.5 mM MgCl_2 , 24 mM

NaHCO₃, 20 mM glucose (Seglen, 1974), 20 mM fructose (Seglen, 1974), 25 mM HEPES, 150 mM sucrose; pH 7.4 at 37°C; osmolality, 300 m Osm.l⁻¹. The dissociation medium used during the isolation procedure consisted of standard medium plus 75 x 10² Units collagenase (Sigma, type 1) and 46 x 10³ Units hyaluronidase (Sigma, type 1) per 100 ml medium (Berry and Friend, 1969).

2.2.3 PREPARATION OF THE PARENCHYMAL CELL SUSPENSION

Under ether anaesthesia, the liver, portal vein and caudal vena cava were dissected free and left *in situ*. A cannula (Braunüle Nr. OG20 for rats up to and including 3 months of age and Nr. 0.5G18 for the rats older than three months of age) was inserted into the portal vein. Three subsequent perfusions were performed with media kept at 37°C and bubbled with 95 percent oxygen and 5 percent carbon dioxide at a flow rate of 0.2 l gas mixture.min⁻¹. The perfusion pressure was about 53 x 10³ Pa (40 mm Hg). The first perfusion of the liver was performed with 15 ml of dissociation medium, after which the liver was blanched evenly. The medium left the body via an incision in the abdominal aorta and was discarded. After the first perfusion, a cannula was inserted into the thoracic portion of the caudal vena cava to carry the efflux; thereafter, the abdominal aorta was clamped off. A second perfusion was performed with standard medium for 15 min to remove Ca²⁺ from the liver, resulting in dissociation of the desmosomes (Berry and Friend, 1969, and Berry, 1976a). The medium used in the second perfusion passed through the liver, left the body via the cannula in the caudal vena cava and was discarded. After the second perfusion, a third perfusion was performed with dissociation medium for 20 min. During this third perfusion, the perfusate returned to the medium reservoir. The perfusion rate of the three perfusions was in the first place adjusted in such a way that the perfusion pressure was never higher than 53 x 10³ Pa (40 mm Hg). In the second place, the perfusion rate was adjusted as high as possible but never higher than 15 ml.min⁻¹. After the third recirculation perfusion, the liver was excised and cut into small pieces with razor blades. The pieces of tissue were incubated for 3 min at 37°C with 50 ml dissociation medium, in which the oxygen tension was at least 50 x 10³ Pa. After incubation, the cell suspension was filtered through five layers of wide maze gauze and through a 50 µm nylon filter. Cellular debris and non-parenchymal cells were removed by centrifugation at 50 g for 5 min. The pellet consisted of parenchymal cells,

which were resuspended in standard or Waymouth MB 752/1 medium. After isolation, which was always performed at the same time of the day, the cells were immediately used for the experiments.

2.2.4 ASSAYS

The permeability of the membrane of the isolated parenchymal cells was checked by incubating these cells with trypan blue at a final concentration of 0.25 percent for about 2 minutes.

The oxygen consumption of the isolated hepatocytes was determined at 37°C with a Clark oxygen electrode. The reaction mixture consisted of 1.2 ml standard medium to which 0.20 ml of a single-cell suspension at a concentration of 1 to 4 million cells.ml⁻¹ was added.

The concentration of the cells was measured by means of a Coulter Counter or a hemocytometer.

The amount of cellular protein was determined by the method of Lowry et al. (1951), using crystalline bovine serum albumin as a standard.

2.2.5 PROCESSING OF THE ISOLATED PARENCHYMAL CELLS FOR ELECTRON MICROSCOPY

Freshly isolated cells were fixed overnight in 2 percent glutaraldehyde (Taab laboratories, Emmer Green, Reading, England) in a 0.1 M cacodylate buffer (pH 7.4). Postosmication for 1 hour in 1 percent OsO₄ in the same buffer followed. The fixed cells were washed in saline and pelleted in a Beckman microfuge at 15,000 g. Thereafter, the pellets were dehydrated in graded ethanol series and embedded in Epon. Ultra-thin sections were cut with a diamond knife on a Reichert ultramicrotome and mounted on uncoated copper grids. Contrast was enhanced by staining with an aqueous solution of uranyl acetate and lead citrate. The sections were examined with a Philips EM 300 electron microscope and photographed with the camera of this instrument.

2.3 RESULTS

2.3.1 LIGHT MICROSCOPIC APPEARANCE OF THE ISOLATED HEPATOCYTES AND THE PERMEABILITY OF THE PLASMA MEMBRANE

Cell suspensions were prepared from the livers of rats of various age groups ranging from 2 weeks to 36 months. The cell yield ranged from 20-40 million isolated cells per gram wet weight of liver. The cell yield appeared to be independent of the age of the rat from which the cells were isolated.

In all cases, light microscopy showed that the isolated parenchymal cells had a round shape and a well defined outline (Figure 2.1). Other cell types such as endothelial and Kupffer cells and cell debris were almost absent. Contamination by nonparenchymal cells was 4 percent at maximum. The percentage of cells which excluded trypan blue amounted

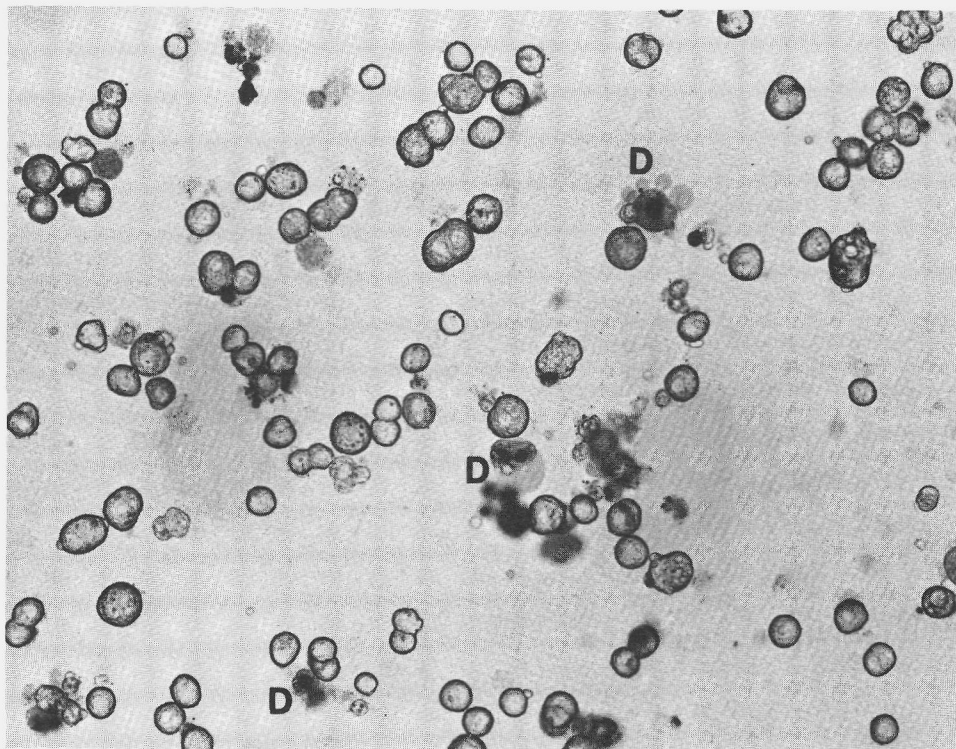


Figure 2.1 Light micrograph of liver parenchymal cells isolated from a 30-month-old rat. The cells were incubated for 30 min in standard medium under 95 percent oxygen and 5 percent carbon dioxide at 37°C. After this incubation period, the cells were suspended in standard medium containing 0.25 percent trypan blue. The darker appearing cells (D) have taken up the vital dye (x 147).

to 95 percent for hepatocytes isolated from livers of young and old rats immediately after isolation. Maintaining the hepatocytes at 37°C under an atmosphere of 95 percent oxygen and 5 percent carbon dioxide with constant shaking (100 oscillations.min⁻¹), which were conditions employed for several of the experiments described in chapters III-VI, did not result in a decrease in the total number of cells. Furthermore, 90 percent of the cells were still viable as determined by trypan blue exclusion (Figure 2.1).

2.3.2 ULTRASTRUCTURAL APPEARANCE

Electron microscopy demonstrated that only severe irreversible damage is discovered by the trypan blue exclusion test. In one of our earlier attempts to isolate hepatocytes, cells which showed a highly damaged ultrastructure were obtained; 90 percent of them still excluded trypan blue. These damaged cells, as shown in Figure 2.2, were characterized by vacuoles scattered all over the cell, the presence of a broad organelle-poor cortical zone and swollen, discontinuous rough endoplasmic reticulum. For comparison, the normal structure of the hepatocytes *in vivo* is shown in Figure 2.3. Comparison of these damaged cells with isolated hepatocytes with an intact ultrastructure (Figures 2.4-2.6), therefore, clearly indicate that the integrity of the isolated hepatocytes which excluded trypan blue must be confirmed by electron microscopic observations.

A low magnification electron micrograph of a suspension of viable liver parenchymal cells isolated from a 3-month-old rat is shown in Figure 2.4. Approximately 85 to 95 percent of the hepatocytes isolated from young and old rats appeared to be structurally well preserved. An electron micrograph of a representative parenchymal cell isolated from the liver of a 30-month-old rat is shown in Figure 2.5. An enlargement of the area indicated in Figure 2.5 is shown in Figure 2.6. Well-preserved organelles as compared with the *in vivo* situation were observed. Even the structures which are more sensitive to the possible damaging effects of the isolation procedure were also intact. For example, the nuclei showed intact chromatin structures, the mitochondria showed unswollen cristae and presented the orthodox *in vivo* configuration and typical glycogen areas were found all over the cell. The strands of the rough endoplasmic reticulum were arranged in parallel nondilated stacks heavily covered by ribosomes. Hepatocytes incubated for 3 hours under 95 percent oxygen and 5 percent carbon dioxide at 37°C still revealed the same ultrastructural features as those of freshly isolated cells.

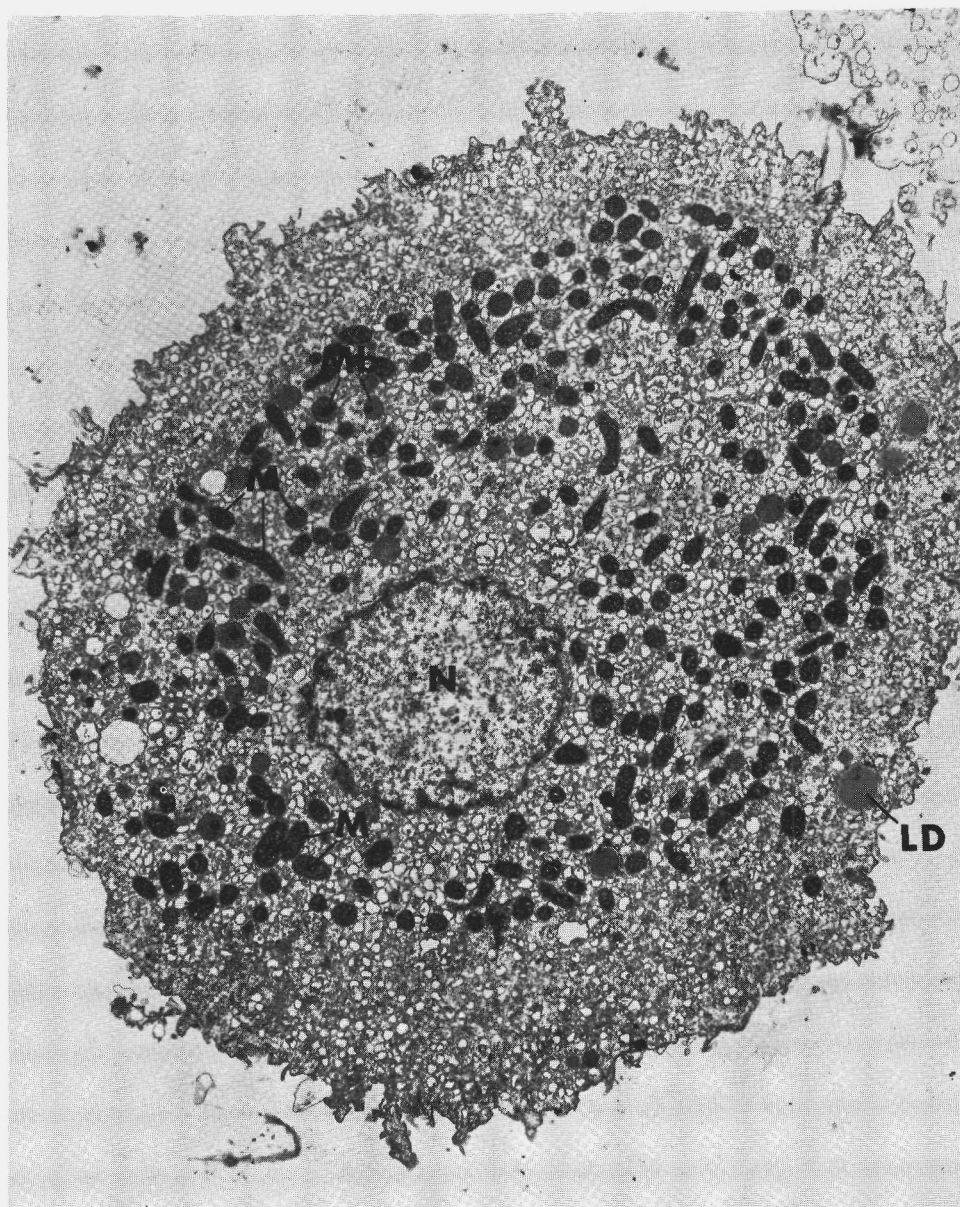


Figure 2.2 Electron micrograph of an ultrastructural damaged cell, which was isolated from a 6-month-old rat and excluded trypan blue. N = Nucleus, M = Mitochondria, Mb = Microbody, and LD = Lipid Droplet. (x 5,320).

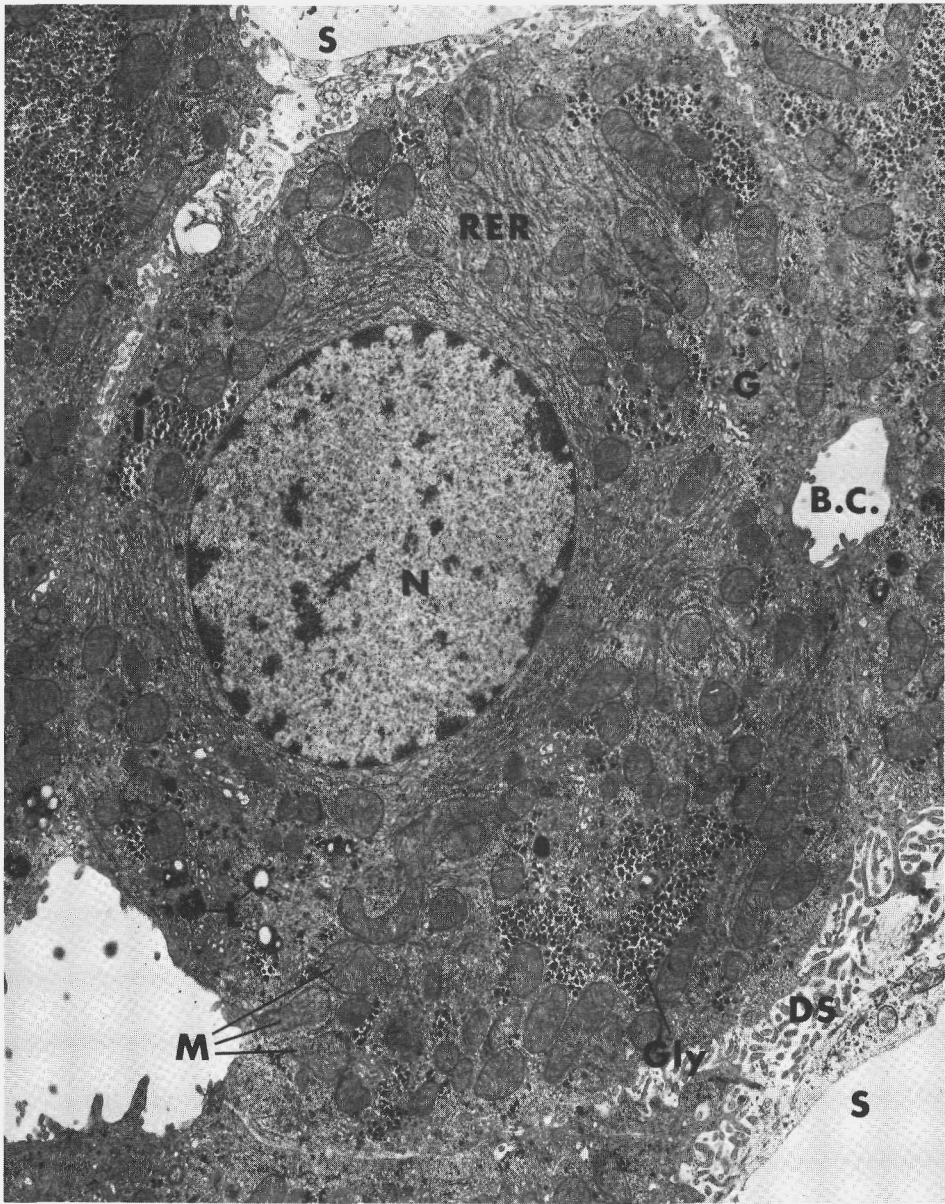


Figure 2.3 Liver parenchymal cells *in vivo* after perfusion fixation with 2 percent glutaraldehyde of the liver of a 3-month-old rat. N = Nucleus; M = Mitochondria; Gly = Glycogen; RER = Rough Endoplasmatic Reticulum; DS = Space of Disse; BC = Bile Canaliculus; L = Lipofuscin; S = Lumen of the sinusoid, and G = Golgi complexes. (x 10,366).

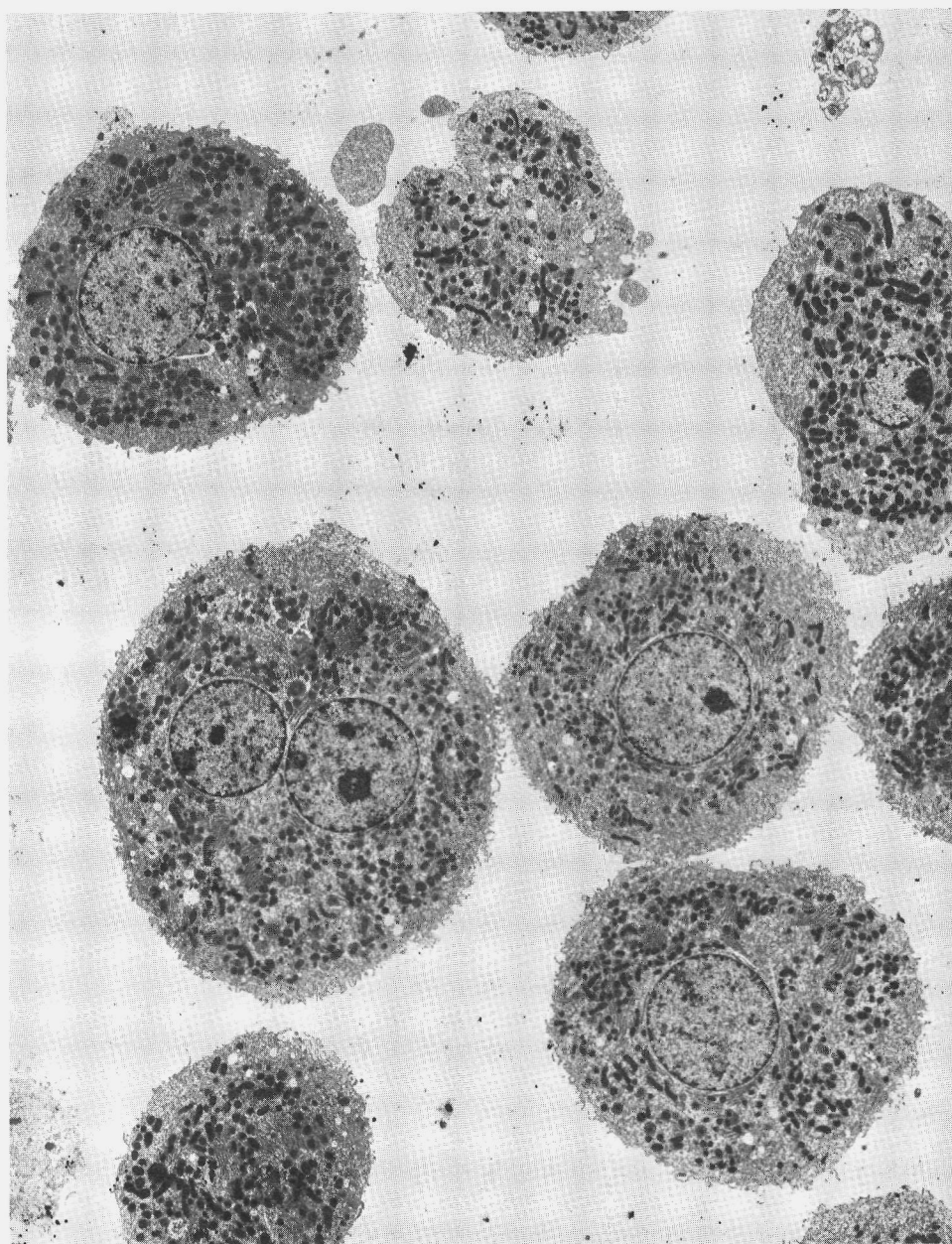


Figure 2.4 Liver parenchymal cells isolated from a 3-month-old rat. (x 4,375).

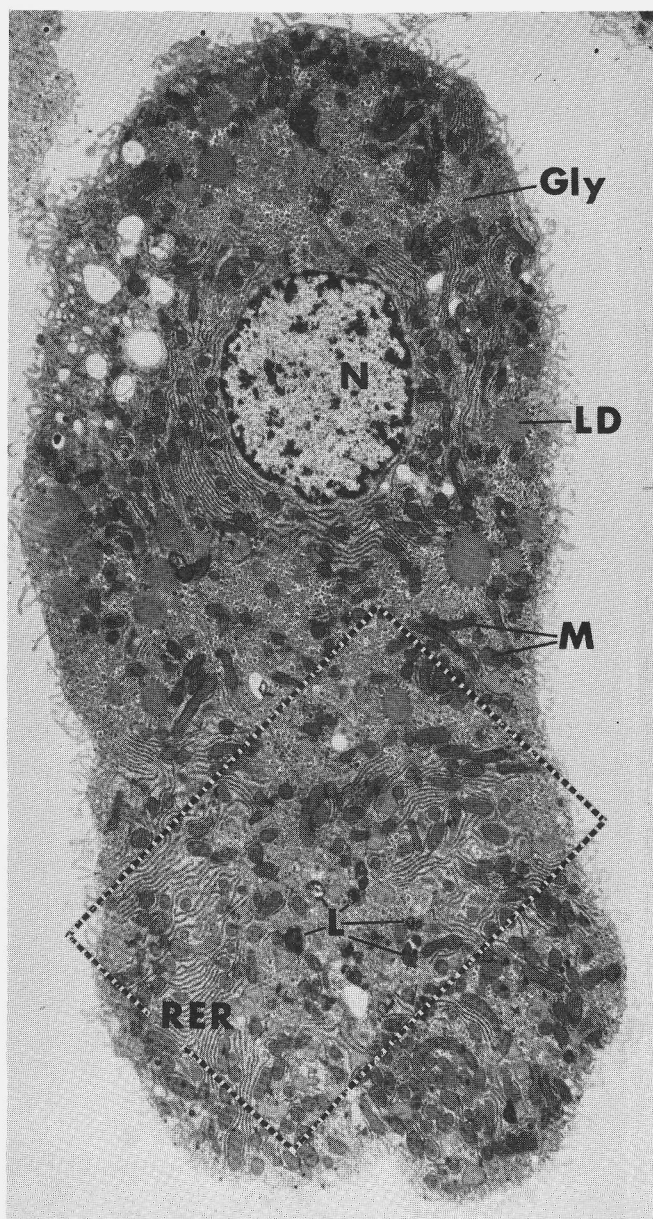


Figure 2.5 Hepatocyte isolated from a rat of 30 months of age. The cytoplasm contains mitochondria (M), strands of rough endoplasmic reticulum (RER), lipid droplets (LD), lipofuscin (L) and areas of glycogen (Gly). Note the intact ultrastructure of the nuclear chromatin (N). An enlargement of the indicated area is shown in Figure 2.6. ($\times 5,875$).

