AGING CHANGES IN THE RAT LIVER

AN EXPERIMENTAL STUDY OF HEPATO-CELLULAR FUNCTION AND MORPHOLOGY

This work represents a thesis for a doctoral degree at the University of Leiden

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TABLE OF CONTENTS

CHAPTER I

INTRODUCTION

For ages, old people have been honoured by their younger contemporaries. The accumulated experiences of their long lives apparently created such great respect that even God was depicted as an old man. Despite such general reverence and this traditional interest for the spiritual accomplishments of the old, the aging process itself has only recently become a subject of systematic study. According to Heron and Chown (1967) this may be explained by the fact that even in the most technologically advanced countries it was not until the present century that considerable numbers of people lived long enough to grow very old. This opinion implies that large numbers of aged people would be the main requirement for the initiation of gerontological research. This view-point has been advanced by many other writers, but it is debatable since it has been shown in other fields of investigation that meaningful research can be accomplished using small numbers of subjects. In fact, the limited occurrence of certain phenomena may attract more attention and stimulate a curiosity. The main reason for the recent development in gerontological research rather seems to be the enormous increase in the proportion of aged people in modern society, which has created a number of completely new problems of both economical and medical significance. Another argument that has been frequently proposed to explain the lack of gerontological investigations, is that this type of research requires an advanced technology. However, this is not supported by the history of science, technology is not a condition for doing research, certainly not for descriptive work. There are numerous cases of beautiful descriptions of aging symptoms that were made long before the emergence of technology. Examples are found in Ecclesiastes 1–18 and Shakespeare's "As you like it". Such artists as Hans Holbein the younger and Delacroix have taken old people as their models. It is obvious that through the years the bodily appearance of old people has held considerable fascination. In the 16th century one turned to systematic observation of the human body during life and after death. During this time Vesalius showed that the human body was of enormous structural complexity. This new method of research required more advanced technological tools. The gradual development of technology facilitated the experimental exploration of phenomena in general. Concerning the scientific study of aging, it can be said that it has really started only in this century. In addition to the slow development of natural science, this can be also explained by a change in mentality towards the older people. Previously one concentrated on the gain of wisdom through experience; recently the focus of interest has been directed to the loss

of youth, capacity and adaptability and also to the disabling diseases which accompany aging. This emphasis of modern society on the disadvantages of old age has induced a demand for measures, effective in preventing or alleviating the aging process. Privately one tries to protect oneself by such means as cosmetics, clothes and various means of preserving one's independence. A scientific measure is the study of aging, from the biological and medical point of view.

Gerontology is defined in Dorland's Illustrated Medical Dictionary (24th edition, 1968) as the scientific study of the problems of aging: clinical, biological, historical and sociological. The present study is within the biological area. The objective of "biological gerontology" is to obtain biological information that can be used for the prevention of disadvantages of old age and for prolongation of the life span of human beings. If prolongation of the human life span would mean prolongation of a life of disability, this objective seems to be contradictory to medical ethics from the outset. Even though this opinion may be held by some individuals, prolongation of life span at any price, is not the primary concern of experimental gerontology. Furthermore, there is no real evidence that a prolongation of the life span necessarily leads to a prolongation of disabilities. It is not unreasonable to assume that the findings of experimental gerontology may lead to a prolongation of the status that exists in the middle years of the human life span. Any manipulation of the time span of human life would lead to problems that at the moment do not exist. If the extention of the human life span should become a reality, it does not necessarily follow that the methods should be universally employed. It would become a matter of choice, that is, on the part of both the physician and the aged or aging patient. At the moment the state of affairs has not advanced to the point where one has to make such a judgement. It cannot be predicted whether artificial prolongation of human life span will ever be a practical possibility. The most urgent task for the present seems to be, to prevent the disadvantages of old age. This will involve three types of efforts: 1) general care; 2) symptomatic relief; 3) control of specific causative factors. The first belongs to the area of geriatrics. The latter two objectives imply increasing our knowledge of aging and the causative factors involving its etiology. This is the concern of "biological gerontology".

So far, the approach in experimental gerontology has not been very systematical, which is probably due to the very limited efforts devoted to this field. In most investigations described in gerontological literature, parameters of a group of old individuals are compared with those of a group of young individuals. In this way many facts are collected, but it is difficult to bring them together in one system. For the latter purpose, it will be helpful if one can arrange conveniently what we want to know about the aging process. It is clear that in the present stage the formulation of the problems has not been completed. One way to approach the problem of aging might be to follow the scheme that

has been found useful in clinical pathology with etiology, pathogenesis, morbid anatomy, symptoms and signs; treatment and prophylaxis. By the pathogenesis of aging is meant the development of the condition after the process has started. Comfort (1968) alludes to this when he declares in a review that "gerontologists are developmental physiologists who deal with the end of a process, namely senescence". He states further that "age processes are unique in physiology, both at the somatic and cellular level in representing increasing instability". Many of these processes start in early adult life or even in adolescence; hearing loss or loss of visual acuity are two of the numerous examples of this. The question as to whether the aging process is or is not an illness, will no be discussed in this context.

There are many theories as to the cause of aging. This is so because investigators have tended to base their hypothesis on their own areas of investigation. The multiplicity of theories indicates that there are still fundamental mysteries to be resolved. For purposes of discussion the possible causes of aging can be classified as endogenous or exogenous in origin. Exogenous causes (factors from the environment) influencing the organism are for example irradiation, pollution of the environment and the composition of food. Endogenous causes are those that arise within the organism itself, from genetically determined mechanisms.

Although this investigation was not designed to prove a special theory, for the sake of fixing one's thought, it was based upon the genetical theory. This theory holds that the cells constituting an organism are programmed by information carried in their genes (Clark, 1964). This programme leads to division and differentiation, as well as to proper function of cell aggregates throughout later life. Since the programme is finite, sooner or later the information is turned off, leading to cellular death (Thung and Hollander, 1967). If the cells are not replaced by mitosis, a decrease in the total number of cells will ensue. It is true that in many organs dying cells are replaced by mitosis. This process itself can also be seen as governed by a time fixed programme at the organ level. Even tissues with continuous cell renewal show aberrations when they grow older. One manifestation of this would be a decrease in mitotic renewal, leading to a decrease in the number of cells, which may be reflected in a decrease in function of that organ. Aging changes may thus be ascribed to exhaustion of genetic programmes (Comfort, 1964), and it has been suggested that each organ is endowed with the possibility of developing its own mechanism and pattern of aging changes (Thung and Hollander, 1967).

One way to understand such a complex system is to find a model that will exhibit similar properties, but is more easily manipulated and to draw analogies from observations on such a model. A model can be misleading if the analogies are stretched too far, but properly used it can help to clarify thought. In that manner theories worked out in an animal model can be applied towards a

better understanding of human aging. The "common denominator in this equation" is the finiteness of life. This does not imply that death has the same cause in different organisms. We do assume, however, that what leads to death in old age, is based on similar principles in animal and in man.

It is preferable to choose experimental animals with a short life span for a gerontological model system. This assures that the aging process that leads to death is faster in the animal than in the researcher and his collaborators, so that the observations imitate, as it were, the procedure of a time-lapse-recording and the conduct of a "long-term" investigation during the life span of the animal becomes feasible with regard to the number of productive years of one researcher. In this connection, sometimes the concept of "biological time" is used, which is related to the concept of "physiological time". Du Nouy (1936) introduced the "physiological time" concept. As an example: "a certain unit of physiological repair work" costs more time in an adult than in a child, therefore it seems that "everything" in an adult takes longer than in a child, in other words, that for a child time goes faster than for an adult. By analogy, "biological time" means that for animals with a short life span, the life processes proceed more quickly than for animals with a long life span. Otherwise formulated, in animals with a shorter life span more events occur in the course of one minute and it seems that the minutes are longer as compared to animals with a long life span. However, according to Comfort (1964) it is meaningless to replace exact time measurements with a vague notion. This is especially true if one wants to make comparisons. Moreover, it is questionable whether it is always true that "a certain unit of physiological repair work" takes more time in an animal with a longer life span than in one with a shorter life span.

In "The laboratory animal in gerontological research" the following criteria are listed that are of particular importance in selecting a species for long-term studies: "1) A well-defined life span. 2) Resistance to epidemics of diseases that cause high mortality or morbidity and that are not readily controlled. 3) Physiological systems and disease entities resembling those of man. 4) Dietary regimen or nutritional requirements resembling those of man. 5) A favourable karyotype and firm information on stem cell populations and immune complement systems. 6) Ease in housing and handling and economical maintenance" (Bustad et al., 1968). These criteria would lead, in the first place, to selection of mice. It is remarkable that mice are not the favourite animals used in experimental gerontology. In the same book Sulkin (1968) reports the results of an enquiry on the question which animals are used most often in gerontological research. The questionnaire was sent to 330 members of the biological and clinical sections of the American Gerontological Society. 110 members returned their questionnaires, hundred of them used rats. Although $^1/_3$ of the questionnaires is not enough to be sure of a conclusion, this result still reflects the situation that the majority of gerontological investigations is done on rats. It is

interesting to note that the investigations of Bustad et al. (1968) were not performed with rats, but with miniature swine.

The present study was done with rats, because part of the estimations appeared to be more feasible in this species than in the mouse. In these animals the influence of aging on the liver was investigated. The liver was chosen for a number of reasons. Data from the literature, indicating that morphological changes take place in the liver during aging, were confirmed by previous studies in this group, using mice (Hollander and Thung, 1966). An obvious next question was, whether such morphological changes should cause functional changes in the liver. In principle, it is possible to quantitate the functioning of the liver using the methods of clinical chemistry. Possibly, functional changes would occur in such a way that they could serve as a parameter for expressing aging of the liver quantitatively.

The theory used as a starting point was that cell death, not compensated by continuous mitosis, would lead to a decrease in the number of cells and this in turn to a decrease in functional capacity. Considering the low mitotic activity found in the liver of normal man, rat and mouse, we may indeed expect to find a decrease in the number of cells and a decrease in liver function during aging.

What has been stated so far may also apply to other organs, for example the kidney, as a general description of the aging process. Experimentally this question may be approached by artificially decreasing the number of cells and observing whether this leads to an earlier appearance or to an acceleration of functional aging changes. For this experiment, the liver has technical advantages over other organs. It is known, for example, that in rats as well as in mice, after a partial hepatectomy (an operation in which two third of the liver is removed) the liver regenerates to its original weight within a few weeks (Chapter II, 5 Partial hepatectomy and aging). However, the histological picture does not return to the original state, but becomes similar to that of an older animal (this will be discussed more fully below). During this period of regeneration extra work will be required from the liver cells, because a smaller number of cells must answer normal metabolic requirements. Moreover, these cells are involved in synthetic activities aimed at regenerative growth. This functional overloading may represent an experimental intensification of the events which already occur in everyday life. Responses to the ever changing demands of daily functional life always involve minute local lesions. Experimental liver regeneration may be said to represent an acceleration and exaggeration of normal life processes. Because of the fact that the liver remnant carries about three times its normal load of work, "its programme" could be expected to run out sooner. If the replacement of lost cells is considered as part of the genetic programming, this activity is also accelerated, with the possible effect that the programme runs out sooner than normally. In this way accelerated aging of

the liver could be induced. The functional overloading of the liver is superimposed upon the normal aging process which in the rat, with its shorter life span, is already faster than in man. The "accelerated aging" that results from all this can possibly like "normal aging" be expressed quantitatively. Theoretically, it was expected that there would be a more rapid or more profound decrease in liver function with time in partially hepatectomized rats. It was postulated that untreated rats and rats with a liver regenerated after a partial hepatectomy should form a suitable model for following functional and morphological changes during aging. This system would also allow an analysis of the pathogenesis of the aging processes in the liver and would be a test for the programmed exhaustion theory of aging (Thung and Hollander, 1967).

CHAPTER II

THE LIVER DURING AGING

(Data from the literature)

Although during the past years much factual material has been accumulated in the gerontological area, it seems remarkable that the liver somehow escaped attention; that is to say there is less literature on the liver during aging than on other certain organs. One might conclude from this that there are less striking changes in the liver during aging than in other organs.

1. Weight

The liver is one of the largest organs of the body. In man, it weighs 1200–1500 g and comprises two percent of the adult body weight (Sherlock, 1968). There are indications that the absolute liver weight decreases during aging (Bürger, 1954). As to relative liver weight, however, DeLand and North (1968) in a series of 625 autopsies of adult humans without evidence of liver diseases, found a linear relationship between liver weight and body weight. The data which Kuipers (1968) obtained from autopsies on deceased persons from the Municipal Old Peoples Home in Rotterdam are in contradiction with this. The admission here is on a social basis after it is shown in a medical examination that the candidate is in a good mental and bodily state. If an inhabitant of the Home becomes ill, he is treated by the family doctor. If a disease of a serious nature occurs he is admitted to a hospital and will, when deceased, not appear in autopsy records of the Home. To counterbalance this selection a control group of the same size was obtained by Kuipers (1968) from the archives of the Central Pathology Laboratory of the Municipal Hospitals in Rotterdam. For each deceased person from the Home an autopsied patient from the archives was chosen of the same age and sex, who had died on or about the same date as the inhabitant from the Home. Since the patients of the control group all died in a hospital, they also represent a selected group, which is, however, selected in a different way than the group from the Home. The groups each consisted of 151 men and 49 women with an age range of 65 to 95 years. The results of the investigation revealed that among the deceased from the Home there were 33 with a liver weight lower than 1000 g. The control group contained only 11 patients with a liver of less than 1000 g in weight. Apparently Kuipers considers that a liver weighing 1000 g or more is in the normal range, while those that weigh less than this are abnormal (he takes no account of the body weight – liver weight ratio). The reason why so many cases from the Home have a

low liver weight remains an open question, according to the author. He wonders if it is a sign of good health, because they got the chance to grow old "physiologically". However, the difference between the groups can also be explained on the basis of a different method of selection. Morphologically Kuipers found a brown atrophy in some of the livers. In brown atrophy which may affect any internal organ and especially heart and liver, there is an increase of the pigment lipofuscin. Brown atrophy is often described in connection with the aging process. According to Strehler (1964) the amount of pigment is proportional to age, at least in the human myocardium. However, this phenomenon is also seen in malnutrition and can therefore be associated with the pattern of feeding. Recently the increase in lipofuscin has been described as an enzyme deficiency (Editorial in The Lancet, 1970). Moreover, although the quantitative accumulation may be associated with the aging process, the occurrence of lipofucsin is not per se an aging phenomenon. Goldfischer and Bernstein (1969) even found lipofuscin as a constant component of newborn human liver. It is possible that the amount of this metabolite occurring in old age, depends upon previous life experiences. The five percent brown atrophy of Kuipers may be a peculiarity of his selected group rather than a characteristic of the general population. This rather extensive discussion of the work of Kuipers has been made in order to stress the difficulties one faces in attempting to draw conclusions from work done with human material.

These difficulties may be overcome by conducting an investigation on animals. Donaldson (1924), in his treatise on the rat reviewed the literature for organ weight up to that year. From the work of Jackson (1913) and Hatai (1913), he gives data for, among others, liver weight, listing organ weight in terms of body weight. It was shown that in rats the growth of the liver was initially very rapid and continued at a nearly uniform rate up to the phase of 400 to 500 g of body weight. Webster et al. (1947) mention the paper of Donaldson but do not find further reliable data in the literature on this subject. Webster et al. therefore determined organ-body weight ratios using 500 albino rats. No attempt was made to determine the age of the rat. However, tables exist which show the correlation between the age and the body weight of rats (Sýkora et al., 1965; Hradil et al., 1966). Webster et al. (1947) grouped their animals in weight classes. In male rats from 200 to 400 g and in female rats from 150 to 350 g the relationship between liver weight and body weight remained on the same level. The data of Addis and Gray (1950) do not agree with this. They find in a study of albino rats a log-log relationship between body weight and liver weight. However, these data have to be considered with reservations. One reason is that not for all body weight groups the number of rats per group is given. Another reason is that the weight of the male rats ranges from 20 to 400 g, while the weight of the female rats ranges from 30 to 169 g. Therefore the age groups are possibly not evenly distributed. Because in

this study we have worked with female rats, Webster's observation of a constant body weight – liver weight relationship between 150 to 350 g was most relevant.

2. Comparative anatomy

In view of the fact that the rat has been used as an experimental animal in this thesis, a few remarks about the comparative anatomy are appropriate. Elias and Sherrick (1969) published what they referred to as anatomical classifications of mammalian livers. This atlas reveals that besides many similarities, there are a few differences between the livers and biliary systems of rat and man. The major differences are: a) the rat liver consists of 5 lobes, while human liver is one mass; b) in man there are two branches of the portal vein, in the rat there is only one; c) the rat has no gall bladder or cystic duct. These macroscopic differences in structure are not accompanied by profound microscopic or functional differences as many studies using the rat have shown.

3. Histology

For a description of the structure of the liver in so far as is pertinent to these discussions, the reader is referred to the work of Elias (1948 a and b) and Elias and Sherrick (1969). After constructing a stereoscopic model of the liver Elias concluded that the former ideas on the arrangement of hepatocytes were apparently in error. Instead of the liver cells being arranged in two-cell thick cords, radiating from a central vein, they formed an irregular almost spongelike entity. This structure was tunneled by a communicating system of cavities, termed lacunae. The lacunae contain the blood capillaries of the liver, the sinusoids (fig. 1).

In sections of the liver the parenchymal cells dominate the field. In man they fill 60 percent of the liver volume (Sherlock, 1968). In the rat they comprise even more than 90 percent of the liver tissue (Harkness, 1957). Due to the two dimensional appearance of the cell plates, present in the plane of section, the cells seem to be arranged in single rows. Between them the sinusoids, sometimes filled with blood and bordered by the flat endothelial and phagocytic Kupffer cells can be seen. Depending upon the haemodynamic relations within the liver, the cell rows seem to radiate from the central veins, the smallest hepatic vein radicles, or from the portal triads. These consist of terminal branches from the portal vein, hepatic artery and bile duct and are surrounded by hepatic tissue. The liver cells are polyhedral, which makes them polygonal in sections. The cytoplasm is basophilic, both finely granulated and cloudy, containing vacuoles. Difficult to see in sections stained with haematoxilin and eosin, but readily detected in unstained sections are the brown or yellow coloured lipofuscin granules. A correlation between the accumulation of lipofuscin and age has been

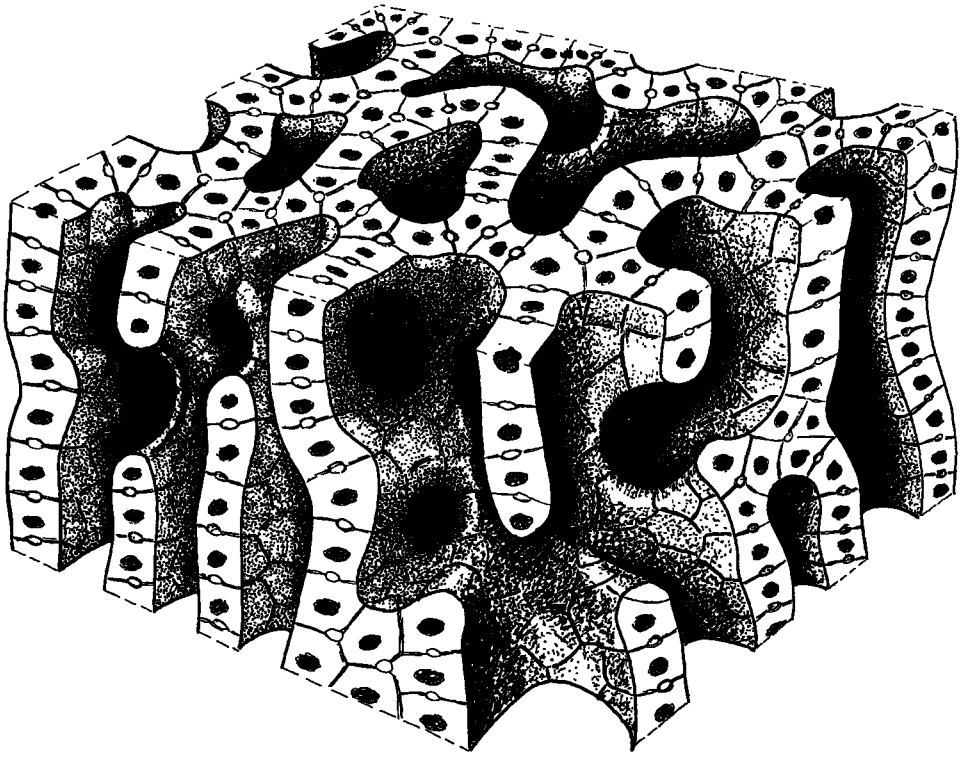

Fig. 1. Stereogram of a portion of the liver illustrating the arrangement of the hepatocytes and lacunae as described by Elias and Sherrick (1969).

established for man (Bachman, 1953; Strehler et al., 1959). As mentioned before, this correlation has been disputed by Goldfischer and Bernstein (1969). Harman (1956) has also shown that there is an increase of lipofuscin in the liver of the rat with advancing age.

Liver cell nuclei are acidophilic, round to oval, located centrally or near the surface of the cell. They have one or more prominent nucleoli. Many cells have two nuclei, but those seen are only part of the total binucleate liver cell population, since both nuclei have to be in the plane of section for recognizing binuclearity. The average number of hepatic nuclei per cubic millimeter of liver tissue is 267×10^3 for 4–6 weeks old rats, 135×10^3 for 4–8 months old rats and 138×10^3 for 21–30 months old rats (Bucher and Glinos, 1950). This change was accompanied by the occurrence of larger nuclei in older rats, which is ascribed to a change in ploidy. As discussed in the next paragraph, the predominantly diploid cell population at birth changes into a predominantly tetra- and octaploid cell population later in life. In view of these changes during life and their importance for our experiments, special attention must

be given to polyploidy. The mechanism by which these nuclei are formed is also of importance.

