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# Cellular Death and Necrosis: Chemical, Physical and Morphologic Changes in Rat Liver

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With 22 figures in the text

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## Introduction

The process of cellular death has received relatively little attention in recent years. Still, if we critically examine our present concepts of necrosis, necrobiosis, autolysis, or even necrolysis (GUILLERY 1939a) and necrophanerosis (MUELLER 1955); and of the so-called degenerations preceding cellular death, with such time-honored names as *cloudy swelling, hydropic degeneration,* or new names such as *pathobiosis* (ALTMANN 1955), there can be no doubt that much of our knowledge pertaining to this field lacks in clarity.

This is not surprising, for the recognition of cellular death lies at the limit—and perhaps just beyond reach—of histological and histochemical methods. Suffice it to mention that the cells which we are accustomed to accept as normal in autopsy material have actually undergone not only death by acute ischemia, but also, in most cases, autolysis for several hours. This very fact, that cells may die without immediately showing any major distortion of their microscopic features, has allowed us to acquire most of our knowledge in the field of morbid anatomy. On the other hand, it has also brought about a peculiar shift in emphasis. The actual process of cellular death, the true catastrophy which is not witnessed in tissue sections, goes almost unmentioned, and the pertinent literature is very limited. In contrast, the secondary changes which transform a dead cell into an obvious lump of debris are brought into prominence under a special name, *necrosis*, and this topic has attracted considerable attention.

The habit of studying tissues in fixed and stained preparations has also helped to eclipse the concept of cellular death. We have become accustomed to ignore the fact that in a tissue section *all* the cells are dead (ZOLLINGER 1948a, GROLL 1949, MUELLER 1955), and this again shifts the emphasis to necrosis, typified by the loss of the nucleus. As a matter of fact, in the contemporary literature, it is often apparent that the distinction between cellular death and necrosis has vanished altogether: the cell is assumed to die when it loses its nucleus. This misconception is to be found even in textbooks (SMITH et al. 1959).

Having opposed the concept of necrosis to that of cellular death, it remains to be stated what is meant by the latter. A satisfactory definition can only be in terms of function, because dead (fixed) cells can be morphologically intact. One may recall that the definition of somatic death is usually stated in terms of prolonged cessation of respiration and circulation. In the case of an individual cell, respiratory activity is not an adequate index of viability, since the debris of crushed or homogenized cells can, under many conditions, be shown to respire. In fact, a number of isolated enzymatic activities may be demonstrated even after the appearance of histologically recognizable "necrosis" (BERENBOM et al. 1955a and b). On the other hand, a cell expends much of its energy in maintaining its

inner environment different from the outer *milieu*. When the cell dies, the distinction between internal and external environment disappears (Lucké and McCutcheon 1932, Davson and Danielli 1952, Barer et al. 1953, Giese 1957, Kaltenbach et al. 1958). A definition of cellular death may be based upon the loss of this general function of "cellular homeostasis": a cell is dead when it is no longer capable of preserving, in a normal environment, the specific composition of its inner *milieu*.

In a literal sense, however, this definition is difficult to apply in any given case without complex quantitative analyses of the intra- and extracellular environments. At the present state of our knowledge, it seems preferable to base a definition of cellular death upon the notion of irreversibility (see also Harman and Gwinn 1949). We will consider cellular death as a process, during which the cell loses ist integrity as a functional unit. This process is characterized by a "point of no return"; that is, by a singular point beyond which the cellular damage is irreversible. The *time of death* can be reasonably identified with the time at which the "point of no return" is reached.

Operationally, the "point of no return" can be determined *a posteriori*, by studying the capacity of the cells to survive a given injury of graded intensity. When the "point of no return" is thus determined, it can be correlated with various biochemical and physical changes, as was done in the present study.

In summary, then, if death of a tissue is brought about by interruption of the circulation, as in our experiments, one should expect to observe the following steps: 1. a period of *reversible alterations*, 2. a point of no return, which we consider the time of *death*, 3. a period of equilibration between the cell and its environment, with little or no changes in the structures visible with the optical microscope (we may refer to this period as *pre-necrotic*), 4. post-mortal changes recognizable with the microscope, or *necrosis*.

To date, there seems to have been no integrated study attempting to define, even at a simple level, the morphologic and biochemical events characteristic of these stages. Much of the data in this field are old and need to be confirmed by modern methods; perhaps this chapter of General Pathology more than any other is obscured by loose terminology and traditions not securely founded on facts. For instance, the early and presumably reversible cellular changes caused by acute injury are usually referred to (see Cameron 1951) with a name coined by Virchow: "trübe Schwellung", literally "opaque swelling", which has been translated under the supervision of Virchow himself as *cloudy swelling* (Virchow 1860). This term, still very much alive 113 years after its birth[1], has been a constant source of argument among scholars and of bewilderment among students. While a swelling is sometimes observed, in the great majority of cases it is vain to search through the microscope for any opacity or "cloudiness". The faint "granules" which sometimes appear in the cytoplasm are generally believed—as will be discussed below—to be swollen mitochondria. Mitochondria can be made to swell quite easily and reversibly *in vitro*; however, as they swell, the opacity of the mitochondrial suspension *decreases* (Cleland 1952, Raaflaub 1953, Price and Davies 1954, Tedeschi and Harris 1955, Lehninger 1956, Beyer et al. 1955, Witter and Cottone 1956,

[1] In his article on Parenchymatous Inflammation, a term which he uses synonymously with Cloudy Swelling, Virchow states that the former term had been used previously by the "older school", but in another sense (Virchow 1852). A footnote then recounts how the new concept was first expressed in a series of lectures at the Charité in 1846: and how two years later there appeared a thesis on *Morbus Brightii*, by one Herr Niemann, announcing "without any reference to the source" that the major changes in this condition lie in the Canaliculi contorti: "... *Epithelii cellulae majores fiunt, endosmosi aucta, eorumque contentum nubilum turbidumque fit* ..." History seems to have done ample justice to the "source".

RECKNAGEL and MALAMED 1958). It is not surprising that some pathologists should now regard "cloudy swelling" as akin to a myth, while others have taken to using the term at the level of gross description, for organs which look large and pale, without referring to any particular histological change (BELL 1913). Despite a century of debate on the nature of "cloudy swelling", there has not been—to our knowledge—a single attempt to pinpoint the elusive cloudiness by a simple measurement of optical density. This we have done, with a rather unorthodox result: before they die, the cells do not become "trüb" at all; under the conditions of our experiments, they actually become more transparent.

In recent times several authors have tried to shed some light on "cloudy swelling" by studying biochemical changes in the livers and kidneys of animals which had been injected with a lethal dose of a toxic drug one or more *days* prior to the time of sacrifice (POPJÁK 1948, FONNESU and SEVERI 1952, 1953, 1954, FONNESU 1954). Data obtained in this fashion must be considered, of course, as averages of regressive and superimposed reparative phenomena, with the added complication that such phenomena are not likely to be synchronous in all types of parenchymal cells, particularly in the kidney. Hence these contributions, while useful as descriptive of a diseased organ as a whole, are of more limited value with regard to events taking place at the cellular level.

"Albuminous swelling" is sometimes used instead of cloudy swelling. This term was proposed by HOPPE-SEYLER who stated that organs "affected by cloudy swelling" also contained abnormally high amounts of protein, a finding allegedly consistent with Virchow's notion of "Trübung" (HOPPE-SEYLER 1921, 1923, 1927, 1928). Perusal of the original papers will show that this oft-quoted contribution is based on erroneous assumptions (see Discussion).