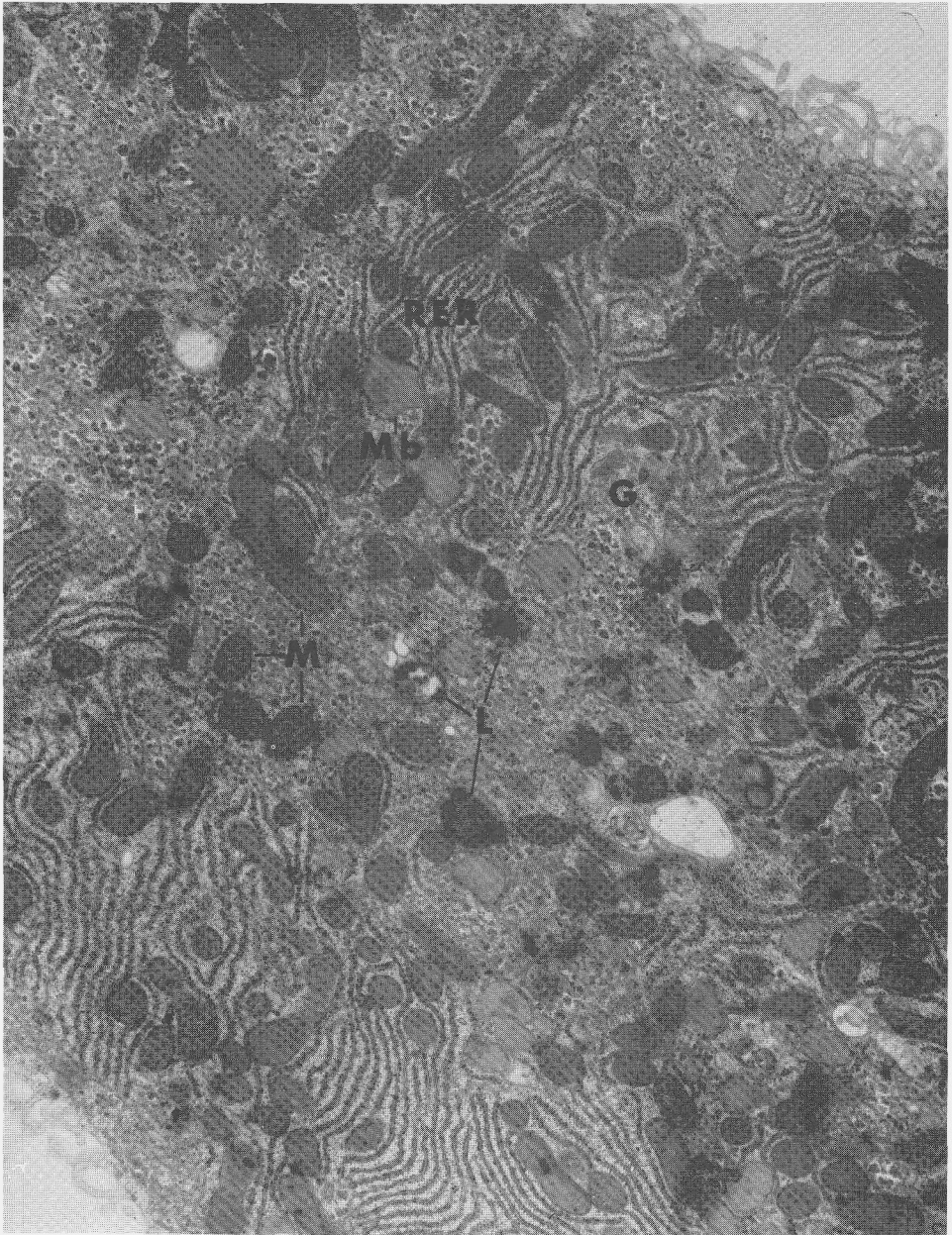


Figure 2.6 A detail of the isolated parenchymal cell of Figure 2.5. Note the well-preserved structure of the mitochondria (M), the rough endoplasmic reticulum (RER), the Golgi complexes (G), the microbody (Mb) and lipofuscin (L). (x 13,880).

Cells obtained from old rats showed ultrastructural characteristics comparable to those of cells prepared from young rats. The cytoplasm of cells from old rats, however, contained larger quantities of lysosome-derived structures, including lipofuscin (Figure 2.6).

2.3.3 METABOLIC INTEGRITY

Measurements of the respiration of the isolated liver parenchymal cells were performed as a test for their metabolic integrity. Stimulation of the respiration by added substrates was studied to test the intactness of the permeability barrier of the plasma membrane. The oxygen consumption of freshly prepared hepatocytes from rats of different ages was not stimulated by the addition of succinate, α -oxoglutarate and sodium citrate (van Bezooijen et al., 1974a). No age-related changes in the endogenous respiration of hepatocytes prepared from young and old rats were observed.

At our Institute Brouwer et al. (1977) performed a more detailed study on the respiratory capacity of the isolated hepatocytes. In agreement with the results mentioned above, no significant differences were observed between the endogenous respiration of hepatocytes from young and old rats. In order to be able to compare the oxygen consumption of the isolated liver parenchymal cells with that of the perfused liver, the respiration of the cells isolated from 3-month-old rats was calculated to be approximately 2.6 μmol of oxygen/min.g wet weight of liver tissue. This calculation was based on the measured protein content of intact liver tissue (211 mg per g wet weight).

The respiratory control ratio is a reliable characteristic of the functional integrity of isolated mitochondria (Chance and Baltscheffsky, 1958). As explained by Brouwer et al. (1977), this ratio cannot be directly measured in the mitochondria inside the isolated hepatocytes. An indirect method for estimating this ratio was developed by blocking the oxidative phosphorylation by addition of oligomycin and by subsequent addition of an uncoupler of the endogenous respiration. By this means, a control ratio is represented by the ratio between the uncoupled and blocked respiration. Our results (Brouwer et al., 1977) revealed that addition of the uncoupler dinitrophenol (DNP) resulted in a stimulation of the respiration rate of liver parenchymal cells prepared from 3-month-old rats by 41 percent. Oligomycin inhibited the oxygen consumption of hepatocytes isolated from rats of 3 months of age by 51 percent. As a result, the ratio between the uncoupled and blocked respiration of the isolated cells had a value of 2.92. No age-related changes were observed for this ratio.

2.4 DISCUSSION

Almost all reports in the literature concerning the isolation of rat liver parenchymal cells refer to the preparation of cells from rats of unstated ages but which probably vary in age from young to adult, as can be judged from their weights. At the beginning of this study, no procedures had been described for the isolation of hepatocytes from both very young and old rats. Since the availability of these cells is a prerequisite for *in vitro* studies on the question as to whether a change in the functional capacity of the liver with age can be partly attributed to change in the functional capacity of the individual liver cells, a method had to be developed for the isolation of parenchymal cells from rats of all ages.

The isolation procedure described in this chapter has been successfully applied to very young rats of two weeks of age up to 36-month-old rats. The cells isolated from 2-week-old rats were used only to determine the ploidy state of these cells. The data obtained will be given in chapter IV.

The cell yield ranged from 20-40 million cells per g wet weight of liver and was independent of the age of the rat. This yield was in the same range as that obtained by Berry and Friend (1969), and Gallai-Hatchard and Gray (1971) but lower than that reported by Seglen (1972).

As far as the viability of the hepatocytes obtained from rats of all ages is concerned, it can be stated that 95 percent of these cells did not stain with trypan blue. Even after a 4-hour incubation period at 37°C, the hepatocytes appeared to have an intact permeability barrier, since about 90 percent of them were still not permeable to trypan blue. From the electron microscopic observations, it was evident that almost all isolated hepatocytes were also ultrastructurally intact.

The endogenous respiration was measured as a characteristic of the metabolic integrity of the cell. The respiration rate of the hepatocytes from 3-month-old rats was 2.6 μmol of oxygen/min.g wet weight of liver. This value is considerably higher than the oxygen uptake of 0.8 μmol /min.g wet weight reported for slices obtained from the livers of young Wistar rats (Thieden and Lundquist, 1967) and comparable with the respiration rate of 2.2 μmol /min.g wet weight observed for the perfused liver (Hems et al., 1966, and Krebs et al., 1974). Consequently, it can be concluded that the isolated hepatocytes were metabolically intact with respect to this characteristic.

No increase in oxygen consumption was found after addition of tri-carboxylic acid cycle intermediates such as succinate. Therefore, it is concluded that intact and viable hepatocytes are not permeable to

succinate. This is in agreement with the existence of permeability barriers for tricarboxylic acid cycle intermediates in the perfused liver or liver slices (Ross et al., 1967, and Hems et al., 1968). However, stimulation of the oxygen consumption of isolated hepatocytes with succinate or other tricarboxylic acid intermediates has been reported by many investigators (Berry and Friend, 1969; Jezyk and Liberti, 1969; Hommes et al., 1970, and Howard et al., 1973). The stimulation by succinate may result from an alteration in the permeability of the plasma membrane caused by the isolation procedure based on mechanical and EDTA treatment such as, for example, was used by Hommes et al. (1970). However, an effect of succinate addition on oxygen consumption has also been reported for cells isolated by procedures based on collagenase and hyaluronidase digestion (Berry and Friend, 1969; Jezyk and Liberti, 1969, and Howard et al., 1973). In these cases, drastic changes in the permeability of the cell membrane and in the viability of the liver cells might be caused by the long incubation periods at 37°C employed for the measurements of the respiration rate and the mechanical shaking of the rather fragile cells in the respirometer.

A critical test for the metabolic integrity of the mitochondria in the isolated hepatocytes appears to be the determination of the ratio between the uncoupled and blocked respiration. The addition of an uncoupler (DNP) stimulated the oxygen consumption of the isolated hepatocytes by about 40 percent (Brouwer et al., 1977), which is in agreement with the data obtained with isolated hepatocytes of which the oxygen consumption was measured after addition of DNP or FCCP (Howard et al., 1973; Krebs et al., 1974; Baur et al., 1975, and Berry, 1976b). An inhibition by 51 percent of the respiration of the isolated liver parenchymal cells was the result of the addition of oligomycin (Brouwer et al., 1977), a percentage also reported by Galeotti (1976). Consequently, the uncoupled versus blocked ratio appeared to be 2.9 for hepatocytes isolated from 3-month-old rats (Brouwer et al., 1977), which is superior to the 1.9 reported for freshly isolated hepatocytes by Schwenk et al. (1976). No age-related changes in this ratio were observed for hepatocytes isolated from rats of different ages which is indicative for an unimpaired energy supply mechanism for liver parenchymal cells up to old age. This is in agreement with the data obtained by measuring the respiratory control ratio of isolated rat liver mitochondria (Gold et al., 1968). Similar data were obtained for old mice by Wilson et al. (1975) who found no significant decrease in mitochondrial enzyme activities with age.

Considering the data presented here, it is evident that an adequate indication of the viability of the isolated liver parenchymal cells cannot be achieved by a single characteristic. The morphological and functional integrity of the isolated hepatocytes can be established only by several criteria.

In this respect, trypan blue staining proves to be a rapid method for a first screening for the viability of the isolated hepatocytes. The ultrastructural intactness of the cells must, however, be checked by electron microscopy.

It can be concluded that the hepatocytes isolated by the present method are comparable to intact liver tissues with respect to their membrane permeability, their ultrastructural appearance and their endogenous respiration. Furthermore, hepatocytes isolated from old rats are not inferior to cells from young rats with respect to the tested viability criteria. In view of these two conclusions, age-related changes in the functional capacity of the isolated hepatocytes cannot be attributed to artifacts of the isolation procedure, but should really represent aging changes.

CHAPTER III

BROMSULFOPHTHALEIN UPTAKE BY ISOLATED HEPATOCYTES

<i>Contents:</i>	3.1	Introduction
	3.2	Materials and methods
	3.2.1	Materials
	3.2.2	Assays
	3.3	Results
	3.3.1	Optimum conditions for BSP storage by isolated liver parenchymal cells
	3.3.2	Nature of the BSP transport across the hepatocyte membrane
	3.4	Discussion

3.1 INTRODUCTION

One of the major functions of the liver, performed by the parenchymal cells, is the excretion of various organic substances. The hepatocytes have the capacity to take up these compounds from the blood, to store them and to excrete them into the bile canaliculi. Among these compounds are organic anions such as bilirubin and corticosteroids and exogenous substances such as bromsulfophthalein (BSP). The excretory function of the liver can be determined *in vivo* by means of the BSP retention test. The principle of this test is as follows. Intravenously injected BSP largely combines with serum albumin and is transported by albumin to the liver parenchymal cells (Baker and Bradley, 1966). After dissociation from albumin, free BSP is transferred from the blood into the parenchymal cells. In the cytoplasm of the cells, the BSP is partially bound by Y protein (ligandin) (Mishkin et al., 1972). Combes and Stakelum (1961) described an enzyme which catalyzed the conjugation of the bound BSP with glutathione. This enzyme, which has been named glutathione S-transferase B, appeared to be identical to ligandin (Habig et al., 1974). Both the unconjugated and the conjugated BSP are finally excreted into the bile canaliculi.

The nature of the processes by which BSP is transported across the cell membrane will be described in more detail in this Chapter as a result of the studies performed with isolated hepatocytes. In general, two types of mechanisms for transport can be distinguished. One is passive diffusion, in which the rate of transfer into the cells is directly proportional to the concentration gradient of the solute across the cell membrane. The other is carrier transport which occurs at a higher rate than would be attributable to diffusion alone. At lower solute concentrations, the rate of transport is related to the concentration of the solute presented to the membrane. However, at higher solute concentrations, the rate of transfer shows evidence of saturation at increasing solute concentrations. Carrier transport can be subdivided into two types, viz., facilitated diffusion and active transport. In contrast to the facilitated diffusion process, the active transport mechanism operates against a concentration gradient and is dependent on temperature and metabolic energy.

The data in the literature on the mechanisms by which BSP or other organic anions are transferred from the plasma into the parenchymal cells are contradictory. The group of Arias and Fleischner has studied this process since 1958 and discovered that Y and Z proteins are intracellular binding sites for organic anions (Mishkin et al., 1972). They postulated that intracellular concentrations of Y and Z determine the diffusion of various organic anions across the plasma membrane (Arias,

1972). Frezza et al. (1974) observed, on the contrary, that the binding capacity for BSP was about three times greater for the proteins present outside of the liver cell than for the intracellular proteins. Furthermore, the affinity of serum proteins for BSP was higher than that of the intracellular liver proteins. Hence, they concluded that their data did not support the hypothesis of the group of Arias that the transfer of BSP from the plasma into the liver cell occurred by diffusion. The explanation of Frezza et al. (1974) for the BSP transfer from the plasma into the liver parenchymal cell was the existence of a carrier mediated process at the level of the liver cell plasma membrane. The same conclusion was reached by Scharschmidt et al. (1975) who injected labeled BSP into intact rats and observed that the amount of BSP cleared by the liver from the blood reached a plateau level at the higher doses of injected BSP.

These studies of Mishkin et al. (1972), of Frezza et al. (1974) and of Scharschmidt et al. (1975) concerning the transport mechanisms of BSP into the parenchymal cells have generally been restricted to the use of whole rats, perfused liver and liver homogenates. These studies have important limitations. Using a whole animal or the perfused liver, a disadvantage with respect to the problem of the BSP transfer across the liver parenchymal cell membrane is that the concentrations of free or bound BSP in and directly outside the liver parenchymal cells cannot be determined with these systems. Liver parenchymal cell suspensions provide a unique system for studying the BSP transfer mechanisms, since the concentration of free and bound BSP inside the cell can be established. Furthermore, there are no BSP concentration gradients in liver parenchymal cell suspensions, which may play a role within the liver lobule from the portal area to the central area.

In addition to the nature of the process by which BSP is taken up by the liver parenchymal cells, the optimum conditions for measuring the functional capacity of isolated hepatocytes with respect to the BSP storage are also studied. The optimum conditions are determined with respect to the time of incubation, the BSP concentration in the incubation medium and the cell concentration. In this way, any small changes in this functional capacity of the isolated hepatocytes with age may be detected.

3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

Three-month-old female WAG/Rij rats weighing 130-160 g were used.

The liver parenchymal cells used for the experiments described in this Chapter were isolated in a slightly different manner from the improved method described in paragraph 2.2.3. The reason for this is that the isolation procedure was improved after the experiments described in this Chapter had been performed. The differences are that the first and second perfusions were not performed and that, after the perfusion with the medium containing the enzymes collagenase and hyaluronidase, the pieces of liver tissue were incubated for 50 min at 37°C in 50 ml dissociation medium under an atmosphere of 95 percent oxygen and 5 percent carbon dioxide with constant shaking.

The standard medium employed during the isolation procedure and in all experiments described in this Chapter was also slightly different from the medium described in paragraph 2.2.2. The standard medium consisted of 0.1 mM L-aspartic acid, 0.2 mM L-threonine, 0.3 mM L-serine, 0.5 mM glycine, 0.6 mM L-alanine, 0.9 mM L-glutamic acid, 0.9 mM L-glutamine, 3 mM KCl, 0.5 mM NaH₂PO₄, 0.5 mM MgCl₂, 24 mM NaHCO₃, 2 mM glucose, 25 mM HEPES, 185 mM sucrose; pH 7.4 at 37°C; osmolality 300 mOsm.l⁻¹. The dissociation medium consisted of the standard medium to which collagenase and hyaluronidase were added.

Electron microscopic examination of the suspensions isolated according to the above described method revealed that the mitochondria often showed a condensation of the matrix. Moreover, the areas of glycogen characteristic of hepatocytes were mostly absent. By measuring the oxygen tension in the incubation medium, it was established that the oxygenation of the parenchymal cells during that incubation period of 50 min was insufficient. Therefore, for subsequent experiments the isolation procedure and standard medium were modified as described in paragraph 2.2.3.

³⁵S-BSP was purchased from the Radiochemical Center Amersham, UK; Soluene[®] 100 from Packard Instrument International, Zurich, Switzerland and bovine albumin fraction V from Sigma, St. Louis, Mo., U.S.A.

3.2.2 ASSAYS

To determine the amount of BSP storage by isolated liver parenchymal cells, a known number of parenchymal cells in 1.5 ml of standard

medium was incubated with ^{35}S -BSP at 37°C under an atmosphere of 95 percent oxygen and 5 percent carbon dioxide with constant shaking (100 oscillations.min $^{-1}$). Immediately after the addition of ^{35}S -BSP to the cell suspension and after various incubation periods, 0.5 ml samples were withdrawn and added to 5 ml ice-cold 0.3 M phosphate buffered saline (pH 7.4). The cells were washed three times with 5 ml ice-cold phosphate buffer with interim centrifugation at 100 g. The cell pellet was then dissolved in 1 ml soluene $^{\text{®}}$. The radioactivity of the samples was counted in 15 ml toluene-based scintillation fluid (composition 50 mg POPOP and 4 g PPO/1 toluene). The radioactivity of ^{35}S -BSP was measured with a liquid scintillation counter. Quenching of the samples was determined by the external standard ratio method (Hayes, 1966). Values obtained after incubation were corrected for the amount of BSP absorbed to the cellular membrane represented by the amount of radioactivity found in the sample withdrawn immediately after the addition of ^{35}S -BSP to the cell suspension.

The method for measuring rat serum albumin was performed according to De Leeuw-Israel et al. (1967). In this method, the amount of albumin was determined in a 5 μl serum sample by means of measuring colorimetrically the capacity of the serum albumin to bind specifically the anionic dye 2-(4'-hydroxy benzene azo) benzoic acid (HBABA).

The amount of BSP bound to cytoplasmic proteins in the parenchymal cells was determined as follows. The first steps of the procedure were exactly identical to the method described above for measuring the total amount of radioactive BSP stored by parenchymal cells. After washing the samples with 5 ml ice-cold phosphate buffer, the cell pellet was resuspended in 5 ml ice-cold 10 percent trichloroacetic acid (TCA) and stored for at least 30 min at 0°C . The resulting precipitate was washed two times with 5 ml ice-cold 5 percent TCA with interim centrifugation at 1000 g. The final pellet was solubilized with 1 ml soluene $^{\text{®}}$ and 15 ml scintillation fluid was added.

Chromatographic separation of free BSP and glutathione-BSP (BSP-GSH) was performed according to a modification of the method of Freundt (1973) employing cellulose powder thin layer chromatography. After precipitation of the protein bound BSP by 10 percent TCA or by 10 ml 96 percent ethanol and 0.72 ml saturated ammonium sulphate solution, the supernatant was taken off and evaporated. The sediment was dissolved in 50 μl 1 N NaOH and thin layer chromatography on cellulose plates was performed with an n-butanol: glacial acetic acid: water (8:1.2:3) mixture as the solvent system. BSP-GSH was made visible by spraying with ninhydrin (0.25 percent) in ethanol and heating at 37°C for 15 min (Stege et al., 1975). BSP was revealed by spraying the plates with 1 N

NaOH. The coloured spots of free BSP and BSP-GSH were scraped off and eluated with 1 ml 1 N NaOH. The eluate was dissolved in soluen[®] and 15 ml scintillation liquid was added.

The diameters of the isolated cells were measured as follows. The cells were placed on a microscope slide by means of a sedimentation chamber. They were dried, stained by the Feulgen reaction followed by counterstaining with a 0.1 percent Light Green solution and photographed with the Ultraphot II apparatus (Carl Zeiss). After standard enlargement of the photographs, the diameters of the cells were measured with a TGZ3 Particle Size Analyzer (Carl Zeiss).

3.3 RESULTS

3.3.1 OPTIMUM CONDITIONS FOR BSP STORAGE BY ISOLATED LIVER PARENCHYMAL CELLS

The time course of BSP storage by isolated hepatocytes in a representative experiment is shown in Figure 3.1. The uptake is linear for the first 10 min and reaches a plateau at about 12 min. Thereafter, the BSP storage does not change up to 45 min of incubation. In a representative experiment, the correlation between the concentration of ³⁵S-BSP

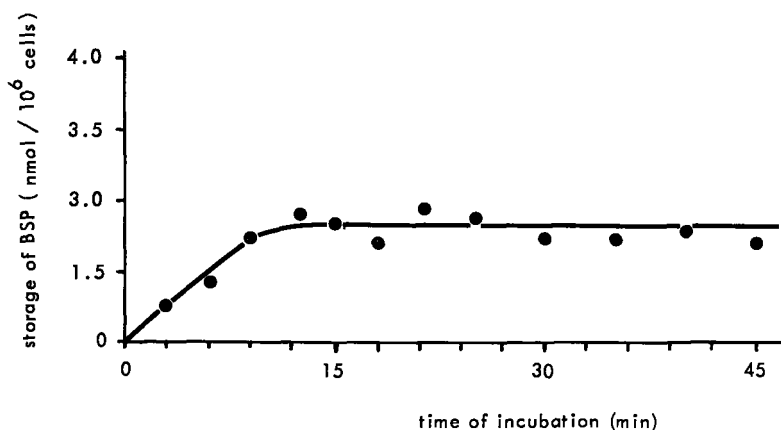


Figure 3.1 Time course of ³⁵S-BSP uptake by isolated liver parenchymal cells. Twelve cell suspensions with a concentration of 290,000 cells.ml⁻¹ were incubated with 30 nmol ³⁵S-BSP/ml incubation medium at 37°C. The amount of BSP uptake was determined at the time intervals indicated in the figure.

in the incubation medium and the amount of BSP stored by the isolated cells in 15 min is shown (Figure 3.2). For cell suspensions consisting of viable parenchymal cells, incubation with BSP concentrations higher than about 25 nmol/ml standard medium do not result in a higher BSP storage. From these results, it can be concluded that the maximum BSP storage takes place at about 25 nmol BSP/ml incubation medium.

On the basis of the results presented in Figures 3.1 and 3.2, the BSP storage in subsequent experiments was measured with 30 nmol ^{35}S -BSP/ml medium after 15 min of incubation. These test conditions guarantee that the hepatocytes function maximally; therefore, small changes in the BSP storage by the cells due to aging can be easily detected.

The plateau level in Figure 3.2 was observed only for suspensions consisting almost entirely of viable cells. Cell suspensions with a high number of nonviable cells (trypan blue stained) did not show a plateau level but the amount of BSP stored by these cells showed a linear increase with increasing concentrations of BSP in the incubation medium.

It was constantly found that the amount of BSP stored by the isolated hepatocytes was proportional to the number of cells present in the incubation medium up to a concentration of 0.4×10^6 cells/ml medium (Figure 3.3). Incubation with cell concentrations higher than

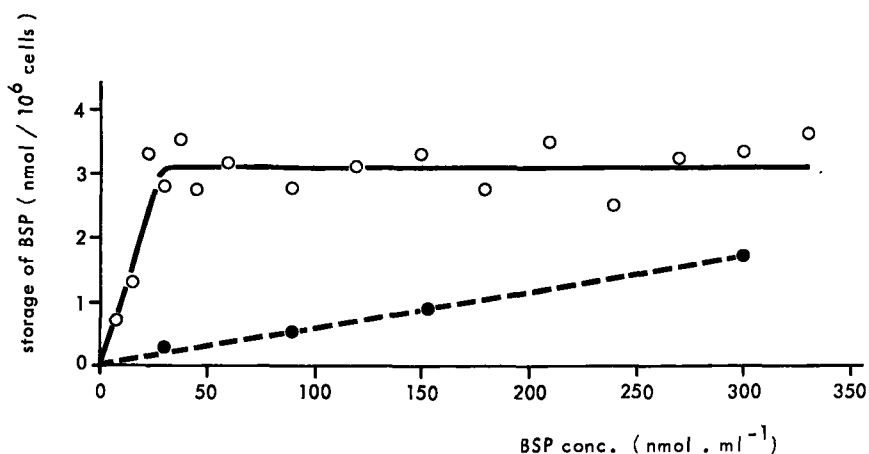


Figure 3.2 The uptake of ^{35}S -BSP by a suspension of viable (O—O) and nonviable (●---●) parenchymal cells as a function of the concentration of BSP in the incubation medium. Sixteen suspensions of viable cells with a cell concentration of $120,000 \text{ cells.ml}^{-1}$ and four suspensions of nonviable cells with a cell concentration of $300,000 \text{ cells.ml}^{-1}$ were incubated at 37°C for 15 min with various amounts of BSP in the medium.

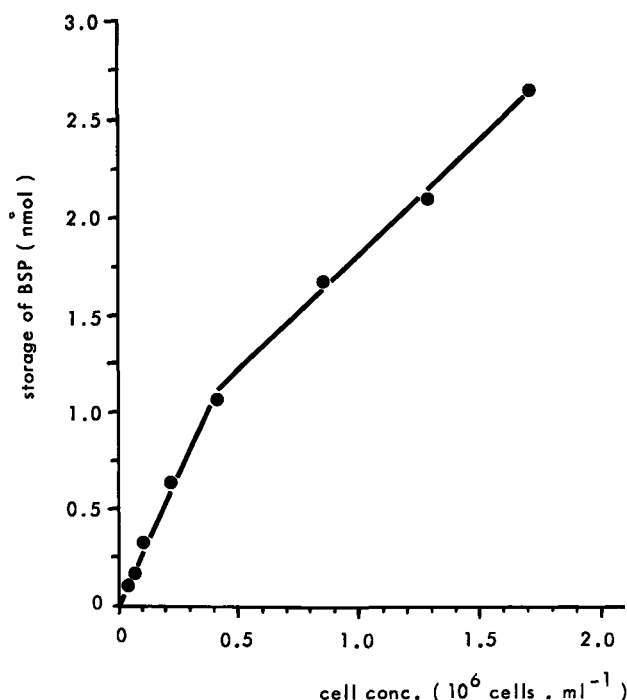


Figure 3.3 Effect of the cell concentration in the incubation medium on the BSP uptake by isolated hepatocytes. Eight cell suspensions with the concentrations indicated were incubated at 37°C for 15 min with the 30 nmol BSP/ml medium.

0.4×10^6 cells/ml medium resulted in a disproportional correlation between the amount of BSP stored by the cells and the number of cells/ml medium. In further experiments, therefore, cell suspensions ranging from 0.5×10^5 to 0.4×10^6 cells.ml $^{-1}$ were used.