4. Polyploidy

The terms diploid, tetraploid and octaploid describe the number of chromosomes per nucleus of a somatic cell in proportion to the number of chromosomes in a germ cell of the same species. Mature germ cells have for each species a constant number of chromosomes, the haploid number. Most of the somatic cells have twice this set and are said to be diploid (double). Nuclei which have four times the haploid number are called tetraploid and nuclei with eight times the haploid number are called octaploid. A nucleus is said to be polyploid if it has more than two full sets of chromosomes.

4a. *Proofs of polyploidy*

Counting of chromosomes

The most direct way to demonstrate the presence of diploid and polyploid nuclei in the liver is by counting the chromosomes in liver cells. Chromosome analyses usually are made on dividing cells in the metaphase, when the chromatin is coiled up in microscopically visible threads. Unfortunately the mitotic index, the number of mitoses found in a stated number of cells, is low in the liver. Nadal and Zajdela (1966a) for instance, found a mitotic index of only $0.06\,^0/_{00}$ in livers of adult rats, most of the liver cells being in interphase. Mitoses are numerous when the liver is in a state of growth: after part of the liver is damaged or removed, or in embryonic liver. These livers can be used for the demonstration of cells of different ploidy in the liver but not to gain information about the frequency distribution of those cells in non-growing liver. In livers of 6–8 weeks old rats, regenerated after a partial hepatectomy, Fabrikant (1967) finds that the degree of polyploidy is increased. This implies that the frequency distribution of the polyploid classes of the replicating nuclei cannot be the same as that of the nuclei of the total cell population of the untreated liver. Post and Hoffman (1965) state that in untreated rats (ranging from one day to 36 months old) the diploid class is the major replicating fraction of the liver cell population. The tetraploids play a minor role. Carrière (1967) found in three months old untreated rats that the dividing cells were predominantly in the tetraploid state with some in the diploid and in the octaploid states. She attributes the discrepancy in the findings to the use of different rat strains. In any event, in both studies one major replicating group of cells is found. Carrière demonstrated that the size distribution of the replicating nuclei is the same as that of the nuclei of the total cell population. She showed this by injecting the rats with tritiated thymidine and measuring the number of la-

belled mitotic cells with autoradiography. A certain proportion of parenchymal liver cells incorporated tritiated thymidine into their DNA. And these were, therefore, presumed to be engaged in mitotic activity. Labelling indexes and nuclear size measurements were made. Twenty-eight hours after injection the diameter of the labelled nuclei showed a distribution similar to that of the total nuclear population. Both size distributions reflected the presence of a large population of tetraploid nuclei and a few diploid and octaploid nuclei. This means that the distribution of ploidy classes of replicating cells in the normal liver can theoretically be used as a parameter of the distribution of ploidy classes of the total liver.

Of course, the determination of the normal chromosome complement of a species is done in tissues that normally exhibit many mitoses such as the skin. In recent years much progress has been made in karyological techniques. This involves the use of tissue culture, colchicine pretreatment and a large number of modifications of the classic squash technique for the differentiation of cells.

Using these methods, chromosomes can be much more clearly demonstrated than by the classic tissue section or squash methods. Moreover, the squash method is liable to technical error. The breaking of metaphase plates into several groups of chromosomes, which are subsequently considered as complete separate plates may give a false impression of aneuploidy. Consequently, what was known about the chromosome complement of most mammals had to be reconsidered. An example is the revision made by Tjio and Levan (1956a) that the human diploid number was not 48, as was assumed, but 46. Tjio and Levan (1956b) demonstrated that the diploid rat complement consists of 42 chromosomes and Levan et al. (1962) found the diploid mouse number to be 40. These values were confirmed by Hsu and Benirschke (1967) who collected an atlas of karyotypes (chromosomal groupings) of different species. However, these karyotypes were not prepared from the liver and above numbers are mentioned here only as a reference.

Hsu and Pomerat (1953) found in cultures of liver cells derived from mouse embryos more than 98 percent diploid nuclei with 40 chromosomes. They also found tetraploid nuclei with 80 chromosomes and several aneuploid nuclei. This latter value could be due to the method employed; normally deviations in chromosome numbers occur in a proportion of somatic cells. Gläss (1957) and Marquardt and Gläss (1957) examined livers of untreated rats aged 12 hours and 6 days. They found that diploid nuclei with 42 chromosomes predominated. In livers of 6 months old rats, after a partial hepatectomy, they found most of the cells to be diploid with 42 chromosomes; some were tetraploid with 84 chromosomes and a few octaploid. In addition, they observed haploid, triploid, pentaploid, hexaploid and also aneuploid nuclei. The latter are most likely artefacts caused by the squash method used. Honda (1964), who counted chromosomes in liver cells sampled by the squash and by the air-dry method

found that most of the nuclei had the normal diploid number of the rat and almost all the others were tetraploid. In this material less than 0.1% aneuploid nuclei were scored. These cells were derived from partially hepatectomized rats. To sum up: literature does not allow a conclusion concerning the existence of polyploid nuclei in untreated non-growing liver, although there are certain indications for their occurrence.

Determination of amount of DNA per cell

There is however indirect evidence available. DNA (desoxyribonucleic acid) is the most important gene material. The DNA of the individual nuclei can be determined by quantitative Feulgen microspectrophotometry. This involves staining the section by Feulgen technique, which is specific for DNA (Pearse, 1960). After this, the proportion of light absorbed, when monochromatic light is passed through the stained nuclei is determined. This provides a measure of the amount of DNA per nucleus. If precautions are taken to ensure maximal dye binding, to match the refractive index of cytoplasm and medium and to reduce stray light, this is a very accurate method for the estimation of the relative number of chromosomes (James, 1965). In 1948 Boivin et al. stated that the amount of DNA in nuclei of different tissues was constant for the same animal and twice that of sperm. This was partly contradicted by Mirsky and Ris (1949) who measured the DNA content in germ cells and various somatic cells and found that in a given organism the DNA content of diploid cells was indeed constant but not twice that of a germ cell. Pasteels and Lisson (1950) found a relationship between the DNA content of diploid nuclei and germ cells that was once more different. Leuchtenberger et al. (1951), however, were able to support the statement of Boivin with evidence. They determined the DNA content of nuclei in preparations of the same organs by two different methods and found similar results. They also found in beef liver nuclei an amount of DNA which was twice that of sperm. In rat liver nuclei three different classes with values of DNA per nucleus representing exact multiples of each other were found.

Since then it has been accepted that all somatic cells contain an amount of DNA depending, in a given species, on the number of chromosomes present. Discrepancies in the standard amount of DNA found in haploid cells are accepted to be due to variation in chromosome number, reduplication of strands of chromatin without separation into distinct daughter chromosomes (polyteny) or synthesis of DNA in preparation for division (Richards et al., 1956). Otherwise the constancy hypothesis is generally accepted, which means that the amount of DNA per nucleus can be used as a parameter for ploidy. Stich (1960) confirmed that the DNA content of adult rat liver cells fell into diploid, tetraploid and octaploid classes. Alfert (1950) demonstrated the existence of multiple DNA classes in human livers.

Nuclear volume

A connection between nuclear volume and ploidy was known for years. In fact this was how one came to know about polyploidy. This started with the publication of Jacobj (1925), who demonstrated that the nuclear volumes in the livers of rats had a multimodal frequency distribution. This distribution indicates that the nuclei were classifiable according to volume, the relative nuclear size in these classes comparing as 1 : 2 : 4 : 8. This ratio was checked by many authors, among others by Clara (1930), who measured human liver nuclei and found that the frequency distribution formed a three-modal curve. The most frequent nuclear sizes compared as 1 : 2 : 4. Helweg-Larsen (1952) confirmed these values for the mouse liver.

Cellular volume

It is more or less taken for granted in the literature that increase in size of nuclei and of cells, within one tissue is directly correlated with an increase in ploidy. Conclusive information came in 1967 from Epstein, who used isolated liver cells from humans, rats and mice and demonstrated that cell size was directly proportional to ploidy. Ploidy was expressed both in DNA concentration and in nuclear volume. Meek and Harbison (1967) confirmed the proportionality of nuclear area and DNA content for human liver cells. Concluding, one might say that polyploidy has been convincingly demonstrated in livers of man, mouse and rat by cytophotometric measurement of Feulgen stained preparations as well as by nuclear volume measurements and by cell size measurements.

4b. *Ploidy at different ages*

The respective nuclear classes are not constant but vary during life. Swartz (1956) determined microspectrophotometrically the DNA content in liver nuclei of 37 human beings ranging in age from 2 weeks to 90 years. He found that up to the age of about 6 years, the liver contains only diploid nuclei. During the years 11–14 a definite tetraploid class appears and about the age of 20 an octaploid class is established. All three DNA classes co-exist in the liver until death. These data are confirmed by Doljanski (1960) and by Meek and Harbison (1967). Swartz points out that the tetraploid class is well established near the beginning of the most active phase of growth of the liver. Because there is no evidence of mitotic growth at this time, it seems justifiable to conclude that increase in cell size through the formation of tetraploid cells plays at least some role in the growth of the liver. However, at about the 20th year the growth curve shows an abrupt drop in rate and it is at this time that the octaploid cells make their appearance. The significance of the appearance of a new poly-

ploid class at a time usually associated with the cessation of growth presents a difficult problem.

In the rat also, the liver cell population changes from diploid at birth to polyploid later in life. Five cytogenetic types of cells develop after birth. These are mononuclear diploids, tetraploids and octaploids and binuclear diploids and tetraploids. The age at which the different classes become established probably depends on the strain of rat studied. Most of the differentiation takes place in the second to fifth week, according to Alfert and Geschwindt (1958). Nadal and Zajdela (1966a) found in one month old rats diploid mononuclear and binuclear cells. James et al. (1966) demonstrated mononuclear diploids and tetraploids and binuclear diploids in 8 weeks old rats. Distribution curves of older animals are scanty in the literature. One report is that of Post and Hoffmann (1965) who discussed replication of liver cells in rats ranging from one day to 36 months of age. Their investigations showed a predominantly tetraploid cell population. This picture was already evident at 8 weeks and remained nearly constant thereafter.

Meinders-Groeneveld (1969) compared the five cell types. She determined cytophotometrically total protein, RNA, acid phosphatase and glycogen concentrations in cells isolated from young adult rat livers. She found that in the mononuclear cells the transition from diploid to tetraploid entails more increase in the substances mentioned above than a mere duplication while the effects of the transition from tetraploid to octaploid fell far short of duplication. When observations were extended to include binuclear cells, the mononuclear tetraploid cells still showed the highest values per haploid complement for all parameters examined. The mononuclear tetraploid cell seemed to her a "successful creation". Possibly the appearance of octaploid cells is to be considered as something detrimental, especially in view of Swartz' (1956) findings that octaploids appear when liver growth stops. Another comparison of rat liver cells was made by Falzone et al. (1959) who compared mean DNA content per nucleus, mean volume of hepatic nuclei and frequency of "giant" cell nuclei from 12–14 months old rats with those from 24–27 months old rats. They found no age or sex differences. Enesco (1967) reported that there was no significant difference in the DNA content of diploid and octaploid liver nuclei from 3 and from 27 months old rats. One concludes that there is no loss of DNA with aging, in cells of the same ploidy.

In the mouse the population is also diploid at birth. Coincident with the time of weaning binuclear diploid nuclei appear. In animals weighing over 26 g there is an increase in the proportions of the higher ploidy classes (Epstein, 1967), but because he does not mention the age of the animals, this information is of little value.

The evolution of the liver cell population in man, rat and mouse seems to proceed along the same lines. This process can be considered as the expression

of a genetic programme. Whether the genes act at a specific time for each animal and whether there is a final characteristic proportion of each cell type in the population, remains a matter of speculation. A comparative study of liver cell populations of different mammals, at different points in their life should clarify the situation. From these, and other studies, it is apparent that man and the rat are comparable in at least one respect. Each starts life with a liver cell population that is largely diploid and this shifts to a predominately polyploid situation later in life.

4c. *Mechanism of formation of polyploid cells and the role of growth hormone*

Many mechanisms have been suggested to elucidate how tetraploid and octaploid nuclei are formed. Most of the explanations associate this doubling of the genetic material with the reduplication of this material in normal cell mitosis. They postulate that a normal cell reproduction cycle is started but not completed. Wilson and Leduc (1948), for example, state that a progressive suppression of the mitotic processes takes place. It starts with an incapability to divide the cytoplasmic material, which would result in the formation of binuclear diploid cells. In the next stage prophase proceeds to telophase, without movement of chromosomes to poles or division of nuclear or cellular materials interposed. This leads to a process called endomitosis, which is a redoubling of coiled condensed chromosomes without breakdown of the nuclear membrane. Finally, replication of chromatin strands, without chromosome formation occurs. These latter two stages result in formation of mononuclear tetraploid cells. Wilson and Leduc based their theory on observations in mouse liver. However, none of the intermediate stages is reported to be seen in human liver (Swartz, 1956). This makes their occurrence improbable, although by no means impossible. It would suggest, however, that a process of replication of chromatin without strand formation accounts for the appearance of tetraploid cells in man. In this way, the theory deals nicely with the formation of mononuclear tetraploid cells. But to account for binuclear tetraploids, one has to assume that the mononuclear tetraploids regain the capacity to follow more of the mitotic cycle than their predecessors. Octaploid cells, however, will be formed along the same line as described above, starting from the tetraploids.

Another hypothesis, proposed by Nadal and Zajdela (1966 a) states that polyploid cells arise from binuclear cells by fusion of nuclear material. They demonstrated that in rat livers already containing binuclear cells, the appearance of polyploid cells could be easily induced by partial hepatectomy. The emergence of the polyploids was strictly correlated to the disappearance of binuclear cells. They found it impossible to induce the formation of polyploid cells in liver tissue which did not previously contain binuclear cells. They saw that during the mitosis of a binuclear cell, although both nuclei participate in the spindle formation, the cell never becomes tetrapolar but always becomes

bipolar. On this observations they based their explanation for the transformation of binuclear to polyploid cells. They hypothesized that "After a simultaneous prophase in both nuclei and the formation of only one spindle, the chromosomic groups fuse, giving a dipolar anaphase with a double chromosomic arrangement". Concluding one might say that the most acceptable explanation is that polyploid cells are formed by the fusion of genetic material of binuclear cells by a particular kind of mitosis. However, this pushes the question back to how binuclear cells are formed. The obvious answer is: by the division of the nucleus without the subsequent division of cytoplasmic materials. It seems therefore, that every explanation is based upon the concept of an unfinished cell division.

In normal cell division, the processes of growth and cell renewal are linked. By analogy, these processes are brought in relationship with polyploid cell formation. At the moment this inference does not bring us much closer to a solution. Our ignorance of the nature of the signals which control the cell cycle and induce, for instance, DNA synthesis or the structural changes at mitosis, is more or less complete (Cell Biology Correspondent of Nature, 1970).

If polyploidization is linked to growth processes, a need for growth hormone for the formation of polyploid cells is to be expected. In a mouse strain with an hereditary defect in the production of growth hormone (the so-called dwarf mouse) Helweg-Larsen (1952) found that the formation of nuclear classes was greatly reduced. Administration of growth hormone to mice of this strain was followed by development of nuclear class series and confirmation of this finding was provided by Swartz (1967) who showed that in 70 days old dwarf mice the liver cells were essentially diploid, while normal mice had a predominantly polyploid liver cell population. After 30 days there was a slight increase in polyploid classes in the dwarf mice while in the normal mice no further change had occurred. This means that the dwarf mice remained arrested. Swartz mentions a personal communication of Leuchtenberger et al. (1954) who demonstrated complete absence of different nuclear classes according to DNA content in the liver of a human adult pituitary dwarf. This establishes a relationship between hereditary hypoplasia of the anterior pituitary lobe and inhibition of nuclear classes formation. In rats, however, no strain with a hereditary lack of growth hormone is known. The situation is imitated by hypophysectomy. This checks polyploidization, according to DiStefano et al. (1955).

So far, the information is in accordance with the theory that growth hormone is needed for polyploid cell formation. Swartz later found that the tetraploid class increased at similar rate in hypophysectomized rats and untreated controls during an observation period of 6 months, starting at the age of $3\frac{1}{2}$ months. This contrasts with the findings of DiStefano, who used younger animals, and followed them for only one week. It also contradicts the findings of Nadal and Zajdela (1966b) who hypophysectomized $1\frac{1}{2}$ and 5 months old rats and found that the polyploid classes remained for 3 months at the same level as was ob-

served at the time of operation. Swartz does not explain this discrepancy, although he mentions the work of Nadal and Zajdela. It is important to note that an increase in polyploid cells in hypophysectomized rats is not in accord with the hypothesis that growth hormone is needed for polyploid cell formation. Moreover, Geschwindt et al. (1958) reported that large numbers of polyploid liver nuclei were observed in hypophysectomized and partially hepatectomized rats, so that the dependence of polyploidy on the pituitary cannot be complete. Swartz (1967), in an attempt to solve this problem, made it in fact a little more intricate. He determined percentages of polyploid nuclei in hereditary pituitary dwarf mice and in hypophysectomized rats, both with intact livers and after a partial hepatectomy. From the work of James et al. (1966) it is known that in normal 8 weeks old rats there is a strong increase in polyploid cells up to eight weeks after the operation. This shifts back in the direction of a normal pattern during the post-operative weeks, but does not reach the previous level consistent with the age of the animal. Swartz found that the percentage of polyploid nuclei in hypophysectomized rats, 53 days after a partial hepatectomy, was at the same level as observed at the time of the hepatectomy and lower than that of hypophysectomized, non-hepatectomized controls. Nadal and Zajdela (1966b), however, performed a partial hepatectomy in 4–6 months old rats three weeks after a hypophysectomy. They observed the liver for 5 days and found that hypophysectomy does not prevent the formation of large numbers of polyploid cells. The confusion is not restricted to the rat. In 100 days old dwarf mice Swartz found 30 days after a partial hepatectomy, an "excess" polyploidization. These conflicting results indicate, that the hypothesis that growth hormone is absolutely necessary for polyploid cell formation cannot be held. One may agree with the conclusion of Swartz that the pituitary regulates the general level of polyploidization by its control over the growth of the body as a whole. Contradictory results may be due to different control mechanisms operating on normally growing and regenerating livers. The final interpretation of the mechanism of polyploidy of the liver must await clarification.

5. Partial hepatectomy and aging

In 1931 Higgins and Anderson showed that extirpation of two third of the liver of rats is followed within 24 hours by cellular proliferation and in approximately two weeks by complete restoration of the original liver weight. Since then, the regenerating liver has been extensively studied and a considerable amount of data has been published. A major portion of this literature has been reviewed, among others by Harkness (1957), Weinbren (1959) and by Bucher (1963). Most authors pay attention to the first days or weeks after operation. They notice that, with respect to dry weight (Harkness, 1957), production of bile

(Weinbren, 1959) and many biochemical aspects (Bucher, 1963) the regenerated liver approximates the normal liver after two to four weeks.

A few hours after a partial hepatectomy, morphological changes are similar to those observed during acute carbon tetrachloride poisoning. Hyaline and lipid droplets appear in the liver cells, while the glycogen content falls (Thung, 1965). In partially hepatectomized animals this picture reflects the inadequacy of the liver remnant to meet normal demands. In poisoned animals this can also be explained by abnormal functional demands. Marked changes in metabolism develop after partial hepatectomy in the reduced liver mass, even before the occurrence of mitotic activity. There is an increased supply of free fatty acids to the liver with simultaneously an increased utilization of these substances. This shift in the metabolism which probably provides the necessary energy for the restoration of the liver tissue, underlines the condition of an increased functional strain (Šimek et al., 1967).

James et al. (1966) noted that shortly after a partial hepatectomy in 7 to 8 weeks old male rats, the distribution of cell types showed some similarities to the pattern of normal rats with an age of one year. Two months after operation they noticed a certain tendency to normalization of the ploidy pattern. However, the picture remained that of a much older animal. Post et al. (1960) and Bucher (1963) mention analogous changes to an "older" population in rat liver after regeneration. Moreover, Hollander and Thung (1966) found that the adult mouse liver after partial hepatectomy showed peculiar nuclear inclusions which were also observed in livers of senile untreated mice. These inclusions were originally observed by Andrew et al. (1943) in senile mice and in senile human liver. These analogies between the picture after partial hepatectomy and hepatic aging lead to the hypothesis that there are similar causative factors. It is thought that the acute inadequacy of the reduced liver to respond to normal functional demands may be compared with an inadequacy of the intact liver to meet normal functional demands throughout life. "The vicissitudes of daily life entail an unremitting stream of minute lesions which may be compared with prolonged application of some minimum level toxicity. Although the ensuing cell decay is in principle compensated by mitosis, it seems that the capacity of the liver to revert to the mitotic state is limited." (Thung, 1965). Following this line of thought one may conclude that in regenerating liver the aging processes are precipitated by acceleration of the normal processes. This should mean that a regenerated liver could be considered as a precipitously aged liver and used as a model for aging studies.