With reference to the late stages, or *necrosis*, a number of points are still open to question. The term "coagulation necrosis" implies that the cellular proteins are denatured and coagulated; this process has not been studied quantitatively, neither is it known whether it precedes, accompanies, or—as is more commonly thought—follows the death of the cell. It is common belief that coagulation necrosis can occur only *in vivo*, whereas tissues preserved *in vitro* merely undergo autolysis (BECHHOLD 1919, BORGER, BAYERLE et al. 1935, BORGER and MAYR 1935, BAUER 1943). This belief, however, has no sound experimental basis, and our data indicate that it is incorrect.

It should not be necessary to emphasize any further the need of additional knowledge with regard to "cloudy swelling", cellular death, and necrosis. In planning our experiments, we did not hesitate to start afresh at a very simple level. It seemed essential, in the first place, to dissociate the cellular changes which are clearly regressive from those of reparative nature. This can be achieved by studying relatively large fragments of an organ, isolated, and therefore allowed to die, either in the incubator or within a living animal. This method has an additional advantage in that the injurious agent is applied to all the cells at essentially the same time; hence a chronologic study of the subsequent events is facilitated, and from the data pertaining to the tissue as a whole it is possible to extrapolate, within reasonable limits, to changes at the level of cells.

The experiments here reported were performed for the most part on fragments of rat liver, implanted in the peritoneum of other rats, or preserved aseptically *in vitro* at 4° or 37° C. Time-curves were constructed for all the variables under study, and particular emphasis was placed on the early stages. Samples were examined with respect to wet weight, dry weight, $p_H$, and extractable protein as measured with a microbiuret method. Protein denaturation was followed by the study of fresh, unfixed sections in the dark field, using visible as well as ultraviolet light; and by measurements of optical density in transmitted light. The oxygen uptake of incubated slices was measured by Warburg respirometry, and morphological changes were studied at all stages.

## Material and methods

Male rats of the Sprague-Dawley strain were used; they were 2—4 months old and weighed 250—350 gr. Each experiment was performed with rats of identical age. All animals were killed by decapitation and bleeding; they were not starved prior to sacrifice.

*Preparation of liver implants.* A donor rat was sacrificed, and its liver was removed to a chilled surface. The median, right, and left lobes were cut into broad strips using two parallel razor blades mounted 8 mm apart, and the strips were subsequently trimmed to an average size of 6 × 8 × 15 mm., corresponding to 0,5—0,7 gr. Each fragment was rapidly weighed and introduced into the peritoneal cavity of a rat previously anesthetized with ether, and then the small abdominal wound closed with nylon thread. After an appropriate time interval the recipient rat was sacrificed, and the implant recovered. When the implantation was performed without surgical asepsis, bacterial cultures taken 24 hours later on blood-agar plates showed that most of the implants had become infected. When sterile implants were required they were prepared from the livers of rats which had been intraperitoneally injected with 20000 units each of penicillin and streptomycin 30 minutes prior to sacrifice. The operation was performed with strict asepsis, and the wound was closed in two layers with metal clips. Infection of these implants (checked by means of smears on blood-agar plates) never occurred.

About half of the operations were performed without sterile precautions. For the first 3—4 hours at least, the effect of bacterial growth on our measurements can be disregarded. For later stages, a number of controls were performed, by comparing aseptic and infected implants with respect to wet and dry weight and optical density, up to 24 hours. Significant differences were not found.

*Dry weights* were obtained by desiccating the samples at 110° C to constant values. In early experiments the whole implant was used, but when it was found that during the first few hours the superficial and deep portions of the implant acquired a different appearance, the specimens were trimmed to separate the surface (to a depth of 1—2 mm.) from the inner core; dry weights were then measured separately on each type of sample.

*Measurements of* $p_H$ were made with a standard Beckman $p_H$ meter. It proved difficult to press fluid out of the liver fragments; the measurements were therefore made on tissue pulp, obtained by rapidly grinding the specimen in a watch-glass with the plunger of a glass syringe. With this method it is possible to obtain values of "intracellular $p_H$" similar to those determined with methods which are in principle more accurate (CALDWELL 1956). The time-lapse between the excision of the specimen and the reading on the $p_H$ meter was less than 2 minutes.

*Extraction of proteins and determination of protein content.* The purpose of this analysis was to determine the amount of soluble protein and of protein breakdown products. Because most of the nuclei disappear in the course of liver necrosis, and the nucleoproteins are either broken down or washed out, it was surmised that this might be reflected in the amount of total extractable protein, and possibly obscure any variations occurring in the amount of protein extractable from the cytoplasm. Extraction was therefore performed with acetate buffer at $p_H$ 5,80 (ionic strength 0,15) which in the case of bovine liver extracts only the soluble proteins, to the exclusion of the nucleoproteins (COHN, SURGENOR, and HUNTER 1951). The concentration was then determined with a microbiuret method[1].

A fragment of tissue was weighed to the nearest mg on a torsion balance, and homogenized in a mortar with a small quantity of sand. Samples from normal livers, used as controls, weighed 30—80 mg; samples from the implants were taken from the inner cores only, as specified above, and weighed 50—100 mg. The ground tissue and sand were diluted with about 0,5 ml of buffer and transferred to a 25 ml Erlenmeyer; the mortar was washed three times with 1 ml of buffer, the washings were added to the contents of the Erlenmeyer, and the latter was brought to 13,5 ml (the volume of the Spinco tubes) with additional buffer. This final extract was then centrifuged for 2 hours at 100000 g in a Spinco ultracentrifuge in order to free it of all particulate matter, and the protein content was determined in the supernatant with the microbiuret method, as described below.

The biuret solution was prepared from 1 liter of 40% NaOH by adding dropwise, while stirring, 200 ml of 1% $CuSO_4 \cdot 5\ H_2O$. One ml of this solution, 1 ml of water and 1 ml of the extract were mixed and let stand 10 minutes (Solution A). Readings were taken in the Beckman Spectrophotometer with constant slit width (approximately 0,3 mm) at 290 and 390 mμ.

[1] We are indebted to Dr. MARGARET HUNTER of the Laboratory of Physical Chemistry, Department of Biological Chemistry, Harvard Medical School, for the details of this unpublished method.

At the same wave lengths, readings were also taken from a solution containing 1 ml of water, 1 ml 40% NaOH and 1 ml of extract (Solution B). The readings obtained from Sol. A were then subtracted from those of Sol. B, at both 290 and 390 m$\mu$. The final reading at 390 m$\mu$ was subtracted from the final reading at 290 m$\mu$. This difference is proportional to the number of peptide bonds present; as little as 20 micrograms per ml protein can be determined with reasonable precision. The biuret should be present in large excess. As a standard, appropriate dilutions of a protein solution containing 0,15 gm human albumin in 50 ml water were used. (Throughout the work we consider that biuret measures peptide nitrogen. It should be pointed out that serine, ethanolamine, threonine, glutamic acid and amides at much higher concentration also give a positive reaction.)