To determine the amount of ^{35}S -BSP stored per 10^6 cells, three different cell concentrations were made from each cell preparation. By plotting the amount of stored BSP, expressed in nmol BSP, against the cell concentration, a straight line based on 4 points was obtained; the origin is the additional point. From this line, the amount of BSP stored per 10^6 cells was read off. Cell suspensions with cell concentrations ranging from 0.5×10^5 to 0.4×10^6 cells/ml medium were incubated with a concentration of 30 nmol BSP/ml medium for 15 min at 37°C under an atmosphere of 95 percent oxygen and 5 percent carbon dioxide. Under these experimental conditions, viable liver parenchymal cells

from 3-month-old rats stored 2.87 ± 0.18 nmol BSP/ 10^6 cells (mean $\pm$ S.E. of 37 experiments). Expressed on a protein basis, 1.95 ± 0.18 nmol BSP/mg protein (mean $\pm$ S.E.) were stored by the isolated hepatocytes. This latter calculation was based on a measured content of 1.47 ± 0.10 mg protein per 10^6 cells (mean $\pm$ S.E. of 11 experiments).

In vivo, BSP is carried to the parenchymal cells by plasma albumin. To determine whether albumin is necessary for the uptake of BSP by the isolated hepatocytes, the physiological concentration of albumin in the serum of 3-month-old female WAG/Rij rats was determined. This concentration appeared to be 4.6 ± 0.1 percent (mean $\pm$ S.E. of 10 experiments). Addition of 5 percent albumin to the incubation medium inhibited the storage of BSP by the isolated cells, as demonstrated in Table 3.1.

3.3.2 NATURE OF THE BSP TRANSPORT ACROSS THE HEPATOCYTE MEMBRANE

In order to discriminate between a passive diffusion process and a carrier mediated mechanism for the BSP transfer, the rate of BSP transport by the cells was measured. The cells were incubated with various amounts of BSP for 5 min, a period during which the storage of BSP is linear with time (cf. Figure 3.1). Figure 3.4, which presents a representative experiment, shows that, with increasing amounts of BSP in the

TABLE 3.1

EFFECT OF BOVINE SERUM ALBUMIN ON THE AMOUNT OF BSP STORED BY ISOLATED LIVER PARENCHYMAL CELLS

Addition	n	Storage of BSP (nmol/ 10^6 cells)		
		mean	S.E.	P
albumin	3	0.07	0.02	< 0.0001
none	2	2.70	0.16	

The parenchymal cell suspensions were incubated at 37°C for 15 min with 30 nmol BSP. ml^{-1} in the presence of 5 percent albumin.

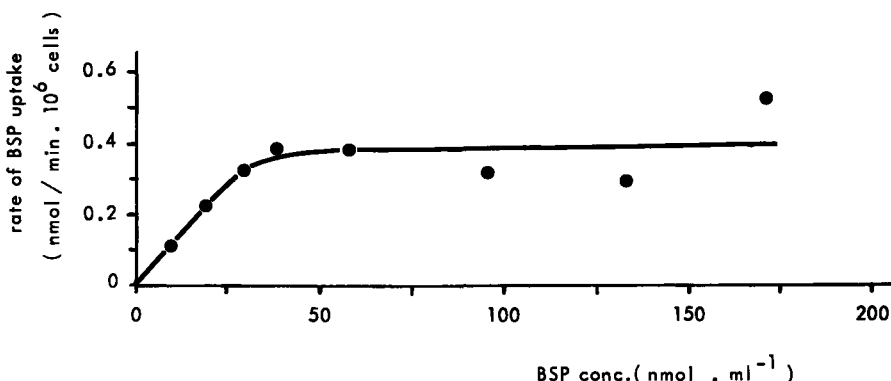


Figure 3.4 The relationship between the rate of BSP uptake by the cells and the concentration of BSP in the incubation medium. Eight cell suspensions were incubated at 37°C for 5 min with different amounts of BSP in the medium.

incubation medium, a plateau level is reached for the rate of BSP transport by the cells. The saturation of the rate of BSP transfer at higher BSP concentrations indicates that the transport of BSP across the cell membrane is of the carrier type.

For discrimination between the two possible types of carrier transport for the BSP uptake, it is necessary to determine whether or not the transport of BSP is operating against a concentration gradient. For this purpose, it is not enough to measure the total amount of BSP in the cells, since that part of the intracellular BSP which is bound to ligandin or conjugated with glutathione will no longer influence the BSP transfer mechanism. Therefore, the amount of free BSP that accumulated in the isolated cells had to be determined. The amount of BSP bound by ligandin, established by measuring the amount of BSP precipitated by TCA, appeared to be 1.06 ± 0.34 nmol BSP/10⁶ cells. The total amount of BSP stored by the hepatocytes was 2.87 ± 0.18 nmol BSP/10⁶ cells, as described above. From these results, it could be calculated that about 40 percent of the total BSP stored by cells appeared to be bound by ligandin.

The BSP not precipitated by TCA or ammonium sulphate was subjected to thin layer chromatography and the amount of free BSP and BSP conjugated with glutathione was determined. These studies revealed that, of the amount of BSP in the parenchymal cells not bound by ligandin, 95 percent and 5 percent could be considered as free BSP and conjugated BSP, respectively.

It was mentioned above that the amount of BSP taken up by the cells was 2.87 nmol BSP/ 10^6 cells. From the measured diameters of 300 hepatocytes a mean volume of $5405 \mu\text{m}^3$ for the parenchymal cells isolated from 3-month-old female WAG/Rij rats, could be calculated. However, this value might be too high because the isolated cells may be flattened during the sedimentation procedure. The concentration of free BSP within a cell could be calculated to be about 310 nmol BSP/ml parenchymal cell volume. In view of the impermeability of various cell organelles for BSP and the possible too high mean volume of the hepatocytes, the actual concentration of free BSP in the cytoplasm of the hepatocytes would be even higher than the mentioned value. Thus, the concentration of free BSP within the cells is at least 10 times higher than the concentration of free BSP in the incubation medium (30 nmol BSP. ml^{-1}). This suggests active transport of BSP into the cell.

Dependency on temperature is another characteristic of active transport. It was found that the amount of stored BSP was significantly higher at 37°C than at lower temperatures (Figure 3.5). A ratio (Q10) of 2.07 ± 0.07 (mean $\pm$ S.E.) for the amount of BSP stored by the parenchymal cells from 3 different cell preparations at 37 and 27°C could be calculated.

To test whether the transfer of BSP is dependent on cellular energy, the rate of BSP transport was measured in the presence of DNP, an uncoupler of oxidative phosphorylation, or KCN, a respiratory inhibitor (Table 3.2). KCN and DNP significantly reduced the rate of BSP uptake.

TABLE 3.2

INHIBITION EFFECT OF KCN AND DNP ON THE RATE OF BSP UPTAKE BY
ISOLATED LIVER PARENCHYMAL CELLS

Addition	Final conc. (M)	n	Rate of BSP uptake (nmol/min. 10^6 cells)		% inhibition	P
			mean	S.E.		
KCN	5×10^{-4}	14	0.138	0.024	56	< 0.01
none		11	0.316	0.064		
DNP	5×10^{-4}	11	0.141	0.017	55	< 0.02

The various cell suspensions were incubated at 37°C for 5 min with 30 nmol BSP. ml^{-1}

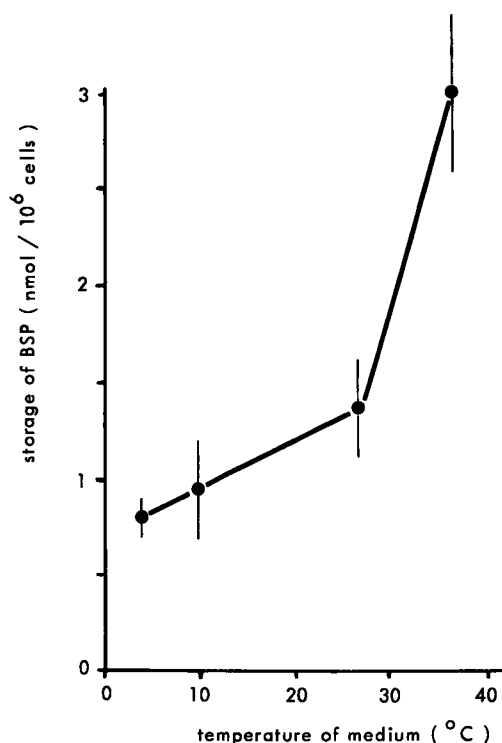


Figure 3.5 Dependency of the amount of BSP stored in 15 min by the isolated hepatocytes on the temperature of the incubation medium. Four cell suspensions were incubated for 15 min with 30 nmol ^{35}S -BSP.ml $^{-1}$ at the indicated temperatures. The data plotted in the figure are the mean $\pm$ S.E. of five experiments.

3.4 DISCUSSION

From the experiments performed to determine the conditions necessary for optimum BSP storage by the isolated liver parenchymal cells (Figure 3.1), it was concluded that the maximum amount of BSP was stored within 15 min of incubation (van Bezooijen et al., 1973; van Bezooijen and Knook, 1974b, and van Bezooijen et al., 1976a). This conclusion has been confirmed by Stege et al. (1975), and Schwenk et al. (1976). The concentration of BSP in the medium necessary to achieve optimum BSP storage appeared to be 30 nmol BSP/ml incubation medium (Figure 3.2). Since Schwenk et al. (1976) did not measure uptake curves at BSP concentrations higher than 26 μM , it is not surprising that they concluded that the uptake of BSP was not saturable.

It was observed (Table 3.1) that a physiological concentration of albumin in the incubation medium inhibited the storage of BSP by the isolated cells by about 98 percent (van Bezooijen et al., 1973, and van Bezooijen et al., 1976a). The same result has been obtained by Stege et al. (1975), and Schwenk et al. (1976) who also incubated isolated hepatocytes with BSP in the presence of albumin. These data with isolated hepatocytes confirmed the results obtained by Brauer and Pessotti (1949) with the perfused rat liver. They noted that addition of albumin to the perfusing fluid greatly retarded BSP uptake by the liver. In man, intravenously injected albumin also decreased the removal rate of BSP. This has been determined in patients with hypoalbuminemia (Grausz and Schmid, 1971) and in babies who were intravenously injected with BSP for diagnostic purpose (Heimann and Roth, 1976). The above described *in vitro* and *in vivo* data with respect to the BSP metabolism in the presence of albumin might be explained by the finding of Pfaff et al. (1975) that free BSP in solution was bound for about 99 percent when albumin was added in a physiological concentration. It can be concluded that, although albumin carries the BSP in the serum, it is unnecessary for the transfer of BSP across the liver parenchymal cell membrane.

The experiments performed to determine the mechanism of transfer of BSP across the cell plasma membrane revealed that the rate of BSP transfer was not proportional to the BSP concentration in the medium but approached a maximum level. This finding is indicative for a carrier type of BSP transport into the viable liver parenchymal cell. A carrier type of transport for BSP into the liver parenchymal cell was suggested on a hypothetical basis by Ketterer et al. (1975), Pfaff et al. (1975), and Erickson and Holtzman (1976). They speculate that the BSP molecule (see formula in Figure 3.6) with the two sulfonic acid groups which are ionized at a physiological pH is not lipid-soluble but water-soluble and might require, therefore, a carrier mediated transport. No saturation of the BSP storage was observed for cells stainable by trypan blue, suggesting a passive diffusion of BSP into damaged cells. It can be concluded from the observations that free BSP was transferred across the cell membrane against a concentration gradient in viable cells and that the BSP storage was dependent on temperature and energy that BSP is transported into the liver parenchymal cells by an active transport system. With respect to the uptake of free BSP against a concentration gradient, it can be mentioned that Schwenk et al. (1976) determined that the concentration of free and bound BSP inside the hepatocyte was much higher, more than 250-fold at a $1 \mu\text{M}$ extracellular BSP concentration, than outside the cell. Nevertheless, they did not conclude that the transfer of BSP occurred by an active transport mecha-

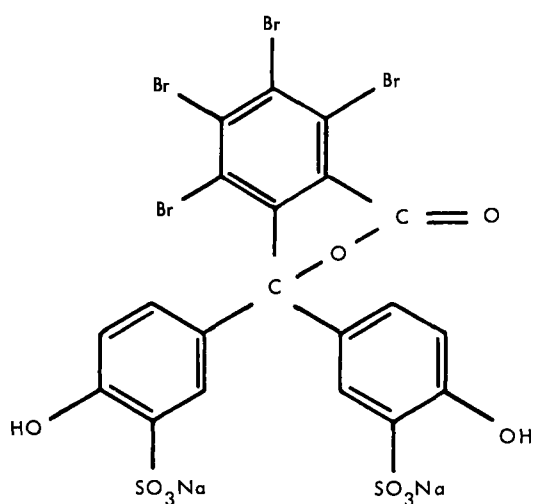


Figure 3.6 Formula of bromsulfophthalein (BSP).

nism. Firstly, because they had not measured the concentration of free BSP inside the cell; therefore, it was impossible for them to ascertain whether or not the free BSP was taken up against a concentration gradient. Secondly, because metabolic inhibitors such as carbonylcyanide *m*-chlorophenylhydrazone and rotenone did not effect the BSP uptake. This last finding seems to be in contradiction with the results of this study, which show an inhibition of the BSP uptake rate by addition of KCN or DNP. Schwenk et al. (1976) did not specify, however, the concentration of the metabolic inhibitors they used. KCN or DNP inhibited the transport of BSP across the cell membrane of the isolated hepatocytes by 55 percent (Table 3.2). Inhibition of the transport of other substances into the isolated hepatocytes by KCN or DNP has also been established by several investigators. For example, an inhibition by KCN of the transport of cholic acid into isolated liver parenchymal cells by 51 percent was reported by Anwer et al. (1976). The uncoupler DNP diminished the transport of carnitine by 58 percent and that of butyrobetaine by 83 percent (Christiansen and Bremer, 1976). The transfer of methotrexate into the isolated hepatocytes was inhibited by DNP by 84 percent (Horne et al., 1976). Another uncoupler, carbonylcyanide *m*-chlorophenylhydrazone, reduced taurocholate uptake by 75 percent (Schwarz et al., 1975).

The dependence of the BSP transfer into the isolated hepatocytes on temperature is expressed by a Q10 of 2.07. This value correlates

well with ratios of 2-3 measured for active transport of amino acids into tissues (Van de Berg, 1974). Harris et al. (1975), who measured the removal rate of BSP from the liver perfusate at 37 and 27°C, found that the rate of removal of BSP was 2.9 times higher at 37°C than at 27°C.

A number of papers have recently appeared which describe the transfer of different substances into isolated liver parenchymal cells. Carrier mediated transport was reported for asialo-fetuin (Tolleshaug et al., 1977), cortisol (Rao et al., 1976), glucose (Baur and Heldt, 1976) and other hexoses (Baur and Heldt, 1977). As observed for BSP, an active transport system was determined for alanine (Edmondson et al., 1977), aminoisobutyric acid (Le Cam and Freychet, 1976, and Chen and Lee, 1977), butyrobetaine and carnitine (Christiansen and Bremer, 1976), cholic acid (Anwer et al., 1976), methotrexate (Horne et al., 1976) and taurocholic acid (Schwarz et al., 1975).

In addition to the studies to determine the mechanisms of uptake and storage of BSP by the isolated hepatocytes, pilot studies on the secretion of BSP were performed by use of thin layer chromatography. These indicated the appearance of glutathione-conjugated BSP in the medium during the incubation of the cells. Thus, it may be concluded that the isolated parenchymal cells were capable of performing all of the various steps of the BSP clearance mechanism.

CHAPTER IV

AGE-RELATED CHANGES IN BROMSULFOPHTHALEIN METABOLISM OF THE LIVER AND ISOLATED HEPATOCYTES

<i>Contents:</i>	4.1	Introduction
	4.2	Methods
	4.3	Results
	4.3.1	BSP metabolism of the liver <i>in vivo</i> in female WAG/Rij rats
	4.3.2	BSP storage by liver parenchymal cells isolated from female WAG/Rij rats of different ages
	4.3.3	The effect of age on the BSP storage by hepatocytes isolated from male WAG/Rij and female BN/Bi rats
	4.4	Discussion
	4.4.1	The BSP metabolism <i>in vivo</i>
	4.4.2	Comparison of <i>in vivo</i> and <i>in vitro</i> data

4.1 INTRODUCTION

The data concerning the effect of age on the functional capacity of the human liver with respect to the BSP retention test are rather conflicting (Table 4.1). Vink (1959) observed an increase in BSP retention during the first 30 years, whereas Thompson and Williams (1965) found that the BSP retention increased up to the age of 80 years. An increase in BSP retention was measured only after 40, 50 and 60 years of age by Calloway and Merrill (1965), Skaunic et al. (1968), and Freston and Englert (1967), respectively. Koff et al. (1973), and Kampmann et al. (1975) failed to demonstrate any age-dependent change in the BSP retention test.

The decline in this human liver function with age appeared to be due to an age-related decrease in the relative storage capacity of the liver for BSP (Thompson and Williams, 1965, and Skaunic et al., 1968). The relative storage capacity was defined as the amount of BSP taken up and stored by the liver as a function of the concentration of BSP in the serum. No change in the maximal excretion capacity, which is a characteristic of the excretion of BSP into the bile, was observed (Thompson and Williams, 1965, and Skaunic et al., 1968).

An increase in BSP retention with age was also found in experiments performed with female RU rats (De Leeuw-Israel, 1971). As in the human situation; the increased BSP retention in the serum of old rats was ascribed to a decrease in the relative storage capacity of the liver for BSP, whereas the maximal excretion capacity remained unchanged (De Leeuw-Israel et al., 1969a).

The decrease in BSP metabolism by the liver with age can be partly explained by a reduction in the number of hepatocytes per unit liver volume with age, which is a consequence of the age-related increase in polyploidy of the liver parenchymal cells (De Leeuw-Israel et al., 1972). In addition, the decline in BSP metabolism with age may be related to several physiological factors. These factors include extracellular factors such as a diminished blood flow through the liver, as suggested by Wilkinson et al. (1968), Clarkson et al. (1970), and Skaunic et al. (1970). Furthermore, the BSP retention might be influenced by competition of BSP with other organic anions for the binding to albumin in the serum (Baker and Bradley, 1966). Morphological alterations may also influence the BSP metabolism, such as in the case of cirrhosis where collagen occupies the space of Disse (Steiner et al., 1965). These complicating factors are excluded in the *in vitro* system offered by the isolated liver parenchymal cells. The use of such an *in vitro* system also enables one to investigate whether a decreased func-

TABLE 4.1

THE EFFECT OF AGE ON THE HUMAN BSP RETENTION TEST

Author(s)	Subjects		Retention time (min)	Age group (years)	BSP retention*
	female (n)	male (n)			
Rafsky and Newman (1949)	26	16	30	65-86	20 percent abnormal**
Vink (1959)	15	28	-	2-50	Increase in half-time of BSP during the first 30 years
Haberman (1962)	55	36	45	60-90	48 percent abnormal***
Calloway and Merrill (1965)	-	73	30	20-90	Increased after 40 years
Thompson and Williams (1965)	-	32	30	20-93	Increased up to 70-80 years
Freston and Englert (1967)	1	83	45	24-72	Increased after 60 years
Skaunic et al. (1968)	-	50	30	18-90	Increased after 50 years
Koff et al. (1973)	-	904	45	20-89	Unchanged with age
Kampmann et al. (1975)	26	17	45	50-88	Unchanged with age

* All investigators injected a dose of 5 mg BSP/kg body weight intravenously

** Abnormal BSP retention means 5 percent or more BSP in the serum after 30 min.

*** Abnormal BSP retention means more than 5 percent BSP in the serum after 45 min.

tion of individual cells plays an important role in the reduction in the functional capacity of the total liver with age. This Chapter concerns investigations on whether a decrease in the storage capacity of the isolated hepatocytes may be the explanation for the age-related decline in the BSP metabolism of the whole liver. Therefore, the storage capacity of hepatocytes isolated from female WAG/Rij rats of different ages for BSP has been determined.

To determine whether the data obtained with the female WAG/Rij rats are sex or strain dependent, the storage capacity of liver parenchymal cells isolated from male WAG/Rij rats and female BN/Bi rats for BSP has also been determined.

4.2 METHODS

The BSP retention test *in vivo* was performed by injecting BSP intravenously into the tail vein. At a given time after injection, a serum sample was withdrawn and the amount of BSP that remained in the plasma was measured. The amount of BSP in the serum samples was measured on an ultramicro scale. This method was developed by De Leeuw-Israel (1971). Twenty μ l serum were mixed with 200 μ l 0.5 N NaOH in saline. A blank was prepared by adding 200 μ l 0.5 N NaOH to 20 μ l normal serum. The BSP in the serum samples and a BSP standard were read against the blank at 580 nm in a Beckman spectro-colorimeter.

The BSP dose necessary to assure that the liver functioned maximally was determined by injecting intravenously 3-month-old female WAG/Rij rats with BSP doses of 4, 5, 6, 7, 8, 9 and 12 mg BSP per 100 g body weight. The BSP retention was measured after 5, 10, 15, 20, 25, 30 and 35 min (Figure 4.1).

In this way, it is possible to construct the so-called disappearance curve. By plotting semilogarithmically the serum concentration against time, a straight line is obtained. Above a certain threshold dose of the injected BSP, however, another curve composed of two straight lines with different slopes is the result. This composite curve represents the saturation curve. The first part of it reflects the storage of BSP by the liver, while the second part gives an indication of the excretion capacity of the liver (De Leeuw-Israel, 1971). To make certain that the storage capacity of the liver is maximally loaded, the dose which causes a saturation curve must be used.

Possible age-related changes in the BSP retention might be explained by changes in the liver uptake of BSP, in the storage of BSP by the liver or in the BSP biliary excretion. In an attempt to discover

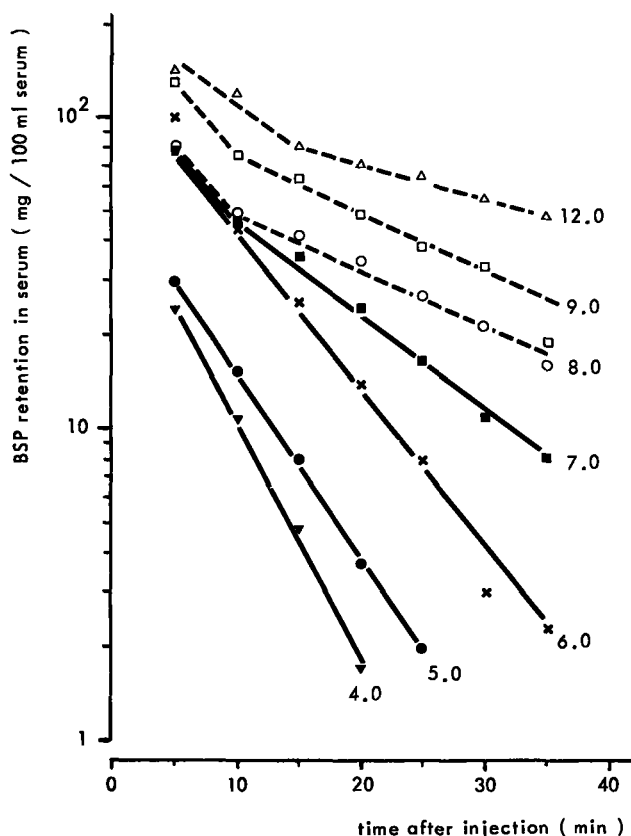


Figure 4.1 Bromsulfophthalein disappearance curves of 3-month-old female WAG/Rij rats at different doses of intravenously injected BSP. The number at the end of the disappearance curve represents the BSP dose injected, expressed in mg/100 g body weight.

which of these phenomena might be involved in the age-related changes in the BSP retention with age, an experiment was designed in which BSP was administered by continuous infusion. According to the method of Wheeler et al. (1960 a and b), it is possible to measure the relative storage capacity (S) and the maximal excretion rate (T_m) by administering a priming dose and infusing the liver of a dog twice at different infusion rates and taking blood samples during both infusions at different time intervals. This method is based on the assumption that the amount of BSP accumulated in the liver is directly related to the BSP plasma concentration. The relative storage capacity (S) is defined as the amount of BSP in mg taken up and stored by the liver per mg BSP

per 100 ml plasma. The maximal excretion capacity (T_m), expressed in mg BSP per min, reflects the rate of BSP excretion into bile, when the liver is saturated with BSP. This method for the dog was adapted on a microscale by De Leeuw-Israel (1971). In the present study, three-month-old female WAG/Rij rats were injected with a priming dose of 8 mg BSP per 100 g body weight. The liver was then infused by means of an infusion pump for one hour with a solution of 250 mg BSP per 100 ml saline at a constant infusion rate of 0.180 mg BSP per min. During this infusion, blood samples were taken at 30, 40, 50 and 60 min. Immediately after the first infusion, a second infusion was performed for a second hour. A solution of 375 mg BSP per 100 ml was infused at a rate of 0.270 mg BSP per min. Again, blood samples were taken after 30, 40, 50 and 60 min. The entire procedure for measuring the relative storage capacity (S) and the maximal excretion rate (T_m) was repeated with doses of 375 and 550 mg BSP per 100 ml saline at infusion rates of 0.270 and 0.396 mg BSP per min, respectively. By varying the BSP concentrations of the solutions infused, it was possible to administer the same amount of saline in all experiments, viz., 2.16 ml. After the second infusion, the plasma volume was determined according to the method described by De Leeuw-Israel et al. (1969b). This method is as follows: after injection of a known amount of Evans blue, 0.1 ml blood samples were taken after 5, 10 and 15 min. The plasma concentrations of Evans blue were measured in a Beckman spectro-colorimeter at 600 nm. From the degree of dilution of the dye, the plasma volume could be calculated.

The storage of BSP by the isolated liver parenchymal cells was established in the same way as described in paragraph 3.2.2, with the exception that the cells were incubated in the standard medium as described in paragraph 2.2.2.

The ploidy state of the hepatocytes isolated from rats of different ages was determined by measuring the diameters of the nuclei of the mono- and binuclear isolated cells as described in paragraph 3.2.2.