6. Liver function and aging

6a. *General aspects*

Hundreds of functions have been shown to be performed by the liver. Usually

they are classified under the headings: bilirubin formation, foreign substance excretion, detoxification and synthesis; carbohydrate metabolism, protein metabolism and lipid metabolism. The many functions of the liver might even be exceeded by the number of methods that have been devised to test them. This does not mean that a large battery of tests is always necessary to arrive at a correct evaluation. On the contrary, too many different laboratory results lead rather to uncertainty than to a clear-cut "diagnosis".

Bertolini (1969) has stated: "Functional exploration of the senile liver is clinically useful also to determine whether or not the diagnostic and prognostic value attributed to the various tests of liver function in younger age groups is retained wholly or in part in liver pathology of the aged." He further comments in his extensive review "Gerontologic Metabolism" that the most striking feature of old age in the liver is not so much the overt failure of certain specific liver functions as the incapacity to meet emergency situations. Confirmation of this is seen in the work of Versnelder et al. (1968) in which it is shown that examinations of liver function in geriatric patients by the methods of clinical chemistry may give results that are of a pathologic nature. In this survey 20% of the patients above 65 years, showed abnormal liver functions such as hyperbilirubinemia. According to Fenster and Porter (1968) this is more likely to be due to extrahepatic obstruction, drug cholestasis, congestive hepatomegalia and cholangitis. This emphasizes the inadequacy of the liver to meet emergency situations, rather than a failure to function in normal circumstances. This means that there may be a fairly good functional activity which is nearing the limits of reserve. It explains why not many abnormal data are found in literature.

With all this in mind it is to be expected that data obtained from loading tests will be of considerable value. For example, in old subjects, abnormal results of several loading tests, among them loading tests with galactose and Rose Bengal have been reported (Rafsky and Newman, 1949).

In rats, there is evidence, some of it conflicting, that liver function declines with age. There are suggestions that liver regeneration is diminished in older animals (Bucher and Glinos, 1950). Reuber (1969) found that there is a decrease in capacity to handle carbon tetrachloride intoxication with age. Von Hahn and Fritz (1966) found that DNA, prepared from the liver of old rats shows different properties as compared to DNA prepared from livers of young rats. For example, DNA in the old rat liver shows an increase in thermal stability which is attributed to the presence and binding of certain proteins (histones). This binding is such that the energy required to separate the strands of the DNA helix is increased. There are also changes in the composition of the liver nucleoproteins which are age associated. Von Hahn (1970) interprets this as a sign of loss of adaptability and decreased capacity of the cell to maintain homeostasis. In contrast to this Beauchene et al. (1970) reviewing the

literature on enzyme activity in the liver, find no consistent changes. They find only evidence for an increased cathepsin activity. Of importance for this study is the work of Klimas (1968) who showed that age affects glucose metabolism. He found that rats over 6 months of age handled glucose less efficiently than younger rats. It was remarkable that from 6 months up to 34 months the ability of the liver to handle glucose did not change. Changes of liver haemodynamics with age in male rats were reported by Wiener and Rabinovici (1961). Actually they measured variations on the pattern of liver circulation in different weight groups. They found that hepatic blood flow per 100 g body weight decreased as the weight of the animal increased. But due to an increased intravascular space, the liver blood flow per gram liver remained unchanged up to a body weight of 300 g. In heavier animals all these values were lower. Portal vein pressures were not affected by variations in weight.

6b. *Albumin*

The albumin level of the serum was chosen to measure liver function. Albumin is one of the plasma proteins that is formed in the liver, along with fibrinogen and some of the alpha and beta globulins. Synthesis outside the liver is insignificant. (For extensive information see Schultze and Heremans, 1966). Because of this fact, changes in the serum proteins form the basis for many tests of hepatic function. A decrease in serum albumin is one of the characteristics of chronic hepatic disease. The serum albumin level is considered a reliable index of the severity in chronic liver disease (Davidsohn and Henry, 1969). In healthy aged people Woodford-Williams et al. (1964) found little difference in the serum albumin level of old people and young adults. This was confirmed by an investigation performed in the Netherlands (Postmus and De Wijn, 1961/63). According to Rafsky et al. (1952), however, the serum albumin level decreases, while that of the globulins increases in old people.

These conflicting data give no definite answer to the question as to whether there is a change in human serum albumin levels during aging. Since in all these investigations the same standard-methods were used, the differences must be caused by incomparability of the groups investigated. In rats, no data on serum albumin changes with age are found in the literature so far.

6c. *Bromsulphalein-tests*

Another useful method measuring liver function during aging utilizes bromsulphalein (BSP). BSP is an indicator dye (colourless at a pH below 7.4 and purple at a pH above 10.4) and a valuable test substance for liver function studies because it represents an activity. For this reason, it is preferable to many other tests, such as determination of transaminases, which are parameters of hepatocellular damage (Hollander et al., 1968). Moreover, it is considered to

be a very sensitive test of hepatocellular function (Zieve and Hill, 1955 a and b). In 1924 Rosenthal and White showed that intravenously injected bromsulphalein is removed from the blood stream almost exclusively by the liver and that the rate of disappearance of this substance is a measure of liver function. It has been widely used since that time. The test is ordinarily performed by injecting a standard dose intravenously and measuring the retention of bromsulphalein at some fixed time after injection. Although this procedure is rather simple, the metabolism of BSP is the result of several complicated processes. These processes include uptake by the liver, conjugation and storage in the liver and excretion of the dye. Since BSP is ultimately excreted with the bile and follows closely the metabolic pathway of bilirubin, some knowledge of bile formation and bilirubin metabolism is important for the understanding of BSP metabolism. Extensive information concerning these mechanisms is given by Hargreaves in his book "The liver and bile metabolism" (1968). This author defines bile as a highly intricate secretion of varying composition. A distinction should be made between the formation of bile and the secretion of substances into it. Brauer (1959) classified substances secreted into the bile, according to their biliary concentration as follows: a) substances with a blood/bile concentration of about 1.0; b) substances with a blood/bile concentration of 1 : 10 to 1 : 100 and c) substances with a concentration ratio of less than 1.0. Bromsulphalein as well as bilirubin are in class b, having a higher concentration in bile than in blood. In the gall bladder, bile is stored and concentrated. In rats, which have no gall bladder, bile flows directly from the ductus choledochus into the intestine. If specific gravity is taken as a parameter of concentration, rat bile is comparable to human liver bile, i.e. before concentration in the gall bladder (Altman and Dittmer, 1968).

Bilirubin is derived from the catabolism of hemoglobin and therefore formed in the reticulo-endothelial system, where red blood corpuscles are destroyed. It is transported via the plasma, as a complex bound to albumin. It passes into the liver cells, where it is conjugated as glucuronate. Conjugated bilirubin is excreted with the bile into the intestine (Hargreaves, 1968).

BSP, which is an exogenous substance, is handled by the liver in a similar manner. On injection into the bloodstream it combines with albumin. Hepatocellular extraction of BSP from the blood entails storage and/or secretion of the dye by the liver cells, after dissolution of the BSP-albumin complex, which has moved across the sinusoidal wall (Baker and Bradley, 1966). BSP is removed from its carrier albumin in the subendothelial space before entering the liver cells (Goresky, 1964). In man BSP is exclusively removed via the biliary pathway (Giges, 1951). In liver disease BSP may appear in urine or eventually in ascites fluid (Leevy et al., 1963). Its selective removal by the liver cells was studied by Cornelius et al. (1967). They found that hepatic cell membranes bind BSP to a high concentration. After entering the liver cells, BSP is conju-

gated with glutathione (Grodsky et al., 1959). In this way, it differs from bilirubin which is conjugated with glucuronic acid. In man some of the bromsulphalein is excreted into the bile in the unconjugated form. This is also in contrast with bilirubin, which has to be conjugated before it can enter the bile (Hargreaves, 1968). An enzyme from rat liver catalysing conjugation of BSP was partially purified by Booth et al. (1961) and identified by Combes and Stakelum (1961). Combes (1965), changing the level of the components of the hepatic BSP-glutathione system of rats by feeding them a protein-free diet, found that intrahepatic conjugation with BSP had an important effect on BSP excretion. Although conjugation did not appear to affect hepatic uptake directly, it did appear to affect the rate, at which BSP is transported from liver cells into bile, in that conjugated BSP was transported more rapidly. Boyland and Grover (1967), however, found evidence that conjugation is not the rate limiting step in hepatic dye clearance.

Administration of BSP can take place in one injection or by infusion. The first method is most often used. One measures the so-called *retention*, viz. the amount of BSP that remains in the plasma at a given time after injection. Serial determinations were introduced by MacDonald (1939). Plotting serum concentration against time, one gets a so-called *disappearance curve*. This curve usually shows an exponential elimination of the BSP from the blood. This means that if the curve is plotted semi-logarithmically, a straight line is obtained (Ingelfinger et al., 1948; Lavers et al., 1949).

Above a certain dose level, however, another type of curve is possible, the so-called *saturation curve*, which will be discussed underneath. In 1942 Wirts and Cantarow found that the excretion of BSP in bile continued for several hours after its disappearance from the plasma. They concluded that the dye is first taken up by the liver and then gradually excreted. Brauer and Pessotti (1949) studied the uptake of BSP by rat liver slices which were incubated in a bromsulphalein solution. They found that the total BSP uptake was dependent on the BSP concentration of the solution. Addition of albumin decreased the uptake. A most important finding was that with a second incubation a short time after a first one, the disappearance of BSP from the solution was less than the first time. Mendeloff et al. (1949) found an analogous phenomenon in vivo in man. They postulated that after a single intravenous injection of BSP, "saturation" of the liver compartment takes place. This was concluded from the fact that a second BSP injection, given shortly after a first one, resulted in a higher BSP retention in the plasma. They thought that the rate of uptake of subsequent doses is limited by the biliary excretion mechanism, after saturation of the liver. Birkenhäger (1956) found that this saturation phenomenon was more pronounced after administration of Probenicid. His explanation was that Probenicid retarded the excretion of BSP. Richards et al. (1959), among others, have shown that after a single intravenous injection of BSP a semi-logarithmic plot

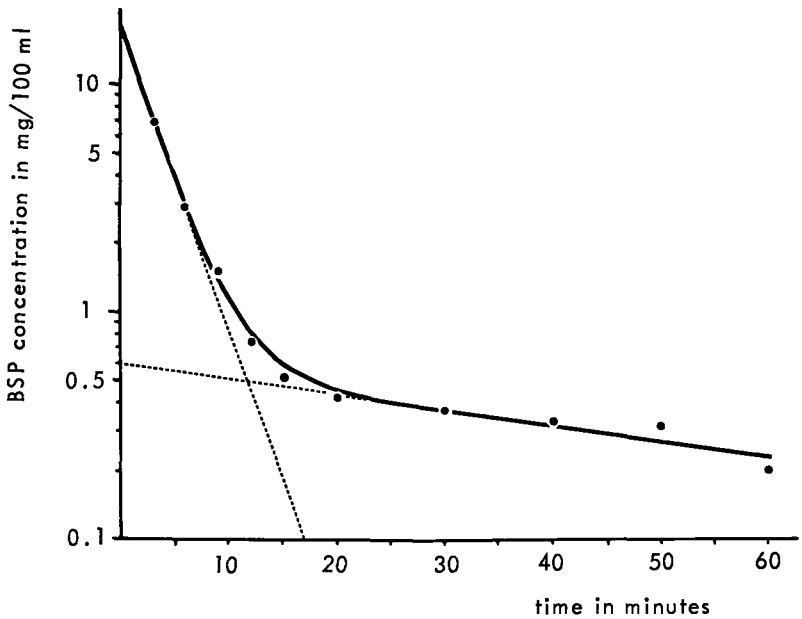

Fig. II. The change in plasma concentration of BSP with time, following a single injection of the dye to a dog (Taken from Richards et al., 1959).

of plasma concentration against time results in a broken line composed of two straight lines with different slopes (fig. II). The first of these lines is thought to be a reflection of hepatic uptake, the second of hepatic excretion. The point of change represents "saturation of the system". Tovey (1967) emphasizes the absolute necessity of giving sufficient bromsulphalein if one wants to load the liver fully. In this way it is possible to measure the "reserve capacity" of the liver, viz. its maximal excretory capacity, rather than a function under "normal" circumstances.

It is thought that the rate of uptake of bromsulphalein from the blood, once the saturation point has been reached, is limited by the maximal rate of BSP excretion. This has been defined by the term T_m, which term is, however, only used in infusion experiments. This maximum transport rate T_m for biliary excretion can be determined by the method of Wheeler et al. (1960 a and b), using mathematical analyses of rates of BSP removal during two intravenous infusions at different rates. In addition to T_m (the maximal excretion rate), the so-called *hepatic removal rate* can also be determined by this method. According to the method of Wheeler et al. (1960 a and b) the BSP is administered in a continuous infusion. During the infusion blood samples are taken from which the rate of change of plasma concentration ($\Delta p/\Delta t$) is determined. The amount of BSP removed from the blood per unit time is the hepatic removal

rate, designated as R. This is equal to the difference between the amount infused per unit time (I) and the amount with which the blood content actually changes (($PV\,\Delta p/\Delta t$) if PV = plasma volume). The complete equation then is $R = I - PV\,\Delta p/\Delta t$.

R, the amount removed per unit time from the blood, is equal to the amount that is excreted into the bile per unit time and the amount that is stored in the liver per unit time. This latter amount depends on the plasma concentration and is defined as $S\,\Delta p/\Delta t$, where S is called the *relative storage capacity*. This means that the removal rate can be expressed by a second formula: $R = T_m + S\,\Delta p/\Delta t$.

The infusion rate can be regulated and the plasma volume can be measured, for example with Evans blue.

The first formula, $R = I - PV\,\Delta p/\Delta t$, therefore, allows the calculation of R. Once R is known, the equation $R = T_m + S\,\Delta p/\Delta t$ still contains two unknowns T_m and S, and can be solved by substituting two sets of values for R and $\Delta p/\Delta t$, obtained during different BSP infusions. This technique is time consuming and tends to be used as a research procedure only.

Concerning the effect of age on BSP metabolism, Thompson and Williams (1965) who assessed liver function in human males ranging from 20–93 years, both by single injection and by infusion methods, showed above the age of 50 years an increasing proportion a reduction in storage capacity. Skaunic et al. (1968) also found that S decreases, beginning with the fifth decade.

7. Considerations for further study

The driving force in gerontology is the desire to prevent or diminish the disadvantages of old age. In order to do this, one has to have information concerning the cause or causes of aging as well as the processes occurring during aging. With respect to the cause of aging we quoted Comfort's hypothesis that somatic cells are genetically programmed for mitosis and differentiation and function for a certain period of time. When this time period has elapsed, the programme "runs out" and deterioration sets in. If insufficient replenishment of cells takes place, a decrease in the number of normal cells will lead to a decrease in function. The rat liver was chosen as a model of an aging organ for the present investigation. The reason for this choice was twofold: a) the rat has been much used in investigations of liver growth and regeneration and b) because this animal has a short life span when compared to man. The liver was also chosen because the events which occur in this tissue during regeneration are thought to be of importance for aging studies. Both after a toxic destruction of liver cells, or after a partial hepatectomy (excision of two third of the liver), the mitotic index increases rapidly. First of all, it should be of interest to know

if this regenerative capacity is maintained in old animals. Secondly, from Comfort's hypothesis, it might be expected that this regenerative activity leads to loss of "programme" and thereby to precocious aging changes. This was indeed suggested by the histological picture of regenerated livers, especially with regard to ploidy.

It has been shown that besides diploid, many tetraploid and octaploid cells may be found in the liver, because of a change from a diploid cell population at birth to a largely tetraploid cell population later in life. Since cell size is directly proportional to ploidy, an "older liver" with a largely polyploid cell population contains fewer cells per unit volume than a "younger liver" with a more diploid cell population. The formation of polyploid cells has been explained in several ways. However, every explanation is based upon the concept of an "aborted" cell division. The universality of the process indicates that it can be considered as the expression of a genetic programme or, if considered as a disadvantageous phenomenon, as the running out of a genetic programme. This would mean that by running out of a genetic programme, cell divisions are started but not completed, giving rise to the polyploid cells. The result is that the number of cells per unit volume decreases, leading to different and possibly deteriorated functional relations. It has been found that, after regenerative growth, the distribution of the cell ploidy in the liver is consistent with that of an older animal. Because of this phenomenon the partially excised liver was chosen as a model for studying the aging process. It was considered that this "accelerated aging" represents essentially what actually happens during normal life. The functioning of an organ in situ can, in principle, be quantified by methods of clinical chemistry. A decrease in the level of serum albumin is considered a reliable index of hepatic dysfunction. Another, and very sensitive method is the measurement of liver function by loading the liver with BSP. According to the literature, the liver function measured with BSP decreases, but only at a very advanced age. There seems to be no unanimous opinion concerning increase or decrease in the serum albumin level with age.

CHAPTER III

PROGRAMME FOR THE STUDY

1. Objectives

The purpose of this study was to investigate:
If there is an effect of aging on liver function.
If there is a relationship between morphological and functional changes in the liver during aging.
If the capacity to restore liver function after partial hepatectomy changes with age.

More specifically our interests were:

A. If there is a change in the results of the bromsulphalein test or in the albumin level of the serum with age.
   If there is indeed a change, further questions arise:
B. Can the nature of this change be specified?
C. Is this change accelerated by a partial hepatectomy?
D. Would the changes in BSP retention and albumin levels be the same in young hepatectomized rats as in older ones under the same conditions?
E. Is this change related to a shift in polyploidy?

2. Experimental design

For our experiments two strains of rats were employed. One of these was the $R_{Amsterdam}$ strain, which is a subline of the Wistar rat. It was introduced into the Netherlands Cancer Institute, Amsterdam, in 1947 and has since been maintained there as a pure line. Other rats used were $R \times U$ hybrids produced by mating female $R_{Amsterdam}$ and male U animals. The U animals originated at the Zootechnical Institute in Utrecht and have been maintained in the Netherlands Cancer Institute since 1958 as a pure line (brother–sister mating).

Chapters V (Serum albumin) and VI (Bromsulphalein) deal with investigations to answer question A which were carried out in two phases:

A. The first consisted of comparative observations on serum albumin and BSP excretion in old rats (30 months) and young rats (3 months).
B. In the second phase, these investigations were continued with the 3 months old group. The experiments were repeated at three months intervals until the rats were 24 or 27 months of age.

In chapter VII (Hepatic storage and maximal biliary excretion) an answer to question B was sought in a comparative investigation using rats of 3 and 24 months of age.

Chapters V (3 Serum albumin) (Gerontological experiments) and VIII (Partial hepatectomy) report on a "longitudinal" experiment, designed to answer the questions C and D. It consisted of 8 parts:

Partial hepatectomy was performed on groups of rats of the ages 3, 6, 9, 12, 15, 18, 21 and 24 months. Every experimental group was matched by a control group of the same sex and age. Liver function was monitored by BSP tests and determinations of serum albumin levels. These tests were initiated prior to the operation and continued throughout the experimentation period.

Chapter IX (Polyploidy) deals with an experiment designed to answer question E and consists of two parts:

1. A comparative investigation in which the degree of polyploidy of the liver cells of 3, 6, 12, 24 and 27 months old rats was determined.
2. An experimental determination of the degree of polyploidy in the liver cells of rats of the age of 27 months. The experiments were started with rats of the age of 3 months. One group was partially hepatectomized at the age of 3 months and allowed to live to the age of 27 months, at which time ploidy again was determined. The second group consisted of rats partially hepatectomized at the age of 24 months in which the degree of ploidy would be assessed at 27 months.

Chapter X (Autopsy findings) gives the results of the histological study of this material.

Furthermore, the plasma volume of old and young rats was determined (Chapter IV, Plasma volume). This was necessary in order to be able to interpret changes in the BSP tests or in the albumin level of the serum.

For details on methodology of BSP tests, plasma volume determinations etcetera, reference is made to the respective chapters.

CHAPTER IV

PLASMA VOLUME

Before starting the experiments it was important to know whether the plasma volume changes during the life of the rats. This was especially relevant, since BSP and protein values were to be expressed in g or mg per 100 ml serum.

Groups of 4 ♀ RU rats were used, aged 3, 6, 12 and 24 months respectively. The mean weight in each group was 210, 230, 215 and 270 g respectively. Plasma volumes were determined by the dye dilution technique (De Leeuw-Israel et al., 1969). The dye used was Evans blue (T 1824). This dye binds to serum albumin (Samson Wright, 1965) and, therefore, leaves the blood vessels very slowly. A known amount of dye (0.3 ml of a 5000 mg per 100 ml solution) in saline was injected into a tail vein. At 5 and 10 minutes following injection, 0.1 ml of blood was obtained by cutting the tail and at 15 minutes 0.1 ml was obtained by heart puncture. Plasma concentrations of Evans blue were determined by use of the Beckman spectro-colorimeter (Ultramicro analytical system) at 600 mμ after dilution of 20 μl plasma with 100 μl saline. The degree of dilution of the dye is a measure of the plasma volume.

The results, presented in table I, show that plasma volume per 100 g body weight did not differ among the age groups ($p > 0.05$). Our values are similar to those of Bond (1958), who measured plasma volumes in adult female Long Evans rats by an Evans blue technique. However, the age of these rats was not specifically stated in this publication. Our data show more specifically that in aging female rats, there is no change in relative plasma volume.

Table I. Plasma volume in ml per 100 gram body weight in untreated female rats of different ages.

Age in months (4 animals per group)	Mean body weight in g ±S.D.	Plasma volume in ml/100 g ±S.D.
3	210 ± 13	5.0 ± 0.4
6	230 ± 7	4.9 ± 0.3
12	215 ± 6	5.1 ± 0.2
24	270 ± 10	5.0 ± 0.4

CHAPTER V

SERUM ALBUMIN

1. General aspects

Before commencing the experimental investigations, techniques had to be worked out by which base line values for serum albumin could be established.