*Measurement of optical density.* The sample to be examined (either normal liver or a portion of an implant) was trimmed to the size of a cube about 6 mm on edge, and cut into slices 0,24 mm thick using a microtome which has been described elsewhere (MAJNO and BUNKER 1957). One such slice was carefully spread out in each of the two chambers of a Thoma-Zeiss blood-counting apparatus moistened with physiological saline solution. When the calibrated coverslip was clamped over the chambers, the slices were uniformly flattened and tended to spread out somewhat, being compressed to the depth of the chamber, i.e. 0,20 mm. The apparatus was then transferred to the stage of a microscope which had been set up for measurements of optical density (white light). This was a binocular microscope, with a vertical tube for a camera attachment; into this tube was fitted the sensitive unit of a Photovolt spot photometer. A 10 × objective and 8 × oculars were used in all experiments. In order to reduce the cross-section of the beam illuminating the specimen, the stage was covered with a metal plaque, perforated by a single hole 1,5 mm in diameter centered on the optical axis of the microscope. A slide was brought into focus, and a field selected using the binocular. By means of a prism the beam was then switched to the photometer, and a reading was taken. The possibility existed that varying amounts of hemoglobin present in the section might introduce an error in the measurements. A second reading was therefore always taken, after a red filter (Corning No. 2418) had been placed below the condenser. Prior to use on each slice the optical system was calibrated by introducing a standard neutral density filter in place of the preparation, and then the source of light was adjusted by means of a rheostat to give a reading of 1,40 on the photometer. Six different fields were selected in each slice. Each field corresponded to 1—2 hepatic lobules. Since the slice was several cells deep, little cytological detail was recognizable; however, the lobules and their radial structure stood out clearly. The fields were selected at random within the central area of the section; fields with one or two central veins, visible as clear round openings representing about 1/150 of the total field, gave the same readings as contiguous areas with no vascular openings.

The measurements of optical density obtained from a given liver were quite consistent. On a single liver slice measurements in white light showed an average variability of 4,3% with a maximum variation of 6,5%. With red light the variability was about 2%. Slices from the same liver gave readings with an average variability of 1,75% with white light and 1% with red light. The variability between different livers was somewhat greater, possibly on account of different glycogen content; hence the optical density of a pathologic specimen was always referred to the initial value for the same liver. Two to 4 slices were cut from each sample, 6 readings were taken from different areas in each slice, and the points shown on the graphs represent the grand average of these figures. When using fresh, normal liver it was found that the readings tended to drop after 10—15 minutes, even if the organ was kept refrigerated; hence the control values had to be obtained within this time.

*Examination in the dark field by visible and ultraviolet light.* Liver implants were prepared, and the recipient animals were killed after intervals of 15, 30, 60 minutes and 2, 4, 8 and 72 hours. When recovered, each implant was halved, and the cut surface of each half was applied against a similar fragment of fresh, normal liver to serve as a control. The two fragments, thereafter treated as one block, were placed in a small beaker, frozen by surrounding the beaker with a mixture of acetone and dry ice, and cut in a cryostat (COONS and KAPLAN 1950). The slices, 5 and 10 micra thick, comprised equal portions of the implanted and control liver, attached along the midline. They were dried with a fan, placed under coverslips with a 7:3 mixture of glycerin and phosphate buffer at $p_H$ 7,4, preserved at —20° C, and examined within a few hours of cutting. *Dark-field* observations in visible light were carried

out with a Zeiss Ultraphot II microscope, using a powerful source of light (high pressure mercury-vapor lamp with no interposed filters) and a dark-field condensor. Photographs were taken at 200× in areas comprising equal portions of the normal and experimental liver. Examination by *ultraviolet light* (autofluorescence) was accomplished under the same conditions as just described, except that a black glass filter (Corning No. 5874/M802, 5,05 mm) was interposed for eliminating the visible portion of the spectrum. Owing to the large dimension of the negatives, and very low ranges of fluorescence, the Ansco Super Hypan plates had to be exposed for 30 minutes.

*Autolysis "in vitro"*. For the study of $p_H$ and optical density during autolysis *in vitro*, liver lobes were allowed to stand aseptically at either 37° or 4° C. The samples were prepared as follows: a donor rat was injected i.p. with 20000 units each of penicillin and streptomycin: 30 minutes later it was sacrificed, the liver was excised aseptically and one lobe was placed in each of 3 sterile Petri dishes; a fourth lobe was kept for the determination of the initial $p_H$ and optical density. The Petri dishes were lined with filter paper moistened with sterile horse serum, and the dishes themselves were maintained—in the constant temperature rooms—inside watertight containers maintaining 100% humidity. At appropriate intervals a sample was excised with sterile precautions, smeared on a blood-agar plate for control of asepsis, and used for the determination of $p_H$ and optical density as usual. All cultures remained sterile with the exception of one from a Petri dish kept at 37° C, which became contaminated after repeated sampling.

*Measurement of oxygen uptake.* Fragments of fresh liver, and inner cores of liver implants prepared as described above, were sliced (Majno and Bunker 1957) to a thickness of 0,4 mm, and transferred to chilled Warburg vessels prepared as follows: main chamber, 3,0 ml of phosphate-buffered Krebs-Ringer medium, with glucose as a source of energy (10 μM/ml); center well, 0,2 ml of 20% NaOH. The flasks were then brought to the constant temperature bath at 37° C, gassed with pure oxygen for 10 minutes, and after an additional 10 minute period of equilibration the first manometric readings were taken. Subsequent readings were taken every 30 minutes for 3 hours. In a typical experiment, triplicate samples of slices from a normal liver were compared with triplicate samples from implants at 3 different stages. The total uptake of oxygen by 100 mg of fresh liver in 3 hours was taken as a control value; results for each implant were expressed as a percentage of this control value.

*Histology.* All tissues were fixed either in 10% phosphate-buffered formalin or in Helly's fluid, embedded in paraffin, and stained with hematoxylin (alone or with eosin), Best's carmine for glycogen, and Cain's or Altmann's methods for mitochondria. Each block of paraffin always contained a fragment of normal liver, fixed and processed together with the experimental sample, in order to make the cytological comparison more reliable. For the study of sequential changes, rats bearing implants at the desired stages, and a control, were killed simultaneously. The implants were fixed, embedded, cut, and stained in one block with the control fragment.

*Planimetric measurements* were made on photomicrographs of material fixed in Helly, in order to verify the existence of any measurable cellular swelling. Sections of implants at the stages of 1, 2, 4 and 8 hours were photographed at 450 × together with a control. In view of the differences between central and peripheral portions, two series of pictures were taken: one in the central part, another about 10 cell-layers below the surface. It was found that on each photograph only about 20 cells could be identified which contained a whole nucleus and had distinct outlines; hence on each print 20 cells of these characteristics were marked off, and their areas were measured with a planimeter.

## Results

*Gross and histologic findings: implanted liver.* After 1 hour in the peritoneal cavity the liver fragments seemed unchanged to the naked eye; by comparison with fresh normal liver, however, they proved to be slightly paler. On the cut surface the peripheral rim was paler than the center. At about 6 hours this difference disappeared, and the entire specimen became progressively paler, until at 18—24 hours it had acquired the characteristic whitish aspect of necrotic

tissue (Fig. 1). Simultaneously there was an increase in the consistency of the tissue. An attempt to quantitate this change with a commercial "penetrometer"[1] was not successful. However, in slicing the samples for respiratory measurements, it was noticed that the slices were quite soft and pliable at $3^1/_2$ hours, while at $4^1/_2$ hours they were stiff and no longer tended to fold.