4.3 RESULTS

4.3.1 BSP METABOLISM OF THE LIVER IN VIVO IN FEMALE WAG/Rij RATS

For the performance of the BSP retention test it is of importance to know that the ratio BSP dose-liver weight is constant or changes with age. Provided that the ratio liver weight-body weight is constant with age, the BSP dose which must be injected can be calculated from

the body weight. Therefore, the liver weight-body weight ratio of female WAG/Rij rats of different ages was determined. A constant ratio with age of about 4 was found, with fluctuations between 3.6-4.5; thus, the BSP dose was based on the body weight (Figure 4.2).

The intention was that the liver would function maximally, so that it would be possible to detect slight changes in the functional capacity of the liver with age. Therefore, the BSP dose which yielded the saturation curve was determined. This was achieved by injecting several doses of BSP intravenously into three-month-old female WAG/Rij rats and plotting the disappearance curves. The results are illustrated in Figure 4.1. With the doses of 4, 5 and 6 mg BSP per 100 g body weight, disappearance curves in the form of straight lines were obtained. After the injection of doses of 8 up to 12 mg BSP per 100 g body weight, saturation curves were observed. This can be deduced from the fact that, about ten minutes after injection, the slope of the last part of the saturation curve is changing from the parallel curves of 4, 5 and 6 mg injected BSP to the more horizontal lines. The disappearance curve of 7 mg injected BSP seems to represent an intermediate curve. In further experiments, the dose of 8 mg BSP per 100 g body weight was used, which guaranteed that the storage capacity of the liver was just loaded maximally. Under these conditions, it can be anticipated that small changes in the BSP metabolism of the liver will be easily detected.

The BSP retention was determined in female WAG/Rij rats of 3, 7, 12, 24 and 36 months of age. Eight mg per 100 g body weight was injected intravenously and the amount of BSP present in the serum was meas-

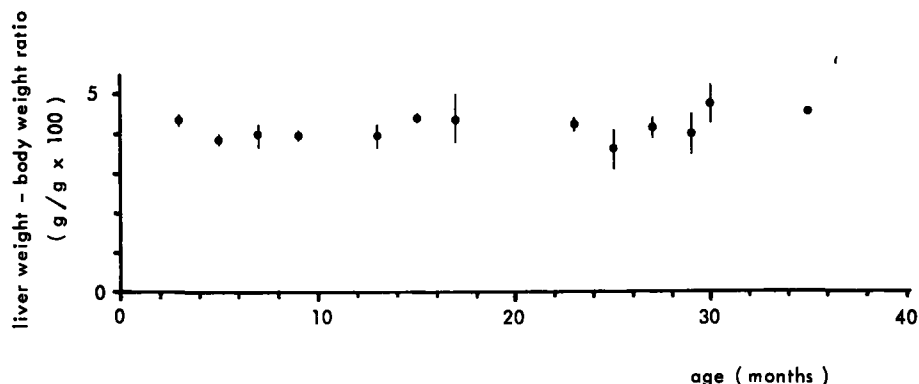


Figure 4.2 The liver weight-body weight ratio as a function of the age of female WAG/Rij rats. For each age group, 3 animals were used, except for the age group of 35 months. The vertical lines represent the standard deviations of the mean.

ured after 35 min. The results are shown in Table 4.2. A significant increase with age was observed during the first 12 months of age.

To determine whether this increase in BSP retention in the older rats was due to a decrease in the relative storage capacity of the liver or to a decline in the maximal excretion capacity, both characteristics were determined in 3- and 30-month-old female WAG/Rij rats. After a priming dose of 8 mg per 100 g body weight, the infusions were performed. It was difficult to obtain reproducible results with this method and even zero relative storage capacities were calculated for the 30-month-old rats. Therefore, no absolute figures will be given. Nevertheless, all data indicated that the relative storage capacity decreased and the maximal excretion rate remained unchanged with age.

4.3.2 BSP STORAGE BY LIVER PARENCHYMAL CELLS ISOLATED FROM FEMALE WAG/Rij RATS OF DIFFERENT AGES

To investigate whether the observed age-related increase in the BSP retention, which might be attributed to a decline in the relative storage capacity of the whole liver, is due to a decreased storage capacity of the individual liver cells, hepatocytes were isolated from

TABLE 4.2

BSP RETENTION IN THE SERUM OF FEMALE WAG/Rij RATS OF DIFFERENT AGES

Age (months)	n	BSP retention (mg/100 ml serum)	
		mean	S.E.
3	9	4.68	0.82
7	4	7.5	1.3
12	8	13.6 ^a	1.2
24	9	14.7 ^a	2.1
36	9	16.5 ^a	2.7

^aValue differs significantly ($p < 0.05$) from the 3- and 7-month value

The BSP retention was determined 35 min after the intravenous injection of 8 mg BSP per 100 g body weight.

the livers of female WAG/Rij rats of different ages. To achieve maximum BSP storage, the parenchymal cells in cell concentrations ranging between 0.05 to 0.4×10^6 cells.ml⁻¹ were incubated with a dose of 30 nmol ³⁵S-BSP.ml⁻¹ for 15 min at 37°C (paragraph 3.3.1). In this respect, the storage capacity of the isolated cells is defined as the amount of BSP, expressed in nmol per 10^6 cells or per mg cellular protein, stored under these optimum conditions.

Liver parenchymal cells from 3-month-old rats appeared to store 11.9 nmol BSP/ 10^6 cells or 8.1 nmol BSP/mg cellular protein (Table 4.3). A sharp decrease was found for the amount of BSP stored by liver parenchymal cells isolated from 12-month-old rats. This was followed by a less pronounced decrease up to 36 months (Table 4.3). The age-related decrease in the BSP storage capacity of the isolated cells was greater when expressed per mg protein instead of per 10^6 cells, which is due to the increase in mg protein/ 10^6 cells with age. This increase in cellular protein might be attributed to an increase in polyploidy in the liver parenchymal cells. To investigate whether an increase in polyploidy takes place in the hepatocytes with age and to what extent this increase might be an explanation for the observed increase in the BSP retention with age, the ploidy state for cells isolated from 0.5-, 1-, 3- and 30-month-old rats was determined. The results shown in Figure 4.3 show that the most pronounced changes in ploidy state occurred

TABLE 4.3

BSP STORAGE CAPACITY OF HEPATOCYTES ISOLATED FROM FEMALE WAG/Rij RATS OF DIFFERENT AGES*

Age (months)	Protein content of parenchymal cells (mg/ 10^6 cells)	BSP storage	
		nmol/ 10^6 cells	nmol/mg cellular protein
3	1.47 ± 0.10 (11)	11.9 ± 1.5 (11)	8.1 ± 1.2
7	1.83 ± 0.09 (4) ^a	10.0 ± 1.3 (4)	5.46 ± 0.76
12	1.80 ± 0.10 (4) ^a	8.28 ± 0.48 (8) ^a	4.65 ± 0.45^a
24	2.03 ± 0.09 (3) ^a	7.4 ± 1.1 (7) ^a	3.62 ± 0.58^a
36	2.01 ± 0.11 (5) ^a	6.4 ± 1.3 (6) ^a	$3.20 \pm 0.70^{a,b}$

*Mean $\pm$ S.E.; number of different cell preparations in parentheses.

^aValue differs significantly ($p < 0.05$) from the 3-month value.

^bValue differs significantly ($p = 0.05$) from the 7- and 12-month values.

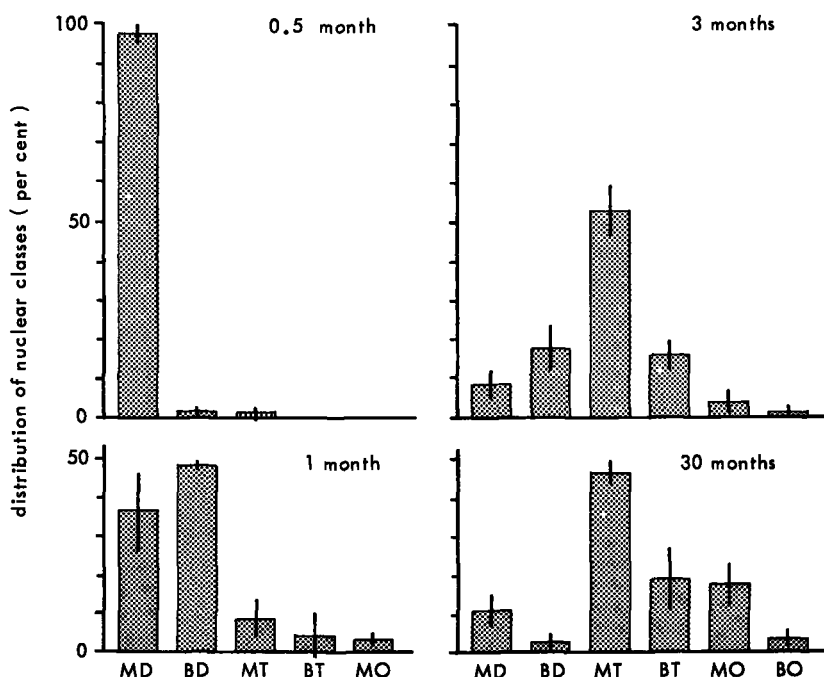


Figure 4.3 Percentage distribution of nuclear classes of parenchymal cells isolated from rats of different ages. Each bar represents the mean of 4 separate experiments. The standard deviation of the mean is indicated by the vertical lines. For each experiment, 200 nuclei were measured; MD, mononuclear diploid; BD, binuclear diploid; MT, mononuclear tetraploid; BT, binuclear tetraploid; MO, mononuclear octaploid; BO, binuclear octaploid.

within the first 3 months of age. There is a clear shift from mononuclear diploid (MD) cells to polyploid cells, particularly to binuclear diploid (BD) cells, between two weeks and 1 month of age. Thereafter, a great decrease in the percentage of mononuclear diploid (MD) and binuclear diploid (BD) cells as well as an increase in the percentage of mononuclear tetraploid (MT) cells occurs. Between 3 and 30 months of age, only relatively small but significant changes occur, viz., a decline in the percentage of binuclear diploid (BD) cells and an increase in the percentage of mononuclear octaploid (MO) cells. Therefore, the increase in cellular protein with age can be partly explained by the shift of the hepatocytes to a higher ploidy state with age.

4.3.3 THE EFFECT OF AGE ON THE BSP STORAGE BY HEPATOCYTES ISOLATED FROM MALE WAG/Rij AND FEMALE BN/Bi RATS

The age-related decrease in BSP storage capacity of the hepatocytes from female WAG/Rij rats might be sex dependent. To investigate this possibility, hepatocytes were also isolated from male WAG/Rij rats of different ages. The amount of BSP stored by these cells was measured. The incubation conditions were identical to those described for the hepatocytes from the female WAG/Rij rats (paragraph 3.2.2). The hepatocytes were isolated from 3-, 12-, and 20-month-old male WAG/Rij rats. The male WAG/Rij rats had a considerably shorter life-span than did the female WAG/Rij rats; the 50 percent survival age of male WAG/Rij rats was 24 months, which was much younger than the 32 months observed for the female WAG/Rij rats. In view of these survival ages, male WAG/Rij rats of absolute younger ages may be comparable with older female WAG/Rij rats. Although the age differences in the BSP storage capacity of the hepatocytes isolated from the male WAG/Rij rats of different ages were not significant (see Table 4.4), they show the same trend of decline as was found for the liver parenchymal cells isolated from the female WAG/Rij rats (Table 4.3).

To detect whether the age-related change in the BSP storage capacity of the hepatocytes from female WAG/Rij rats was a strain dependent phenomenon, hepatocytes were also isolated from female rats of another strain, viz., the BN/Bi rat. The cells were prepared from female BN/Bi rats of 3, 12, 24 and 31 months of age. Thus, about the same age groups were studied as with the female WAG/Rij rats, which is in agreement with the fact that the 10, 50 and 90 percent survival ages of female BN/Bi and WAG/Rij rats are almost identical (Table 2.1). An age-related decrease in the BSP storage capacity of the isolated cells was found for female BN/Bi rats (Table 4.5), just as was found for hepatocytes prepared from female WAG/Rij rats of different ages (Table 4.3).

4.4 DISCUSSION

4.4.1 THE BSP METABOLISM IN VIVO

Many authors (Rafsky and Newman, 1949; Haberman, 1962; Thompson and Williams, 1965; Freston and Englert, 1967, and Skaunic et al., 1968) have observed in man that the liver function as determined from the BSP retention test decreased in late age (Table 4.1). Only Vink

TABLE 4.4

BSP STORAGE CAPACITY OF HEPATOCYTES ISOLATED FROM MALE WAG/Rij
RATS OF DIFFERENT AGES*

Age (months)	Protein content of parenchymal cells (mg/10 ⁶ cells)	BSP storage nmol/10 ⁶ cells	nmol/mg cellular protein
3	2.65 ± 0.10 (12)	9.4 ± 1.2 (9)	3.55 ± 0.47
12	2.01 ± 0.22 (4) ^a	6.9 ± 1.4 (7)	3.43 ± 0.79
20	2.12 ± 0.17 (5) ^a	6.7 ± 1.4 (8)	3.16 ± 0.71

*Mean ± S.E.; number of different cell preparations in parentheses.

^aValue differs significantly (p < 0.05) from the 3-month value.

TABLE 4.5

BSP STORAGE CAPACITY OF HEPATOCYTES ISOLATED FROM FEMALE BN/Bi
RATS OF DIFFERENT AGES*

Age (months)	Protein content of parenchymal cells (mg/10 ⁶ cells)	BSP storage nmol/10 ⁶ cells	nmol/mg cellular protein
3	1.49 ± 0.11 (11)	7.53 ± 0.88 (10)	5.03 ± 0.71
12	1.94 ± 0.20 (3)	4.4 ± 1.5 (4) ^a	2.27 ± 0.81 ^a
24	2.18 ± 0.11 (4) ^a	4.2 ± 1.3 (3) ^a	1.92 ± 0.60 ^a
31	2.27 ± 0.13 (5) ^a	6.6 ± 1.7 (7)	2.90 ± 0.77 ^a

*Mean ± S.E.; number of different cell preparations in parentheses.

^aValue differs significantly (p < 0.05) from the 3-month value.

(1959) mentioned that this decrease occurred during the early stage of life. To study the BSP retention test in rats of different ages, it is of importance that the injected dose is based on the liver weight. If the ratio between liver weight and body weight with age is known, the dose of injected BSP can be based on body weight. A constant liver weight-body weight ratio was observed with age for the female WAG/Rij rat (Figure 4.2). This is in agreement with the data of Webster et al. (1947) for female albino rats, with those of Zumoff and Pachter (1964) for female Sherman albino rats and with those of De Leeuw-Israel (1971) for female RU rats. The liver weight-body weight relationship of male rats, however, deviated from linearity in late age (Webster et al., 1947, and Zumoff and Pachter, 1964). A similar trend of decrease in the BSP retention with age as observed for man by Vink (1959) was found with female RU rats by De Leeuw-Israel (1971), who observed an increase in BSP retention in the serum of female RU rats between 3 and 12 months of age. After 12 months, there was no significant change up to 27 months. The data presented in this Chapter on the BSP retention test for the female WAG/Rij rats which revealed an increase in BSP retention between 3 and 12 months while no change in BSP retention was observed between 12 and 36 months, are in full agreement with those for the female RU rat.

The decrease in the BSP metabolism of the liver *in vivo* may be attributable to changes in the ploidy state of the liver parenchymal cells with age (De Leeuw-Israel, 1971). Although the main changes in the ploidy state of the hepatocytes from female WAG/Rij rats appeared to occur within the first 3 months of age, some smaller changes took place between 3 and 30 months of age. Assuming that a mononuclear diploid cell has half the volume of a binuclear diploid and a mononuclear tetraploid cell, which have half the volume of a binuclear tetraploid or mononuclear octaploid cell, it could be calculated that the small but significant changes in the ploidy state of the hepatocytes between 3 and 30 months are responsible for an increase of about 20 percent of the mean cell volume. Consequently, the number of hepatocytes per unit liver volume (i.e. the amount of cell surface per unit volume) decreased between 3 and 30 months. Since BSP is transported across the parenchymal cell membrane, the liver function with respect to the BSP retention test may decline in a small measure between 3 and 30 months due to the observed increase in the polyploidy of the hepatocytes. This increase in polyploidy of the liver parenchymal cells with age is strain dependent. Although the pattern of the shift in ploidy is mostly identical, viz., a shift from mononuclear diploid to binuclear diploid to mononuclear tetraploid to binuclear tetraploid to mononuclear octa-

ploid cells, as described in detail for the female WAG/Rij rat in paragraph 4.3.2, the onset and end of the ploidy changes differed from strain to strain. In Table 4.6, the changes in the ploidy state of hepatocytes of different rat strains with age are shown. The female WAG/Rij rat showed an age pattern which differed from the patterns observed by Alfert and Geschwind (1958), and De Leeuw-Israel (1971). Both observed a decrease in the percentage of mononuclear diploid cells and an increase in mononuclear tetraploid cells after 3 months, which was not determined for female WAG/Rij rats. The data of Nadal and Zajdela (1966) also differ from those for the female WAG/Rij rat. Nadal and Zajdela (1966) observed no significant changes after 4 months, whereas, with female WAG/Rij rats, significant changes were observed between 3 and 30 months of age.

The increase in BSP retention with age might also be due to an age-related decrease in the relative storage capacity (S) or in the maximal excretion rate (T_m). Doubts about the reliability of the determination of these characteristics have been expressed by McIntyre et al. (1973). For example, they observed that although the BSP plasma concentration increased during the second half hour infusion period, the plasma concentration of BSP during that second infusion was lower than it had been at the end of the first infusion. Furthermore, they calculated that the relative storage capacity could not represent a constant as was defined by Wheeler et al. (1960 a and b). Nevertheless, McIntyre et al. (1973) concluded that the value for the maximal excretion rate (T_m) must be of the right order. By perfusing the liver only once and measuring directly the amount of BSP excreted via the bile, the T_m ranged from 62-113 percent of the value measured with the method of the two infusion rates; the mean value for the direct measurement of the BSP excretion via the bile appeared to be 88 percent of the value obtained with the indirect method of the two infusions rates (Wheeler et al., 1960b). The calculation of T_m by use of the method of Wheeler gave results which tend to overestimate the BSP output into the bile. In spite of the above mentioned complications, the determination of the relative storage capacity (S) may still give an indication of the functional capacity of the liver parenchymal cells (McIntyre et al., 1973).

The doubts concerning the validity of the determination of T_m and S expressed by McIntyre et al. (1973) have been strengthened by the experiments performed with the female WAG/Rij rats. For example, the determinations with four 30-month-old female WAG/Rij were questionable. By plotting the BSP concentrations in the serum during both second half hours against the time of infusion, linear lines were obtained. This is

TABLE 4.6

PROPORTIONS OF DI- AND POLYPLOID HEPATOCYTES IN LIVERS
OF RATS OF DIFFERENT AGES

Strain Sex Authors	Age (months)	MD (%)	BD (%)	MT (%)	BT (%)	MO (%)	BO (%)
Long-Evans female Alfert and Geschwind (1958)	0.5 1 2 3 12	86 55 33 21 6	5 32 26 16 1	4 7 35 52 66	 1 2 7 11	 1 14	
Wistar male Post et al. (1960)	0.75 2 4 6 16 24	79 26 20 13 12 10		21 73 78 86 87 73		0 2 2 2 1 17	
Wistar sex not mentioned Nadal and Zajdela (1966)	0.75 1 2 4 9	92 58 10 5 2	5 32 25 15 11	2 8 51 68 72	 9 12 13	 2 1 2	
RU female De Leeuw-Israel (1971)	3 6 12 24 27	72 54 30 29 27		25 42 61 63 66		3 4 9 8 7	
WAG/Rij female this study	0.5 1 3 30	97 36 9 11	2 48 18 3	1 9 53 46	 4 16 19	3 4 18	 2 4

MD, mononuclear diploid; BD, binuclear diploid; MT, mononuclear tetraploid; BT, binuclear tetraploid; MO, mononuclear octaploid; BO, binuclear octaploid

a prerequisite for the performance of this test. Nevertheless, zero values for the relative storage capacities were calculated. Therefore, it was decided that no absolute figures of the results of this test would be given in this study. The data obtained only indicated that the maximal excretion rate (T_m) remained unchanged with age, while the relative storage capacity (S) decreased.

4.4.2 COMPARISON OF *IN VIVO* AND *IN VITRO* DATA

As mentioned in paragraph 4.4.1 the significant increase in BSP retention during the first year of life of female WAG/Rij rats is only slightly influenced by the changes in the ploidy of the liver parenchymal cells. Another possibility is a decrease in the relative storage capacity of the liver for BSP, while the maximal excretion capacity might remain unchanged. An age-related decline in the storage capacity of the whole liver might be due to a decreased storage capacity of the individual liver cells. In figure 4.4, data on the BSP retention test in female WAG/Rij rats of different ages, which have been presented in Table 4.2, are plotted against the age of the rats in months. In this figure are also plotted the data on BSP storage by the cells isolated from rats of different age groups. These data were already given in Table 4.3. The curve for the BSP retention test is an exact mirror image of the curve which reflects the storage capacity of the hepatocytes isolated from female WAG/Rij rats of different ages for BSP. This strongly suggests that the decrease in the function of the whole liver, which is mainly observed during the first year of life, is at least partly due to an age-related decline in the storage capacity of the individual hepatocytes for BSP. With the BSP retention test an increase in the standard of error of the mean with age was observed (Table 4.2). Bafitis and Sargent (1977) have also recently stressed the increase in the coefficient of variation for various functions in aging man. This increase in variability may obscure what really happens. It is tempting to speculate that there might be subpopulations of aging individuals, or even aging organs, so that one could separate out those within the normal range, those above this range and those below the normal range. In contrast, it was observed that the standard error of the data concerning the BSP storage capacity of the isolated hepatocytes did not increase with age (Table 4.3). This might be explained by the fact that many influences which may change *in vivo* with age are excluded or kept constant in the system of the isolated hepatocytes. These findings emphasize that the system of the isolated hepatocytes is a suitable one to study the cellular basis of organ aging.

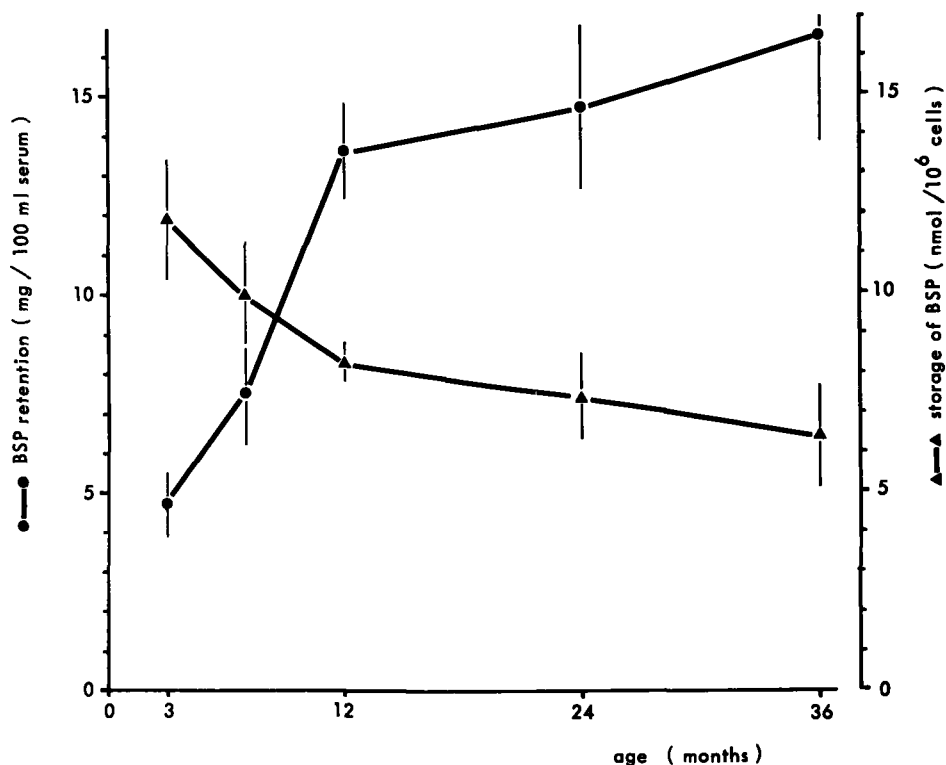


Figure 4.4 The effect of age on the BSP metabolism *in vivo* and *in vitro*. The BSP retention in the serum of female WAG/Rij rats of 3, 7, 12, 24 and 36 months of age (●—●). BSP storage in liver parenchymal cells isolated from female WAG/Rij rats of the same age groups (▲—▲). The values represent the mean and the standard error of the mean.

The decrease in BSP storage capacity of the isolated hepatocytes might be explained as follows. As established in Chapter III, BSP is transported into the hepatocytes by an active transport system. This means that the transport is dependent on cellular energy. Therefore, a decrease in the energy supply of the isolated liver parenchymal cells may be the explanation for the age-related decline in the storage capacity of the isolated liver parenchymal cells. However, we (van Bezooijen et al., 1974a, and Brouwer et al., 1977) have obtained indications that hepatocytes isolated from old rats had an unchanged energy supply, as described in paragraph 2.3.3. It could be concluded that the age-related decline in BSP storage by the isolated hepatocytes could not be explained by a defective energy supply mechanism with age. Other

possibilities to explain the observed decrease in the storage capacity of the isolated hepatocytes with age are decreased transport of BSP across the cell membrane or a decrease in the binding capacity of the intracellular proteins for BSP. These possibilities will be discussed in more detail in Chapter VII.

It can be concluded that the most pronounced changes in the ploidy state of the hepatocytes from female WAG/Rij rats occurred within the first three months of age and that the decline in the BSP metabolism of the whole liver observed after 3 months of age is only slightly influenced by the shift in ploidy and is mainly due to the decline in the storage capacity of the individual hepatocytes for BSP.