It might be appropriate here to elaborate about the scale of the chemical measurements. All tests were performed on an ultra micro scale. Ultra micro methods and techniques for the clinical laboratory have been described by many authors, for example Sanz (1957) and O'Brien et al. (1968). These methods are often used in pediatrics. The term "ultra micro methods" is a reference to the volume of serum used for a determination (rather than to the quantity of the material to be measured). Volumes up to 50 µl (0.05 ml) are considered as being in the ultra micro range (Sanz, 1957). Methods suitable for these small volumes are very advantageous in work with small animals. It becomes possible to keep the animals alive and to repeat the test several times in the same animals. This also makes it possible to do life span studies on individual animals.

The known methods for determining albumin on the ultra micro scale all involve electrophoretic fractionation or salt fractionation. In salt fractionation, the method employed, the total serum proteins are measured by means of a colorimetric reaction. This is most often the biuret test. Following this the globulins are precipitated by means of organic salts. A second colorimetric test is performed on the supernatant. The value obtained is assumed to represent the albumin. The difference between the total protein and albumin is reported as globulin. This method proved to be unreliable on an ultra micro scale, because the supernatant following salt fractionation still contained some globulins. Electrophoretic separation proved to be equally unreliable. Therefore, a dye binding method, that had been used on a macro scale was adapted to micro level (De Leeuw-Israel et al., 1967).

Serum albumin specifically binds the anions of acid dyes. Based upon this, macro methods were developed which permitted direct quantitative determination of albumin without prior fractionation. The specific binding of an indicator dye 2–(4''-hydroxybenzene azo) benzoic acid (HBABA) was used by Rutstein et al. (1954). Ness et al. (1965) evaluated the method and improved it. Martinek (1965) also introduced further refinements. All these methods were developed for measurement of human albumin. Pastewka and Ness (1965) found different dye binding capacities for human and bovine albumin. That means that bovine albumin cannot be used as a standard for human albumin measurements and by inference the same applies to rat albumin. To adopt this method

for measuring rat serum albumin it had to be scaled down to micro level. In view of the findings of Pastewka and Ness, purified rat albumin was chosen as an initial standard. The albumin was purified as previously reported (De Leeuw-Israel et al., 1967). This was later substituted by another standard as will be discussed below.

2. Method

Reagents:

Stock dye solution: 0.726 g of 2–(4''-hydroxybenzene azo) benzoic acid (HBABA) dissolved in 5 ml of 1 N NaOH and diluted to 500 ml with distilled water.

Phosphate buffer: (pH 6.2–6.4) 2.35 g of K_2HPO_4 and 8.37 g of KH_2PO_4 dissolved in and diluted to 1000 ml with distilled water.

Working dye solution: 9 ml of phosphate buffer added to 1 ml of stock dye solution.

Procedure:

Sample: 200 µl of the working dye solution is added to 5 µl of serum.

Blank: 200 µl of the working dye solution is added to 5 µl of distilled water.

Readings were made in the Beckman spectro-colorimeter of the Ultramicro analytical system, at 510 mµ.

Because purified albumin was difficult to obtain in sufficient quantities, a second standard was chosen. This consisted of the pooled serum from 10 female rats of 4 months of age. The dye binding capacity of the pool was compared with that of the purified rat albumin. The results indicated that the pool contained 4.2 g% albumin. In a previous publication (De Leeuw-Israel et al., 1967) we had shown that the pooled rat serum can be stored in the freezer at −15 °C for a period up to three months, without a significant change in the albumin concentration. This was advantageous because it permitted repeated use of the pool as a standard.

Total protein was determined by the biuret method. The ultra micro adaptation of the method of Kingsley (1942) and of Gornall et al. (1949) was used (Technical Bulletin no 6074 C, Beckman Instruments, Inc. 1960).

3. Gerontological experiments

Total protein and albumin levels of the serum were selected as parameters in an attempt to determine the effect of aging on liver function. The specific goal was to find out if there were changes in the level of these substances with increasing age. These changes might reflect the capacity of the liver to produce proteins. All experiments were done with female rats. Albumin and total pro-

tein levels of thirty 14 months old R (Wistar) rats were compared with those of 8 three months old rats of the same sex. From the results obtained the mean values and standard deviations were calculated. The total protein values were 6.2 $\pm$0.2 g% and 6.2 $\pm$0.1 g% for old and young rats respectively. The albumin levels were 3.4 $\pm$0.6 g% and 3.9 $\pm$0.1 g% for old and young rats respectively. It can be seen that there were no significant differences between total protein and albumin levels in old and young rats (Wilcoxon $p > 0.01$).

In the second phase the investigation was continued with the 3 months old group. The total protein and albumin levels were determined every 3 months, until the death of the animals at the age of 24 months. The mean values and the standard deviations were calculated. The results are shown in table II. It can be seen that again there was no significant difference (Wilcoxon $p > 0.01$).

Table II. Albumin and total protein levels in the serum of a group of 8 untreated R rats at different ages. It can be seen that no significant changes occur as the animals age.

Age in months	Albumin (mean $\pm$S.D. in g%)	Total protein (mean $\pm$S.D. in g%)
3	3.9 $\pm$ 0.1	6.2 $\pm$ 0.1
6	3.6 $\pm$ 0.2	6.2 $\pm$ 0.1
9	3.9 $\pm$ 0.1	6.6 $\pm$ 0.2
12	4.2 $\pm$ 0.4	6.4 $\pm$ 0.2
15	3.7 $\pm$ 0.2	6.4 $\pm$ 0.1
18	3.4 $\pm$ 0.1	6.5 $\pm$ 0.2
21	3.6 $\pm$ 0.1	6.6 $\pm$ 0.2
24	3.7 $\pm$ 0.2	6.7 $\pm$ 0.3

So we concluded that aging alone leads to no changes in the serum levels of these proteins.

The next consideration was to study the effect of partial hepatectomy on total protein and serum albumin levels. Of immediate concern was the albumin level. We wanted to see if a change occurred and whether it was of the same magnitude in young rats and in older ones.

Partial hepatectomy was performed in groups of 3, 6, 9, 12, 15, 18, 21 and 24 months old RU rats. Each experimental group consisted of 8 female rats and was matched by a control group of the same number, age and sex. The follow up was continued until the rats were moribund, or until they were sacrificed at the age of 24 months or 27 months (Table III). Albumin and total protein levels were determined before partial hepatectomy and at different intervals thereafter. The results are listed in table IV.

Table III. Number of animals present in the partially hepatectomized and normal control groups at the various time intervals following partial hepatectomy.

	Time interval after operation																	
		after	weeks				months					months						
Partial hepatectomy	before	1 day	1	2	3	4	2	3	4	5	6	9	12	15	18	21	24
Partial hepatectomy at 3 months	8												8		7	7	3
Controls	8														8	6	4
Partial hepatectomy at 6 months	8											8	7	6	5		
Controls	8													8	7		
Partial hepatectomy at 9 months	8											8	7	6			
Controls	8												8	7			
Partial hepatectomy at 12 months	8											8	7				
Controls	8											8	7				
Partial hepatectomy at 15 months	8											8					
Controls	8											8					
Partial hepatectomy at 18 months	8					8	6	6	4		4						
Controls	8										8						
Partial hepatectomy at 21 months	8		8	7			7										
Controls	8						8	6									
Partial hepatectomy at 24 months	8					8	6	5									
Controls	8							8									

Table IV. Serum albumin and total protein levels at different time intervals after operation in animals, partially hepatectomized at various ages as compared to normal controls. Up to one week after operation the values were derived from determinations on groups consisting of eight animals each. After this, due to deaths, the number in each group ranged from seven down to three. For the actual number of living animals in each experimental group see table III.

		Time interval after operation															
		after	weeks				months					months					
Partial hepatectomy	before	1 day	1	2	3	4	2	3	4	5	6	9	12	15	18	21	24
Partial hepatectomy at 3 months																	
Albumin																	
mean ±S.D. in g%	3.3±0.1	3.6±0.1	3.4±0.1	3.6±0.3	3.1±0.6	3.2±0.5	4.2±0.2	4.6±0.2	3.9±0.3	4.0±0.5	4.5±0.4	4.2±0.3	4.5±0.3	3.6±0.3	3.4±0.3	3.9±0.2	3.9±0.3
Total protein																	
mean ±S.D. in g%	6.3±0.3	5.4±0.1	6.0±0.1	6.7±0.4	6.8±0.2	6.9±0.2	7.0±0.3	6.6±0.2	6.8±0.1	6.6±0.4	6.7±0.4	7.3±0.3	7.3±0.3	7.1±0.2	7.0±0.3	7.1±0.2	7.3±0.2
Controls																	
Albumin																	
mean ±S.D. in g%	3.5±0.2	4.2±0.2	4.0±0.1	4.5±0.2	4.0±0.2	3.7±0.2	4.6±0.3	4.6±0.2	4.2±0.3	4.2±0.2	4.3±0.4	4.5±0.2	4.5±0.3	3.8±0.3	3.4±0.2	3.9±0.2	3.8±0.1
Total protein																	
mean ±S.D. in g%	6.2±0.3	6.5±0.2	6.5±0.1	7.0±0.2	6.9±0.2	7.0±0.3	7.0±0.2	6.7±0.3	7.3±0.3	6.5±0.3	7.0±0.1	7.2±0.3	7.1±0.2	7.2±0.4	7.2±0.4	7.1±0.2	7.2±0.2
Partial hepatectomy at 6 months																	
Albumin																	
mean ± S.D. in g%	3.7±0.2	3.5±0.2	3.3±0.1	3.2±0.1	3.1±0.1	3.1±0.1	3.2±0.2	3.9±0.2	4.1±0.1	3.8±0.2	4.3±0.1	4.1±0.1	3.5±0.3	3.5±0.1	3.5±0.2		
Total protein																	
mean ±S.D. in g%	7.0±0.2	5.7±0.3	6.0±0.2	6.2±0.2	6.9±0.2	7.0±0.2	7.0±0.2	7.0±0.3	7.2±0.2	7.1±0.3	7.3±0.5	7.1±0.2	7.0±0.2	7.0±0.1	7.0±0.2		
Controls																	
Albumin																	
mean ±S.D. in g%	3.7±0.2	3.8±0.2	3.8±0.2	3.7±0.2	3.8±0.2	3.6±0.2	3.7±0.2	4.0±0.2	3.9±0.3	4.0±0.4	4.2±0.3	4.1±0.2	3.4±0.2	3.5±0.3	3.5±0.4		
Total protein																	
mean ±S.D. in g%	7.0±0.2	6.9±0.2	7.0±0.1	7.0±0.1	7.0±0.2	7.0±0.2	7.0±0.3	7.0±0.3	6.8±0.3	6.7±0.1	7.3±0.2	7.2±0.1	7.1±0.1	7.0±0.4	6.9±0.3		
Partial hepatectomy at 9 months																	
Albumin																	
mean ±S.D. in g%	3.6±0.1	3.2±0.2	3.5±0.2	3.9±0.2	3.8±0.3	4.2±0.2	4.4±0.4	4.1±0.3	4.1±0.3	4.2±0.2	4.2±0.3	3.9±0.2	3.6±0.2	3.4±0.2			
Total protein																	
mean ±S.D. in g%	6.8±0.3	5.9±0.4	5.8±0.2	6.6±0.2	6.7±0.3	7.0±0.3	7.0±0.3	6.9±0.2	6.9±0.3	7.0±0.2	7.0±0.2	7.2±0.2	6.9±0.2	6.9±0.3			
Controls																	
Albumin																	
mean ±S.D. in g%	3.8±0.2	3.8±0.3	4.3±0.3	4.3±0.2	4.4±0.3	4.1±0.3	4.3±0.3	4.4±0.3	4.5±0.3	4.1±0.2	4.1±0.1	3.7±0.2	3.8±0.2	3.6±0.4			
Total protein																	
mean ±S.D. in g%	7.0±0.2	7.0±0.2	6.6±0.2	6.5±0.2	6.9±0.3	7.2±0.2	6.5±0.2	6.9±0.3	6.9±0.2	6.9±0.2	6.9±0.1	7.1±0.2	7.1±0.2	6.9±0.3			
Partial hepatectomy at 12 months																	
Albumin																	
mean ±S.D. in g%	4.2±0.4	3.6±0.1	3.1±0.2	3.7±0.3	3.7±0.2	4.2±0.4	4.1±0.2	4.0±0.1	3.7±0.3	3.6±0.3	3.6±0.2	3.9±0.2	3.5±0.2				
Total protein																	
mean ±S.D. in g%	7.0±0.4	6.3±0.3	6.4±0.2	6.3±0.1	6.8±0.1	7.1±0 2	7.0±0.1	7.0±0.1	6.9±0.3	7.0±0.3	7.2±0.1	7.2±0.1	7.3±0.1				

Controls													
Albumin													
mean ±S.D. in g%	4.1±0.3	4.0±0.3	4.3±0.2	4.3±0.2	4.2±0.1	4.2±0.3	4.1±0.3	4.0±0.1	3.5±0.6	3.5±0.3	3.5±0.2	4.2±0.3	3.7±0.2
Total protein													
mean ±S.D. in g%	7.2±0.3	7.3±0.4	7.1±0.3	7.2±0.2	7.0±0.1	7.2±0.7	7.1±0.2	7.1±0.1	6.9±0.4	7.0±0.2	7.1±0.3	7.0±0.3	7.2±0.2
Partial hepatectomy at 15 months													
Albumin													
mean ±S.D. in g%	3.9±0.2	3.1±0.2	2.3±0.2	2.5±0.3	2.8±0.1	3.0±0.2	3.4±0.1	3.3±0.3	3.3±0.1	4.2±0.6	3.6±0.2	3.5±0.1	
Total protein													
mean ±S.D. in g%	7.0±0.2	5.7±0.2	6.0±0.2	6.9±0.2	6.9±0.4	7.0±0.3	7.2±0.2	7.2±0.2	6.9±0.3	7.3±0.5	7.1±0.2	7.1±0.2	
Controls													
Albumin													
mean ±S.D. in g%	3.6±0.2	3.5±0.2	3.6±0.2	3.7±0.2	3.8±0.1	3.5±0.3	3.4±0.2	3.4±0.4	3.3±0.3	3.5±0.4	3.5±0.2	3.4±0.1	
Total protein													
mean ±S.D. in g%	7.0±0.2	7.0±0.1	6.9±0.4	6.8±0.3	6.9±0.3	6.7±0.4	6.8±0.3	6.7±0.2	7.2±0.2	7.2±0.4	7.2±0.2	7.2±0.2	
Partial hepatectomy at 18 months													
Albumin													
mean ±S.D. in g%	3.7±0.2	3.1±0.2	2.3±0.1	2.9±0.1	2.8±0.2	2.7±0.1	3.4±0.1	3.6±0.4	3.6±0.4	3.5±0.2	4.0±0.3		
Total protein													
mean ±S.D. in g%	6.9±0.3	6.1±0.3	6.4±0.1	6.9±0.1	6.9±0.2	6.7±0.2	7.1±0.4	7.3±0.1	7.3±0.2	7.0±0.4	7.3±0.2		
Controls													
Albumin													
mean ±S.D. in g%	3.6±0.3	3.5±0.1	3.3±0.1	3.4±0.1	3.5±0.1	3.6±0.1	3.4±0.1	3.7±0.2	3.6±0.2	3.9±0.2	3.9±0.2		
Total protein													
mean ±S.D. in g%	7.0±0.2	7.0±0.1	7.1±0.3	7.0±0.2	6.9±0.1	6.7±0.2	7.3±0.5	7.2±0.2	6.9±0.4	7.2±0.3	7.3±0.2		
Partial hepatectomy at 21 months													
Albumin													
mean ±S.D. in g%	3.5±0.2	2.3±0.1	2.1±0.2	2.7±0.3	2.9±0.2	3.5±0.2	3.6±0.2	3.6±0.2					
Total protein													
mean ±S.D. in g%	7.0±0.3	5.9±0.3	6.1±0.2	6.8±0.2	7.0±0.4	7.2±0.2	7.3±0.2	7.3±0.2					
Controls													
Albumin													
mean ±S.D. in g%	3.6±0.2	4.6±0.3	3.5±0.2	3.5±0.2	3.5±0.2	3.5±0.3	3.5±0.3	3.4±0.2					
Total protein													
mean ±S.D. in g%	6.9±0.2	7.2±0.2	7.0±0.4	7.4±0.2	7.2±0.2	7.1±0.3	7.2±0.2	7.2±0.1					
Partial hepatectomy at 24 months													
Albumin													
mean ±S.D. in g%	3.7±0.1	2.4±0.2	2.6±0.2	2.8±0.2	2.9±0.1	3.2±0.1	3.7±0.1	3.7±0.3					
Total protein													
mean ±S.D. in g%	7.2±0.1	5.7±0.4	6.6±0.4	6.7±0.3	6.8±0.2	7.3±0.1	7.3±0.2	7.0±0.4					
Controls													
Albumin													
mean ±S.D. in g%	3.7±0.3	3.5±0.1	3.6±0.2	3.7±0.3	3.8±0.3	3.9±0.2	3.7±0.2	3.7±0.3					
Total protein													
mean ±S.D. in g%	7.3±0.1	6.8±0.3	6.9±0.3	7.2±0.3	7.3±0.3	7.4±0.3	7.3±0.1	7.2±0.2					

When interpreting these findings we first of all noted that in untreated rats total serum protein and albumin levels do not show a significant change with increasing age. This is illustrated in fig. III.

Subsequent comparison between operated and untreated rats, shows significant changes in these values during the observation period as illustrated in table V.

In the partially hepatectomized animals there is a decrease in the total protein as well as in the albumin level, as can be expected. After 1–3 weeks the total protein level is back to values that do not differ significantly from those of untreated controls. However, the albumin values have not returned to the control levels at this time. This indicates an impairment in the ability of the liver to synthesize albumin for some time after the operation. This observation may be interpreted as follows. Albumin is only formed in the liver while globulin is formed in other sites (Schultze and Heremans, 1966). Therefore the albumin level of the serum is considered to be a parameter of hepatic function (Davidsohn and Henry, 1969) (cf. chapter II). The primary post-operative decrease in both albumin and total protein levels may be explained through an increased hydration following blood loss at operation. In later phases, the albumin level is restored at a slower rate than the total protein level. This suggests an impaired albumin synthesis, temporary compensated for by an increase in globulins. The albumin levels return to control values between one to three months after the operation. This was not correlated with the age of the animal at the time of

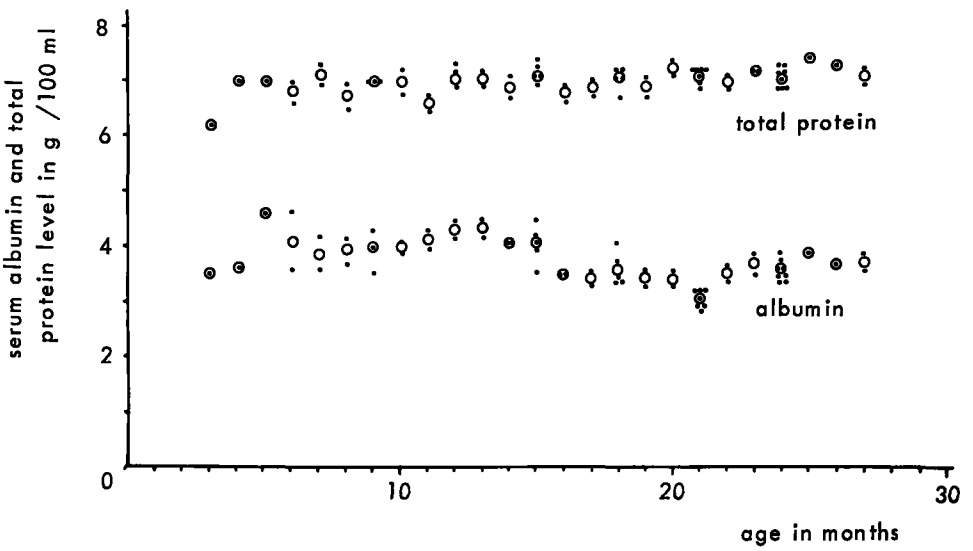

Fig. III. Albumin and protein levels in the serum of untreated rats of different ages. These represent the normal controls of the partial hepatectomy experiments described in chapter V. Each point represents the mean of a group of 8 animals. Open circles represent the means of the different groups of the same age.

Table V. *Statistical analysis of the data shown in table IV illustrating the differences in albumin and total protein levels between partially hepatectomized animals and normal controls.*

Partial hepatectomy		Time interval after operation																
		before	after 1 day	weeks 1	2	3	4	months 2	3	4	5	6	months 9	12	15	18	21	24
Partial hepatectomy at 3 months versus controls	Albumin	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	?
	Total protein	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	?
Partial hepatectomy at 6 months versus controls	Albumin	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−		
	Total protein	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−		
Partial hepatectomy at 9 months versus controls	Albumin	−	+	+	+	+	−	−	−	−	−	−	−	−	−			
	Total protein	−	+	+	−	−	−	−	−	−	−	−	−	−	−			
Partial hepatectomy at 12 months versus controls	Albumin	−	+	+	+	+	−	−	−	−	−	−	−	−				
	Total protein	−	+	+	+	−	−	−	−	−	−	−	−	−				
Partial hepatectomy at 15 months versus controls	Albumin	−	+	+	+	+	+	−	−	−	−	−	−					
	Total protein	−	+	+	−	−	−	−	−	−	−	−	−					
Partial hepatectomy at 18 months versus controls	Albumin	−	+	+	+	+	+	−	−	−	−	−						
	Total protein	−	+	+	+	−	−	−	−	−	−	−						
Partial hepatectomy at 21 months versus controls	Albumin	−	+	+	+	+	−	−	−									
	Total protein	−	+	+	+	−	−	−	−									
Partial hepatectomy at 24 months versus controls	Albumin	−	+	+	+	+	+	−	−									
	Total protein	−	+	+	+	−	−	−	−									

+ = significant difference ($p < 0.05$, Wilcoxon 1945)
− = no significant difference ($p > 0.05$, Wilcoxon 1945)
? = not enough animals surviving for statistical comparison

the operation. It is also clear that the age at which partial hepatectomy is done, has no effect on the final albumin level. Finally, normal albumin levels are maintained in partially hepatectomized, as well as in normal rats up to 24 or 27 months of age.