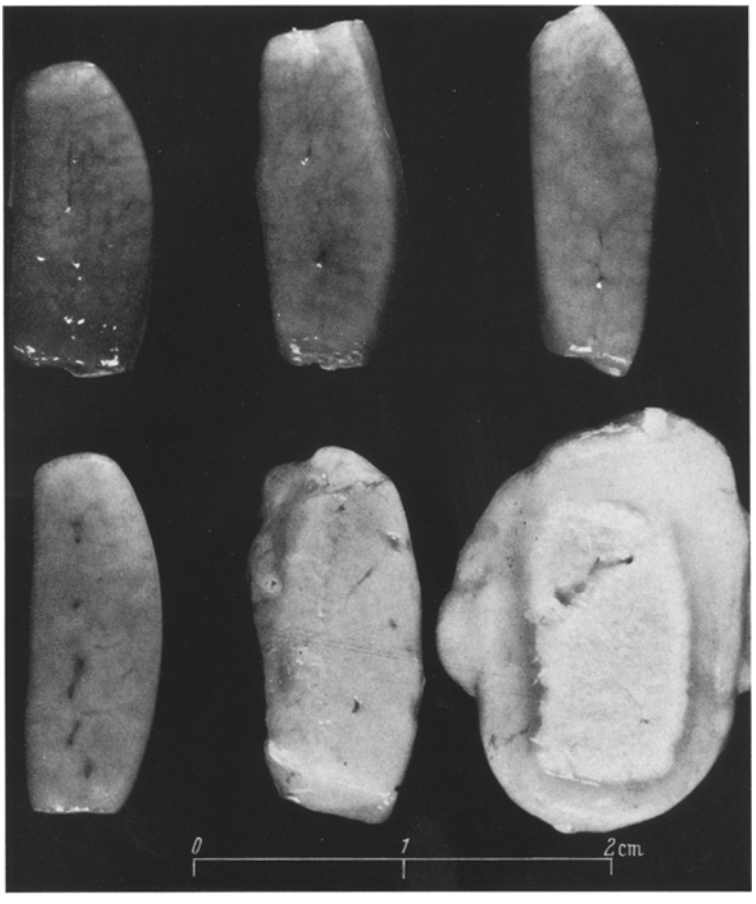



Fig. 1. Gross changes in fragments of rat liver which have been left in the peritoneal cavity for varying periods (hereafter referred to as *liver implants*). *Above*, left to right: normal control, stages of 2 and 4 hours. *Below:* stages of 12 hours, 24 hours and 9 days. Paleness is already apparent in the 2-hour specimen. At the 24-hour stage the tissue is very similar to a white infarct. Note adhesion at 24 hours (at top of specimen) and thick layer of granulation tissue at 9 days

Beyond 12—18 hours the implants became anchored by adhesions, usually to the omentum; an envelope of granulation tissue rapidly appeared, while the superficial portion of the liver fragment became bright yellow to a depth of about 1 mm. Histologically, this zone contained liver cells loaded with lipide droplets and necrotic polymorphs. In a few animals killed 2—3 months later the implant was reduced to a calcified nodule 2—3 mm in diameter.

[1] A device for measuring the consistency of bituminous materials, lubricating greases, etc. (Macalaster Bicknell Co., 243 Broadway, Cambridge, Mass.).

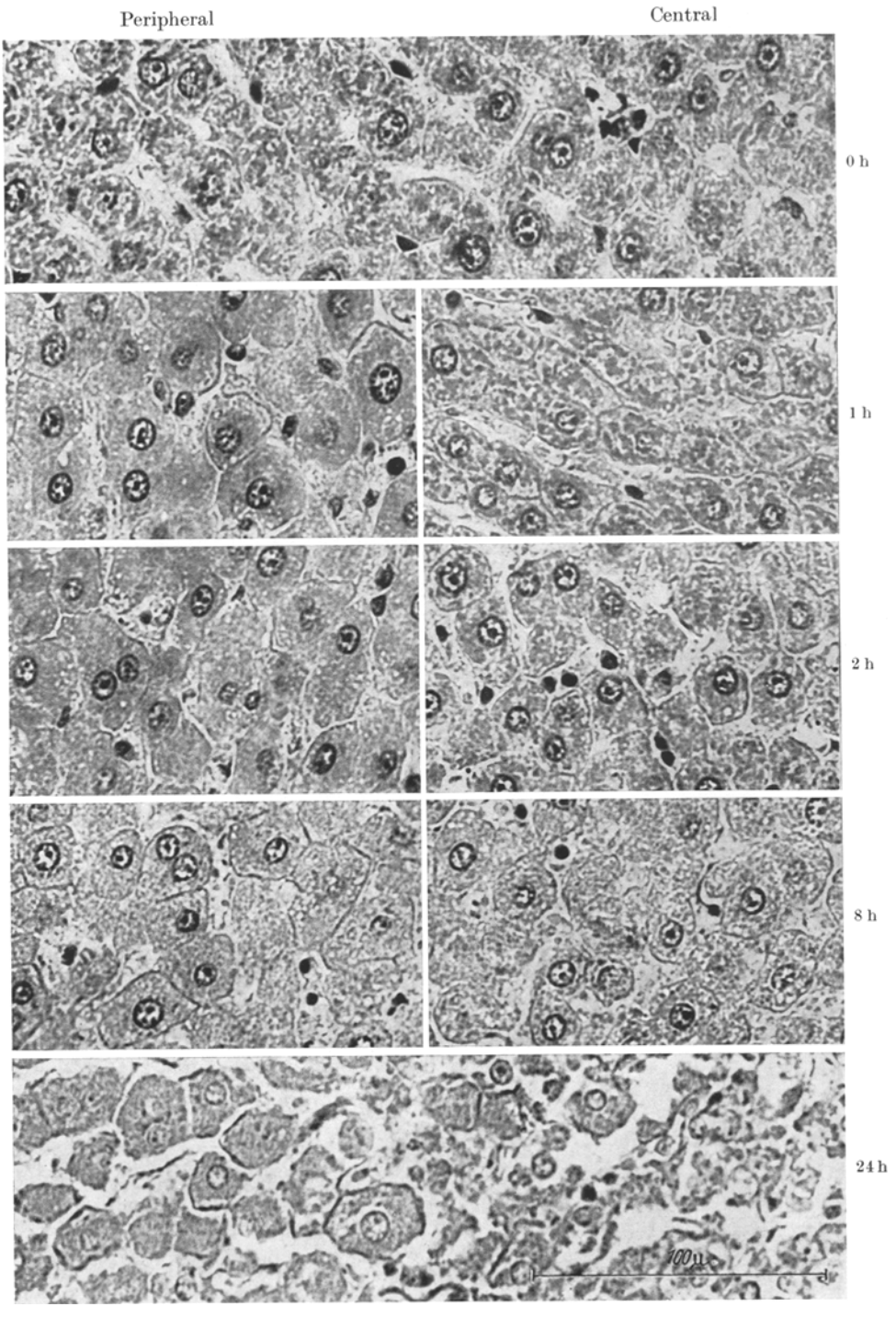



Fig. 2. Histological changes in liver implants. *Fixation:* Helly. H. and E., 450 ×. *Top:* control; other stages as indicated. After 1 and 2 hours the cells at the periphery of the specimen (*left*) stained more evenly with eosin, and the cytoplasm was filled with very fine semitransparent "granules", probably

In *Helly-fixed material* stained with H. and E. (Fig. 2) the cells in the central core remained essentially normal in aspect up to 4 hours, with a mottled cytoplasm similar to that of the controls. At the periphery of the specimen, cells filled with small vacuoles were found already at 15 minutes on the surfaces not lined by liver capsule. At 30 minutes, and more clearly at 45 and 60 minutes, there was a continuous peripheral layer 10—20 cells deep consisting of cells staining more uniformly with eosin. Some of these were visibly swollen, and at a high power all were distinctly „foamy" in aspect, with occasional small vacuoles (Fig. 2, 1 hour, left; Fig. 3 b). The cytoplasm appeared as if it were filled with small, closely packed, semitransparent spherules of very uniform diameter. Scattered among these cells, others could be found in which the cytoplasm was honeycombed with larger vacuoles. The size and number of vacuoles increased at 2 and 4 hours (Fig. 3 c); at 8 hours the cytoplasm was very pale and obviously abnormal throughout the section. The cellular outlines, however, became progressively *more* distinct up to 8 hours, and only at 24 hours were there clear indications of fragmentation (Fig. 2, bottom). The nuclei did not seem to change in a consistent fashion; at 1 hour in the "foamy" cells some of the nuclei were pale and swollen, others were smaller and stained more intensely (Fig. 3 b); at 24 hours they failed to stain. Hence necrosis could not have been diagnosed earlier than the 8th hour.