CHAPTER V

THE EFFECT OF AGE ON ALBUMIN SYNTHESIS BY ISOLATED HEPATOCYTES

<i>Contents:</i>	5.1	Introduction
	5.2	Materials and Methods
	5.3	Results
	5.3.1	Incubation conditions for optimum albumin synthesis by isolated liver parenchymal cells
	5.3.2	Albumin synthesis by hepatocytes isolated from rats of different ages
	5.4	Discussion
	5.4.1	Optimum incubation conditions for albumin synthesis by isolated hepatocytes
	5.4.2	Albumin synthesis by liver parenchymal cells isolated from rats of different age groups

5.1 INTRODUCTION

The synthesis of serum albumin is a specific liver function (Miller and Bale, 1954, and Kukral et al., 1961). Albumin is a highly flexible molecule with a molecular weight of about 65,000 (Hughes, 1954; Charlwood, 1961, and Peters, 1962) and containing about 580 amino acids (Peters, 1962). The amino acid composition of human serum albumin has been determined by Tristram (1953), and Schultze et al. (1962). The amino acid composition of bovine (Tristram, 1953, and Schultze et al., 1962), rat (Peters, 1962) and rabbit albumin (Jacobs and Koj, 1969) is strikingly similar to that of human serum albumin. Recently, the complete amino acid sequence of human (Behrens et al., 1975; Meloun et al., 1975, and McLachlan and Walker, 1977) and bovine (Brown, 1975) albumin has been determined. No enzymatic function of albumin is known, but it is the major contributor to the maintenance of the osmotic pressure of the plasma and serves as a carrier of metals, anions, fatty acids, bilirubin, hormones and drugs (Rothschild et al., 1972).

Albumin has been demonstrated in liver parenchymal cells by immunofluorescent staining (Gitlin et al., 1953; Hamashima et al., 1964; Chandrasakharam et al., 1967; Lane, 1969; Brozman, 1971; Feldmann et al., 1972, and Guillouzo et al., 1976). Some workers have also found albumin in sinusoidal cells (Gitlin et al., 1953; Wollensak and Seybold, 1957; Hamashima et al., 1964, and Brozman, 1971) or in the bile duct epithelial cells (Brozman, 1971). The reason that albumin was also observed in sinusoidal and bile duct cells might be that the preservation by freeze drying (Gitlin et al., 1953) or the fixation by ethanol (Gitlin et al., 1953, and Hamashima et al., 1964) caused diffusion of the albumin. Another explanation might be that the albumin observed in the sinusoidal cells had been taken up from the blood. Unfortunately, it is impossible to distinguish between albumin that has been newly synthesized or taken up by the cells from the blood with the immunofluorescent technique which was used in the above-mentioned studies.

Only a fraction of the liver parenchymal cells contain albumin. The percentages of hepatocytes in which albumin could be demonstrated by immunofluorescent staining appeared to be 10 percent (Hamashima et al., 1964) or 36 percent (Feldmann et al., 1972) for the human liver and 25 percent (Lane, 1969) for the rat liver. The distribution of the albumin containing hepatocytes is considered by some authors to be random throughout the liver lobule (Hamashima et al., 1964; Chandrasakharam et al., 1967; Lane, 1969; Feldmann et al., 1972, and Lin and Chang, 1975), whereas others have described a preference for the central vein area (Hamashima et al., 1964). Schreiber et al. (1970), who measured

the albumin synthesis of hepatocytes, concluded that the capacity to synthesize albumin is randomly distributed throughout the lobule. With isolated hepatocytes, Valet et al. (1977) found that 17-18 percent of these cells secreted albumin into their culture medium. This finding that only a proportion of the hepatocytes is engaged in albumin synthesis might be explained by a differentiation among the hepatocytes, i.e., the competence to synthesize albumin is limited to certain of these cells. Another explanation might be that, although all hepatocytes are capable of synthesizing albumin, not all are doing so at the same time. An increase in albumin production by the whole liver can be explained by increased synthesis of albumin by a constant number of specialized cells and/or by an increase in the number of hepatocytes that synthesize albumin. A study of Feldmann et al. (1972) in patients with a nephrotic syndrome may support the first hypothesis. In these patients, albumin is supposed to be excreted via the urine due to insufficient kidney function. Consequently, the serum albumin concentration is decreased, which might be compensated for by the liver by means of increased albumin synthesis. Since the number of albumin forming hepatocytes in the liver of these patients is identical to that of the normal liver, an increase in albumin synthesis might be the result of an increased synthetic capacity of the same number of hepatocytes (Feldmann et al., 1972). A criticism is that Feldmann et al. (1972) did not determine whether increased albumin synthesis took place in these patients with a nephrotic syndrome. The other possibility, that increased albumin synthesis is the result of an increase in the number of albumin synthesizing hepatocytes, is supported by experiments performed by Chandrasakharam et al. (1967), who found that the number of parenchymal cells containing albumin was dependent on the protein content of the diet. In addition, strong evidence for this possibility has been reported by Peters et al. (1968) who found a correlation between the number of albumin containing hepatocytes and the amount of albumin synthesized by liver slices prepared from normal and nephrotic rats and from rats with a regenerating liver.

It was shown with subcellular fractions of liver homogenates obtained after intravenous injection of radioactive leucine that albumin is synthesized on the membrane bound ribosomes of the hepatocytes (Hicks et al., 1969, and Takagi et al., 1970). Maximum radioactivity of the albumin was observed for the rough microsomes after 4 to 5 min (Glaumann and Ericsson, 1970). Corresponding maximum activities were measured after 15 min in smooth microsomes and after 20 min in Golgi-rich fractions (Glaumann and Ericsson, 1970, and Peters et al., 1971). On the basis of these data, it was concluded that the apparent pathway of secretion is from rough endoplasmic reticulum (RER) to smooth endo-

plasmic reticulum (SER) through the Golgi apparatus (GA) and then through the cell membrane to the sinusoid. This conclusion is supported by the finding of Guillouzo et al. (1976) that albumin is located at the ultrastructural level in the RER, the SER and the GA. With the methods used by Glaumann and Ericsson (1970), Peters et al. (1971), and Guillouzo et al. (1976), no distinction was made between albumin and proalbumin. Purification of the newly formed "albumin" from the cytoplasmic organelles makes it possible to show that the labeled amino acids first appear as a so-called proalbumin, which is a precursor of albumin (Geller et al., 1972; Judah et al., 1973; Dorling et al., 1975; Edwards et al., 1976a; Schreiber et al., 1976, and Urban et al., 1976). Two groups have independently determined that the proalbumin differs from albumin by an oligopeptide attachment at the aminoterminal. The sequence of this peptide has been described as arginine, glycine, valine, phenylalanine, arginine and arginine (Quinn et al., 1975, and Russell and Geller, 1975) and as glycine, valine, phenylalanine, serine and arginine (Urban et al., 1974). Edwards et al. (1976b), working with subcellular fractions of the liver and separating the precursor albumin and albumin by means of DEAE-cellulose chromatography, showed that only proalbumin was found in the RER. The ratio of albumin to albumin precursor increased from RER to SER to GA. Edwards et al. (1976b) therefore suggest that albumin precursor is synthesized on the RER and converted into albumin in the SER and the GA. The secretion product consists only of albumin.

The first objective of this study of the albumin synthesis was to develop a method for gathering baseline data on the albumin synthesizing capacity of cells isolated from young female WAG/Rij rats. An important prerequisite for the method was that the capacity of the isolated cells to synthesize albumin should approach the *in vivo* synthesizing capacity of the liver, so that any small changes due to aging could easily be detected. The second objective was to measure the amount of albumin synthesized by hepatocytes isolated from female WAG/Rij rats of different ages under optimal conditions.

5.2 MATERIALS AND METHODS

From the hepatocytes isolated from rats of different ages, one portion of the cell suspension was used to measure the BSP storage capacity; the other was used to determine the amount of synthesized albumin.

The following method was used for determining the amount of albumin synthesized by the hepatocytes. A known number of cells was incubated in 4 ml of a modified Waymouth MB 752/1 medium. The modification of the Waymouth's medium was that, instead of adding 34.5 mM of NaHCO_3 to the Waymouth MB 752/1 medium (Grand Island Biological Company, Grand Island, U.S.A.) 25 mM HEPES, 0.56 mM L-alanine and 0.21 mM serine were added. The osmolality of the medium was adjusted to 308 mM by adding NaHCO_3 . The incubation of the hepatocytes took place at 37°C in an atmosphere of 95 percent O_2 and 5 percent CO_2 for various time intervals. The oxygen tension of the incubation medium was maintained at at least 30×10^3 Pa (Jeejeebhoy et al., 1975). After incubation, the cells were lysed by addition of Triton-X-100 in a final concentration of 1.3 percent to release the albumin. The samples were dialyzed against distilled water for two days to remove the Triton-X-100; thereafter, the albumin was concentrated by lyophilization. The final residue was dissolved in 200 μl 0.3 M phosphate buffered saline (PBS) per 10^6 cells originally present per ml incubation medium, if the cells had been isolated from a 3-month-old rat. Since the average cell size increases with age (see Figure 4.3), the final residue of the cells isolated from older rats was dissolved in 400 μl PBS per 10^6 cells originally present per ml incubation medium. The amount of albumin was measured by the radial immunodiffusion method of Mancini et al. (1965) in a combined modification of Kalff (1968), and Rádl et al. (1970). In this modification, a 1.5 percent solution of Special Agar Noble (Difco Laboratories, Detroit, Michigan, U.S.A.) in veronal buffer (3.68 g diethylbarbituric acid, 20.6 g Na-veronal, distilled water up to 2,000 ml) was boiled in a water bath until the agar solution was clear and then cooled to 50°C . A specific antiserum to rat albumin (RARA/Alb, Nordic Diagnostics, Tilburg, The Netherlands) was added to the agar solution in a final dilution varying from 1/16 to 1/151, depending on the antibody titer of the antiserum. This antiserum was tested in the Ouchterlony plate. The antisera against rat serum albumin formed a single precipitation band with purified rat serum albumin and rat serum. Fourteen ml of the agar-antiserum mixture were applied to a glass slide of 8.5 x 8.5 cm in a level position. After solidification of the agar, 34 wells (2.5 mm in diameter) were punched out in the agar layer with a needle and a template. They were filled with 5 or 10 μl of the samples by means of an Eppendorf pipette. Ten different dilutions of a reference serum (rat serum albumin, Nordic Diagnostics), and duplicate samples of the dissolved residue obtained with three different dilutions of the original cell suspensions were included in each plate (Figure 5.1). The development of the precipitin rings in the agar plates was completed in a moist chamber at room temperature within three days. The plates were

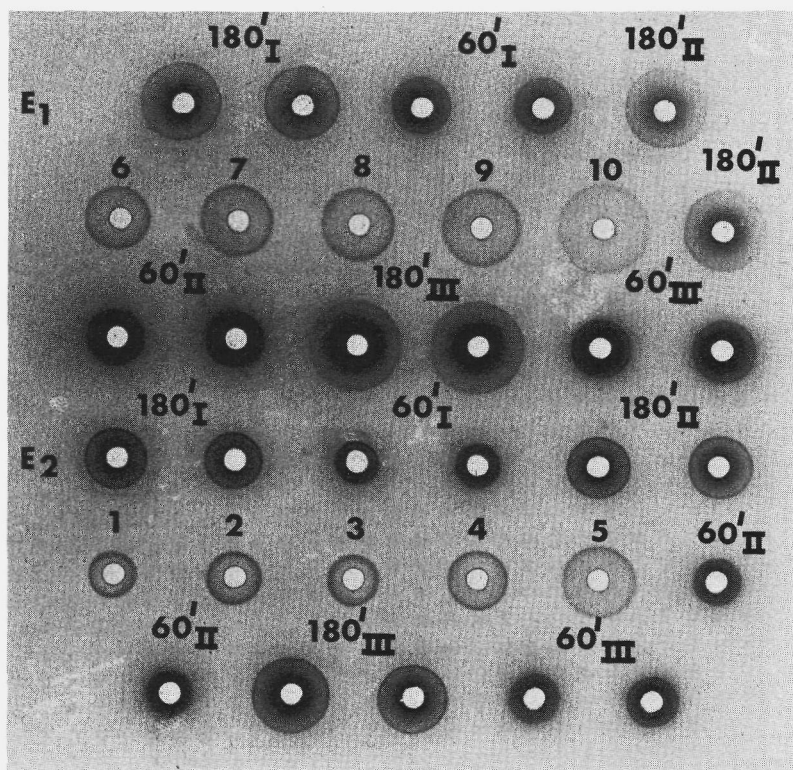


Figure 5.1 Mancini plate. The numbers 1 to 10 represent precipitin rings of the 10 different solutions of the reference serum. E_1 represents the duplicate samples of the dissolved residues after 180 and 60 minutes of incubation obtained for three different dilutions of the original cell suspensions, indicated by the numbers I, II and III. E_2 represents the data of another cell preparation.

next extensively washed in buffered saline for two days, covered with moistened filter paper and dried at 37°C . After removing the dry paper and washing away the paper fibres under tap water, the plates were stained by a mixture of Amido black 10 B and Thiazine red (0.25 g each) dissolved in 50 ml of glacial acetic acid and 450 ml of methanol. De-staining of the background was done by washing the plates in a washing solution (100 ml glacial acetic acid, 500 ml methanol, 400 ml distilled water) three times for 2 min. Standard curves were made by plotting the square of the mean diameter of the rings against the dilution of the reference serum.

With this immunological technique, both proalbumin and albumin are measured (Urban et al., 1974); therefore, no distinction is made between albumin and proalbumin. Consequently, the term albumin always means albumin together with proalbumin.

5.3 RESULTS

5.3.1 INCUBATION CONDITIONS FOR OPTIMUM ALBUMIN SYNTHESIS BY ISOLATED LIVER PARENCHYMAL CELLS

The influence of the pH on the amount of albumin synthesized by hepatocytes isolated from 3-month-old female WAG/Rij rats was investigated over a pH range of 7.4 to 8.2 (Figure 5.2). Maximum synthesis was found at pH 7.8. Figure 5.3 shows the time course of the amount of albumin synthesized by the isolated cells. This figure, based on experiments which were repeated many times, reveals a lag period of a maximum of 30 min. After the lag period, the curve is linear up to at least 3 h of incubation. On the basis of this result, samples were always taken after 60 min and 180 min of incubation and the capacity for albumin synthesis per time unit was calculated by subtracting the 60 min value from the 180 min value. For practical reasons, the amount of albumin present in and secreted by the cells was measured together at the different incubation times. The total number of cells did not decrease during the incubation period of 180 min, whereas the percentage of viable cells as determined by trypan blue exclusion changed only slightly from 94 to 88 percent. A linear relationship between cell concentration and the amount of synthesized albumin was observed up to a cell concentration of at least 3×10^6 cells/ml medium (Figure 5.4). The data of

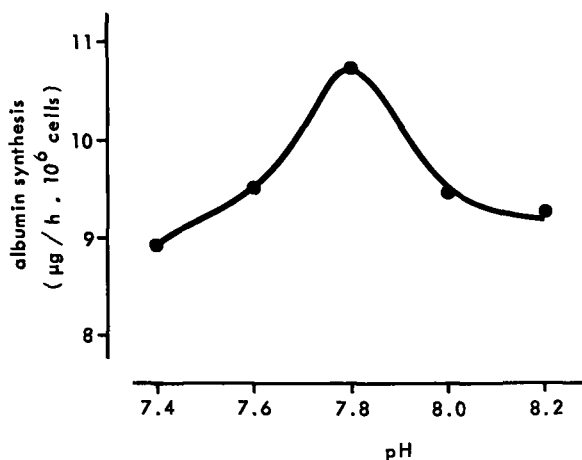


Figure 5.2 Influence of the pH on the amount of albumin synthesized by hepatocytes isolated from a 3-month-old female WAG/Rij rat.

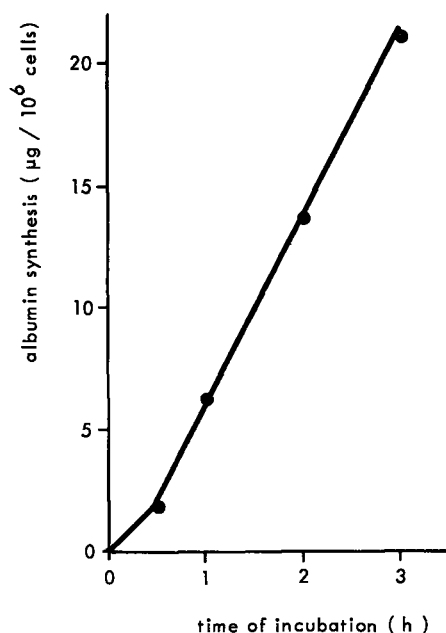


Figure 5.3 Time course of albumin synthesis by hepatocytes isolated from a 3-month-old rat.

Figure 5.4 are representative, since each measured value for the amount of albumin synthesized by hepatocytes isolated from rats of different ages (see Tables 5.1, 5.2, and 5.3) was determined from 3 different dilutions from the original cell suspensions and each time a straight line between the cell concentration and the amount of synthesized albumin was observed.

With due observance of the experimental conditions mentioned above, the quantity of albumin synthesized by hepatocytes isolated from 3-month-old female WAG/Rij rats amounted to 5.8 µg albumin/h.10⁶ cells.

5.3.2 ALBUMIN SYNTHESIS BY HEPATOCYTES ISOLATED FROM RATS OF DIFFERENT AGES

The second objective of the studies described in this chapter was to compare the capacity for albumin synthesis of parenchymal cells iso-

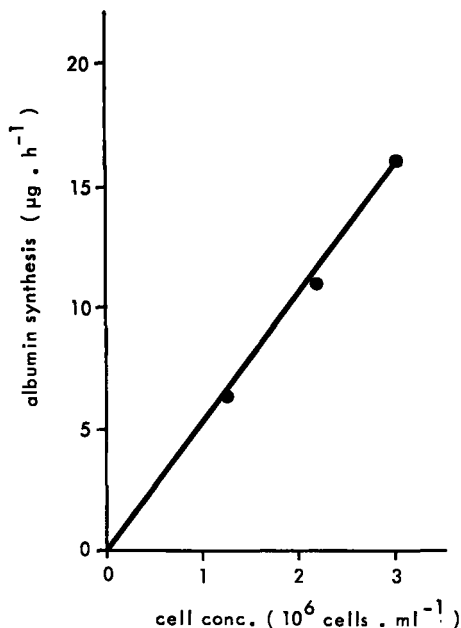


Figure 5.4 Effect of cell concentration in the incubation medium on the albumin synthesis by parenchymal cells isolated from a 3-month-old rat.

lated from rats of different ages. Table 5.1 shows a significant decrease in the amount of albumin synthesized per 10^6 liver parenchymal cells isolated from female WAG/Rij rats between 3 and 24 months of age. On the contrary, a sharp increase was found after 24 months up to 36 months. When the albumin synthesizing capacity was expressed on a protein basis, approximately the same pattern of age-related changes was observed, with the exception that liver parenchymal cells isolated from 36-month-old female WAG/Rij rats did not significantly synthesize more albumin than cells isolated from 3-month-old female WAG/Rij rats. This latter phenomenon was due to an increase in the protein content of the cells with age. Since the cells were from the same suspensions (with the exception of the 27-month-old male WAG/Rij rats) as those which were prepared for the determination of the BSP storage capacity, the protein content of the hepatocytes used for the determination of the albumin synthesis and the BSP storage is exactly the same (see Table 4.3).

Under the incubation conditions which were optimal for the liver parenchymal cells isolated from female WAG/Rij rats, the capacity of liver parenchymal cells isolated from male WAG/Rij and female BN/Bi

TABLE 5.1

ALBUMIN SYNTHESIS BY HEPATOCYTES ISOLATED FROM FEMALE WAG/Rij RATS
OF DIFFERENT AGES*

Age (months)	Protein content of parenchymal cells (mg/10 ⁶ cells)	Albumin synthesis μg/h.10 ⁶ cells	μg/h.mg cellular protein
3	1.47 ± 0.10 (11)	5.77 ± 0.79 (12)	3.94 ± 0.60
12	1.80 ± 0.10 (4) ^a	3.73 ± 0.34 (8) ^a	2.06 ± 0.20 ^a
24	2.03 ± 0.09 (3) ^a	2.29 ± 0.30 (7) ^{a,b}	1.13 ± 0.15 ^{a,b}
31	-	6.36 ± 0.78 (3) ^{b,c}	-
36	2.01 ± 0.11 (5) ^a	9.8 ± 1.7 (7) ^{a,b,c}	4.88 ± 0.89 ^{b,c}

*Mean ± S.E.; number of different cell preparations in parentheses

^aValue differs significantly (p < 0.05) from the 3-month value

^bValue differs significantly (p < 0.05) from the 12-month value

^cValue differs significantly (p < 0.05) from the 24-month value.

TABLE 5.2

ALBUMIN SYNTHESIS BY HEPATOCYTES ISOLATED FROM MALE WAG/Rij RATS
OF DIFFERENT AGES*

Age (months)	Protein content of parenchymal cells (mg/10 ⁶ cells)	Albumin synthesis μg/h.10 ⁶ cells	μg/h.mg cellular protein
3	2.65 ± 0.10 (12)	1.75 ± 0.31 (7)	0.66 ± 0.12
12	2.01 ± 0.22 (4) ^a	2.19 ± 0.22 (7)	1.09 ± 0.16
20	2.12 ± 0.17 (5) ^a	2.19 ± 0.50 (7)	1.03 ± 0.25
27	2.18 ± 0.24 (5) ^a	1.93 ± 0.70 (4)	0.88 ± 0.34

*Mean ± S.E.; number of different cell preparations in parentheses

^aValue differs significantly (p < 0.05) from the 3-month value

TABLE 5.3

ALBUMIN SYNTHESIS BY HEPATOCYTES ISOLATED FROM FEMALE BN/BI RATS
OF DIFFERENT AGES*

Age (months)	Protein content of parenchymal cells (mg/10 ⁶ cells)	Albumin synthesis	
		$\mu\text{g/h.}10^6$ cells	$\mu\text{g/h.mg}$ cellular protein
3	1.49 $\pm$ 0.11 (11)	3.15 $\pm$ 0.56 (10)	2.11 $\pm$ 0.41
12	1.94 $\pm$ 0.20 (3)	1.88 $\pm$ 0.34 (4)	0.96 $\pm$ 0.20
24	2.18 $\pm$ 0.11 (4) ^a	2.9 $\pm$ 1.2 (4)	1.34 $\pm$ 0.55
31	2.27 $\pm$ 0.13 (5) ^a	3.8 $\pm$ 1.7 (4)	1.67 $\pm$ 0.76

*Mean $\pm$ S.E.; number of different cell preparations in parentheses

^aValue differs significantly ($p < 0.05$) from the 3-month value

rats of different ages to synthesize albumin was determined. The amount of albumin synthesized by liver parenchymal cells isolated from 3-, 12-, 20- and 27-month-old male WAG/Rij rats (see Table 5.2), was much less than that measured with the liver parenchymal cells from female WAG/Rij rats (Table 5.1). No significant changes in the capacity to synthesize albumin were observed for the different age groups of male WAG/Rij rats when the rate of albumin synthesis was expressed per 10⁶ cells or per mg protein. The amount of albumin synthesized by hepatocytes isolated from female BN/BI rats of comparable ages, expressed in μg albumin per hour per 10⁶ cells or mg protein (see Table 5.3), was less than that of hepatocytes isolated from female WAG/Rij rats but more than that of hepatocytes from male WAG/Rij rats. Although the differences were not significant, approximately the same pattern with age for the albumin synthesis was observed as was found for the female WAG/Rij rats.

5.4 DISCUSSION

5.4.1 OPTIMUM INCUBATION CONDITIONS FOR ALBUMIN SYNTHESIS BY ISOLATED HEPATOCYTES

The finding that incubation of the cells isolated from female WAG/Rij rats in modified Waymouth MB 752/1 medium results in considerable albumin production is in agreement with the data of Jeejeebhoy et al. (1975). The importance of the composition of the incubation medium for albumin synthesis by isolated hepatocytes can be deduced from experiments performed, among others, by Jeejeebhoy et al. (1975). They found that hepatocytes suspended in Waymouth MB 752/1 medium synthesized significantly more albumin than did hepatocytes incubated in Ham's F10 medium. Another proof of the importance of the incubation medium was reported by Grant and Black (1974), who determined that the amount of albumin synthesized by the isolated hepatocytes was two times higher in HEPES buffered Eagle medium as compared to Krebs-Ringer phosphate, with supplementation of amino acids to physiological rat serum levels. In addition, Dich and Gluud (1976) found an increase in the albumin synthetic rate by a factor of 3 by switching from Hanks solution medium containing 1 percent bovine albumin to Krebs-Henseleit buffer with 0.5 percent gelatin. These last authors observed, as was also found in this study, that albumin was synthesized by the isolated hepatocytes at a slower rate during the first 15 to 30 min of incubation. Such a lag period of at the most 30 min had also been observed by Weigand and Otto (1974a), and Feldhoff et al. (1977) with isolated liver parenchymal cells. With liver slices, a lag period of about 20 min was found by Judah and Nicholls (1971).