Summarizing these results, we may conclude that there is no change in protein synthesizing capacity of the liver with increasing age. Furthermore, the restoration of albumin synthesizing capacity after partial hepatectomy was identical at all age levels. This would seem to indicate a marked reserve capacity of the liver up to old age, which probably is related to the capacity for regenerative growth (cf. chapter VIII Partial hepatectomy).

CHAPTER VI

BROMSULPHALEIN

1. Methods

Before commencing the experimental investigations, techniques had to be perfected by which base line values for BSP retention could be established.

The bromsulphalein was obtained from Merck-Darmstadt (Bromthalein® 0.5 g in 10 ml). As was the case with the total protein and serum albumin, measurements of BSP concentrations in serum were conducted on an ultra micro scale. For this purpose a method of Van der Vies (Pharmacologisch Research Laboratorium, N.V. Organon, Oss; unpublished results) was used. BSP, the formula of which is shown in fig. IV is an indicator dye (colorless at a pH below 7.4, purple at a pH above 10.4, cf. chapter I). All the methods used for the determination of BSP take advantage of this fact and consist in principle of dilution of an aliquot of serum with an alkaline solution and measuring the BSP concentration colorimetrically. The following modification was adopted in order to make measurements on micro liter scale. The *sample* consisted of 20 micro liters of serum obtained after injection of the dye +200 micro liters of 0.5 N NaOH in saline. This was read against a blank prepared by adding 20 micro liters of normal serum to the 0.5 N NaOH solution. The *standard* was prepared by first diluting the BSP solution to a concentration of 10 mg per 100 ml.

This was accomplished by adding saline to an ampule containing 5000 mg/ml,

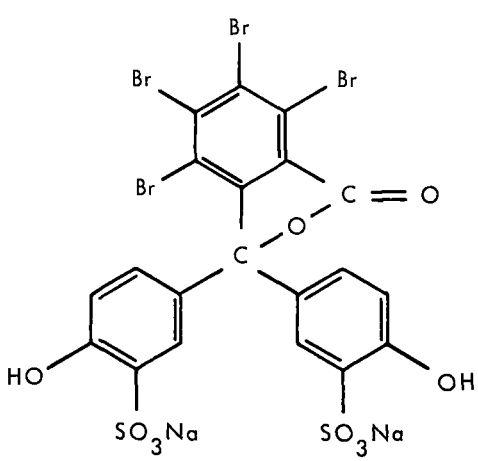

Fig. IV. Formula of bromsulphalein (BSP).

until the desired concentration was obtained (the same ampule was used for injections). Twenty micro liters of this standard were then added to 200 micro liters of serum. Readings were made at 580 mμ in the Beckman spectro-colorimeter of the ultra micro system. The small volume of serum needed when working at the ultra micro level makes it possible to obtain a sufficient amount without bleeding a rat to death or making it anemic. For this reason it is possible to repeat a test several times in the same animal and to conduct a life span study (cf. chapter II).

Such a study, involving loading of the liver in vivo, requires that the dose be based on unit liver weight. Provided the liver has a constant weight relationship to total body weight, the dose can be calculated from the body weight. (In investigations on humans, BSP dosage is also based on body weight.) In this connection the observations of Webster et al. (1947) are most important,

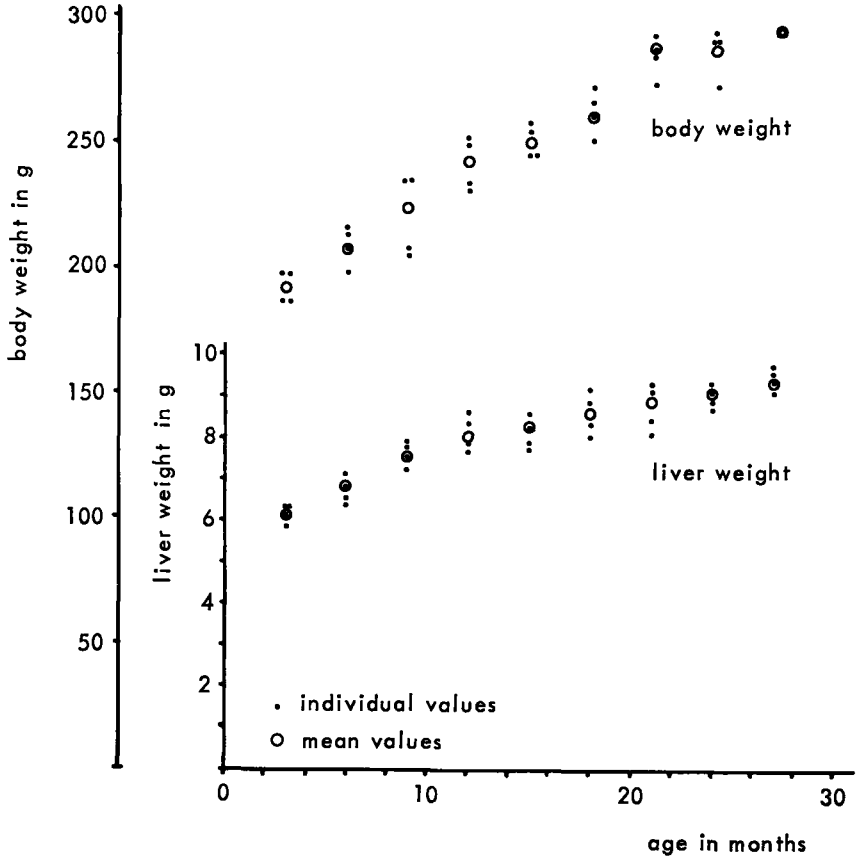

Fig. V. Liver weight and body weight in groups of four ♀ RU rats of different ages. The liver weight – body weight ratio remains constant from 3 months through 27 months of age.

since he showed that in female rats from 150 to 350 g the body weight – liver weight relationship remains the same (cf. chapter I). In the present investigation this was verified by determining the liver weight – body weight ratio of female rats of the ages of 3, 6, 9, 12, 15, 18, 21, 24 and 27 months (fig. V). It was found that the liver weight – body weight ratios varied between 3.0 and 3.2 percent in all age groups. Since this is a nearly constant ratio the dose of BSP may be calculated from the body weight.

2. BSP saturation of the liver in normal young rats

In connection with the BSP dose level, it was necessary to determine the dose at which the liver of a healthy young animal would be maximally loaded. It was reasoned that at this level, functional impairment of the liver would lead to increased BSP retention in the blood. Maximal loading would imply the measurement of the reserve capacity of the liver rather than a function under "normal" circumstances (cf. chapter II). It is possible that under normal circumstances old animals do not function less efficiently than young animals, but that in the presence of an extra burden an impairment becomes evident. Aging may become apparent in the beginning by a gradual loss in reserve capacity of organs, followed later on by a functional loss under normal conditions of daily life. It was reasoned that by choosing the dosage which "fills the liver completely" and uses up the reserve capacity, functional impairment would become evident through increased BSP retention in the blood. If so, the chosen dose level should be sensitive enough to detect even a slight change in the reserve capacity of the liver with respect to handling BSP. With this knowledge one could determine the degree of functional impairment as well as the extent of functional regeneration in partially hepatectomized animals. In other words, a base line value was to be found against which the liver function of old and of partially hepatectomized animals could be compared.

In order to establish the dosage regime, the following experimental procedure was employed: 1) The dye was repeatedly administered to the same group of four 3 months old female RU rats on different days. Injections were given through one of the tail veins. The dye dosages used were 8, 7, 6, 5, 4, 3 and 2 mg per 100 g body weight. 2) Blood samples were taken 15 minutes after each injection (this time was chosen rather arbitrarily) and the dye concentration was determined. It appeared that after the administration of 2, 3, 4 or 5 mg per 100 g body weight, no difference in retention was found, but after injecting higher doses, there was a significantly higher retention of BSP in the blood after 15 minutes. It was found that with dosages above 5 mg per 100 g body weight the retention increased. This is shown in fig. VI. A plot of retention against dose shows a more or less flat line up to 5 mg per 100 g body weight,

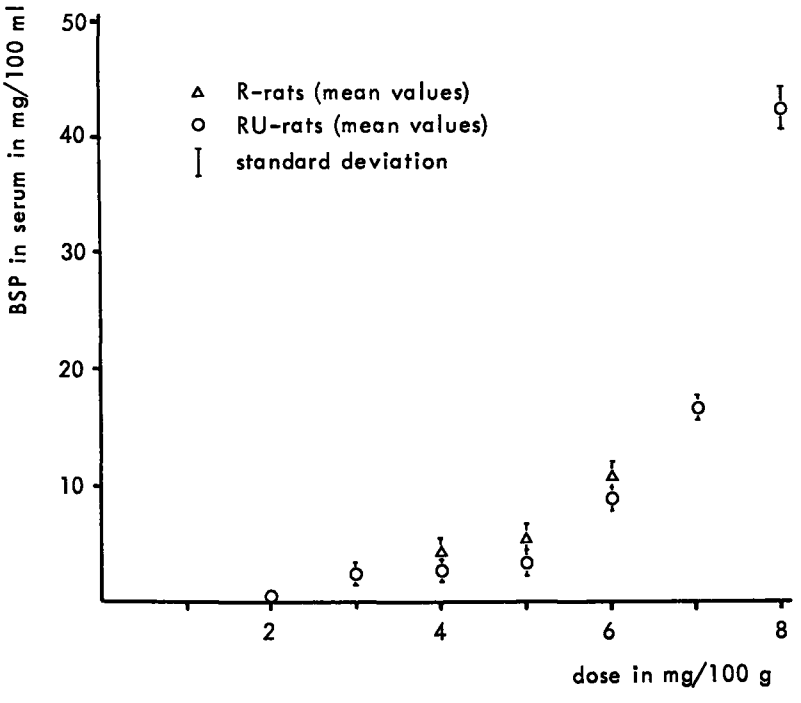

Fig. VI. BSP levels in the serum of 3 months old ♀ rats 15 minutes after injection of different doses of BSP. Mean values and standard deviation for groups of 4 rats are shown. At the 6 mg dose level, BSP retention rises sharply, indicating maximal loading of the liver at 5 mg.

above which dose level there was a sharp rise in retention.

We concluded that with a dose of 5 mg BSP per 100 g body weight, the liver of 3 months old female RU rats might be maximally loaded.

Therefore, an experiment was designed to test the validity of this conclusion, i.e. to verify that we were indeed measuring reserve capacity, that is whether the chosen dose of 5 mg per 100 g body weight would lead to maximal uptake of the dye by the liver cells. For this purpose, complete disappearance curves were constructed, using different time intervals. The following experiment was devised: a) groups of 4 young female RU rats of 3 months age were injected with 2, 3, 4, 5, 6, 7 and 8 mg BSP per 100 g body weight, the same dye dosages as employed in the previous experiment. Injections were given through one of the tail veins; b) blood samples were collected at 5, 10, 15, 20, 30 and 45 minutes after injection and the BSP level was determined. The results are illustrated in fig. VII. Analysis of the curves revealed that with doses of 2, 3 and 4 mg per 100 g body weight a straight line function was obtained. However, with doses of 5 and 6 mg per 100 g body weight it takes the form of a

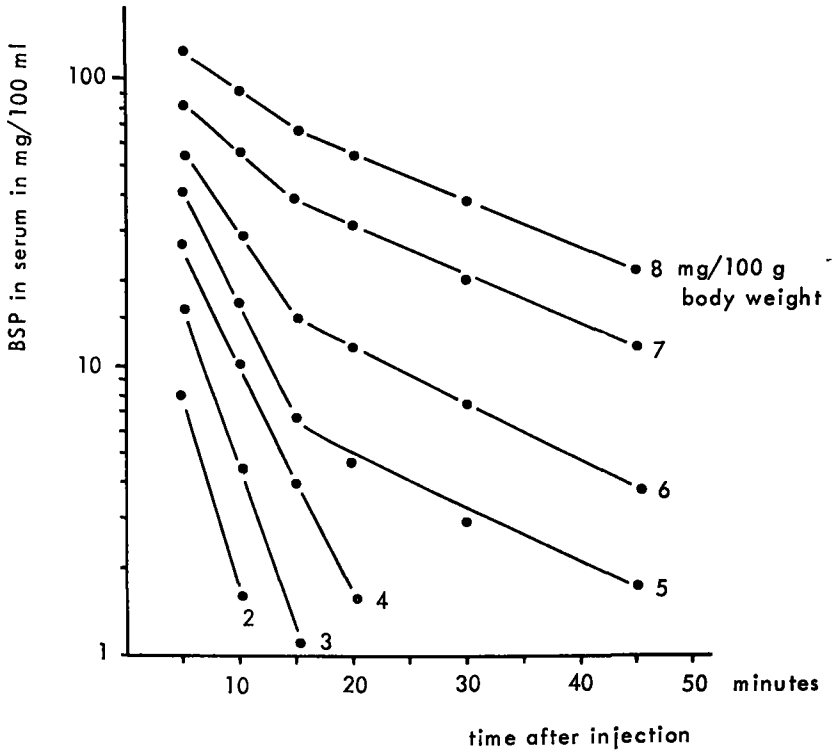

Fig. VII. Disappearance curves of BSP in the serum of 3 months old ♀ RU rats. Each point represents the mean value for a group of 4 animals, following injection of different doses of BSP. Critical dose level is 5 mg, as shown by deflection occurring in this disappearance curve.

saturation curve. As discussed in chapter II, the point of deflection of the curve means that saturation occurs, the first phase of the curve being predominantly due to the rapid uptake of BSP by the liver. After the "saturation point", which was reached 15 minutes after injection of the dye, we find a decrease in the slope of the disappearance curve. This second part of the curve reflects the continued excretion of BSP in the bile by the saturated liver. This is referred to as the "excretion curve". With doses of 7 and 8 mg per 100 g body weight no point of deflection was found with the time intervals used, but the slope of the curve was parallel to the excretion curve with doses of 5 and 6 mg per 100 g body weight. At these dose levels saturation may occur much earlier and the deflection of the curve may therefore not become apparent in the present experiment. However, we may conclude that BSP saturation for the liver of 3 months old female rats is indeed reached at 5 mg per 100 g body weight. In further experiments, we have used the 5 mg dose level and have focused on

the excretion part of the curve, for which purpose we have always taken blood samples at 15, or 15, 30 and 45 minutes following injection of BSP. This decision was based on the following arguments:

3. Arguments for experimental use of the excretion curve

The BSP disappearance curve at the 5 mg dose level in a functionally impaired liver can theoretically take one of two forms (or, of course, a combination of these): 1) The bromsulphalein serum level may decrease at a slower than normal rate. The disappearance curve then is a straight line, at a higher than normal serum level and with less slope than normal in the first part of the disappearance curve. This is to be expected when the liver cannot absorb the normal amount of BSP per time unit. 2) The alternative is a saturation curve with a decreased slope of the second part. This would indicate that the uptake of BSP from the blood by the liver is unimpaired, but the excretion into the bile is impaired. The resulting stagnation of BSP in the liver would lead to a decreased slope of the second part of the curve.

It takes a rather large volume of blood to construct a disappearance curve as compared to the total blood volume of a rat, even on an ultra micro scale. This being the case, we did not attempt to construct the whole curve in those animals that we wished to keep alive for life span studies. We had to choose, therefore, part of the curve. We focused on the latter part of the curve, because this would be higher than normal in both of the hypothetical cases mentioned above. The information so obtained is not complete, because it does not reveal whether the impairment is of the uptake mechanism or of the excretion process or both. It simply indicates whether some kind of impairment has taken place. This procedure, however, assures that the animals remain alive for the life span study. Experiments which will be described later were designed to detect the nature of the impairment.

4. The use of rats of different types and age

All experiments up to this point were performed with RU rats. Older female R (Wistar) rats were also available for use in some of the gerontological observations. Since we wanted to take advantage of this fact, it was necessary to repeat some of the previous experiments. The body weight – liver weight ratio was checked for 3, 6, 9, 21 and 24 months old ♀ R rats (four in each group). Again all observations were within the range of 3.0 and 3.2%.

To verify the dose of BSP to be administered, four ♀ R rats (3 months old) were injected with 6, 5 and 4 mg BSP per 100 g body weight on different days and blood samples were taken after 15 minutes. Again, it appeared that after 5 and 4 mg per 100 g body weight, there was little difference in retention of

the dye, while retention was significantly higher after 6 mg per 100 g body weight (fig. VI). The conclusion was the same as in the previous experiment, that is, that with 5 mg per 100 g body weight the liver was fully loaded.

In an attempt to ascertain that we were indeed measuring the reserve capacity and to determine the interval for taking blood samples, groups of four (3 months old) R female rats were injected with respectively 4, 5 and 6 mg BSP per 100 g body weight. Blood samples were collected at 5, 10, 15, 20 and 30 minutes after injection. A plot of the results was again consistent with the conclusion that saturation occurred 15 minutes after injection (fig. VIII). On the basis of these results it was decided that a standard dose of 5 mg BSP per 100 g body weight was most satisfactory and that 15 minutes, or 15, 30 and 45 minutes after injection were optimal for taking blood samples in order to determine the "excretion curve".

All of the previously described experiments were performed with 3 months old rats. Next, it was necessary to verify whether 15, or 15, 30 and 45 minutes were the optimal time intervals for taking blood samples in older rats. In order

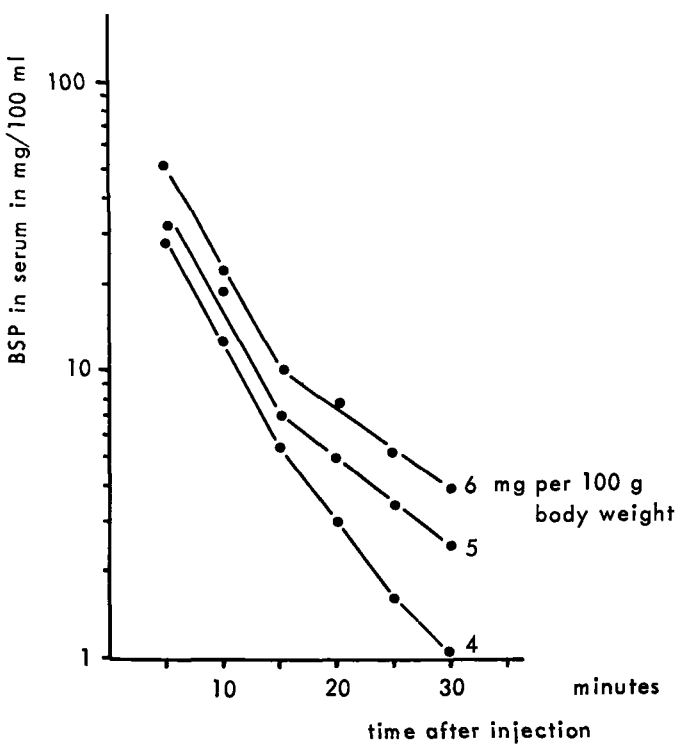

Fig. VIII. Disappearance curves of BSP in the serum of 3 months old R rats. Each point represents the mean value for a group of 4 animals, following injection of different doses of BSP.

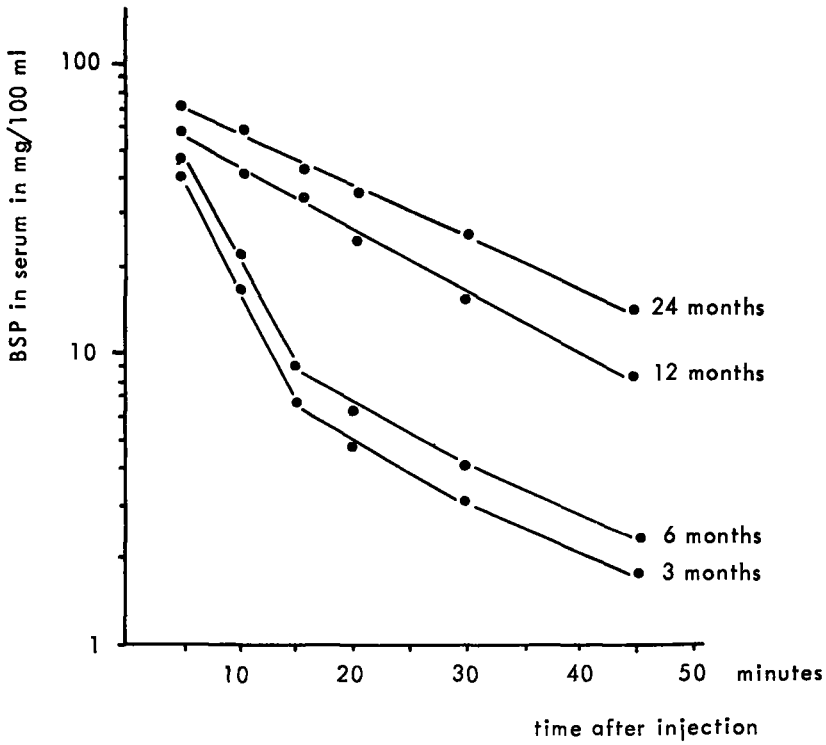

Fig. IX. Disappearance curves of BSP in the serum of ♀ RU rats of different ages. Each point represents the mean value for a group of 4 animals, following injection of a standard dose of 5 mg BSP per 100 g body weight.

to do this, 4 female RU rats of 6, 12 and 24 monts of age were injected with 5 mg BSP per 100 g body weight. Blood samples were taken at 5, 10, 15, 20, 30 and 45 minutes and BSP levels were determined. From the data obtained, disappearance curves were constructed. The results are shown in figure IX. It can be seen that with 6 months old rats the disappearance curve has the characteristics of a saturation curve, showing a point of deflection at 15 minutes after injection of the dye. This time is precisely the same as was found with the 3 months old group. But the entire curve is on a higher serum BSP level. In animals older than 6 months no point of deflection was found, but the curve was parallel to the "excretion curve" of the 3 and 6 months old rats on a higher level of BSP. Therefore, these curves were considered to indicate that the liver was saturated within 5 minutes at this dose level, indicating distinct functional impairment. Whatever the case may be, it was felt that valid results could be obtained by taking blood samples from these animals at 15, or 15, 30 and 45 minutes following the injection of the dye.