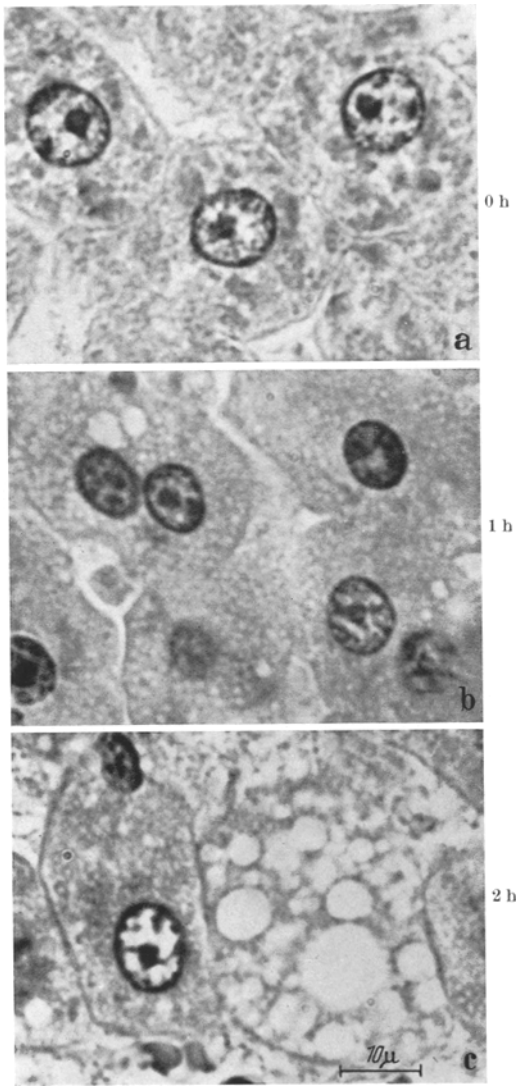



Fig. 3

swollen mitochondria (compare with Fig. 3 b, 3 c). Towards the center of the specimen (*right*) the cytoplasm retained a clumped aspect similar to that of the control. At the 8th hour this topographic difference is no longer apparent. A diagnosis of necrosis would not be warranted before the 8-hour stage; cellular death, however, occurs much earlier, probably towards the end of the first hour. (All the specimens were simultaneously fixed and embedded, then cut and stained as a single block. The same comment applies to Fig. 3, 4, 5)

Fig. 3 a—c. Histological changes in liver implants. *Fixation:* Helly. Oil immersion, 1160 ×. a *Control,* Normal rat liver. b Implant, peripheral portion, *1-hour stage.* Note "foamy" aspect of cytoplasm: probably due to swollen mitochondria; and vacuoles. c Implant, *2-hour stage,* peripheral portion. Note cell with foamy cytoplasm, side by side with another which has become riddled with vacuoles

*Formol-fixed material* (Fig. 4) was considerably less informative than that fixed in Helly. In normal cells, the cytoplasm was filled with large, irregular basophilic masses. At 1 and 2 hours, the cytological picture was essentially the same as in the controls. Of the pathologic changes in the superficial layer, so

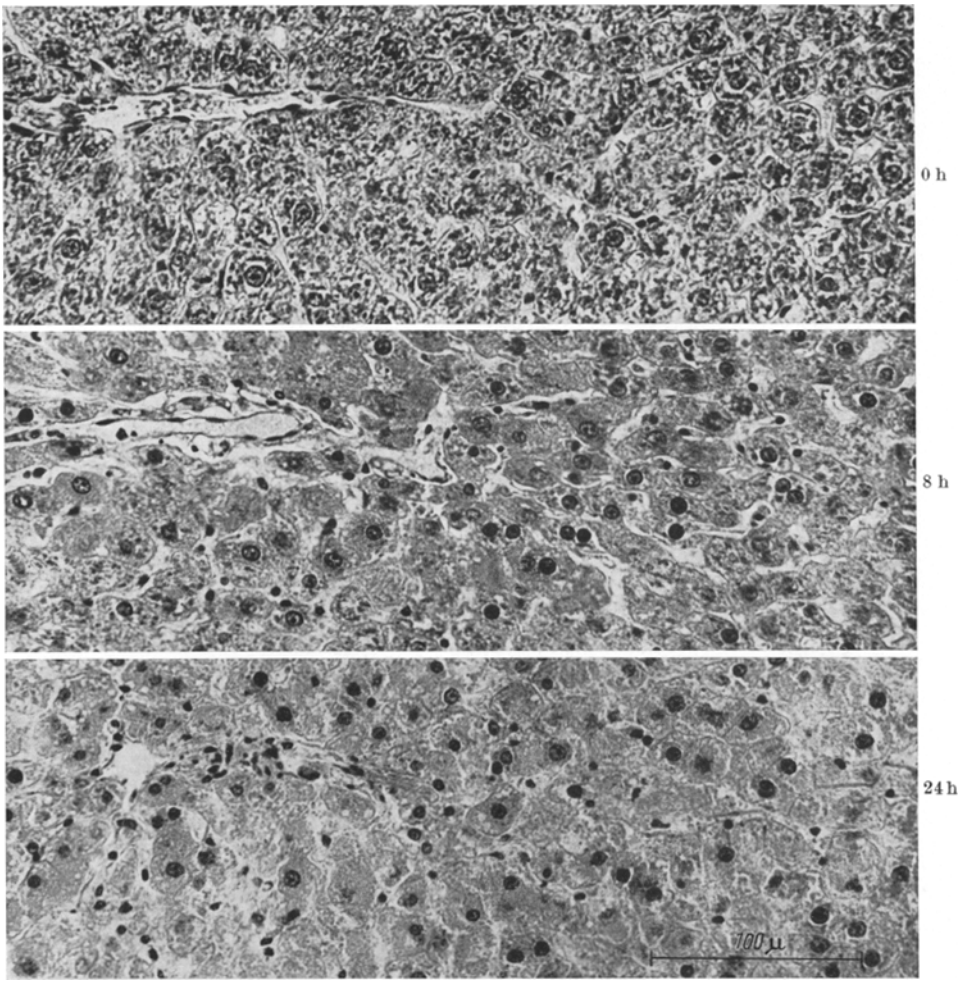



Fig. 4. Histological changes in liver implants. Formalin fixation, H. and E., 288 ×. *Top: Normal control.* Note abundance of basophilic clumps obscuring the nuclei. *At the 8-hour stage* much basophilia is lost, but the nuclei stand out clearly; the diagnosis of necrosis is still debatable. *At the 24-hour stage,* the nuclei are better stained than after fixation in Helly's fluid (Cf. Fig. 2)

clearly preserved by Helly's fixative, only traces were recognizable: along the surface some cells contained unconspicuous vacuoles, while very few retained the foamy aspect earlier described. At 4 hours the cytoplasm stained more evenly with eosin, and at 8 hours no clusters of basophilic material were visible. Hence at 4 and 8 hours, to the unprepared eye, the dead cells were in some respects "better looking" than the shaggy normal cells (Fig. 4). A large number of nuclei remained stainable at 24 hours (Fig. 4); the diagnosis of necrosis would have been warranted somewhat later than the 8th hour. With *Best's carmine stain*