The amount of albumin synthesized by hepatocytes isolated from 3-month-old female WAG/Rij rats appeared to be $5.8 \mu\text{g albumin/h.}10^6 \text{ cells}$, which is higher than that found for 3-month-old male WAG/Rij and female BN/Bi rats. An explanation for that difference might be that the optimal incubation conditions determined for hepatocytes from female WAG/Rij rats are suboptimal for hepatocytes from male WAG/Rij and female BN/Bi rats. Another possible explanation is that the albumin capacity of hepatocytes is strain and sex dependent. The albumin synthesizing capacity of 3-month-old female WAG/Rij rats appeared to be much higher than reported in all of the data published up to now (Table 5.4). For the sake of convenience, to compare the literature data with each other and with that of the 3-month-old female WAG/Rij rat, general conversion factors were used so that the albumin synthetic capacity of the isolated hepatocytes could be expressed in $\mu\text{g albumin/h.}10^6 \text{ cells}$ (Table 5.4). However, the comparison of all data is complicated by the fact that rats of different strains, sex and age have been used in these

TABLE 5.4

COMPARISON OF RATES OF ALBUMIN SYNTHESIS BY ISOLATED RAT HEPATOCYTES
DETERMINED BY VARIOUS AUTHORS

Author	Rate of albumin synthesis	
	unit used by author	$\mu\text{g}/\text{h} \cdot 10^6$ cells
East et al., 1973	$0.48 \mu\text{g}/\text{h} \cdot 10^6$ cells	0.48
Weigand and Otto, 1974a	$0.63 \mu\text{g}/\text{h} \cdot 10^6$ cells	0.63
Grant and Black, 1974	$1.2 \mu\text{g}/\text{h} \cdot 10^6$ cells	1.2
Jeejeebhoy et al., 1975	$0.32 \text{ mg}/\text{h} \cdot \text{g}$ hepatocytes	2.4^{a-b}
Dich and Gluud, 1976	$0.28 \text{ mg}/\text{h} \cdot \text{g}$ liver	2.4^b
Crane and Miller, 1977	$15 \text{ pmol}/\text{h} \cdot 10^6$ cells	1.0^c
Feldhoff et al., 1977	$0.38 \text{ mg}/\text{h} \cdot \text{ml}$ packed cells	2.9^{d-a-b}
This study	$5.8 \text{ mg}/\text{h} \cdot 10^6$ cells	5.8

^a a factor of 1.18 was used to convert g hepatocytes to g liver weight (Striebich et al., 1953; Weibel et al., 1969, and Greengard et al., 1972)

^b a conversion factor of 1/114 was used to convert g liver weight to 10^6 cells (Seglen, 1973)

^c a factor of $65,000 \cdot 10^{-6}$ was used to convert pmol albumin to μg albumin (Hughes, 1954; Charlwood, 1961, and Peters, 1962)

^d 1 ml packed cells were taken to be the equivalent to 1 g hepatocytes

studies. In the study of East et al. (1973), the albumin synthesis amounted to $0.48 \mu\text{g}$ albumin/h $\cdot 10^6$ cells. A slightly higher amount has been claimed by some other authors (Grant and Black, 1974; Weigand and Otto, 1974a, and Crane and Miller, 1977). In contrast to the albumin synthesis mentioned by those authors, a higher albumin synthesis was measured by Jeejeebhoy et al. (1975); Dich and Gluud (1976), and Feldhoff et al. (1977). The $5.8 \mu\text{g}$ albumin/h $\cdot 10^6$ cells reported for albumin synthesis of 3-month-old female WAG/Rij rats is also higher than the values reported for liver slices (Table 5.5) and for the perfused liver (Table 5.6). Only very high synthetic rates were observed by Katz et al. (1967) and Gordon and Humphrey (1960) (see Table 5.6). The synthesis of albumin by the hepatocytes isolated from 3-month-old female WAG/Rij rats nearly equals the values reported for the *in vivo* situation

TABLE 5.5

COMPARISON OF RATES OF ALBUMIN SYNTHESIS BY RAT LIVER SLICES
DETERMINED BY VARIOUS AUTHORS

Author	unit used by author	Rate of albumin synthesis
		$\mu\text{g/h.}10^6$ <i>in vivo</i> cells
Campbell and Stone, 1957	360 $\mu\text{g/h.g}$ liver	3.0 ^b
Marsh and Drabkin, 1958	365 $\mu\text{g/h.g}$ liver	3.0 ^b
Huberman and Soberon, 1970	115 $\mu\text{g/h.g}$ liver	0.96 ^b
Peters, 1973	150 $\mu\text{g/h.g}$ liver	1.3 ^b

^b see table 5.4

(Table 5.7). This finding indicates that the isolation procedure did not negatively influence the functional capacity of the isolated cells and that the incubation conditions for the isolated hepatocytes with respect to the albumin synthesis were optimal.

5.4.2 ALBUMIN SYNTHESIS BY LIVER PARENCHYMAL CELLS ISOLATED FROM RATS OF DIFFERENT AGE GROUPS

A decrease in the albumin synthesizing capacity of hepatocytes isolated from young (3 months) and adult (12 months) rats has been observed for female WAG/Rij rats. Such a decrease has also been observed *in vivo* (Wise and Oliver, 1967, and Peters and Peters, 1972) and with liver slices (Peters et al., 1968 and 1973). This decrease might be attributable to the fact that the turnover rate of albumin decreased during the maturation of the rat (Jeffay, 1960). Peters et al. (1968) correlated this decrease in albumin synthesis with a decline in the number of cells containing albumin. The increase in albumin synthesis observed in late age for the female WAG/Rij rats seems to be in agreement with *in vivo* results obtained by Beauchene et al. (1970), Salatka et al. (1971), Ove et al. (1972), and Obenrader et al. (1974). All of these investigators found increased albumin synthesis in what they

TABLE 5.6

COMPARISON OF RATES OF ALBUMIN SYNTHESIS BY ISOLATED PERFUSED RAT LIVER
DETERMINED BY DIFFERENT AUTHORS

Author(s)	unit used by author	Rate of albumin synthesis
		$\mu\text{g/h.}10^6$ <i>in vivo</i> cells
Gordon and Humphrey, 1960	11 mg/h.300 g rat	8.0 ^{b-e}
Marsh, 1961	0.56 mg/h.g liver	4.8 ^b
John and Miller, 1966	0.47 mg/h.g liver	4.0 ^b
Katz et al., 1967	10 mg/h.100 g rat	22.0 ^{b-e}
Kirsch et al., 1969	1.64 mg/h.300 g rat	1.2 ^{b-e}
Hoffenberg et al., 1971	1.7 mg/h.100 g rat	3.6 ^{b-e}
Matern et al., 1972	0.39 mg/h.g liver	3.3 ^b
Saunders et al., 1973	1.75 mg/h.300 g rat	1.3 ^{b-e}
Tavill et al., 1973	6.3 mg/h.300 g rat	4.7 ^{b-e}
Nováková et al., 1974	0.52 mg/h.100 g rat	1.1 ^{b-e}
Lloyd et al., 1975	2.66 mg/h.300 g rat	1.9 ^{b-e}
Feldhoff et al., 1977	0.54 mg/h.g liver	4.6 ^b

^b see table 5.4^e a factor of 1/4 was used to convert 100 g body weight to g liver weight (Webster et al., 1947; Zumoff and Pachter, 1964; De Leeuw-Israel, 1971, and this study, figure 4.2).

called "old" rats. However, Beauchene et al. (1970), and Salatka et al. (1971) called rats of about 24 months of age old rats. The old female rats used by Ove et al. (1972), and Obenrader et al. (1974) were even 17-20 months of age. Since those investigators failed to give any information about the mean survival time of the rat strains they used, it is very difficult to determine whether the rats were really old. In addition, Salatka et al. (1971), Ove et al. (1972), and Obenrader et al. (1974) compared the albumin synthesizing capacity of young (1 month) and "old" rats but not of intermediate age groups. Therefore, it is difficult to discover whether the rats used by these authors might also reveal an increase in albumin synthesis in late age. Only Beauchene et

TABLE 5.7

COMPARISON OF RATES OF ALBUMIN SYNTHESIS BY THE RAT LIVER *IN VIVO*
DETERMINED BY DIFFERENT AUTHORS

Author(s)	Rate of albumin synthesis	
	unit used by author	$\mu\text{g/h.}10^6$ <i>in vivo</i> cells
Kirsch et al., 1968	11.7 mg/h.300 g rat	8.7 ^{b-d}
Haider and Tarver, 1969	17.1 mg/h.200 g rat	19 ^{b-d}
Morgan and Peters, 1971	0.74 mg/h.g liver	6.4 ^b
Jeejeebhoy et al., 1972	0.88 mg/h.g hepatocytes	6.6 ^{a-b}

a-b-d see table 5.4 and 5.6

al. (1970) compared the albumin synthesizing capacity of 11-14 and 23-28 month old rats. This last study indicates an increase in albumin synthesis in late age.

An explanation for the observed increase in albumin synthesis for female WAG/Rij rats in late age might be that the liver had to compensate for increased albumin excretion via the urine due to kidney insufficiency as suggested by Beauchene et al. (1970). An increase in albumin excretion in rats due to senescent albuminuria was observed by Beauchene et al. (1970). This finding has been confirmed by Obenrader et al. (1974), who observed, however, that after an experimentally induced increase in albumin excretion, no increase in albumin synthesis was evident. The same phenomenon was found by Peters and Peters (1972). They experimentally induced nephrosis in rats. Consequently, a marked urinary albumin loss resulting in a decrease in the amount of serum albumin took place. Nevertheless, they observed that the rate of albumin synthesis was not increased. Furthermore, if there is an excess amount of albumin circulating in the blood, albumin synthesis should decrease. However, no such decrease in albumin synthesis was observed after infusion of albumin in rabbits (Rothschild et al., 1964). Therefore, it was concluded that albumin synthesis and serum albumin concentration are not directly interdependent. Rothschild et al. (1969a) studied patients with cirrhosis of the liver. All of these patients revealed a

decreased serum albumin level, whereas the albumin production was found to be normal, elevated or even diminished. It was concluded that albumin synthesis did not bear any relationship to serum albumin levels. . The only relationship which has been found between albumin synthesis and serum composition is that the albumin synthesis is sensitive to changes in osmotic concentrations of the plasma, as was established in the isolated perfused liver of the rabbit (Rothschild et al., 1969b).

An interesting phenomenon was observed by Ove et al. (1972) with *in vivo* experiments involving the intact liver and by Chen et al. (1973) with isolated liver microsomes. These authors observed, in contradiction to Rothschild et al. (1969b), that when the albumin concentration of the blood was decreased by bleeding the rats by heart puncture and immediately injecting i.p. the same amount of 0.15 M NaCl solution equal to the amount of blood removed, the capacity of young rats for albumin synthesis increased with respect to non-bled young rats, whereas bleeding did not influence the capacity for albumin synthesis in old rats.

In analogy to these phenomena, the high values for the albumin synthesis by the cells isolated from old rats might be explained by the fact that these cells may function at their maximum albumin synthesizing capacity. However, hepatocytes isolated from younger rats may not function maximally. An alternative explanation may be that all cells capable of synthesizing albumin are active in the old rats, while, in the younger rats, some of these cells are held in reserve.

The finding that the amount of albumin synthesized by the hepatocytes isolated from male WAG/Rij rats and female BN/Bi rats did not change significantly with age might be explained as follows. Since the optimal incubation conditions are determined with hepatocytes isolated from the female WAG/Rij rats, it is possible that those conditions are not the optimal ones for hepatocytes isolated from male WAG/Rij and female BN/Bi rats. This hypothesis is supported by the observation that the amount of albumin synthesized by hepatocytes isolated from male WAG/Rij and female BN/Bi rats (Tables 5.2 and 5.3) is smaller than that measured for female WAG/Rij rats (Table 5.1). Consequently, if hepatocytes of old male WAG/Rij or female BN/Bi rats functioned maximally *in vivo*, this maximum functioning cannot be visualized *in vitro*, since only small amounts of albumin can be synthesized by those isolated hepatocytes due to the suboptimal incubation conditions.

CHAPTER VI

AGE-RELATED CHANGES IN PROTEIN SYNTHESIS BY ISOLATED LIVER PARENCHYMAL CELLS

- Contents:*
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 - 6.2 Materials and Methods
 - 6.3 Results
 - 6.3.1 Optimum conditions for protein synthesis by isolated liver parenchymal cells
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6.1 INTRODUCTION

The process of protein synthesis is of great importance for the maintenance and functioning of cells and tissues. Age-related deficiencies in protein synthesis are often considered as being among the major causes of aging (Hrachovec, 1971; Ove et al., 1972; Buetow and Gandhi, 1973, and Florini and Sorrentino, 1976). The liver, as an organ which exhibits high protein synthesizing activity, has been chosen by many investigators to study aging changes in the synthesis of protein (Mainwaring, 1969; Beauchene et al., 1970; Hrachovec, 1971; Ove et al., 1972; Buetow and Gandhi, 1973; Chen et al., 1973; Blok et al., 1974; Junghahn and Bielka, 1974; Péñzes, 1975; Hellthaler et al., 1976; Layman et al., 1976, and Goswami, 1977).

An age-related increase in liver protein synthesis *in vivo* was reported by Beauchene et al. (1970) when they compared 11-14 and 23-28-month-old male and female rats. However, other investigators observed no detectable changes in liver protein synthesis between female rats of 4-6 weeks and 17 months (Ove et al., 1972) or between those of 6, 12 and 25-28 months of age (Péñzes, 1975), as shown in Table 6.1. Literature data on aging changes in protein synthesis by isolated liver microsomes are also contradictory (Table 6.1). Chen et al. (1973) found no difference in protein synthesis with liver microsomes isolated from 6-week and 24-month-old male rats. In contrast, an age-related decrease in protein synthesizing capacity was observed by other investigators using microsomes isolated from 2-3, 7-11 and 19-25-month-old rats (Hrachovec, 1971), from 12 and 20 to 31-month-old female rats (Buetow and Gandhi, 1973), from 3-5, 8-12 and 18-26-month-old female rats (Hellthaler et al., 1976) and from 3-week- to 12-month-old male rats (Goswami, 1977).

Layman et al. (1976) conducted a detailed study on the onset of the decline in protein synthesis. They used the postmitochondrial supernatant fraction which was prepared from livers of rats of 1, 3, 6, 8, 13 and 18 months of age by centrifuging the homogenate at 15,000 g for 10 min. They observed a sharp decrease during the first 9 months of the rat's life; no significant changes were observed in the period of 9 to 18 months.

Many of the discrepancies found for the age influences on protein synthesis may be the consequence of using rats of different strains, sex and age groups. With respect to the age of the animals used, it is remarkable that many of these gerontological studies are lacking in the survival data of the animals used, so that it is impossible to establish the 50 percent survival age. According to Burek and Hollander (in

TABLE 6.1

TOTAL PROTEIN SYNTHESIS BY LIVER PREPARATIONS FROM RATS OF DIFFERENT AGES

Author(s)	Strain	Sex	Organization level	Ages (months)	Change*
Beauchene et al., 1970	Wistar	F+M	<i>in vivo</i>	11-14; 23-28	I
Ove et al., 1972	Fischer	F	<i>in vivo</i>	1-1.5; 17	NC
Péñzes, 1975	Wistar	F	<i>in vivo</i>	6; 12; 25-28	NC
Hrachovec, 1971	Albino	M	isolated microsomes	2-3; 7-11; 19-25	D
Buetow and Gandhi, 1973	Wistar	F	isolated microsomes	12; 20-31	D
Chen et al., 1973	Fischer	M	isolated microsomes	1-1.5; 24	NC
Hellthaler et al., 1976	Wistar	F	isolated microsomes	3-5; 8-12; 18-26	D
Goswami, 1977	Wistar	M	isolated microsomes	0.7; 12	D
Layman et al., 1976	Sprague-Dawley	F	postmitochondrial supernatant fraction	1; 3; 6; 9; 13; 18	D NC

*D means decrease, I means increase and NC means no change.

press), old animals are those which live longer than the 50 percent survival age. Since the 50 percent survival ages are unknown, one cannot determine whether the above-mentioned gerontological studies were performed with really old animals. Furthermore, for the *in vivo* studies, age-related deficiencies in several physiological factors such as hormones and some nutrients, especially amino acids, will directly influence the protein synthesizing capacity of the liver *in vivo* (Munro, 1970, and Sidransky, 1972). The usefulness of the system of isolated microsomes for studying aging changes in protein synthesis is also limited. The capacity of the isolated microsomes to synthesize total protein is linear for only about 5 min and a plateau is reached after about 20 minutes of incubation (Mainwaring, 1969; Hrachovec, 1971; Buetow and Gandhi, 1973; Chen et al., 1973, and Layman et al., 1976). This may be due to several factors, such as the absence of the genetic control mechanism located in the nucleus and the lack of several components present in the cytoplasm. This last possibility may be deduced from experiments performed by Junghahn and Bielka (1974). They observed that the synthesis of protein by polysomes isolated from 3-month-old rats was increased by the addition of a cytosol fraction from rats of the same age. The stimulation of the protein synthesis by the polysomes was less when the cytosol from 2-year-old rats was used. Another disadvantage of the use of isolated microsomes is that the microsomal system synthesizes at only about 4 percent of the *in vivo* rate of protein synthesis by the total liver (Richardson et al., 1971).

As will be shown in this chapter, the disadvantages of both *in vivo* studies and studies with isolated microsomes can be overcome with the *in vitro* system offered by suspensions of hepatocytes isolated from livers of rats of different ages.

In the previous chapter, it was observed that the albumin synthesizing capacity of liver parenchymal cells isolated from female WAG/Rij rats decreased between 3 and 24 months of age and increased afterwards. It seemed of interest to determine whether this change in albumin synthesis with age reflects an overall change in the protein synthesis of the hepatocytes or only a change in the capacity to synthesize a liver-specific protein such as albumin.

6.2 MATERIALS AND METHODS

The method for determining the protein synthesis was as follows. A known number of parenchymal cells in 1.5 ml Waymouth MB 752/1 medium, which was modified as described in paragraph 5.2, was incubated with

various concentrations of L-(1- ^{14}C) leucine (Radiochemical Center Amersham, UK) and unlabeled leucine. The incubation was carried out at 37°C under an atmosphere of 95 percent O_2 and 5 percent CO_2 with constant shaking ($100 \text{ oscillations} \cdot \text{min}^{-1}$). Immediately after the addition of the leucine mixture and after various incubation periods, 0.5 ml samples of the cell suspension were withdrawn, added to 5 ml ice-cold 10 percent TCA and stored for at least 30 min at 0°C . The resulting precipitate was washed two times with 5 ml ice-cold 5 percent TCA with interim centrifugation at 300 g. The pellet was then dissolved in 1 ml soluene[®]. The radioactivity of the samples was counted with a liquid scintillation counter. Values obtained after incubation were corrected for the amount of radioactivity in a sample withdrawn immediately after the addition of the leucine mixture to the cell suspension.

To investigate the possible breakdown of newly synthesized protein during the 2 h incubation period, parenchymal cells were incubated with 8 μmol of a mixture of ^{14}C - and unlabeled leucine per ml under the conditions mentioned above. After 30 min of incubation, the hepatocytes were washed 3 times with phosphate buffered saline (PBS) and resuspended in the incubation medium with 8 μmol of unlabeled leucine. Samples of the cell suspensions were withdrawn after 0, 30, 60, 90 and 120 min of incubation. After TCA precipitation, the radioactivity of the samples was determined as described above.

When the effect of age on the protein synthetic capacity of the isolated hepatocytes was studied, three different cell concentrations were made of each cell preparation. By plotting the synthesis of protein (expressed in nmol leucine incorporated per hour) against the cell concentration, a straight line could be drawn through 4 points, representing the 3 obtained values and the origin. From this line, the amount of synthesized protein (expressed in nmol leucine/h. 10^6 cells) was read off.

6.3 RESULTS

6.3.1 OPTIMUM CONDITIONS FOR TOTAL PROTEIN SYNTHESIS BY ISOLATED LIVER PARENCHYMAL CELLS

The development of a method for measuring protein synthesis by isolated parenchymal cells required preliminary experiments to determine several conditions necessary for optimum synthesis. As a first condition, the pH dependence of the incorporation of leucine into protein was determined. No differences in protein synthesis were observed

between pH 7.4 and 8.0. In further experiments, the cells were incubated at pH 7.8, since optimum albumin synthesis occurred at this pH (see paragraph 5.3.1). The hepatocytes were incubated in the modified Waymouth MB 752/1 medium, the same medium which was used for measuring the albumin synthesizing capacity of the isolated hepatocytes. This means that the albumin and protein synthesis could be compared under the same experimental conditions.

Using hepatocytes isolated from 3-month-old female WAG/Rij rats, the time course of the incorporation of leucine into protein appeared to be linear for at least 4 hours from the start of the incubation (Figure 6.1). In subsequent experiments, the protein synthesis was determined during the first two hours of incubation.

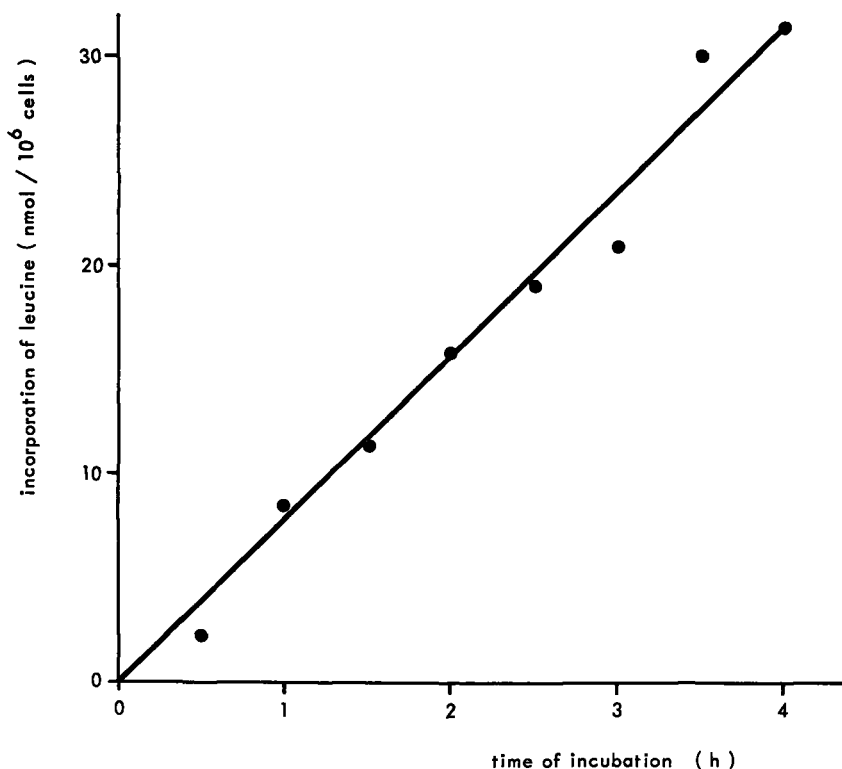


Figure 6.1 Time course of the protein synthesis by isolated liver parenchymal cells from 3-month-old female WAG/Rij rats. Eight cell suspensions of a cell concentration of 0.67×10^6 cells.ml⁻¹ were incubated with 8 μ mol leucine/ml medium. At the time intervals indicated in the figure, the amount of leucine incorporation was measured.

The effect of the concentration of leucine in the incubation medium on the rate of protein synthesis by the isolated liver parenchymal cells was investigated. For cells isolated from 3-month-old female WAG/Rij rats, the protein synthesis increased with increasing amounts of leucine in the incubation medium until a plateau level was reached at about 6 μmol leucine/ml incubation medium (Figure 6.2). In further experiments, a concentration of 8 μmol leucine. ml^{-1} was added to the incubation medium. This concentration guaranteed that the cells functioned maximally, so that any small changes in the protein synthesizing capacity with age could be detected.

Figure 6.3 shows that, for hepatocytes isolated from 3-month-old female WAG/Rij rats, the rate of leucine incorporation into protein is proportional to the number of cells present in the incubation medium between 0.25 and at least 4×10^6 cells/ml medium. To determine the rate of leucine incorporation into protein, liver parenchymal cell suspensions with cell concentrations ranging from 0.25 to 3.5×10^6 cells/ml medium were incubated with 8 μmol leucine/ml medium for 2 h at 37°C . An oxygen tension of at least 30×10^3 Pa was maintained in the incu-

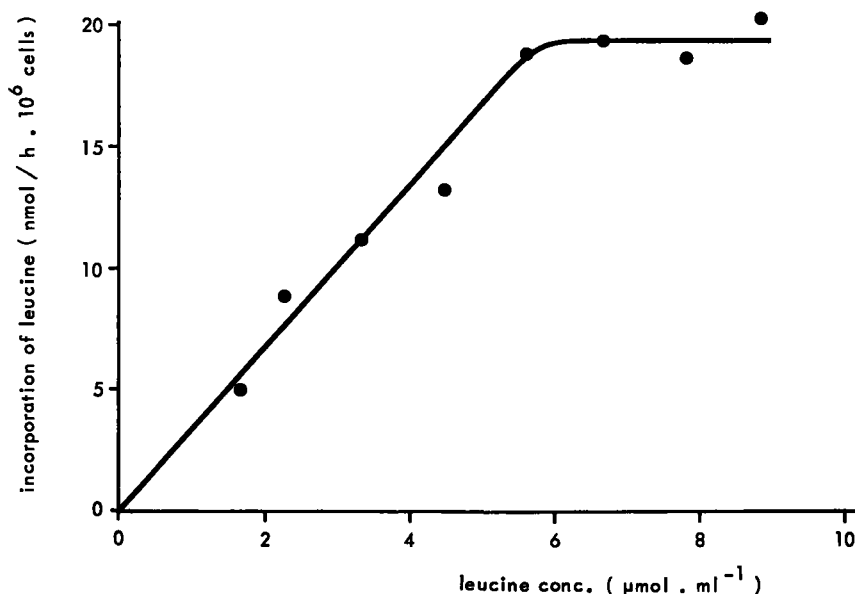


Figure 6.2 The incorporation of leucine into protein by liver parenchymal cells isolated from 3-month-old female WAG/Rij rats as a function of the concentration of leucine in the incubation medium. Eight cell suspensions with a cell concentration of $0.6 \times 10^6 \cdot \text{ml}^{-1}$ were incubated for 2 h with various concentrations of ^{14}C - and unlabeled leucine in the medium.

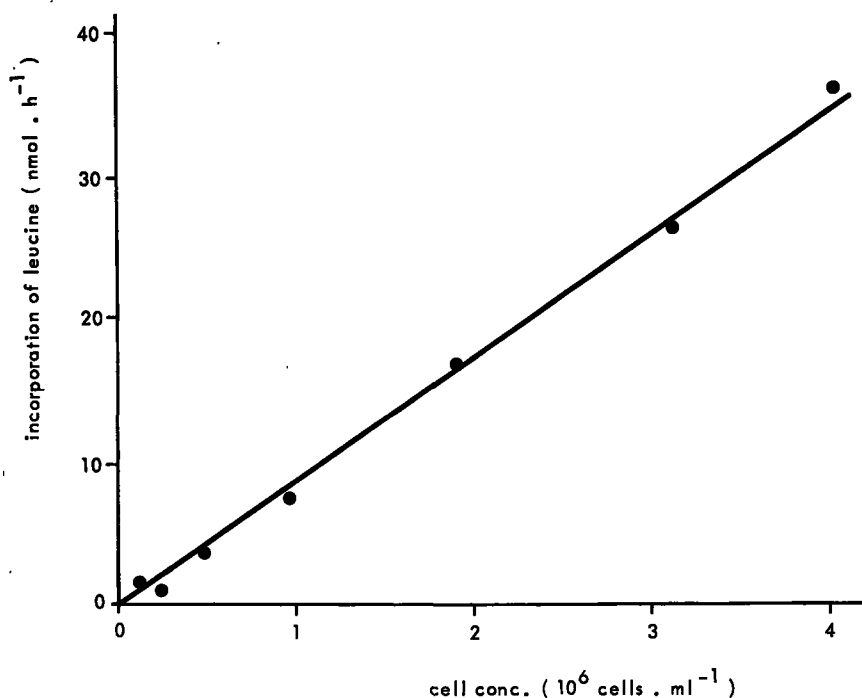


Figure 6.3 Effect of the cell concentration in the incubation medium on the incorporation of leucine into protein by hepatocytes isolated from 3-month-old female WAG/Rij rats. Seven cell suspensions at the concentrations indicated were incubated for 2 h with 8 μ mol leucine/ml medium.

bation medium with 95 percent O_2 and 5 percent CO_2 (paragraph 5.3.1). Under these experimental conditions, the incorporation of leucine into protein by hepatocytes isolated from 3-month-old female WAG/Rij rats was 14.4 nmol leucine/h. 10^6 cells.