5. Orientation concerning BSP retention in rats

In order to determine the range within which BSP retention values could be expected, observations on a larger number of RU rats were required.

The rats were of the same age as the youngest group contemplated for gerontological experimentation. The group consisted of 100 female 3 months old RU rats. Using the standard dose of 5 mg per 100 g body weight, blood samples were taken 15 minutes after injection. From the results obtained, the mean value and standard deviation were calculated: 6.1 $\pm$1.2 mg%.

6. Gerontological experiments

These were again done concomitantly with the albumin determination of chapter V. The specific purpose was to see whether there was a change in the bromsulphalein test with age. Such a change should reflect a changed reserve capacity of the liver with regard to its ability to excrete a substance from blood to bile.

In order to investigate this possibility, a study consisting of two phases was begun. 1) BSP retention values of fourteen 30 months old ♀ R rats were compared with those of eight 3 months old ♀ R rats. The observations were carried out concomitantly with the observations on serum proteins described in chapter III. The mean weight of the old rats was 235 g and of the young rats 180 g. The rats were injected with the standard dose of 5 mg BSP per 100 g body weight. Blood samples were taken at 15, 30 and 45 minutes after injection (in the excretory phase). The results are shown in table VI. In the 30 months old rats, the BSP retention, as measured 15, 30 and 45 minutes after a single injection of the dye was significantly higher than in the 3 months old rats (Wilcoxon $p = 0.01$). This indicates an impairment of the liver function in the old rats, which should be confirmed, however, by longitudinal studies on rats during aging.

For this purpose, and also to discover at which age liver function changes and whether this change is sudden or gradual, the investigation was continued with the 3 months old group. Excretion curves were determined at intervals of 3 months until all the animals were sacrificed at the age of 24 months. The results are shown in table VII. It can be seen that the 15 minute retention

Table VI. BSP retention in the serum of old and young rats (mean values $\pm$S.D.) in mg per 100 ml.

Minutes after injection	15′	30′	45′
Old rats 30 mo (14 animals)	14.1 $\pm$ 3.3	5.3 $\pm$ 1.8	2.1 $\pm$ 1.1
Young rats 3 mo (8 animals)	4.8 $\pm$ 2.2	2.3 $\pm$ 0.9	1.1 $\pm$ 0.9

Table VII. BSP retention in the serum of rats of different ages (mean value ±S.D.) in mg/100 ml; 8 animals per group.

Age in months	Minutes after injection		
	15′	30′	45′
3	4.8 ± 2.2	2.3 ± 0.9	1.1 ± 0.9
6	12.1 ± 1.9	4.5 ± 1.2	1.6 ± 0.5
9	14.6 ± 2.6	7.0 ± 2.3	3.6 ± 2.1
12	16.7 ± 2.2	6.6 ± 2.9	3.0 ± 0.7
15	16.1 ± 1.0	5.8 ± 2.7	2.7 ± 1.5
18	16.9 ± 2.2	7.0 ± 1.5	3.1 ± 0.6
21	16.2 ± 2.1	6.5 ± 1.5	2.8 ± 0.7
24	17.1 ± 2.3	7.2 ± 1.2	3.2 ± 1.2

values increase in rats over 3 months of age. The values found in 3 months old rats correspond to those found previously. It can also be seen that the BSP retention values at 30 minutes after injection are significantly higher at 6 months (and older) than at 3 months of age. Between the values for 6, 9, 12, 15, 18, 21 and 24 months, there are no significant differences. These data show that despite the fact that the liver weight – body weight ratio and the dose of BSP per g liver weight was the same in each experiment, differences exist between the younger and older ages. It is obvious that the livers of rats over 3 months of age, were not capable of handling the dye in the same manner as those of the younger ones. It should be noted, however, that although the "excretion curves" of the older animals lie on a higher level, they are practically parallel to each other and to the "excretion curve" of the 3 months old animals. Since this indicates a similar rate of excretion of dye into the bile, the conclusion is suggested that the decrease in liver function may be due to a decrease in uptake or storage capacity. This question will be dealt with in chapter VII (Hepatic storage and maximal biliary excretion).

Whatever the cause, a change that occurs between the ages of 3 and 6 months can, in general, hardly be called an aging phenomenon. Any changes that are to be interpreted as a manifestation of aging should have at least some detrimental or involutionary aspects (cf. chapter I, Introduction). In fact, we have demonstrated a decreased ability to handle BSP, occurring during a life period of growth, rather than of aging in the involutionary sense. Since this indicates a decrease in reserve capacity, it might be interpreted as another example of early aging changes, comparable to the decreasing number of cells of the central nervous system or the increasing rigidity of the optic lens. This interpretation, however, is dubious because the change in this case is not progressive: with increasing age, the functional capacity of the liver remains constant from the age of 6 months onwards.

CHAPTER VII

HEPATIC STORAGE AND MAXIMAL BILIARY EXCRETION

In reference to the changes in BSP retention demonstrated in chapter VI, 6 (Bromsulphalein, gerontological experiments) several possible explanations may be involved. Among these are: a) decreased liver uptake, b) decreased storage of BSP in the liver and c) decreased biliary excretion.

In an attempt to discover which of these phenomena was involved an experiment was designed in which BSP was administered by continuous infusion (De Leeuw-Israel et al., 1969). For this purpose the method which Wheeler et al. (1960 a and b) had applied to dogs and humans was adapted to the rat. This has been described in chapter II. By this method, relative storage capacity and maximal excretion by the liver can be determined separately. The relative storage capacity (S) is a measure of the processes, responsible for the uptake and storage of BSP in the liver. The amount stored is related to the plasma concentration and S is defined as the number of mg BSP taken up into storage per mg BSP in 100 ml of plasma. The subsequent excretion of BSP into the bile shows a maximal rate (T_m) under conditions where the liver is saturated. This is true, at least for man and the dog. For the purpose of this experiment, it was assumed that this is also true for the rat and that the same procedure as used by Wheeler et al. (1960 a and b) could be employed. The experiment was performed in the following manner:

Hepatic storage and excretion values were determined in groups of four 3 months old ♀ RU rats and four 24 months old ♀ RU rats. After a priming dose of 5 mg an infusion of BSP was administered via the jugular vein. This was accomplished by the use of an infusion pump. For the duration of one hour, a solution of 250 mg BSP per 100 ml saline was infused at a constant rate of 0.18 mg BSP per minute. During this first hour blood samples were collected at 30, 40, 50 and 60 minutes and the BSP levels were determined. The procedure was continued for a second hour with a solution of 375 mg BSP per 100 ml saline at a constant infusion rate of 0.27 mg per minute. At the completion of the study the plasma volume of each animal was determined by the use of Evans blue as described in chapter IV (Plasma volume). S and T_m were calculated according to the formulae previously described (cf. chapter II, 6 c, Bromsulphalein-tests). The results are shown in tables VIII and IX. A typical time course of BSP concentrations in plasma in 3 months old rats is shown in fig. X. It can be seen that during the second half hour of each infusion period the plasma BSP concentration increased in a linear fashion with time in both age

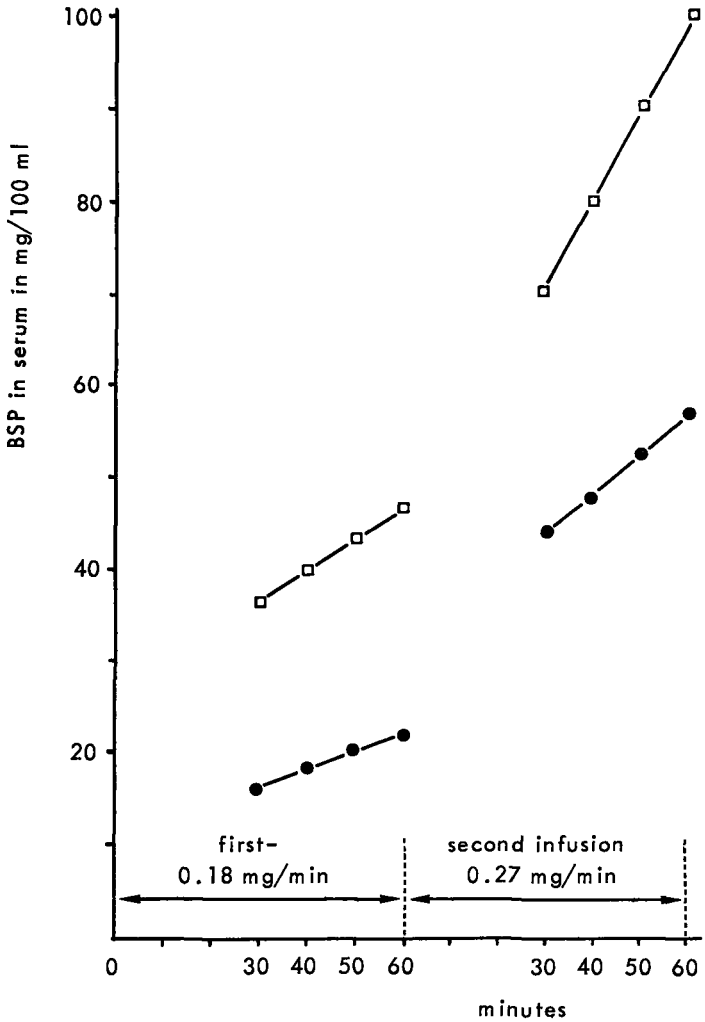

Fig. X. Typical time course of BSP concentrations in plasma during a continuous BSP infusion experiment in a 3 months old rat (rat no 1, ●) and a 24 months old rat (rat no 5, □). (From De Leeuw–Israel et al., 1969).

groups. As in the case of Evans blue, BSP is largely bound to albumin in the blood plasma. As a result, only insignificant amounts are excreted into the urine. What we are measuring, therefore, is the concentration of BSP remaining in the blood plasma following storage of liver cells and biliary excretion.

Plasma volume was the same in both groups of rats (table VIII; Wilcoxon $p > 0.05$). If one examines the data in table X, it will be seen that, in the case of the young rats, there was an increase in the calculated plasma volume following the two hour infusion period. The method used for calculating the plasma

Table VIII. Body weight and rate of change in serum BSP ($\Delta p/\Delta t$) during the continuous infusion experiment and plasma volume as determined after the experiment.

Rats	Body weight in g	First infusion $I = 0.18$ mg/min. BSP in serum mg/100 ml 30′	40′	50′	60′	$\Delta p/\Delta t$ mg/min.	Second infusion $I = 0.27$ mg/min. BSP in serum mg/100 ml 30′	40′	50′	60′	$\Delta p/\Delta t$ mg/min.	PV ml
3 mo.												
no. 1	195	16.1	18.6	20.3	22.4	0.21	44.5	50.3	53.8	58.5	0.46	12
2	195	9.3	11.5	13.6	16.1	0.22	47.8	52.6	56.8	61.4	0.45	11
3	200	9.6	11.7	13.9	16.4	0.22	45.6	51.5	55.0	59.6	0.46	12
4	195	16.4	19.2	22.9	25.7	0.36	44.5	50.0	56.1	64.0	0.65	10
24 mo.												
no. 5	250	36.3	39.8	43.3	46.8	0.35	70.0	77.6	90.1	99.5	0.99	10
6	260	32.0	35.1	39.8	43.9	0.40	66.2	75.8	85.4	90.5	0.96	12
7	260	36.2	41.0	45.0	49.2	0.43	77.2	86.6	96.0	103.0	0.86	13
8	265	36.3	39.8	43.5	48.0	0.39	49.5	58.5	67.2	76.7	0.90	13

volume, prior to the infusion, is described in chapter IV (Plasma volume). But, if one compares the plasma volume of untreated young and old rats, there are no significant differences, (cf. chapter IV table I). The differences between the pre-infusion and post-infusion values for the young rats may be due to the excretion of a smaller volume of the infused saline in the urine of the young rats as compared to the old ones.

The S of the old rats was found to be significantly lower than in the young rats (Wilcoxon $p = 0.05$). The two groups did not differ significantly with respect to T_m.

Table IX. Hepatic Removal Rates (R_1: First Infusion; R_2: Second Infusion), Relative Storage Capacity (S) and Maximum Biliary Excretion (T_m).

Rats	$R_1 \frac{\text{mg}}{\text{min.}}$	$R_2 \frac{\text{mg}}{\text{min.}}$	$S \frac{\text{mg}}{\text{mg/100 ml}}$	$T_m \frac{\text{mg}}{\text{min.}}$
3 months				
no. 1	0.16	0.22	0.24	0.11
2	0.16	0.22	0.20	0.12
3	0.15	0.21	0.25	0.09
4	0.14	0.20	0.20	0.07
24 months				
no. 5	0.14	0.17	0.05	0.12
6	0.13	0.16	0.05	0.12
7	0.12	0.16	0.09	0.08
8	0.12	0.15	0.06	0.10

Note: R_1 and R_2 were calculated by the equation $R = I - PV\ \Delta p/\Delta t$. Substitution of the two sets of values for R and $\Delta p/\Delta t$ in the equation $R = T_m + S\ \Delta p/\Delta t$ provided a set of simultaneous equations that could be solved for both T_m and S.

These facts may be illustrated by the use of the Wheeler formulae (cf. chapter II, 6 c, The liver during aging; Bromsulphalein).

$$R = T_m + S\,\Delta p/\Delta t$$

$$R = I - PV\,\Delta p/\Delta t$$

where R = removal rate, T_m = excretion rate, S = relative storage capacity, PV = plasma volume, Δp and Δt represent changes in BSP retention with changes in time and I = infusion rate. Then:

$$I - PV\,\Delta p/\Delta t = T_m + \Delta p/\Delta t, \text{ and consequently}$$

$$I = T_m + (S+PV)\,\Delta p/\Delta t.$$

Since I and T_m are the same in both groups, it follows that:
$(S+PV)\,\Delta p/\Delta t$ has similar values for old and young rats. On comparing the values for the two groups, it was found that in old animals S was smaller and PV was the same, while $\Delta p/\Delta t$ was greater then in the young rats. If there had been no change in the plasma volume of the young rats following infusion (as was the case for the old ones), the final PV would be even smaller than was actually found. In that event, the higher value for S in young as compared to old rats would be even more marked.

The results indicate that the changes in BSP retention during life are due to a decline in S. This decline stands in contrast to the uniformity in T_m in the old and young rats. A change in S, while T_m remains the same is also seen in elderly humans (cf. chapter II, 6 c, Liver function and aging; Bromsulphalein). The mechanism responsible for the selective reduction in S requires further discussion. One factor which may safely be excluded as a cause for decreased storage capacity in 24 months as compared to 3 months old rats is a reduced liver size between 3 months and 24 months of age, since the liver weight – body weight ratio is fairly constant (cf. chapter VI, Bromsulphalein) and in the present experiment the older animals were significantly heavier than the young rats (table X). It is of interest that the findings of Thompson and Williams (1965) in man also show a lack of correspondence between decrease of S with increasing age on the one hand and liver size on the other.

The diminished capacity of the liver of older rats to remove and store BSP from the blood might be related to various other factors such as: 1) Diminished blood flow through either the hepatic artery or the portal vein. 2) A slower transfer from the blood into the hepatic cell as is the case in cirrhosis (Popper and Schaffner, 1957) where collagen occupies the space of Disse or, by a decreased effective cell surface. 3) A change in the capacity of the cell to metabolise BSP. 4) A decrease in the number of cells.

Table X. Body weight, and plasma volume before and after the two hours infusion period in young and old rats.

Rats	Body weight in g	Plasma volume in ml before the experiment (calculated)	Plasma volume in ml after the experiment (determined)
3 months			
no. 1	195	9.8	12.0
2	195	9.8	11.0
3	200	10.0	12.0
4	195	9.8	10.0
24 months			
no. 5	250	12.5	10.0
6	260	13.0	12.0
7	260	13.0	13.0
8	265	13.3	13.0

With respect to point 1: We have no data on blood flow to the rat liver in our experiments. The following reasoning may, however, help to exclude this factor. According to Maddrey et al. (1969) there is no correlation (in man) between the blood flow through the hepatic artery and either T_m or S. As to portal blood flow it has been shown that in cases of portal hypertension, the disturbance is the same as in aged individuals namely a decreased S and a normal T_m. The portal vein input is reduced in portal hypertension (Maddrey et al., 1968). The explanation of Maddrey et al. (1969) therefore is that diminished portal blood flow might lead to a slower turnover of cells which would result in an older and less efficient hepatic population, explaining a decreasing S in cases of portal hypertension. However, cellular turnover in old rats most probably is not slower than in young ones. Regeneration of function after partial hepatectomy is even faster in old than in young rats. The explanation of Maddrey et al., therefore, is not applicable in our case.

With respect to point 3: Metabolism of BSP in the liver. This includes conjugation with glutathione, an enzyme catalyzed process. According to Boyland and Grover (1967), this is not the rate limiting step in BSP metabolism. This is confirmed by Cook et al., (1968) who in a study on active chronic hepatitis, showed that there occurred impairment in both S and T_m. There was, however, no significant correlation between change in T_m and conjugation. Whelan et al. (1970) found that intrahepatic conjugation in vivo is important for biliary transport of BSP in the rat. They also showed that excretion is impaired when conjugation is impaired. This explanation does not apply here since we have found that storage capacity is decreased but that there is no change in excretion rate. Of the four factors mentioned above, two remain to be considered, viz. a decreased BSP uptake from the blood per liver cell or a decrease of the number of liver cells. These factors will be discussed in connection with the morphological findings in old and young rats as presented in chapter X.

CHAPTER VIII

PARTIAL HEPATECTOMY

Although the results of the previous experiments suggested a change in functional capacity they did not offer insight into the mechanisms involved. Moreover, strictly speaking, no real aging changes were observed, since these changes took place at such an early age and remained more or less at a plateau afterwards. This made us even the more curious as to the effects of partial hepatectomy. The original objective had been, to find out whether a change in liver function was accelerated by a partial hepatectomy; another was to see if the change in BSP retention with age was the same in young partially hepatectomized rats as in old ones under the same conditions. In view of the results obtained in the previous investigation some new questions arose. Some of these were:

a. Since normal aging does not seem to entail changes in liver function, is there any permanent impairment of liver function after partial hepatectomy?
b. Is there a difference between rats partially hepatectomized at young ages and at old ages?
c. If repair occurs, at what time does it happen?
d. Is return to normal liver function correlated with return to normal liver weight?
e. Does repair occur to the same extent in young and in older animals?
f. Is restoration of normal liver function in the partially hepatectomized rats of a permanent nature or does diminished capacity reappear in old age?

In order to answer adequately the questions posed, we had to return to a consideration of the liver weight – body weight relationship. As has been mentioned we have determined that the liver weight – body weight ratio in an intact animal is $\pm 3.1\%$. With this knowledge it would be possible to ascertain when complete regeneration had occurred. And we could also detect whether normal function had been regained. According to the literature (Bucher, 1963) restoration of liver weight after partial hepatectomy takes place 2–4 weeks after the operation. Bucher and Glinos (1950) showed that following partial hepatectomy in adult and old rats, regeneration took place to the same extent. This was true when measurements were made two weeks following the operation.

To establish whether this applied to our strain of rats, the following experiment was performed: Four ♀ 3 months old RU rats (weighing 200 g) and four ♀ 24 months old RU rats (weighing 270 g) were partially hepatectomized and the

excised liver was weighed. After 4 weeks the animals were sacrificed and the regenerated livers were weighed. The liver weight – body weight ratios were found to be 3.0 and 3.2% respectively. These were the same as seen in unoperated animals. This suggested that the liver weight – body weight ratio is restored in old and in young animals within 4 weeks after partial hepatectomy.

On the basis of this preliminary experiment, the investigation of the effect of partial hepatectomy was continued. Female RU rats of 3, 6, 9, 12, 15, 18, 21 and 24 months were divided into groups of eight according to age. Each experimental group was matched by a control group of the same number, age and sex. All rats were followed up to the end of their life at 24 or 27 months. In this way the controls themselves represented a life span study which begins at different ages, i.e. at 3, 6, 9, 12, 15, 18, 21 and 24 months.