*for glycogen*, all cells of the control fragment contained large amounts of red-staining material; none of this was present in the extracellular spaces (Fig. 5a). At 1 hour there was a clearcut picture: a thin rim of peripheral cells still contained

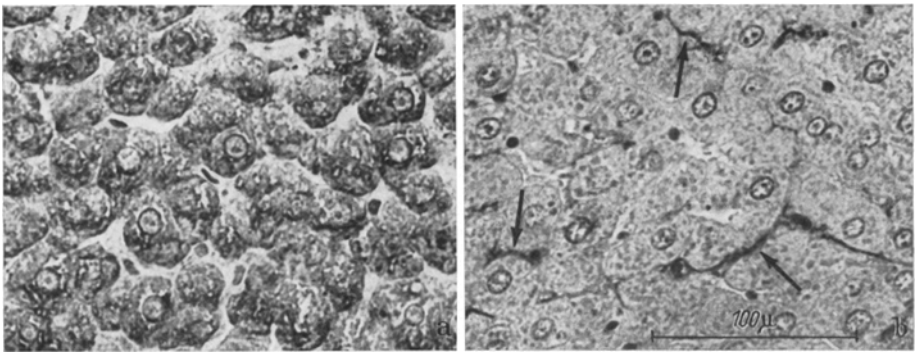



Fig. 5a and b. Extracellular migration of glycogen in liver implants. Best's carmine, 288 ×. a *normal control*. The dark areas in the cells correspond to material staining a deep red. b *implant at 4-hour stage*. No glycogen is visible inside the cells; but rows of granules can be found in the extracellular spaces (arrows) where traces remain stainable up to the 8th hour

some glycogen (though less than the controls); progressing towards the interior, the intracellular glycogen vanished, while some granules appeared to have been spilled into the extracellular spaces. By 4 hours only traces of glycogen remained, also confined almost exclusively to the extracellular spaces (Fig. 5b).

The result of *planimetric measurements* on cells from material fixed in Helly is shown in Fig. 6 (the photographs used for

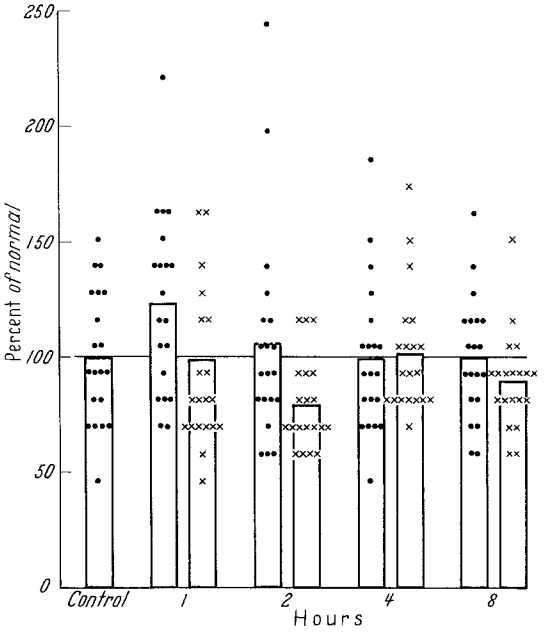



Fig. 6. Illustrating the fact that cellular swelling, though easily observed in the fresh state (Fig. 10), may be practically impossible to recognize in tissue sections of paraffin-embedded material. Planimetric measurements of the cross-sectional area of single cells, in a normal liver (first bar to the left) and in liver fragments which had been left in the peritoneal cavity for varying periods. Each point refers to one cell, • from the periphery, × from the center of the specimen. Results expressed as a per cent of the control value. Occasional swollen cells are found, but there is no significant general trend

this study are the same which are partially reproduced in Fig. 2). Enlarged cells are found with some consistency only after 1 hour, and then only in the superficial portion of the specimen: in these cells, the average increase in the cross section area is 23 per cent. In formalin-fixed material no significant changes were found.

Mitochondrial stains were too inconsistent to give useful information.

*Gross and histologic findings: livers incubated in vitro.* The liver lobes kept at 4° C remained soft (Fig. 7); after several weeks they had acquired a brownish


Fig. 7. Effect of temperature on the gross aspect of rat liver preserved *in vitro*. Slices from lobes of rat liver which were maintained for 12 days under sterile conditions and in a humid atmosphere at 4° C or at 37° C. *Left: control.* Two slices of fresh, normal liver. *Middle: Liver maintained in the refrigerator at 4° C.* The color was reddish brown, and not very dissimilar from the controls except for a pale outer rim in which the cells were swollen. The consistency remained soft, as demonstrated by the shape taken by the two juxtaposed slices. *Right: Liver incubated at 37° C.* The tissue is very similar to that of a white infarct, and the slices are stiff

discoloration, and after 3 months on cross section the outer rim was brownish, the portion in contact with the bottom of the container was white. Histologically, after 3 and 12 days the preservation was excellent (Fig. 8). After 3 months, at the periphery the tissue pattern was well preserved and with it much cellular detail (Fig. 9a); even the tissue eosinophils retained their cha-

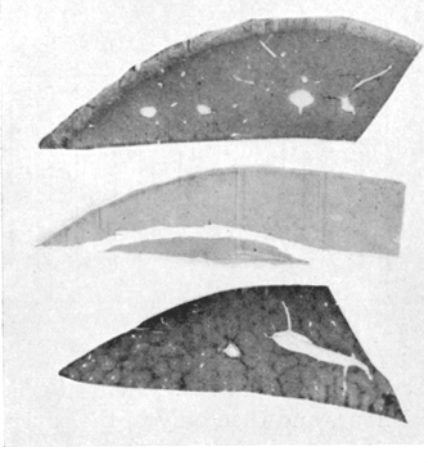


Fig. 8. Effect of temperature on the histological aspect of rat liver preserved *in vitro*. Paraffin sections from three fragments of rat liver, which were formalin-fixed and embedded simultaneously, cut and stained as one block (H. and E., 3,5 ×). The gross picture emphasizes the difference in staining with hematoxylin. *Below: control* (normal liver). *Middle: 12 days of incubation at 37° C. Top: 12 days in the refrigerator at 4° C.* Basophilia is almost the same as the control. A pale rim has developed (see Fig. 7) where the tissue had been exposed to air

racteristic staining affinity. In contrast, the deep "white" portion was devoid of stainable nuclei (Fig. 9b).

The lobes maintained at 37° C rapidly became stiff and pale, with patches of greenish discoloration undoubtedly due to the presence of blood pigments.