6.3.2 PROTEIN SYNTHESIS BY HEPATOCYTES ISOLATED FROM RATS OF DIFFERENT AGES

The influence of age on the capacity to synthesize protein was determined with liver parenchymal cells isolated from female WAG/Rij rats of 3, 12, 24, 31 and 36 months of age. A significant decrease in the capacity for protein synthesis was observed between 3 and 12 months (Table 6.2). Thereafter, no significant change was measured up to 24 months of age. A considerable increase in protein synthesis by the

hepatocytes was measured after 24 months of age up to the age of 36 months. The value obtained for the protein synthesizing capacity of 36-month-old rats did not differ significantly from that obtained for 3-month-old rats.

To investigate whether the results obtained for the protein synthesis might be negatively influenced by proteolytic degradation, the amount of radioactivity incorporated into the cell suspension during a 30 minute incubation period was followed during an additional incubation period of 2 hours without radioactive leucine in the incubation medium. With hepatocytes isolated from both 3- and 31-month-old rats, no loss in TCA precipitable radioactivity in the cell suspension was observed during that second incubation period. Reutilization of labeled amino acids which might be released during proteolysis of the newly synthesized proteins had been minimized because the cells were incubated with an at least 500-fold excess of unlabeled leucine during the subsequent incubation period of 2 hours. The observed differences in the incorporation rates of leucine into protein with age, therefore, represent age-related changes in protein synthesis.

The data on the protein synthesis by hepatocytes isolated from male WAG/Rij rats are presented in Table 6.3. The chosen incubation conditions for determining the protein synthesis were the same as those which had been proved to be optimal for hepatocytes isolated from female WAG/Rij rats. The amount of protein synthesized by liver parenchymal cells isolated from male WAG/Rij rats appeared to be in the same range as that observed for the female WAG/Rij rats, with the exception that the 3-month value for the male WAG/Rij rat is lower than the 3-month value for the female WAG/Rij rat (Table 6.2 and 6.3). Consequently, a difference between the protein synthesizing capacity of female and male WAG/Rij rats is that, with male WAG/Rij rats, no decrease in the rate of protein synthesis between 3 and 12 months of age was observed. The sharp increase in protein synthesis by male WAG/Rij rats in late age (between 20 and 27 months) is in agreement with the data of the female WAG/Rij rats. This indicates that this increase is not sex dependent.

Table 6.4 shows the findings on the protein synthesizing capacity of the liver parenchymal cells prepared from female BN/Bi rats. The chosen incubation conditions were identical to those which were optimal for liver parenchymal cells from the female WAG/Rij rats. The protein synthesizing capacity of the female BN/Bi rat showed the same age pattern as that of the male WAG/Rij rat. No decrease in the capacity to synthesize protein was observed between 3 and 12 months. As observed for the hepatocytes isolated from the female and male WAG/Rij rat, the

TABLE 6.2

TOTAL PROTEIN SYNTHESIS BY LIVER PARENCHYMAL CELLS ISOLATED FROM FEMALE
WAG/Rij RATS OF DIFFERENT AGES*

Age (months)	Protein content of parenchymal cells (mg/10 ⁶ cells)	Incorporation of leucine	
		nmol/h.10 ⁶ cells	nmol/h.mg cellular protein
3	1.47 ± 0.10 (11)	14.4 ± 1.4 (10)	8.70 ± 1.10
12	1.80 ± 0.10 (4) ^a	9.8 ± 1.4 (6) ^a	5.53 ± 0.91 ^a
24	2.03 ± 0.09 (3) ^a	8.4 ± 1.2 (5) ^a	4.14 ± 0.62 ^a
31	-	12.9 ± 2.8 (4)	-
36	2.01 ± 0.11 (5) ^a	14.7 ± 1.2 (7) ^{b,c}	7.32 ± 0.70 ^c

*Mean ± S.E.; number of different cell preparations in parentheses.

^aValue differs significantly ($p < 0.05$) from the 3-month value.

^bValue differs significantly ($p < 0.05$) from the 12-month value.

^cValue differs significantly ($p < 0.05$) from the 24-month value.

capacity of hepatocytes prepared from female BN/Bi rats to synthesize protein sharply increased in late age, namely, between 24 and 31 months. This suggests that this phenomenon is not strain and sex dependent.

6.4 DISCUSSION

6.4.1 PREREQUISITES FOR OPTIMUM PROTEIN SYNTHESIS BY ISOLATED HEPATOCYTES

From the time course of protein synthesis of hepatocytes isolated from 3-month-old female WAG/Rij rats (Figure 6.1) it could be concluded that protein was synthesized in a linear way for at least four hours. Craig and Porter (1973), Schreiber and Schreiber (1973), Dich and Gluud (1976), and Seglen (1976) also observed that protein synthesis occurred in a linear way for the period they studied, which was 1.5; 1; 1 hour and 2 hours, respectively.

TABLE 6.3

TOTAL PROTEIN SYNTHESIS BY LIVER PARENCHYMAL CELLS ISOLATED FROM MALE
WAG/Rij RATS OF DIFFERENT AGES*

Age (months)	Protein content of parenchymal cells (mg/10 ⁶ cells)	Incorporation of leucine nmol/h.10 ⁶ cells	nmol/h.mg cellular protein
3	2.65 ± 0.10 (12)	11.3 ± 1.2 (9)	4.26 ± 0.48
12	1.80 ± 0.22 (4) ^a	10.5 ± 1.4 (8)	5.22 ± 0.90
20	2.12 ± 0.17 (5) ^a	9.2 ± 1.6 (8)	4.33 ± 0.83
27	2.18 ± 0.24 (5) ^a	15.7 ± 1.5 (4) ^{a,b,c}	7.2 ± 1.1 ^{a,c}

*Mean ± S.E.; number of different cell preparations in parentheses.

^aValue differs significantly ($p < 0.05$) from the 3-month value.

^bValue differs significantly ($p < 0.05$) from the 12-month value.

^cValue differs significantly ($p < 0.05$) from the 20-month value.

TABLE 6.4

TOTAL PROTEIN SYNTHESIS BY LIVER PARENCHYMAL CELLS ISOLATED FROM FEMALE
BN/Bi RATS OF DIFFERENT AGES*

Age (months)	Protein content of parenchymal cells (mg/10 ⁶ cells)	Incorporation of leucine nmol/h.10 ⁶ cells	nmol/h.mg cellular protein
3	1.49 ± 0.11 (11)	6.38 ± 0.71 (9)	4.29 ± 0.57
12	1.94 ± 0.20 (3)	6.7 ± 1.7 (4)	3.45 ± 0.95
24	2.18 ± 0.11 (4) ^a	9.1 ± 2.0 (4)	4.18 ± 0.94
31	2.27 ± 0.13 (5) ^a	17.5 ± 2.4 (7) ^{a,b}	7.7 ± 1.2 ^{a,b}

*Mean ± S.E.; number of different cell preparations in parentheses.

^aValue differs significantly ($p < 0.05$) from the 3-month value.

^bValue differs significantly ($p < 0.05$) from the 12- and 24-month value.

The linear relationship between the cell concentration and the amount of synthesized protein observed in the range of 0.25 to at least 4.0×10^6 cells/ml medium fully agrees with the concentrations of 0.26 to 4.7×10^6 cells.ml⁻¹ used by Schreiber and Schreiber (1973).

A saturation curve obtained by plotting the leucine concentration in the medium against the amount of synthesized protein was also observed by Schreiber and Schreiber (1972), who measured that optimal protein synthesis took place at a concentration of 1.5 μ mol leucine.ml⁻¹, instead of the 6 μ mol leucine.ml⁻¹ observed in this study (Figure 6.2).

The rate of incorporation of 14.4 nmol leucine/h.10⁶ hepatocytes isolated from 3-month-old female WAG/Rij rats is, to our knowledge, at least 20 times higher than the values reported in the literature up to now (Jezyk and Liberti, 1969; Weigand et al., 1974b, and Igarashi, 1977). A possible explanation may be that in these studies non-optimal doses of leucine were used during the incubation.

The rate of protein synthesis by the isolated hepatocytes from female WAG/Rij rats is at least 2 times higher than that of liver slices (Huberman and Soberon, 1970; Judah and Nicholls, 1971; Peters, 1973, and Perin et al., 1974). The *in vivo* rate of protein synthesis, expressed in nmol leucine incorporated per hour per 10⁶ *in vivo* cells, reported by Maeno et al. (1970) is also lower than that found for the isolated hepatocytes. The main reason may again be that, for the liver slices and the *in vivo* studies, sub-optimal concentrations of labeled leucine were used.

6.4.2 POSSIBLE EXPLANATIONS FOR THE OBSERVED INCREASE IN PROTEIN SYNTHESIS IN LATE AGE

The increase in protein synthesis after 24 months of age might be due to compensation by the liver for an increase in protein excretion via the urine attributable to kidney insufficiency, as was proposed as a possible explanation by Beauchene et al. (1970). To investigate whether senescent proteinuria occurred in female WAG/Rij rats, the amount of total protein excreted in the urine was determined (Table 6.5). The amount of excreted protein per 24 hours did not change between 3 and 12 months, but increased sharply between 12 and 24 months. Thereafter, no significant change was observed up to 30 months. Considering these observations, the increase in protein synthesis between 24 and 36 months cannot be attributed to increased excretion of protein

TABLE 6.5

THE EXCRETION OF URINARY PROTEINS BY
FEMALE WAG/Rij RATS OF DIFFERENT AGES*

Age (months)	Total protein excretion (mg/24 h)
3	1.26 $\pm$ 0.31 (5)
12	1.18 $\pm$ 0.16 (5)
24	14.0 $\pm$ 5.8 (5) ^a
30	16.7 $\pm$ 6.5 (5) ^a

*Mean $\pm$ S.E.; number of determinations in parentheses

^aValue differs significantly ($p < 0.05$) from 3- and 12-month value

via the urine. Firstly, because the value for the amount of excreted protein obtained for the 24-month-old female WAG/Rij rats (14.0 ± 5.8 mg total protein/24 h) is 12 times lower when compared with that for the 23-28-month-old female and male Wistar rats (173 ± 40 mg total protein/24 h) used by Beauchene et al. (1970). Secondly, although increased protein excretion via the urine was observed in female WAG/Rij rats, this increase took place between 12 and 24 months, while the increase in protein synthesis occurred between 24 and 36 months. Furthermore, chronic progressive glomerulonephropathy as described by Snell (1969) was histopathologically seldom observed in aging WAG/Rij rats (Burek, 1978).

A second possibility to explain the increase in protein synthesis in late age is that the liver compensates for proteolytic activity in the liver. An increase in the activity of cathepsin D - the main lysosomal proteolytic enzyme - in the liver was observed with rats (Barrows et al., 1962, and Beauchene et al., 1967) and mice (Leto et al., 1976) of 12 and 27 months of age. However, a decrease in the cathepsin activity of the liver was found with rats aged 1 month and 20 months (Ross and Ely, 1954, and Wiederanders et al., 1976a). These conflicting data were obtained with homogenates of the whole liver. A more clear-cut method is to separately determine the age-related changes in the liver parenchymal and nonparenchymal cells, a technique developed by Knook

and Sleyster (1976). They measured the specific activity of cathepsin D in liver parenchymal cells isolated from 3-, 12-, 24- and 30 to 35-month-old female BN/Bi rats. The enzymatic activity increased after 12 months and even doubled between 24 and 30 to 35 months. The increase in protein synthesis observed for the female BN/Bi rats between 24 and 31 months occurred in the same age period as the increase in cathepsin D activity. This may suggest a possible relationship between increased protein synthesis and increased proteolytic activity. It must be mentioned, however, that some authors (Huisman et al., 1974, and Wiederanders et al., 1976b) attribute to cathepsin D a predominant role in the degradation of foreign, extracellular proteins.

A third possible explanation for the increased protein synthesis includes a compensatory synthetic activity by the liver due to the accumulation of "altered" malfunctioning proteins. Several reports in the literature mention the accumulation with age of structurally or functionally altered proteins. Altered inactive adolase has been demonstrated in the livers of 31-month-old mice (Gershon and Gershon, 1973) and altered glucose-6-phosphate dehydrogenase in the livers of 750-day-old mice (Wulf and Cutler, 1975). An age-associated accumulation of faulty lactic dehydrogenase has been observed in the livers of 29-month-old rats (Schapira et al., 1975). Inactive forms of superoxide dismutase appeared to be present in the livers of rats and mice of 32 and 28 months of age, respectively (Reiss and Gershon, 1976). However, Yagil (1976), who studied the livers of young (10 weeks), middle aged (24 months) and very old (39 months) mice, could not detect any sign of altered glucose-6-phosphate dehydrogenase as determined by electrophoresis, heat inactivation or electroimmunodiffusion. From the data mentioned above, it can be concluded that several "altered" malfunctional proteins accumulate in late age. In further experiments, it will be investigated whether this last explanation may account for the observed increase in protein synthesis in late age.

6.4.3 THE RATIO OF ALBUMIN SYNTHESIS VERSUS PROTEIN SYNTHESIS BY ISOLATED HEPATOCYTES WITH AGE

A reason of choosing the protein synthesizing capacity as a functional characteristic of the isolated hepatocytes was to find out whether the changes in the albumin synthesizing capacity with age are representative for changes in all proteins. The results for the albumin synthesis have been described in paragraph 5.3.2. To some extent, the

age-related pattern of the albumin synthesis by hepatocytes isolated from female WAG/Rij rats was comparable with that observed for protein synthesis by these hepatocytes. A decrease in albumin synthesis between 3 and 24 months was followed by a considerable increase between 24- and 36-months. In contrast to the results obtained for the protein synthesis, cells isolated from 36-month-old female WAG/Rij rats synthesized significantly more albumin than did cells isolated from 3-month-old female WAG/Rij rats. As a consequence, the ratio between albumin and protein synthesis by hepatocytes isolated from female WAG/Rij rats did not change during the first 24 months and increased significantly between 24 and 36 months (Table 6.6). An increase in albumin synthesis without

TABLE 6.6

THE RELATIONSHIP BETWEEN THE RATIO OF ALBUMIN VERSUS TOTAL PROTEIN SYNTHESIS AND THE AGE OF THE RATS FROM WHICH THE HEPATOCYTES WERE ISOLATED

Age (months)	Ratio albumin versus total protein synthesis*		
	female WAG/Rij	rat strain male WAG/Rij	female BN/Bi
3	0.40 $\pm$ 0.07 (11)	0.15 $\pm$ 0.03 (8) ^a	0.49 $\pm$ 0.10 (10)
12	0.38 $\pm$ 0.06 (7)	0.21 $\pm$ 0.03 (8) ^b	0.28 $\pm$ 0.09 (4)
20		0.24 $\pm$ 0.07 (8)	
24	0.27 $\pm$ 0.05 (6)		0.32 $\pm$ 0.15 (4)
27		0.12 $\pm$ 0.05 (4) ^c	
31	0.49 $\pm$ 0.12 (4)		0.22 $\pm$ 0.10 (6)
36	0.67 $\pm$ 0.13 (7) ^d		

*The data are expressed as: $\frac{\mu\text{g albumin/h.}10^6 \text{ cells}}{\text{nmol leucine/h.}10^6 \text{ cells}}$

Mean $\pm$ S.E.; number of different cell preparations in parentheses.

^aValue differs significantly ($p < 0.05$) from 3-month value of female WAG/Rij and BN/Bi rats

^bValue differs significantly ($p < 0.05$) from 12-month value of female WAG/Rij rat

^cValue differs significantly ($p < 0.05$) from 31-month value of female WAG/Rij rat.

^dValue differs significantly ($p < 0.05$) from 24-month value.

a concomitant increase in protein synthesis is possible, since the albumin synthesis represents only about 7 to 10 percent of the protein synthesis (Peters and Peters, 1972, and Edwards et al., 1976a).

A change in the ratio between albumin and protein synthesis with age was not observed for hepatocytes prepared from male WAG/Rij and female BN/Bi rats (Table 6.6). From the data presented in Table 6.6, it can be concluded that the ratio between the albumin and protein synthesis by liver parenchymal cells isolated from male WAG/Rij rats of different ages is lower in comparison with that of the hepatocytes from female WAG/Rij rats of the same ages. This is due to the fact that the amount of albumin synthesized by hepatocytes from male WAG/Rij rats is less than that of female WAG/Rij rats (as discussed in paragraph 5.3.2) without a concomitant difference in the protein synthesizing capacity of the liver parenchymal cells prepared from the female and male WAG/Rij rats. No significant differences were observed between the ratio albumin synthesis versus protein synthesis by the hepatocytes prepared from female WAG/Rij and BN/Bi rats when the ratios calculated for the same age groups of those rat strains were compared.

CHAPTER VII

GENERAL DISCUSSION

The objective of this study was to determine the cellular basis of organ aging or, more specifically, to investigate whether a change in the functional capacity of an organ with age might be attributed to a change in the functional capacity of the cells constituting that organ. To achieve this objective, hepatocytes were isolated from rats of different ages and the functional capacity of these cells was determined on the basis of their competence to store BSP and to synthesize albumin and protein. The determination of these functional attributes was accomplished by incubating the isolated hepatocytes under optimal conditions. In this way, any small changes in one of these measured functional activities of the isolated hepatocytes with age may be detected. For the determination of these functional activities it is of general importance that the hepatocytes isolated from young and old rats are viable. In this way, any observed changes in these functions of the cells with age can be really considered as age-related phenomena and cannot be attributed to artifacts resulting from the isolation procedure. The viability of the isolated hepatocytes was determined from their capacity to exclude trypan blue, from their ultrastructural appearance and from their metabolic activity based on their oxygen consumption. It could be concluded that the hepatocytes isolated from young as well as from old rats were viable with respect to these characteristics. In addition to these viability criteria, the data obtained by the determination of the BSP storage proved that these cells were also viable with respect to their functional capacity. For, it was found that the uptake of BSP by isolated hepatocytes viable according to the criteria mentioned above was by means of an active transport system, while nonviable isolated hepatocytes took up BSP by passive diffusion. In addition, the amount of albumin synthesized by the hepatocytes isolated from 3-month-old rats was in the same range as that observed for the *in vivo* situation; this is further evidence of an undiminished functional capacity of the isolated liver parenchymal cells. Furthermore, the rate of incorporation of leucine into protein by the hepatocytes isolated from 3-month-old rats was at least 20 times higher than other values reported in the literature for the rate of protein synthesis in isolated hepatocytes. The rate of synthesis of proteins by liver slices or by the whole liver as reported in the literature is also lower than that observed in this study for the isolated cells. Although these comparisons are complicated by the fact that most investigators used sub-optimal leucine concentrations, it can be concluded that the isolated hepatocytes were also in an excellent viable state with respect to this physiological characteristic. Consequently, it can be considered that the isolated liver parenchymal cells compare favorable to the cells in the *in vivo* situation.

The reason for choosing the BSP storage capacity as a determinant of the functional capacity of the isolated hepatocytes is as follows. De Leeuw-Israel (1971) from our Institute had already studied the metabolism of BSP by the rat liver *in vivo* by means of the BSP retention test. Her choice of this functional characteristic had been based, among others, on the data that the metabolism of BSP as determined by means of the BSP retention test decreased with age in man. De Leeuw-Israel (1971) also found a decreased liver function with age as determined by means of the BSP retention test in intact female RU rats. However, the most pronounced changes occurred between 3 and 12 months of age. The data of De Leeuw-Israel (1971) could be reproduced with the female WAG/Rij rats used in this study. An increase in BSP retention of female WAG/Rij rats with age, significant between 3 and 12 months of age, was observed. An increase in BSP retention might be due to changes in the ploidy state of the liver parenchymal cells (De Leeuw-Israel et al., 1972). The important changes in the ploidy state of the parenchymal cells occurred within the first three months of age in the female WAG/Rij rats. Therefore, the changes in the ploidy state of the hepatocytes between 3 and 36 months influenced the observed age-related changes in the BSP retention test only in a small measure. A more important contribution to the observed decrease in liver function may be that the functional capacity of the individual hepatocytes decreased with age. To investigate this possibility the BSP storage capacity of hepatocytes isolated from rats of different ages was determined. It was found that the storage of BSP by the isolated hepatocytes decreased with age, especially during the first year of life. It was remarkable that the decrease in the BSP storage capacity of the isolated cells with age fully paralleled the observed decrease in the capacity of the whole liver to remove BSP. Consequently, for the BSP clearance of the liver, it can be concluded that a change in the functional capacity of the liver with age can be at least partly attributed to a change in the function of the individual hepatocytes.

The effect of age on the capacity of the isolated hepatocytes to synthesize albumin and protein was also determined. It was found that the amount of albumin and protein synthesized by the isolated hepatocytes increased sharply between 24 and 36 months of age. Therefore, the functional capacity of the whole liver with age with respect to albumin and protein synthesis may also be influenced by the observed aging changes in the capacity of the individual hepatocytes to synthesize albumin and protein.

A cellular basis of liver aging has been established in this study. The next step is to elucidate the mechanisms which cause the

observed aging changes in the individual hepatocytes. The observed age-related decrease in the capacity of the isolated hepatocytes to store BSP might be due to age-related changes in the mechanisms for transport of BSP across the cell membrane. It was mentioned above that the transport of BSP across the cell membrane is of the active transport type. This was based among others on the finding that the rate of BSP uptake by the isolated cells is energy dependent. A decrease in the amount of BSP stored by hepatocytes isolated from the older rats might be a result of a decrease in the energy supply of those hepatocytes. However, experiments performed in our Institute by Brouwer et al. (1977) showed an unchanged energy supply of the isolated hepatocytes with age. Whether age-related changes in the transport mechanism of BSP might influence the BSP storage capacity of the isolated parenchymal cells in another way will be tested by analyzing the kinetics of the BSP uptake mechanism at different ages. Another possibility might be that the transport is not the limiting factor but that the binding capacity for BSP inside the cell determines the amount of BSP stored by the hepatocytes. This hypothesis can be verified by measuring the amount of ligandin, the intracellular protein which mainly binds the stored BSP, in hepatocytes isolated from rats of different ages.

The increase in albumin and protein synthesis by the isolated hepatocytes in late age might be explained as a compensation by the liver for increased excretion of protein via the urine or for increased proteolytic activity. It was observed that the increase in protein synthesis by the hepatocytes isolated from rats older than 24 months was not due to changes in the amount of protein excreted via the urine in that period. In addition, Knook and Sleyster (1976) of our Institute determined whether the activity of cathepsin D, a lysosomal proteolytic enzyme, was increased after 24 months of age. Indeed, an increase in the cathepsin D activity of isolated hepatocytes was measured in that period. However, a problem with the interpretation of the correlation between the increase in albumin and protein synthesis and the increase in the cathepsin D activity is that cathepsin D is supposed to mainly degradate foreign proteins. Another explanation might be that the liver in late age must compensate for increased synthesis of "altered" malfunctional proteins. This possibility will be studied in the future.

The observed aging changes in the functional capacities of the isolated hepatocytes may first seem to be contradictory. The BSP storage capacity of the isolated hepatocytes decreased with age, while the synthesis of albumin and protein increased. The finding that the metabolism of BSP decreased with age *in vivo* as well as *in vitro* may be explained by the fact that, with this functional test, the whole liver as well as the isolated hepatocytes are expected to function maximally.

If there is a decline in this function of the liver and that of the individual hepatocytes with age, this should be made evident by use of this test. This last argument does not necessarily apply to the measurement of the albumin and protein synthesis. For, hepatocytes in 3-month-old rats do not have to compensate for the possible infirmities of old age such as an increase in proteolytic activity or the occurrence of "altered" malfunctional proteins. Hence, hepatocytes in 3-month-old rats do not need to make use of their reserve capacity. Therefore, it may be supposed that hepatocytes isolated from 3-month-old rats, although incubated under optimal conditions, do not function maximally. However, hepatocytes in rats older than 24 months might have to compensate for increased protein degradation or an increase in the amount of "altered" malfunctional proteins. Consequently, the hepatocytes in rats older than 24 months are forced to draw upon their functional reserve capacity and therefore synthesize a greater amount of albumin and protein. Since the incubation conditions for the isolated hepatocytes were optimal, the *in vivo* induced increase in albumin and protein synthesis could be expressed by the hepatocytes isolated from rats older than 24 months.

Summarizing, it can be concluded that the functional capacity of the liver *in vivo* with respect to the metabolism of BSP decreases with age. This decrease is mainly due to an age-related decline in the functional capacity of the individual hepatocytes. The observed changes in albumin and protein synthesis may result from a decreased reserve capacity of the hepatocytes.

SUMMARY

The objective of this study was to determine whether changes in the functional capacity of an organ with age are caused by age-related changes in the function of the individual cells constituting that organ. Gerontological research using intact organs has mainly resulted up to now in a mass of descriptions of aging phenomena. From these data, one does not get a clear insight into the cellular basis of organ aging. This is partly due to the fact that the influence of complicating extracellular factors such as neurological, endocrinological and circulatory influences cannot be excluded in studies with intact organs. Experimental studies using homogenates or organelle suspensions prepared from organs of animals of different ages have also revealed many changes with age. Reintegration of these gerontological data into the *in vivo* situation is difficult. One of the problems is that homogenates or organelle suspensions are prepared from different cell populations constituting an organ. Another problem with these *in vitro* studies is the absence of various cytoplasmic components and of the genetic control mechanisms located in the nucleus. In this way, clarification of the cellular mechanisms of organ aging with the aid of these *in vitro* data is difficult.