The BSP test was performed as outlined (chapter VI, 2, BSP saturation of the liver in normal young rats). A standard dose of 5 mg BSP per 100 g body weight was injected. Blood samples were taken 15 minutes after injection and BSP levels were determined.

The results are shown in tables XI and XII and summarized here. First the results obtained with the untreated rats were compared to the base line values. It can be seen that the levels of 3 months old rats, and also those of 6 months of age were within the normal range (cf. table VIII). There is an increase at the age of 9 months, and this level is maintained in older animals. This means that the picture is the same as in the previous investigation on R rats except that the change occurs around the age of 9 months instead between 3 and 6 months. Possibly this is due to the use of RU rats in the present series of experiments.

If one compares the values obtained in control animals at different ages within each individual group as well as between the different groups, it can be seen that in every instance this change is found between 9 and 12 months of age and that from 12 months on there are no significant differences. This indicates a true change that is not caused by the BSP nor due to its accumulation. If it was a matter of simple accumulation one would expect the BSP level to be much lower in the group of animals when BSP tests were started at an older age, for instance approximating that found in the 3 months old group.

If the data for the partially hepatectomized animals are examined, it is seen that in the group of animals operated at 3 months of age and followed to the end of the experiment, there was an abrupt rise in BSP retention one day after the operation. Eight days later the value had fallen somewhat but was still above the initial level. The sudden rise, following partial hepatectomy is not surprising since two third of the liver had been removed. Return to "normal values", that is to values in controls of the same age, did not take place until two months after operation. This is somewhat later than the restoration of normal liver weight, which has taken place at least four weeks after operation.

Table XI. Bromsulphalein retention in serum (mg per 100 ml ±S.D.) at different time intervals after operation, in animals partially hepatectomized at various ages as compared to normal controls. The determinations were made on the same animals as were used for the albumin and total protein levels. Up to one week after operation, the values were derived from determinations on groups consisting of eight animals each. After this, due to deaths, the number in each group ranged from seven down to three. For the actual number of living animals in each experimental group see table III.

		Time interval after operation															
		after	weeks				months					months					
Partial hepatectomy	before	1 day	1	2	3	4	2	3	4	5	6	9	12	15	18	21	24
Partial hepatectomy at 3 months	4.1±1.4	31.7±5.7	11.7±2.7	6.7±1.8	8.6±2.0	8.8±2.0	8.1±1.3	8.2±2.6	10.5±1.4	12.2±2.2	12.9±2.0	16.2±2.0	18.0±1.3	18.1±2.3	17.7±2.1	16.9±0.9	18.9±4.0
Controls	5.0±1.2	4.1±1.4	3.6±1.2	4.1±0.9	4.2±1.6	5.3±1.6	6.8±1.8	7.2±1.9	11.6±2.1	11.3±1.9	12.0±1.0	16.7±1.0	18.4±0.5	17.5±0.6	17.1±3.2	18.9±1.3	17.3±1.2
Partial hepatectomy at 6 months	6.9±2.5	34.4±4.0	16.1±4.7	14.6±2.5	14.3±3.0	15.3±2.4	14.0±1.4	14.2±1.9	14.7±2.4	16.9±2.0	17.9±2.5	18.2±2.3	18.8±2.4	18.9±2.4	17.6±0.4		
Controls	7.1±1.0	6.8±2.0	8.1±1.5	6.4±3.6	10.4±2.4	9.1±1.5	10.8±1.8	11.9±2.8	12.6±2.1	15.6±1.8	16.8±3.4	18.2±1.2	18.9±1.4	18.6±1.8	17.2±2.8		
Partial hepatectomy at 9 months	11.9±1.3	33.5±3.7	26.0±4.4	21.4±3.2	20.8±3.0	19.5±2.8	19.8±3.0	22.4±3.0	18.2±2.6	18.7±1.7	19.2±2.1	17.7±2.9	18.6±1.7	18.4±1.8			
Controls	11.8±1.1	13.5±1.1	13.9±1.6	14.5±3.1	15.1±1.5	14.5±1.9	15.2±1.0	15.5±1.3	17.2±1.5	17.4±1.4	20.1±1.5	16.8±1.7	18.6±1.9	21.7±2.7			
Partial hepatectomy at 12 months	18.7±3.7	43.3±2.5	28.6±1.7	23.0±1.4	20.8±1.4	19.1±0.6	17.8±1.2	17.3±2.2	17.2±2.7	18.0±2.6	16.2±3.7	16.8±2.3	18.4±2.2				
Controls	16.7±1.5	17.7±0.3	18.4±2.0	18.7±1.1	16.5±1.9	16.9±2.3	19.0±0.8	16.5±0.9	16.9±2.7	17.1±2.2	17.8±3.3	17.9±1.2	18.4±0.1				
Partial hepatectomy at 15 months	17.4±4.0	53.6±4.3	21.6±2.0	19.1±1.2	19.0±2.0	19.8±2.4	17.6±2.6	17.7±1.9	19.1±3.6	19.3±1.7	19.0±1.5	19.0±3.0					
Controls	17.8±3.4	18.2±1.5	18.9±1.4	19.4±3.0	18.2±2.6	18.6±2.3	16.6±1.5	16.4±2.4	16.1±3.5	18.1±2.5	18.0±0.5	18.3±1.9					
Partial hepatectomy at 18 months	18.5±2.6	52.5±4.0	22.0±3.1	20.9±0.9	19.7±2.5	20.2±1.5	21.3±2.0	19.6±2.0	19.9±2.6	18.6±2.1	18.2±1.2						
Controls	17.0±2.6	17.5±1.7	17.1±2.5	18.4±2.0	19.4±1.6	18.9±1.8	18.4±2.0	18.6±1.5	19.5±2.4	18.9±2.0	18.5±1.0						
Partial hepatectomy at 21 months	19.7±2.0	42.1±3.0	23.4±1.9	19.6±1.7	19.0±1.9	19.7±1.8	19.9±1.2	20.0±3.8									
Controls	17.4±3.2	19.3±2.8	18.4±1.6	18.0±1.4	19.3±1.5	20.6±1.9	19.4±1.0	18.6±2.1									
Partial hepatectomy at 24 months	18.9±1.4	50.4±4.4	24.5±3.5	19.8±1.3	19.8±1.0	19.7±1.7	19.6±2.7	18.9±3.7									
Controls	19.5±2.1	19.0±0.8	18.7±1.1	19.2±1.0	19.6±2.3	18.2±1.5	19.4±1.3	19.2±2.0									

Table XII. Statistical analysis of the data shown in table XI, illustrating the differences in BSP retention between partially hepatectomized animals and normal controls.

	Time interval after operation																	
		after	weeks				months					months						
Partial hepatectomy	before	1 day	1	2	3	4	2	3	4	5	6	9	12	15	18	21	24
Partial hepatectomy at 3 months versus controls	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	?
Partial hepatectomy at 6 months versus controls	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−		
Partial hepatectomy at 9 months versus controls	−	+	+	+	+	+	+	+	−	−	−	−	−	−			
Partial heparectomy at 12 months versus controls	−	+	+	+	+	+	−	−	−	−	−	−	−				
Partial hepatectomy at 15 months versus controls	−	+	+	−	−	−	−	−	−	−	−	−					
Partial hepatectomy at 18 months versus controls	−	+	+	−	−	−	−	−	−	−	−						
Partial hepatectomy at 21 months versus controls	−	+	+	−	−	−	−	−									
Partial hepatectomy at 24 months versus controls	−	+	+	−	−	−	−	−									

+ = significant difference ($p \leq 0.05$ Wilcoxon)
− = no significant difference ($p > 0.05$ Wilcoxon)
? = not enough animals surviving for statistical comparison

Once the liver function as measured by BSP was restored the BSP retention values remained at control levels even in "aged" rats.

It can also be seen that in the 6 and 9 months old animals restoration of the liver function took longer than was the case for the 3 months old group. The 9 months old group exhibited the slowest return to normal. This finding was to be expected, that is, the older the animal the slower the restoration. If one follows the results it is found that this view is not completely supported by the experiments. One sees that in twelve months old rats restoration of function after partial hepatectomy occurred more rapidly than in the 9 months old rats. For animals that are 15 months of age, at the time of the operation, the liver function is restored two weeks after partial hepatectomy. Once this function was restored, it remained stable. The same phenomenon as observed in the 15 months old group, was also seen in older animals.

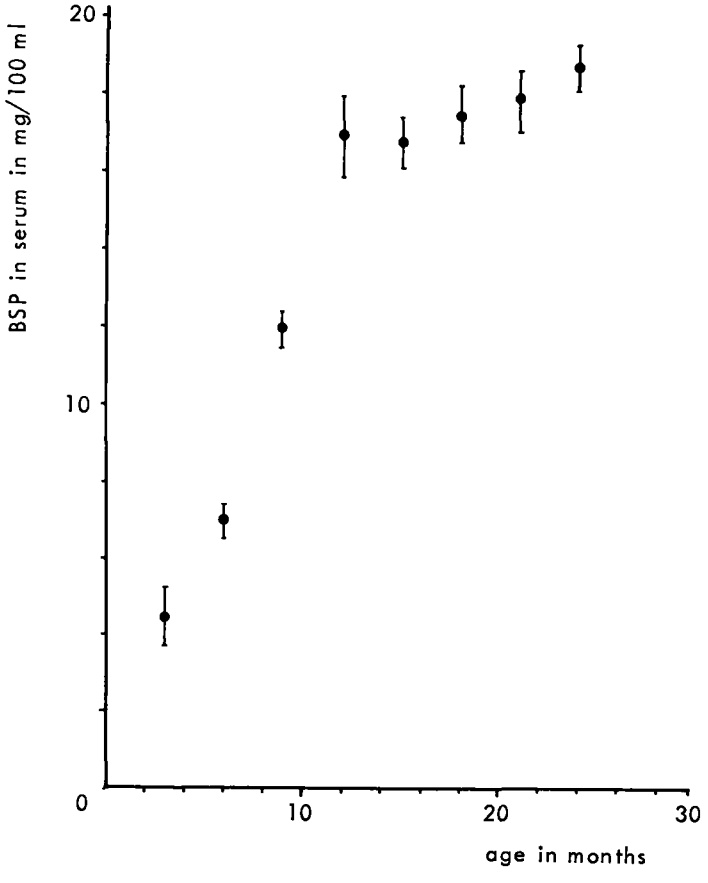

Fig. XI. Bromsulphalein retention values in untreated ♀ RU rats of different ages. These represent the initial values of the controls of the partially hepatectomized rats.

A detail from table XI is shown in fig. XI. Here the initial BSP values are plotted against the ages of the animals in each group. This reveals that the BSP retention reaches its peak between 9 and 12 months and that its value is essentially the same for the groups above this age.

Liver function of rats, as measured by BSP retention, regenerates up to 12 months of age, in 2–4 months after partial hepatectomy. Above 12 months of age, the function regenerates much more rapidly and from 15 months on, complete restoration occurs in two weeks in all age groups. Thus the important changes occur at 9 months of age and beyond this age little of immediate interest is seen. Later on, an attempt will be made to explain the underlying mechanisms of these phenomena, (cf. chapter IX Polyploidy).

Since BSP levels of aged partially hepatectomized rats did not differ from those of untreated, age-matched controls, one cannot conclude that partial hepatectomy induced any permanent functional impairment or acceleration of aging processes. It is evident that whatever aging changes affect the liver function between 6 and 9 months of age, they are not modified by the fact that two third of the liver has been extirpated and regenerated. The regeneration process itself does not seem to be influenced by age-dependent changes. The changing speed of functional regeneration demands an explanation which may be relevant to the overall evaluation of changes in liver function with advancing age.

CHAPTER IX

POLYPLOIDY

A survey of the literature revealed that there is a shift from a diploid to a largely polyploid cell population in the rat during life (cf. chapter II). According to Post and Hoffman (1965) this shift can occur rather early in life. However, it was not clear whether this shift is gradual or abrupt and at which age it occurs in the strain of rat, used in our investigation. In an attempt at clarification, the distribution of diploid, tetraploid and octaploid nuclei was determined in ♀ RU rats.

1. Methods

The cells were differentiated from each other by use of tetraphenyl boron (TPB) as described by Rappaport and Howze in 1966. This technique offers a distinct advantage when compared to others in that prior infusion of the organ is unnecessary. The principle is the following. Sodium tetraphenyl boron is a specific agent for complexing potassium ions which are the major cations involved in the aggregation of cells in liver tissue. Removal of K^+ results in a dissociation of liver tissue in vitro to a suspension of single cells. The suspensions were prepared in the following manner. The rats were killed with ether and the liver was quickly removed. It was then sliced into small pieces using razor blades. The pieces were placed in a dissociating solution of 3 mM sodium tetraphenyl boron in a 5 mM sodium phosphate buffer, pH 7.8, containing 0.05 M sucrose and 0.14 M NaCl. They were stirred in this solution for 1 hour at 25 °C. The cell suspensions were then washed three times with cold "dissociation solution" without TPB. They were resuspended in the same solution to which trypan blue had been added at a concentration of 1 : 1000 for the purpose of staining the nuclei. Finally, drops of the suspension were placed on a microscope slide and air-dried. Permanent mounts were then prepared by the application of Malinol and a cover-slip. Next they were photographed with the Ultraphot II apparatus (Carl Zeiss). The objective employed was a plan-achromatic. The exposures were made on Ilford Pan-F 35 mm film which was developed for 4 minutes and 30 seconds at 20 °C in Agfa-Gevaert "Rodinal" in a dilution 1 : 30. Those photographs which were to be used for measurements were enlarged on a special light weight projection paper (Kodak "Kodagraph P 84"). The final magnification was 370 ×. The diameters of the photographed nuclei were measured with a Carl Zeiss TGZ3 Particle Size Analyzer. Examples are shown in fig. XII. According to Swartz (1967) at least 1000 nuclei per rat should be

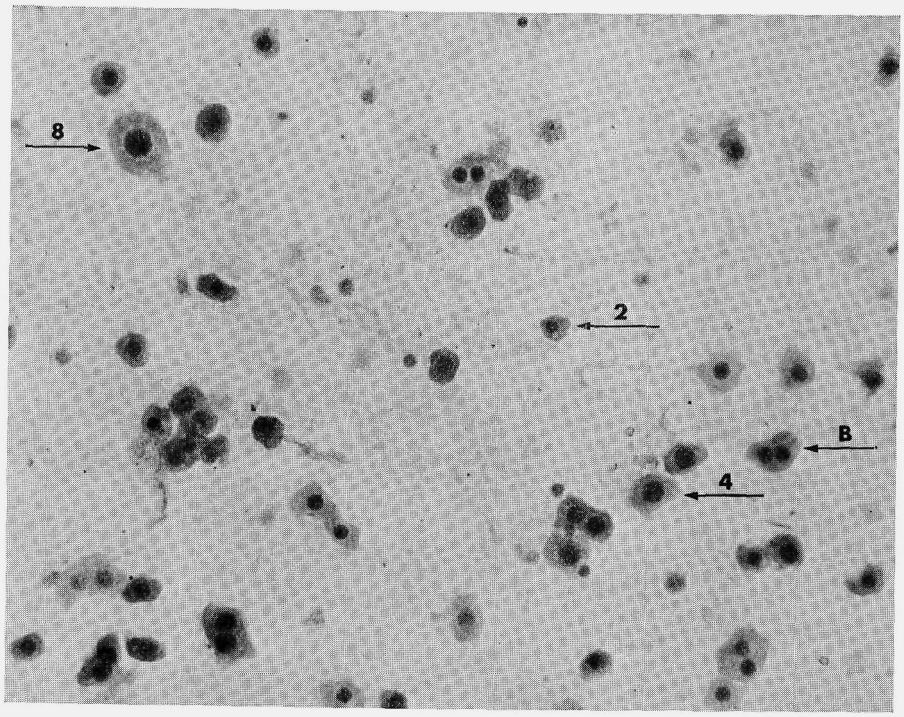

Fig. XII. Example of a liver cell suspension. This suspension was prepared from liver of a ♀ 27 months old rat, partially hepatectomized at the age of 3 months. (Magn. 360×).
2 = diploid; 4 = tetraploid; 8 = octaploid; B = binucleate

measured in order to obtain significant results. James et al. (1966) measured 2000 nuclei per rat. We decided to choose the latter figure.

2. Results

Measuring 2000 nuclei per animal, we first of all found that the frequency distribution of cell diameters shows distinct peaks at relative sizes of 1.00, 1.24 and 1.56 (fig. XIII). This is in agreement with the assumption that the tetraploid has twice the volume of the diploid nucleus and that the octaploid nucleus is twice the volume of the tetraploid. Theoretically the relative diameter sizes would be 1 : 1.26 : 1.58. Since our findings closely agreed with the theoretical values, nuclei within the relative size range of 0.88 to 1.10 were counted as diploid, between 1.11 and 1.34 as tetraploid and from 1.35 to 1.62 as octaploid.

The distribution of diploid, tetraploid and octaploid nuclei was thus determined in 3, 6, 12, 24 and 27 months old RU rats, using two animals at each age level.

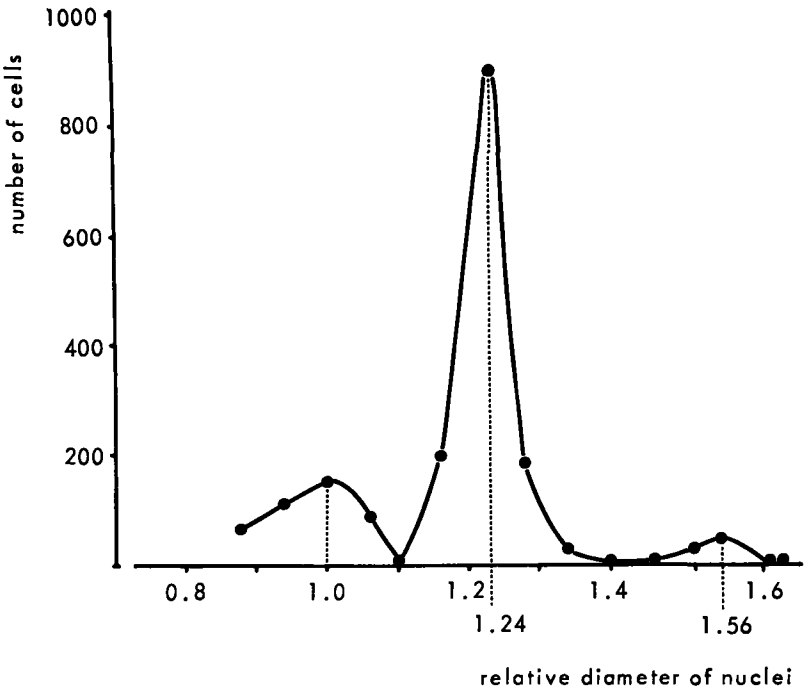

Fig. XIII. Distribution of liver cell nuclei over relative size classes in an untreated 27 months old ♀ RU rat.

The results are shown in table XIII. It is evident from this that between 12 months and 27 months no significant differences occur. There is however a shift to polyploidy between the ages of 3 and 6 months and between 6 and 12 months which correlates with the results obtained in the function tests (cf.

Table XIII. Distribution of nuclear classes in livers of untreated ♀ RU rats. Per age group two animals were used, 2000 nuclei being measured per animal.

Age in months	Diploid (%)	Tetraploid (%)	Octaploid (%)
3	73.6	24.2	2.2
	70.6	26.2	3.2
6	55.7	40.9	3.4
	52.1	43.7	4.2
12	31.3	59.2	9.5
	28.7	63.4	7.9
24	27.1	64.7	8.2
	30.5	60.5	9.0
27	28.3	63.0	8.7
	25.7	68.4	5.9

Table XIV. Distribution of nuclear classes in livers of 27 months old ♀ RU rats after liver regeneration.

	Diploid (%)	Tetraploid (%)	Octaploid (%)
Untreated			
27 months old rats	28.3	63.0	8.7
(cf. table XIII)	25.7	68.4	5.9
Partial			
hepatectomy at 3 months	28.9	63.4	7.7
	26.1	67.2	6.7
Partial			
hepatectomy at 24 months	25.8	64.5	9.7
	24.2	62.3	13.5

chapter VIII, Partial hepatectomy). The latter period coincides also with the time of more rapid restoration of liver function following partial hepatectomy (cf. chapter VIII).

The next experiments were designed in order to determine the distribution of polyploid cells in regenerated livers after partial hepatectomy. Female RU rats were used which were sacrificed at 27 months of age. One group was partially hepatectomized at 3 months, while in the other group the operation had taken place at 24 months of age. The experimental groups can be compared with normal control rats taken from the previous experiment. As can be seen in table XIV there was no significant change in the distribution of the nuclei after partial hepatectomy at three months of age. Animals operated at 24 months of age, however, showed some (but no significant) increase in the number of octaploid cells, when sacrificed three months later. This corresponds to the findings of James et al. (1966) discussed in chapter II, The liver during aging (Data from literature), indicating a tendency towards increased ploidy after partial hepatectomy. Furthermore, as confirmed by the group operated at 3 months, regenerative growth of the liver has no significant effect in the final state of the liver as expressed by the degree of ploidy.

CHAPTER X

AUTOPSY FINDINGS

Autopsies were performed for the express purpose of excluding from our studies possible complications not arising from the experimental treatment. These effects would tend to mask the effect of aging or experimental aging, which was our main interest.

1. Material

For the experiments described in this thesis, we used 64 normal control rats and 64 partially hepatectomized animals. Whenever possible, autopsy was done on these animals and tissues were preserved for histological observations. The animals were allowed to complete their life span and were autopsied when they were moribund. This occurred mostly between 24 and 27 months. The remaining animals were killed by ether anesthesia.