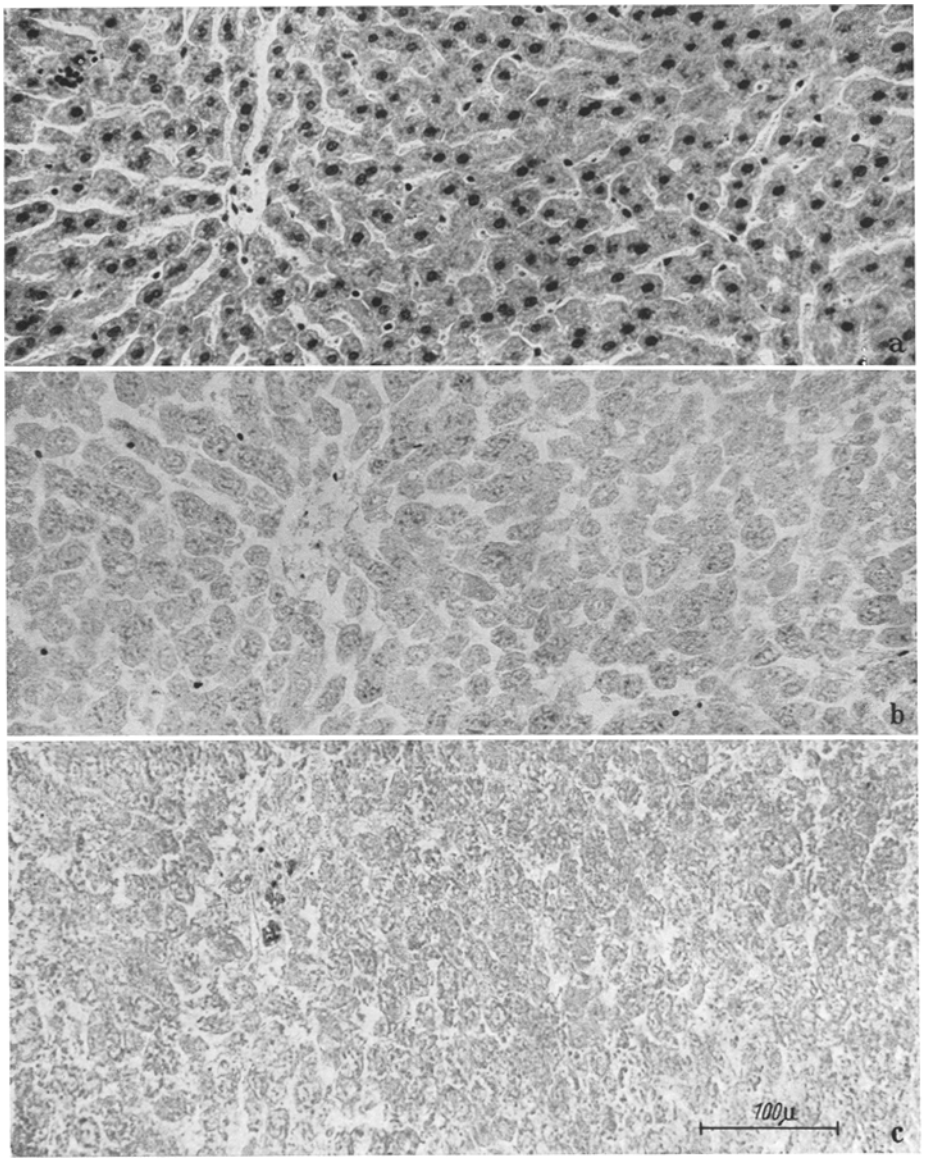



Fig. 9a—c. Rat liver preserved *in vitro*; histological changes. a *Specimen maintained for 3 months at 4° C*; superficial portion. The general architecture and nuclear staining are retained. b Same specimen, deeper portion which appeared grossly as white. Nuclear staining has disappeared. c *Specimen maintained for 3 days at 37° C*. The nuclear staining of parenchymal cells has disappeared throughout the specimen. It is clear that the histological changes typical of coagulation necrosis, as well as the gross changes (Fig. 7), can develop also *in vitro*

Though they never acquired the bright white aspect of liver tissue undergoing necrosis *in vivo*, their appearance at 3 and 12 days was that of "coagulated tissue" and quite close to that of a white infarct (Fig. 7). Histologically, at 3 days the

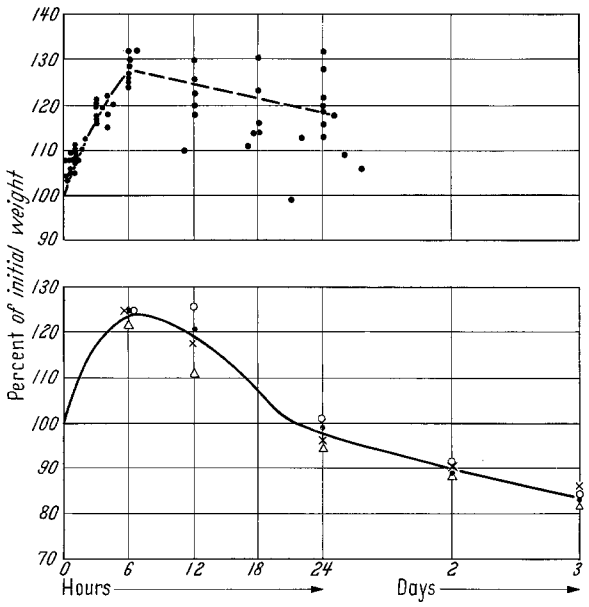



Fig. 10. Liver implants: progressive changes in *total weight*, expressed as a percentage of the initial weight. *Above:* results obtained with 60 liver fragments implanted without sterile precautions. Note consistent rise up to 6 hours. Thereafter (and approximately at the same time as adhesions begin to develop) there is a considerable scatter of values. *Below:* weight changes of 4 liver implants, introduced with sterile precautions; at the intervals shown the samples were reweighed and transferred to new recipient animals. The formation of adhesions was largely prevented. Note consistent decline in weight after 6 hours

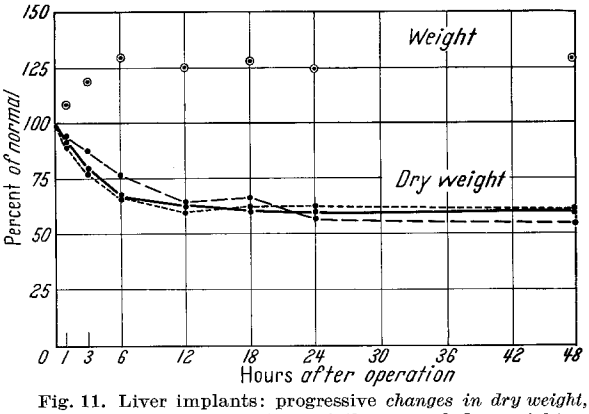



Fig. 11. Liver implants: progressive *changes in dry weight*, expressed as a percentage of the normal dry weight. — dry weight of a fullthickness sample of the fragment; ··· dry weight of the outer 1 mm layer; - - - dry weight of the inner core. For each stage, one liver fragment was used; the *total weight* of each of these fragments is indicated above the 100 % line (⊙) as a percentage of the initial weight

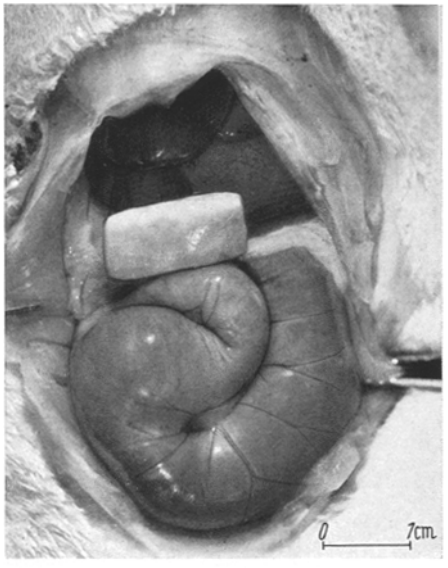
architecture of the tissue was still recognizable, but all the nuclei failed to stain with the exception of a few pertaining to endothelia or bile ducts. The picture was indistinguishable from that of coagulation necrosis; the same was true at 12 days, when no traces of nuclear staining remained (Fig. 8 and 9c).