A system based on the isolation of cells from organs of individuals of different ages has the advantages that extracellular influences can be excluded and that the data obtained can probably be used to explain the *in vivo* observed aging phenomena. By measuring the functional capacity of the isolated cells, it can be determined whether age-related changes in the functional capacity of an organ can be attributed to changes in the function of the cells constituting that organ. Organs consisting of long-lived postmitotic cells are supposed to play an important role in the aging process of the individual. To these organs belong brain and heart. Isolating cells from brain or heart in an intact state and measuring the functional capacity of possible isolated neurons or heart muscle cells is still difficult to achieve. Another group of long-lived cells are the reverting postmitotic cells. These cells can undergo mitotic activity if they receive an appropriate stimulus; under normal circumstances, they live as long as the individual. Liver parenchymal cells, also called hepatocytes, belong to the reverting postmitotic cells. The isolation of liver parenchymal cells and the measurement of functions of these isolated cells are possible. By isolating liver parenchymal cells from rats of different ages and measuring the functional capacity of these isolated cells, the aging process of cells which live as long as the animal can be studied.

A prerequisite for achieving the objective of this study was that the hepatocytes isolated from young and old rats should be viable, so that any observed changes in the functioning of cells isolated from

young and old rats would not be caused by artifacts due to the isolation procedure. The method used to isolate hepatocytes was a combination of modifications of already existing methods. In this modified method, the enzymes collagenase and hyaluronidase were used. The viability of the isolated hepatocytes was determined by the trypan blue exclusion test, by electron microscopical examination and by the oxygen consumption with or without the addition of different substrates. The determination of the exclusion of trypan blue by the isolated hepatocytes is a handy and quick test to determine the viability of the cells. It appeared that trypan blue stained cells had a damaged cell membrane. For a further evaluation of the structural intactness of the nonstained cells, electron microscopic studies are necessary. From data obtained with the viability criteria mentioned above, it was concluded that hepatocytes isolated from young as well as from old rats had an intact cell membrane, revealed similar ultrastructural features as those of parenchymal cells in the *in vivo* situation and had an intact energy supply mechanism.

The choice of the functional characteristics of the isolated hepatocytes was based mainly on the following considerations. Firstly, research on aging changes in the functional capacity of the intact liver in the rat had already been conducted in the Institute for Experimental Gerontology TNO by F.R. de Leeuw-Israel. She determined the influence of age on the functional capacity of the liver *in vivo* on the basis of two characteristics: the metabolism of bromsulfophthalein (BSP) and the concentration of albumin in the serum (De Leeuw-Israel, 1971). Secondly, the functions to be measured should be specific for the liver parenchymal cells. Consequently, as functional characteristics of the isolated hepatocytes to be studied, the storage of BSP and the synthesis of albumin were chosen. To determine whether possible age-related changes in the capacity of the isolated hepatocytes to synthesize albumin are representative for changes in the synthesis of all proteins, the influence of age on the synthesis of protein by isolated hepatocytes was also studied.

To measure the storage capacity for BSP in the correct manner, the mechanism by which the isolated hepatocyte takes up BSP was studied. It was observed that the rate of BSP uptake by isolated cells reached a plateau level with increasing concentrations of BSP in the incubation medium. It was concluded that the uptake of BSP took place by a carrier type of transport mechanism. Since the uptake of free BSP by the isolated hepatocytes took place against a concentration gradient and was temperature and energy dependent, it was concluded that the BSP is transported into the liver parenchymal cells by an active transport

system. The optimal incubation conditions for the BSP storage were also determined. In this way, the liver parenchymal cells must function maximally and any small age-related changes in the capacity of the hepatocytes to store BSP may be detected. Maximum storage of BSP by hepatocytes isolated from 3-month-old female WAG/Rij rats was obtained by incubating the hepatocytes with 30 nmol ^{35}S -BSP per ml medium for 15 min at 37°C under an atmosphere of 95 percent oxygen and 5 percent carbon dioxide. The influence of age on the removal of intravenously injected BSP had already been studied by De Leeuw-Israel (1971). She observed a decreased liver function with age as determined by means of the BSP retention test. The *in vivo* data of De Leeuw-Israel obtained with female RU rats could be reproduced with female WAG/Rij rats used in this study. Thereafter, hepatocytes were isolated from rats of different ages and the BSP storage capacity of these hepatocytes was measured under the above-mentioned optimal incubation conditions. It was observed that the storage capacity of the isolated hepatocytes for BSP decreased with age, especially during the first year of life. The observed age-related decrease in the capacity of the liver to remove injected BSP fully paralleled the age-related decrease in the storage capacity of the isolated hepatocytes.

The functional capacity of the isolated hepatocytes was also determined on the basis of albumin synthesis. The amount of albumin was measured by a radial immunodiffusion method. Optimum albumin synthesis was obtained by incubating the hepatocytes in cell concentrations up to 3×10^6 cells per ml in a modified Waymouth MB 752/1 medium with an oxygen tension of 30×10^3 Pa in the medium, a pH of 7.8 and a temperature of 37°C . Thereafter, the albumin synthesis by hepatocytes isolated from rats of different ages was determined. The capacity to synthesize albumin decreased between 3 and 24 months. Following that, a sharp increase was measured between 24 and 36 months of age.

The capacity of the isolated hepatocytes to synthesize protein was also determined. By incubating the hepatocytes in cell concentrations ranging from 0.25×10^6 and 4×10^6 cells.ml $^{-1}$ in a modified Waymouth MB 752/1 medium with 8 μmol of a mixture of unlabeled and ^{14}C -leucine per ml at a pH of 7.8 and a temperature of 37°C , optimum protein synthesis was determined. The synthesis of protein by hepatocytes isolated from rats of different ages was determined. A sharp increase in protein synthesis was measured between 24 and 36 months of age, which is in agreement with the data on the albumin synthesizing capacity.

The observed changes in the functional capacity of the individual hepatocytes between 3 and 36 months of age might be caused by shifts in the ploidy states of the hepatocytes in that period. However, the most

important shifts in the ploidy state of the hepatocytes in the female WAG/Rij rat occurred within the first 3 months of life.

From the above mentioned data on the BSP storage and the synthesis of albumin and protein by hepatocytes isolated from rats of different ages, it can be concluded that the individual cells constituting an organ show pronounced age-related changes. The conclusion can be drawn that observed changes in organ function with age can, at least partly, be explained by changes in the functional capacity of the cells constituting the organs.

With respect to the decrease in the BSP storage capacity of the isolated hepatocytes with age, the following may play a role. Firstly, this decrease may reflect the fact that the transfer of BSP across the parenchymal cell membrane is age-dependent. This possibility can be investigated in various ways. As mentioned above, the transport of BSP takes place by means of an active transport system. This conclusion was based, among others, on the observation that the rate of BSP uptake by the isolated hepatocytes was energy-dependent. A decrease in the amount of BSP stored by hepatocytes isolated from the older rats might be explained by the fact that the energy supply of cells from older rats is less efficient than that of cells from young rats. However, experiments performed in our Institute by Brouwer et al. (1977) revealed that the energy supply of isolated hepatocytes was independent of age. It is of importance to determine whether age-related changes in the transport system influences the storage of BSP by isolated hepatocytes in another way or whether the capacity of the hepatocytes to bind BSP intracellular to cytoplasmatic proteins changes with age.

The increase in albumin and protein synthesis by the individual hepatocytes in late age might be explained as a compensation by the liver for, firstly, increased excretion of protein via the urine or, secondly, increased proteolytic activity. The increase in protein synthesis by the hepatocytes isolated from rats older than 24 months could not be explained by changes in the amount of protein excreted via the urine. In our Institute, Knook and Sleyster (1976) determined whether the activity of cathepsin D, the main lysosomal proteolytic enzyme, increased after 24 months. Indeed, they found that the cathepsin D activity in isolated hepatocytes increased during that period. However, a problem with the interpretation of the correlation between the increase in albumin and protein synthesis and the increase in cathepsin D activity is that cathepsin D is supposed to mainly degrade foreign proteins. A third explanation might be that the liver must compensate for the occurrence of altered, malfunctional proteins in late age. This possibility will be studied in the future.

In the first instance, the observed aging phenomena of the isolated liver parenchymal cells seem to be contradictory. The storage capacity of the isolated hepatocytes for BSP decreased with age, while the synthesis of albumin and protein increased. The fact that the metabolism of BSP decreased with age *in vivo* as well as *in vitro* might be explained by the fact that, in the determination of this functional capacity, care was taken that the liver and the isolated hepatocytes functioned maximally. A decrease in this function of the liver or of the individual hepatocytes with age will be made evident by this functional test, since it is impossible for the cells to draw upon a reserve capacity during the testing. This last argument does not necessarily apply to the measurement of the functional capacity to synthesize albumin and protein. Hepatocytes from 3-month-old rats do not have to function maximally with respect to their protein synthesizing capacity, since they do not have to compensate for the possible infirmities of old age such as an increase in the proteolytic activity or the occurrence of "altered" malfunctional proteins. The hepatocytes in young animals have no need to make use of their reserve capacity. Therefore, it may be supposed that hepatocytes isolated from 3-month-old rats, although incubated under optimal conditions, do not function maximally. However, hepatocytes in rats older than 24 months might have to compensate for increased protein degradation or for an increase in "altered" malfunctional proteins. Consequently, hepatocytes in rats older than 24 months are forced to draw upon their reserve capacity and have to synthesize more protein and albumin. Since the incubation conditions were optimal, the isolated hepatocytes could express this *in vivo* induced increase in the albumin and protein synthesis. With this argument in mind, it is rational to assume that the functional capacity of the liver *in vivo* not only with respect to the metabolism of BSP but also with respect to the synthesis of albumin and protein may decrease with age. This decrease can be at least partly attributed to a decreased functional capacity of the individual hepatocytes with age.

SAMENVATTING

De doelstelling van het uitgevoerde onderzoek is de cellulaire basis voor het slechter functioneren van organen bij het ouder worden na te gaan. Verouderingsonderzoek aan intacte organen heeft tot nu toe voornamelijk geresulteerd in een scala van beschrijvingen van verouderingsverschijnselen. Aan de hand van deze gegevens heeft men echter nog geen inzicht verkregen in de cellulaire mechanismen van orgaanveroudering. Dit wordt mede veroorzaakt doordat de invloed van storende extracellulaire factoren, zoals neurologische, endocrinologische en circulatoire invloeden in studies met intacte organen niet uitgesloten kunnen worden. Experimentele studies waarbij gebruik gemaakt werd van homogenaten of van organelsuspensies bereid uit organen van dieren met verschillende leeftijden hebben eveneens vele veranderingen bij het ouder worden aangetoond. Het inpassen van deze verouderingsgegevens in de *in vivo* situatie stuit echter op vele problemen. Een van deze problemen is dat de homogenaten of de organelsuspensies bereid worden uit de verschillende celsoorten waaruit een orgaan is opgebouwd. Een ander probleem bij deze *in vitro* studies is de afwezigheid van verscheidene cytoplasmatische componenten en van de genetische in de kern gelocaliseerde, controlemechanismen. Hierdoor wordt de opheldering van de cellulaire mechanismen van orgaanveroudering met behulp van deze *in vitro* gegevens bemoeilijkt.

Een systeem, gebaseerd op het isoleren van cellen uit organen van individuen van verschillende leeftijden, biedt als voordelen dat extracellulaire invloeden uitgesloten kunnen worden en dat de verkregen resultaten waarschijnlijk gebruikt kunnen worden om *in vivo* waargenomen verouderingsfenomenen te verklaren. Door nu de functionele capaciteit van de geïsoleerde cellen te meten kan worden nagegaan of een aan de leeftijd gerelateerde verandering in de functionele capaciteit van een orgaan kan worden toegeschreven aan veranderingen in het functioneel vermogen van de cellen waaruit dat orgaan is opgebouwd. Organen die opgebouwd zijn uit langlevende postmitotische cellen worden verondersteld een belangrijke rol te spelen in het verouderingsproces van het individu. Tot deze organen behoren de hersenen en het hart. Het isoleren van cellen uit de hersenen en het hart in een intacte staat en het meten van de functionele capaciteit van eventueel geïsoleerde neuronen of hartspiercellen zijn moeilijk te verwezenlijken. Een andere groep van langlevende cellen zijn de "reverting" postmitotische cellen. Deze cellen zijn in staat om zich te delen nadat zij een geschikte prikkeling ontvangen hebben. Onder normale omstandigheden leven zij echter even lang als het individu zelf. Tot de "reverting" postmitotische cellen behoren de leverparenchymcellen, ook wel genoemd hepatocyten. De isolatie van leverparenchymcellen en het meten van functies van deze

geïsoleerde cellen zijn de laatste tijd mogelijk gebleken. Door leverparenchymcellen te isoleren uit ratten van verschillende leeftijden en de functionele capaciteit van deze geïsoleerde cellen te meten, kan het verouderingsproces van cellen die even lang leven als het dier zelf, worden bestudeerd.

Een eerste vereiste voor het welslagen van dit onderzoek is dat de hepatocyten geïsoleerd uit jonge zowel als oude ratten vitaal zijn, zodat eventueel gevonden verschillen in het functioneren van cellen geïsoleerd uit jonge en oude ratten niet kunnen worden veroorzaakt door artefacten als gevolg van de isolatieprocedure. De gebruikte methode om hepatocyten te isoleren was een combinatie van modificaties van reeds bestaande technieken. Bij deze gemodificeerde methode werd gebruik gemaakt van de enzymen collagenase en hyaluronidase. De vitaliteit van de geïsoleerde hepatocyten werd bepaald aan de hand van de trypaanblauw uitsluiting, van elektronenmicroscopische opnamen en van de zuurstofconsumptie al of niet in aanwezigheid van verschillende substraten. De uitsluiting van trypaanblauw is een snel werkende test voor het bepalen van de vitaliteit van de cellen. Het is gebleken dat trypaanblauw gekleurde cellen zeker een beschadigde celmembraan bezitten. Voor een verdere evaluatie van de structurele intactheid van de niet gekleurde cellen zijn echter elektronenmicroscopische studies noodzakelijk. Aan de hand van de resultaten verkregen met de drie hierboven genoemde vitaliteitscriteria kon worden geconcludeerd dat hepatocyten geïsoleerd uit jonge zowel als oude ratten een intact celmembraan hebben, een ultrastructuur vertonen die sterk op het *in vivo* beeld van de leverparenchymcel lijkt en kunnen bogen op een intacte energievoorziening.

De keuze van de te meten functionele eigenschappen van de geïsoleerde levercellen was voornamelijk gebaseerd op de volgende redenen. Ten eerste was er reeds verouderingsonderzoek aan de intacte rattelever in het Instituut voor Experimentele Gerontologie TNO uitgevoerd onder leiding van F.R. de Leeuw-Israel. Zij bepaalde *in vivo* de invloed van veroudering op het functionele vermogen van de lever aan de hand van twee parameters: de verwerking van broomsulfofthaleïne (BSP) en de concentratie van albumine in het serum (De Leeuw-Israel, 1971). Ten tweede zouden de te meten functies specifiek voor de leverparenchymcellen moeten zijn. Dientengevolge zijn als te bestuderen functionele eigenschappen van de geïsoleerde hepatocyten gekozen de stapeling van BSP en de synthese van albumine. Om na te gaan of eventuele leeftijdsafhankelijke veranderingen in de capaciteit van de geïsoleerde hepatocyten om albumine te synthetiseren representatief zijn voor veranderingen in de synthese van alle eiwitten is eveneens de invloed van de leeftijd op de synthese van eiwit door geïsoleerde hepatocyten bepaald.

Om de stapelingscapaciteit voor BSP op een juiste wijze te kunnen meten werd allereerst het mechanisme waarmee de geïsoleerde hepatocyt BSP opneemt bestudeerd. Gevonden werd dat de snelheid van BSP opname door geïsoleerde hepatocyten bij toenemende concentraties van BSP in het incubatiemedium een plateauwaarde bereikte. Hieruit kon worden geconcludeerd dat de BSP opname geschiedde door middel van een "carrier" transport. Daar de opname van vrij BSP door de geïsoleerde cellen bovendien plaatsvond tegen de concentratie gradient in en temperatuur en energie afhankelijk bleek te zijn, kon worden geconcludeerd dat de opname van BSP door de geïsoleerde hepatocyten plaatsvond door middel van een actief transport systeem. De optimale incubatie condities voor de BSP stapeling werden bepaald om er voor te zorgen dat de leverparenchymcellen maximaal zouden functioneren. Eventuele kleine met de leeftijd gerelateerde veranderingen in het vermogen van de hepatocyt om BSP te stapelen zouden op deze wijze toch kunnen worden waargenomen. Een maximale stapeling van BSP door hepatocyten geïsoleerd uit 3 maanden oude vrouwelijke WAG/Rij ratten kon worden verkregen door de hepatocyten gedurende 15 min te incuberen met 30 nmol ^{35}S -BSP per ml medium bij 37°C onder een atmosfeer van 95% O_2 en 5% CO_2 . Zoals hiervoor vermeld was de invloed van veroudering op de verwerking van intraveneus geïnjecteerd BSP door de lever reeds bestudeerd door De Leeuw-Israel (1971). Zij nam een aan de leeftijd gerelateerde achteruitgang in de leverfunctie met het ouder worden waar bepaald aan de hand van de BSP retentietest. De *in vivo* resultaten van De Leeuw-Israel verkregen met vrouwelijke RU ratten konden worden gereproduceerd met vrouwelijke WAG/Rij ratten, de ratten die voor dit onderzoek gebruikt zijn. Uit vrouwelijke ratten van verschillende leeftijden werden vervolgens hepatocyten geïsoleerd en de BSP stapelingscapaciteit van deze hepatocyten werd gemeten onder de hierboven vermelde optimale incubatiecondities. Waargenomen werd dat de stapelingscapaciteit van de geïsoleerde hepatocyten voor BSP afnam met het ouder worden, met name gedurende het eerste levensjaar. Opmerkelijk was dat de waargenomen leeftijdsafhankelijke achteruitgang in de capaciteit van de lever om geïnjecteerd BSP te verwijderen volledig parallel liep met de aan de leeftijd gerelateerde achteruitgang in de BSP stapelingscapaciteit van geïsoleerde hepatocyten.

De functionele capaciteit van de geïsoleerde hepatocyten werd eveneens bepaald aan de hand van de albumine synthese. De hoeveelheid albumine werd gemeten met behulp van een enkelvoudige radiaal immunodiffusie techniek. Optimale albumine synthese werd verkregen door de hepatocyten, in celconcentraties tot aan 3×10^6 cellen per ml, te incuberen in een gemodificeerd Waymouth MB 752/1 medium bij een zuurstofspanning in het medium van 30×10^3 Pa, een pH van 7,8 en een tempera-

tuur van 37°C. Vervolgens werd de albumine synthese van hepatocyten geïsoleerd uit ratten van verschillende leeftijden gemeten. De capaciteit om albumine te synthetiseren nam af tussen 3 en 24 maanden, om daarna sterk te stijgen tussen 24 en 36 maanden.

Zoals reeds vermeld werd ook de capaciteit van de geïsoleerde hepatocyten om eiwit te synthetiseren bepaald. Door de hepatocyten, in celconcentraties tussen 0.25×10^6 tot 4×10^6 cellen per ml, te incuberen in gemodificeerd Waymouth MB 752/1 medium met 8 μmol van een mengsel van ongelabeld en ^{14}C -gelabeld leucine per ml bij een pH van 7.8 en een temperatuur van 37°C, werd een optimale eiwit synthese gemeten. Voor de synthese van eiwit bepaald aan hepatocyten geïsoleerd uit ratten van verschillende leeftijden werd eveneens een sterke toename tussen 24 en 36 maanden waargenomen.

De waargenomen veranderingen in de functionele capaciteit van de individuele hepatocyten tussen 3 en 36 maanden zouden kunnen zijn veroorzaakt door verschuivingen in de ploïdiegraad van de hepatocyten gedurende deze periode. Gevonden is echter dat de belangrijkste verschuivingen in de ploïdiegraad van de hepatocyten in de WAG/Rij rat plaats vonden voor de leeftijd van 3 maanden.

Uit de hierboven vermelde resultaten over de BSP stapeling en de synthese van albumine en eiwit door hepatocyten geïsoleerd uit ratten van verschillende leeftijden, kan worden geconcludeerd dat de individuele cellen waaruit een orgaan is opgebouwd duidelijke leeftijdsafhankelijke veranderingen vertonen. Hieruit kan de conclusie worden getrokken dat waargenomen veranderingen in orgaanfuncties met het ouder worden, tenminste gedeeltelijk kunnen worden verklaard door veranderingen in de functionele capaciteit van de cellen waaruit de organen zijn opgebouwd.

Wat betreft de afname in de BSP stapelingscapaciteit van de geïsoleerde hepatocyten met het ouder worden kunnen de volgende oorzaken een rol spelen. Ten eerste zou deze afname kunnen worden veroorzaakt doordat het transport van BSP over het parenchymcelmembraan mogelijk leeftijdsafhankelijk is. Deze mogelijkheid kan op verscheidene manieren worden nagegaan. Zo is reeds vermeld dat het transport van BSP geschiedt door middel van een actief transport systeem, wat mede geconcludeerd werd uit de waarneming dat de opnamesnelheid van BSP door de geïsoleerde cellen energie afhankelijk was. Een afname in de hoeveelheid BSP gestapeld door hepatocyten geïsoleerd uit de oudere ratten, zou kunnen worden veroorzaakt doordat de energievoorziening van cellen uit oudere ratten minder efficiënt verloopt dan die van cellen uit jonge ratten. Experimenten uitgevoerd in ons Instituut door Brouwer et al. (1977) toonden echter aan dat de energievoorziening van geïsoleerde hepatocyten onafhankelijk van de leeftijd was. Of het transport systeem

op een andere wijze een aan de leeftijd gerelateerde invloed uitoefent op de BSP stapeling door geïsoleerde hepatocyten ofwel dat de capaciteit van de hepatocyt om BSP intracellulair te binden aan cytoplasmatische eiwitten verandert met het ouder worden, zou verder nagegaan moeten worden.

De toename in albumine- en eiwitsynthese op latere leeftijd door de individuele hepatocyten zou kunnen worden verklaard als een compensatie van de lever voor ten eerste, een toegenomen uitscheiding van eiwit via de urine of ten tweede, een toegenomen proteolytische activiteit. De toename in de eiwit synthese van de hepatocyten geïsoleerd uit ratten ouder dan 24 maanden kon niet worden verklaard door veranderingen in de hoeveelheid eiwit uitgescheiden via de urine. In ons Instituut is door Knook en Sleyster (1976) nagegaan of de activiteit van cathepsine D, een lysosomaal proteolytisch enzym, toenam na 24 maanden. Inderdaad werd gevonden dat de cathepsine D activiteit in geïsoleerde hepatocyten in die periode toenam. Een moeilijkheid bij de interpretatie van de correlatie tussen de toegenomen synthese van albumine en eiwit en de toegenomen cathepsine D activiteit is echter dat verondersteld wordt dat cathepsine D voornamelijk lichaamsvreemde eiwitten zou afbreken. Een derde verklaring zou kunnen zijn dat de lever op latere leeftijd moet compenseren voor het ontstaan van veranderde, slecht functionerende eiwitten. Deze mogelijkheid moet nog worden onderzocht.

De waargenomen verouderingsverschijnselen van de geïsoleerde leverparenchymcellen lijken in eerste instantie tegenstrijdig. De stapelingscapaciteit voor BSP van de geïsoleerde hepatocyten neemt af bij het ouder worden, terwijl de synthese van albumine en eiwit toeneemt. Dat de verwerking van BSP zowel *in vivo* als *in vitro* afneemt bij het ouder worden zou kunnen worden verklaard doordat bij de bepaling van deze functionele capaciteit ervoor gezorgd is dat zowel de lever als de geïsoleerde hepatocyten maximaal moeten functioneren. Neemt deze functie van de lever of van de individuele hepatocyten nu af met het ouder worden dan zal de afname door deze functionele test tot uiting komen, omdat er bij deze test geen aanspraak meer gemaakt kan worden op een aanwezige reserve capaciteit. Dit laatste hoeft echter niet het geval te zijn bij de meting van de functionele capaciteit om albumine en eiwit te synthetiseren. Immers, het is waarschijnlijk dat hepatocyten in 3 maanden oude dieren niet maximaal behoeven te functioneren wat betreft hun eiwit synthetiserend vermogen omdat zij nog niet behoeven te compenseren voor de eventuele gebreken van de oude dag zoals een mogelijke toename in proteolytische activiteit of het ontstaan van veranderde, slecht functionerende eiwitten. De hepatocyten in jonge dieren

behoeven hun reserve capaciteit nog niet aan te spreken. Verondersteld kan dan ook worden dat hepatocyten geïsoleerd uit 3 maanden oude ratten, hoewel geïncubeerd onder optimale condities, niet maximaal functioneren. Hepatocyten in ratten ouder dan 24 maanden zouden dan echter wel moeten compenseren voor een hogere eiwitafbraak of voor een toename van veranderde, slecht functionerende eiwitten. Dientengevolge worden deze hepatocyten *in vivo* gedwongen hun reserve capaciteit aan te spreken en moeten zij meer eiwitten en albumine synthetiseren. Daar de incubatie condities optimaal zijn zullen de geïsoleerde hepatocyten deze *in vivo* geïnduceerde toename in de albumine en eiwit synthese kunnen uiten. In feite zou dus de functionele capaciteit van de lever *in vivo* zowel wat betreft de verwerking van BSP als de albumine en eiwit synthese, verminderen met het ouder worden. Deze vermindering kan tenminste gedeeltelijk worden toegeschreven aan een afgenomen functionele capaciteit van de individuele hepatocyten.

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CURRICULUM VITAE

Geboren op 27 januari 1944 te Breda. In 1963 eindexamen HBS-B aan het Onze Lieve Vrouwe Lyceum te Breda. Van 1963 tot 1969 studie in de biologie aan de Katholieke Universiteit te Nijmegen. Doctoraal examen met hoofdvak Zoölogie bij Dr. A.M.Th. Beenakkers. Bijvakken: Farmacologie onder leiding van Prof.Dr. J. van Rossum en Chemische Cytologie onder leiding van Prof.Dr. Ch.A.M. Kuyper. Tijdens militaire dienst als ornitholoog gestationeerd op de vliegbasis Ypenburg, afdeling Wetenschappelijk Onderzoek. Sinds 1971 werkzaam als wetenschappelijk medewerker van het Instituut voor Experimentele Gerontologie van de Gezondheidsorganisatie TNO waar het onderzoek dat ten grondslag ligt aan dit proefschrift onder leiding van Dr. D.L. Knook werd uitgevoerd.