Tissues were fixed in formalin, imbedded in paraffin, mounted on slides and stained by haematoxylin, phloxin and saffron.

2. Results

Chronic respiratory disease (CRD) is endemic in many rat colonies (Innes et al., 1956; Ganaway and Allen, 1969). The disease can be regarded as a complex of two separate entities namely infectious catarrh caused by Mycoplasma pulmonis and enzootic bronchiectasis (endemic pneumonia) caused by a virus (Nelson, 1967). These diseases are of a long duration and death often occurs late in life. Since morbidity can be high in conventional colonies, CRD can be fatal for long term experiments. We anticipated no infections, because the animals were derived from a SPF colony which is quarterly tested for CRD. This was confirmed when autopsies were performed on the experimental animals. The respiratory organs showed no signs of chronic respiratory disease. In some animals the lungs were congested. At times grey white spots were visible on the surface of the visceral pleura. These consisted of clumps of foamy cells containing fat, such as those described by Bullock et al. (1968). In one case an alveolar cell carcinoma of the lung was found.

Many animals showed tumors of the pituitary resembling those seen by Kim et al. (1960). These consisted mainly of chromophobe cells along with a small number of eosinophils. In addition these animals exhibited fibro-adenomata of the mammary glands, stimulation of the mammae and ovarian abnormalities (cystic hyperplasia).

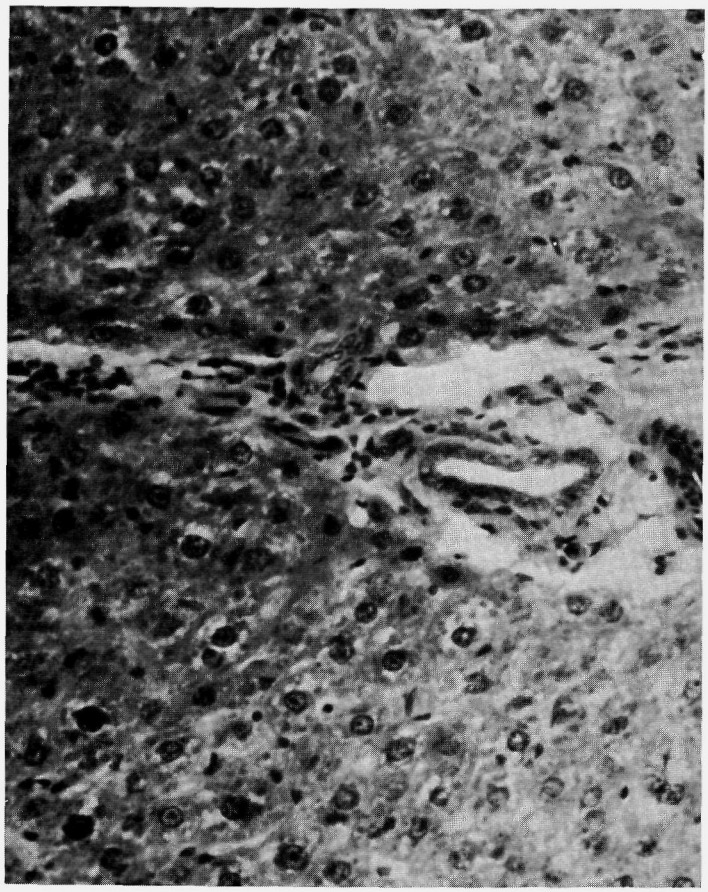

Fig. XIV. Liver of a ♀ 3 months old untreated RU rat. Magnification 360×

Eleven cases of an alveolar cell carcinoma or of a micro carcinoma of the thyroid such as described by Lindsay et al. (1968) were found. In seven cases a thyroid tumor was associated with a chromophobe adenoma of the pituitary. In the other four cases the pituitary presented the normal picture.

In one animal an adrenal cortical carcinoma associated with a chromophobe adenoma of the pituitary was seen.

Other abnormalities included one carcinoma of the salivary gland and one uterus carcinoma.

Except for tumors, the principal spontaneous disease seen in this rat strain was a chronic nephritis of the type described by Snell (1967). Macroscopically the kidneys were brown and in a few cases enlarged. Microscopically there were focal lesions. The glomerular membrane was thickened and there was proliferation of the epithelial cells in Bowmans space. There was atrophy and

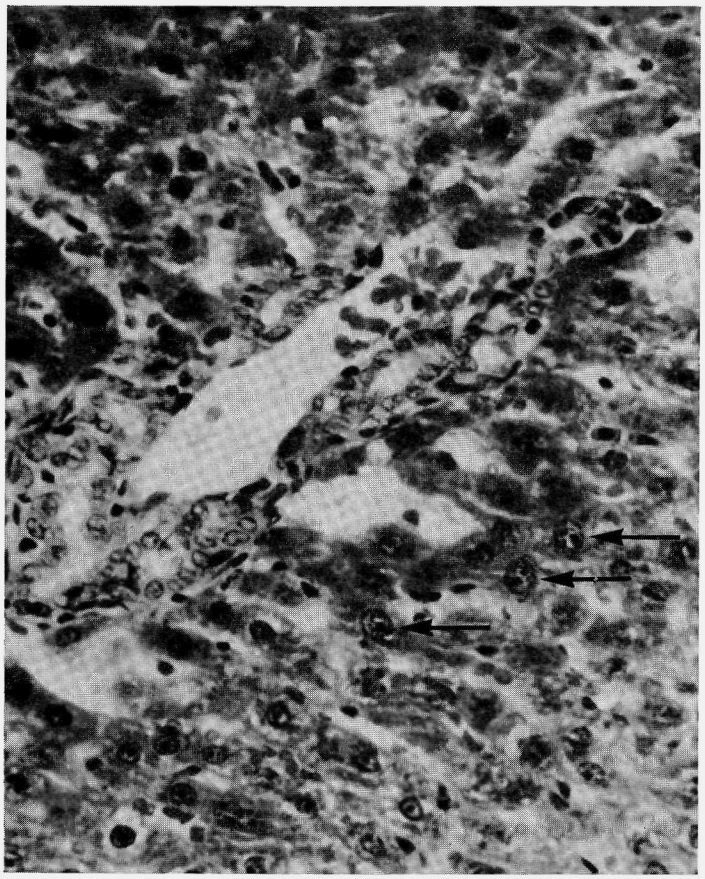

Fig. XV. Liver of a ♀ 27 months old untreated RU rat. Bile duct proliferation and polyploidy are evident. (←) Magn. 360×

hyalinization of the glomerular tufts and adhesions between glomerular tufts and Bowmans capsule. Atrophic and dilated tubules which were sometimes filled with proteinous casts were also seen. At times the stroma was slightly infiltrated with lymphocytes. Often only a few of such areas were found in a kidney. In one case the kidneys were distinctly hydronephrotic. On a few occasions, a chronic pyelonephritis was seen and one kidney carcinoma resembling a Grawitz tumor was found.

The pancreas sometimes showed atrophy of some of the lobules and shrinkage of acini.

The gross appearance of the stomach and intestines was normal. These organs were not investigated microscopically.

Myocardial lesions were sometimes observed, corresponding to the description by Fairweather (1967). These were characterized by slight fibrosis and in

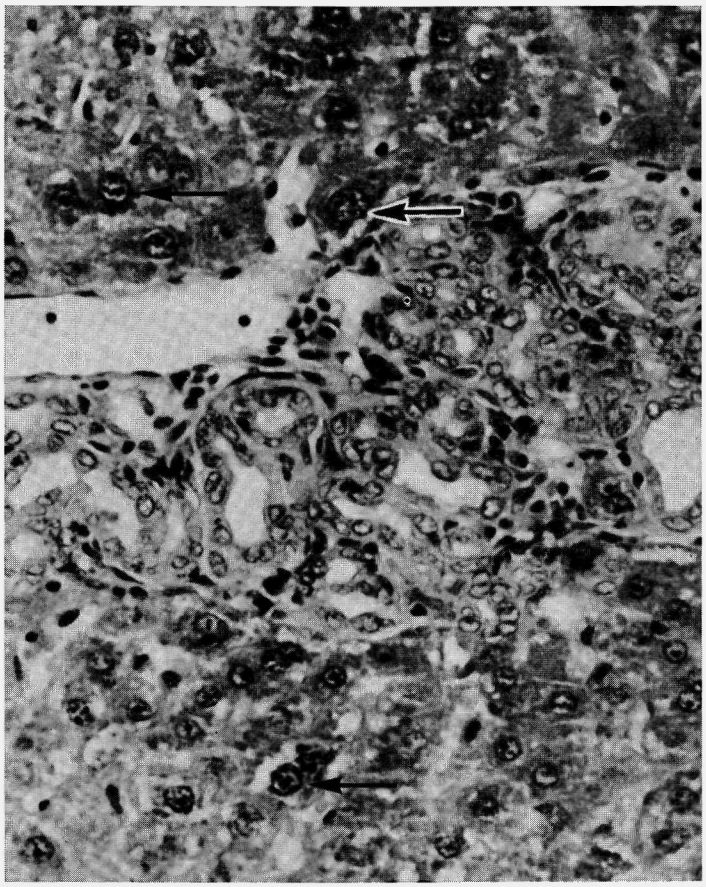

Fig. XVI. Liver of a ♀ 27 months old RU rat, partially hepatectomized at the age of 3 months. Polyploidy (←) and extensive bile duct proliferation are seen. (Magn. 360×)

one case in the left ventricle a focus which showed infiltration with mononuclear leucocytes around degenerating muscle fibers was found.

Special consideration was given to the liver. The livers of the untreated animals showed the usual number of lobes. The operated animals posessed only the two lobes remaining following partial hepatectomy but these were markedly increased in size. The liver weight – body weight ratio was assessed (cf. table XV). The occurrence of polyploidy was important and many binucleate hepatic cells were seen (fig. XIV, XV and XVI). The nuclei were round or oval and filled with heavily stained chromatin. Multiple nucleoli were evident and intranuclear inclusions were frequently seen. Sometimes there were islands of haematopoiesis. No signs of infection were apparent. Parasitic lesions were absent. Occasional cysts were present. Areas of bile duct proliferation were

Table XV. Summary of the most important autopsy findings.

	Number of rats	Age of rats at autopsy (months)	Liver weight body weight ratio mean ±S.D.	Bile duct proliferation	Pituitary adenoma	Mammary fibro-adenoma
Controls	50	18–24	3.2 ± 0.4	15×	19×	11×
	14	24–27	3.1 ± 0.2	7×	8×	7×
Partial hepatectomy	49	15–24	3.2 ± 0.3*	25×	22×	18×
	13	24–27	3.2 ± 0.4	7×	4×	3×

* One rat with a liver tumor not included.

observed, lying in a fibrous stroma, which appeared to be statistically more abundant in the partially hepatectomized rats than in the controls.

One partially hepatectomized rat showed a hepatoma. This exhibited a trabecular structure with wide spaces filled with blood between individual trabeculae. The cells of the hepatoma, as well as the cells of the rest of the liver showed a vacuolar degeneration of the cytoplasma.

No further microscopic differences between partially hepatectomized and untreated animals were noted.

The most important of these autopsy findings are summarized in table XV.

CHAPTER XI

SUMMARIZING DISCUSSION AND CONCLUSIONS

On reviewing the literature, one is impressed by the multitude of theories of aging and many random facts collected concerning aged people and animals. There is no clear picture as to what exactly occurs during the aging process or why the events take place. There seems to be, however, general agreement that as an individual ages, processes occur that lead to less efficient functioning of many organs. This is reflected in the fact that the efficiency or reserve capacity of many functions are diminished. Many of these processes seem to develop perhaps slowly during the life of the individual. The age at which they first become apparent, and the rate at which they develop, may differ in various organs. For the systematical development of gerontological knowledge, it is of interest, therefore, to inventorize data and changes throughout life, in all vital organs. One organ on which little gerontological work has been done is the liver.

The present study, therefore, was aimed at analyzing functional and structural changes of the rat liver with age. It seemed that at the age of three months the liver had already attained its full size and functional reserve capacity. Between three and nine months this reserve capacity as measured through BSP tests decreased markedly. This indicates an early onset of functional decline which can hardly be called an effect of old age. It exemplifies, however, how each organ may have a life cycle of its own, with crucial changes occurring at different ages (cf. chapter I).

Within the context of these studies, the early functional decline, however, is limited to the way in which the rat liver handles BSP. In other respects the liver may be just as active at 24 months as at 3 months of age. It was actually found that functional regeneration after partial hepatectomy as measured through BSP tests occurred more rapidly in 12 months old rats than in 9 months old rats. For animals that were 15 months of age or older at the time of the operation, this aspect of liver function was already restored after two weeks.

When we determined the percent distribution of nuclear ploidy classes in the liver, there were significant differences in ploidy percentages between the livers of 3 and 6 as well as between 6 and 12 months old rats. With increasing age there was a shift from a diploid to a polyploid cell population. After 12 months, no further changes in ploidy distribution were found.

When one compares these findings with the results of the BSP studies, it might be concluded that there is a relationship between the proportions of various nuclear classes and the handling of BSP. It was demonstrated that in

livers with a high degree of polyploidy, plasma retention of BSP was increased and relative storage capacity was decreased. Polyploid cells are larger than diploid ones. Thus, old rat livers have fewer cells and a lower total cell surface per unit volume than is found in the young animals. This provides the following explanation for the observed decrease in BSP clearance.

According to Cornelius et al. (1967) hepatic cell membranes have a specific BSP-binding capacity and may thus play a crucial role in the processes of BSP-handling by the liver. These authors actually calculated quantitative relations between the amount of cell membrane material and the BSP clearing capacity of rat liver. Average liver cell size and the relative cell surface as a function of cell size, may thus be an important determinant for liver function as measured by BSP tests.

The polyploid state of the cells may also explain why in animals older than 12 months, "functional regeneration" takes place much more rapidly than in young ones. As mentioned above the old rat liver is composed largely of polyploid cells while that of young animals contains few such cells. According to James et al. (1966) the regenerated liver of the young rat contains more polyploids than that of the controls. The appearance of those large polyploid cells might lead to impairment in BSP uptake, since there is an overall decrease in cell surface-liver volume ratio. The livers of the old animals, however, will regain more rapidly the original degree of ploidy than the young ones. This return to original state is accompanied by a return to the original level of function.

Since both liver function and cell ploidy stabilize between 6 and 12 months and since no further changes occur with aging or following partial hepatectomy, it seems that the liver is not a suitable model for the study of accelerated aging. As was mentioned earlier, however, each organ may have a life cycle of its own and the rat liver seems to show little senile degeneration up to the age of 27 months. Its capacity for regenerative growth confirms this conclusion and our findings can be considered as representative of the life cycle of an organ which has great reserve capacity up to old age. This cycle is completely different from that of other organs such as the gonads which show an early decline, or the CNS which shows a steady decrease of reserve capacity with increasing age. In terms of our original hypothesis, that aging involves the exhaustion of genetic programmes for the structure and functions of cells and organs, we have not been able to demonstrate the time-limits of this programme in the case of the rat liver. We have, however, found a number of age related changes in both structure and function of the rat liver which may be summarized as follows:

1. Relative liver weight as well as albumin production remain constant from 3 months up to 27 months of age.
2. Both aspects show considerable reserve capacity, since partial hepatectomy

is followed by a complete recovery in these respects, whether the operation takes place at younger or at older ages.

3. Relative storage capacity for BSP decreases during the first part of the total life span, but it stabilizes from 6 or 9 months onwards.
4. This latter aspect is related to a shift in liver cell population towards increased polyploidy, which also stabilizes during the first half of the total life span and shows no further change from 12 months onwards.
5. The latter two aspects are also not affected by partial hepatectomy. After a period of regeneration, both parameters regain original values whatever the age at which the animals are operated.

These findings, of course, raise further issues of gerontological relevance, such as the functional significance of liver cell polyploidy. This and other questions may be a starting point for further study of the life cycle of the rat liver.

SAMENVATTING

De wens om de ongemakken van de oude dag te verminderen of, nog liever, te voorkomen vormt het voornaamste motief om zo veel mogelijk informatie over verouderingsprocessen te verzamelen en de losse informatie zo goed mogelijk in een systeem te rangschikken.

De rattelever, versneld verouderd, naar wij postuleerden, door de regeneratie die optreedt na een partiele hepatectomie, leek ons een geschikt model voor verouderingsonderzoek. Het was namelijk uit de literatuur bekend dat in onbehandelde levers van mens, rat en muis de celpopulatie gedurende het leven verandert van hoofdzakelijk diploid naar vooral polyploid. Bovendien was bekend dat, bij muizen en jonge ratten, korte tijd na een partiele hepatectomie de geregenereerde lever het polyploid aspect heeft van een veel oudere lever. Wij vroegen ons af of de veroudering en de eventuele versnelde veroudering te kwantificeren waren, terwijl onze proefdieren in leven bleven. Dit zou ons de mogelijkheid geven om de leverfunctie gedurende het hele leven van één groep proefdieren te vervolgen. En bovendien biedt een besparing aan proefdieren economische en ethische voordelen. Om de leverfunctie te meten gebruikten wij methoden uit de klinische chemie, waar men immers gewend is het functioneren van organen te meten, terwijl de subjecten in leven blijven. Omdat de rat een klein proefdier is en dus weinig substraat voor onderzoek kan missen, werden ultramicromethoden gebruikt.

Op twee manieren werd de leverfunctie getest. Ten eerste door een bepaling van het serum albumine gehalte. Dit meet het „eiwit producerend vermogen", omdat albumine alleen in de lever geproduceerd wordt. Ten tweede werd een bromsulphaleine (BSP) belastingstest uitgevoerd, waarmee het vermogen van de lever wordt gemeten om een stof die in het bloed aanwezig is, op te nemen en naar de gal toe uit te scheiden.

Het onderzoek bestond uit twee delen. In het eerste werd een groep oude ratten vergeleken met een groep jonge. Daarna werd het onderzoek voortgezet met de jonge ratten. In het tweede deel werden verschillende groepen van 8 ratten op steeds hogere leeftijd partieel gehepatectomeerd en werd bij hen en bij hun even oude en even grote controlegroepen de leverfunctie vervolgd tot aan hun natuurlijke dood, of tot het eind van het experiment. De overgebleven ratten waren toen 24 of 27 maanden oud.

Voordat met het eigenlijke onderzoek begonnen werd, werd eerst vastgesteld dat er geen verandering in het relatieve plasmavolume (Hoofdstuk IV) noch in de lichaamsgewicht-levergewicht ratio optrad (Hoofdstuk VI).

De eerste vraag die wij ons stelden was of er een verandering van leverfunctie gevonden werd bij verouderende onbehandelde levers (met onze meetmethoden). Dit bleek niet zo te zijn, wat de albumine produktie betrof (Hoofdstuk V).

Het vermogen om BSP uit het bloed te verwijderen, was echter bij de oude ratten minder groot dan bij de jonge (Hoofdstuk VI). Toen het onderzoek echter met de jonge ratten voortgezet werd bleek de verandering al op tamelijk jonge leeftijd op te treden namelijk op 6 en 9 maanden (Hoofdstuk VI en VIII).

Bij een poging om de oorzaak van deze verandering na te gaan werd BSP per infuus toegediend. Met deze methode, die beschreven was voor mensen en honden, en door ons aangepast werd aan de rat, bleek dat het vermogen om BSP te stapelen bij oudere ratten verminderd was, maar het vermogen om BSP uit te scheiden niet.

Verder hielden wij ons bezig met de vraag of partiele hepatectomie het verouderingsproces versnelt (Hoofdstuk VIII). Dit bleek niet zo te zijn. Althans noch het albumine gehalte van het serum, noch het vermogen om BSP uit het bloed te verwijderen waren na een partiele hepatectomie op de lange duur veranderd ten opzichte van de controle ratten. We vonden echter wel iets wat ons aanvankelijk verbaasde. De leverfunctie, gemeten met BSP, was na een partiele hepatectomie bij oude ratten sneller hersteld dan bij jonge.

Dit verschil en ook de verandering in BSP retentie bleek gecorreleerd te kunnen worden aan een verandering in ploidie van de levercelpopulatie. De toename van het aantal tetra- en octaploide celkernen bij stijgende leeftijd bleek parallel te lopen met de afname van het BSP stapelingsvermogen (Hoofdstuk IX).

Onze verklaring voor de vermindering van het vermogen om BSP te kunnen stapelen bij stijgende leeftijd is, dat de polyploide cellen van de oudere lever relatief een minder groot celoppervlak hebben terwijl juist de celmembranen essentieel zouden zijn om BSP aan de cel te binden.

Tenslotte menen wij dat ieder orgaan „op eigen wijze oud wordt" en dat de lever beschouwd kan worden als een orgaan dat lang zijn reserve capaciteit bewaart. Daardoor was het ons onmogelijk de tijdsgrenzen van dit proces te vinden, hoewel we wel een aantal aan de leeftijd gebonden veranderingen zagen.

Tenslotte laat dit onderzoek nog heel wat vragen open. Wat is bijvoorbeeld de functionele betekenis van polyploidie in het leverweefsel? Moet het beschouwd worden als een rijpingsverschijnsel of is het daarentegen als een vroeg optredende veroudering (deficiente celvermeerdering) te beschouwen? Deze en nog heel wat andere vragen zouden een uitgangspunt kunnen zijn voor verdere studie van de levenscyclus van de rattelever, wat ons weer moet leiden naar uitbreiding van onze gerontologische kennis.

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