*Changes in wet and dry weight.* a) When non-aseptic liver fragments were placed into the peritoneal cavity, their absolute weight rose steadily for about 6 hours (Fig. 10, above); thereafter the changes were less consistent, but most of the specimens tended to lose some of the weight they had acquired. Beyond 30 hours the surrounding adhesions prevented accurate determinations. In some cases the dry weights were also taken (Fig. 11); percentage-wise these tended to drop faster than the wet weights rose, indicating that there was net loss of solids. b) In order to follow the weight changes for longer than 30 hours, an experimental procedure was devised whereby the implants could be maintained free from adhesions. To this end a set of four sterile fragments were aseptically implanted into 4 rats, recovered, reweighed, and reimplanted 8 times during 6 days (Fig. 10, below). New recipient animals were used for every other operation. The weight of these fragments rose by 25% as in the previous series, but after 24 hours it dropped to subnormal values. Adhesions were found consistently at 6 and 12 hours, later the fragments were

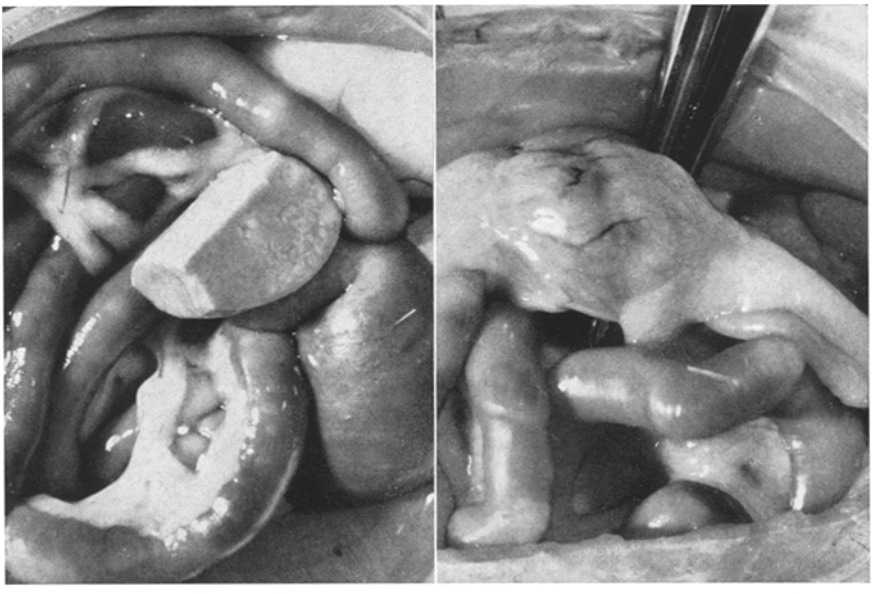
either free (Fig. 12a) or bound by very small, loose adhesions. In this respect, the reimplanted fragments behaved in the same way as pieces of liver tissue which had been dipped for 1 minute in boiling water (Fig. 12b).

*Changes in $p_H$.* Fig. 13 shows the changes in $p_H$ found in liver implants up to 12 days as well as in livers autolyzing *in vitro* at 4° and 37° C. Each point represents the average of 2 determinations on 1 fragment.

*Changes in optical density.* Fig. 14 shows the results obtained from 60 implants. The optical density was significantly decreased as early as 20 minutes after implantation; it reached a minimum at 1—2 hours, returned to the initial value at 4—5 hours and rose above it thereafter. The changes were clearly recognizable also by direct examination through the microscope (Fig. 15). When the measurements




a



b c

Fig. 12a—c. Fate of various kinds of liver fragments 1 week after they were introduced into the peritoneal cavity. a *Sterile liver implant, which was transferred through 5 recipient animals* until it reached an advanced stage of coagulation necrosis and failed to evoke the formation of adhesions. b Fragment of liver which was *coagulated by boiling* prior to implantation. This specimen also remained free in the peritoneal cavity. c Fragment of *normal, fresh liver:* note complete wrapping by the omentum

were performed on livers autolyzing *in vitro*, the curves were again biphasic but the variations were slower at 37° (Fig. 16) and especially at 4° C (Fig. 17). With livers autolyzing at 4° C, the rise in optical density was observed after 92 days, and in the deep or "white" portion only (Fig. 17).

*Observations in the dark field.* The cells of normal liver were only faintly visible (Fig. 18). The nucleus had a weak opalescence, and the cytoplasm contained numerous fine granules, visible but not bright, in which Brownian motion could be discerned at high power. Implants of the 15 minute stage appeared the same as the controls, but from 30 minutes on there was a progressive increase in brightness, which proceeded faster in the nucleus than in the cytoplasm. After 1 hour some of the nuclei appeared as bright rings, as if there were a perinuclear zone free of particles, and a precipitation against (perhaps inside) the nuclear membrane. From 4 hours onwards the cells of the pathologic liver had a truly snow-white appearance, in striking contrast with the control (Fig. 18).

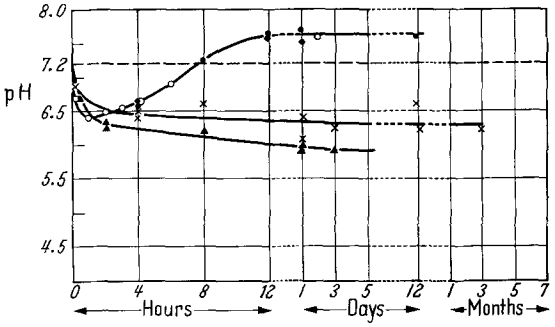



Fig. 13. Changes in $p_H$ in liver tissue, isolated under various conditions. *Upper curve:* changes occurring *in vivo*, in peritoneal implants (sterile ● and non-sterile ○ fragments gave consistent results). *Middle curve* ×: fragments incubated *in vitro* at 37° C, under sterile conditions. *Lower curve* ▲: fragments maintained *in vitro* in the refrigerartor at 4° C under sterile conditions. All determinations were made on tissue brei with a Beckman $p_H$-meter

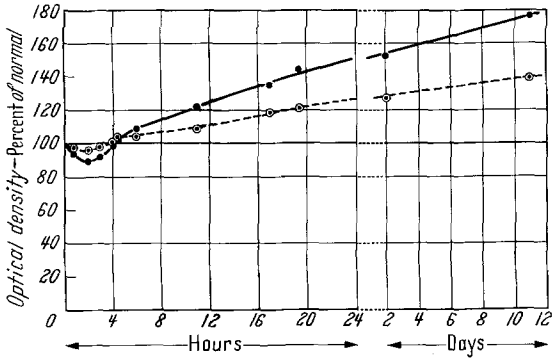



Fig. 14. Optical density of liver implants in peritoneum at various stages. The measurements refer to unfixed slices, of a standard thickness of 0,2 mm., examined with (●) and without (⊙) red filter. Compare with Fig. 15

*Observations by ultraviolet light (autofluorescence).* At 15 minutes the field appeared entirely dark to the naked eye; on the photographic plate there appeared a faint outline of the tissues, with no difference between experimental and control (Fig. 19). In subsequent stages the experimental liver developed a slight, but progressively increasing autofluorescence, which was also noticeable by direct examination. On the photographic plates much of the cellular detail was unfortunately lost, probably on account of Brownian motion during the long exposure.

*Changes in extractable protein.* The amount of "extractable peptide bond" obtained from normal, control samples corresponded to that of a 5—6% solution of albumin, in agreement with results of previous workers (Cohn et al. 1951). In livers implanted into the peritoneal cavity this quantity varied as shown in Fig. 20.

*Oxygen uptake by liver implants.* The results are shown in Fig. 21, in which each point represents the average of 3 values.

## Discussion

The experimental method for the reproduction of cellular death, which was used in this study, is not original. It was observed more than half a century ago

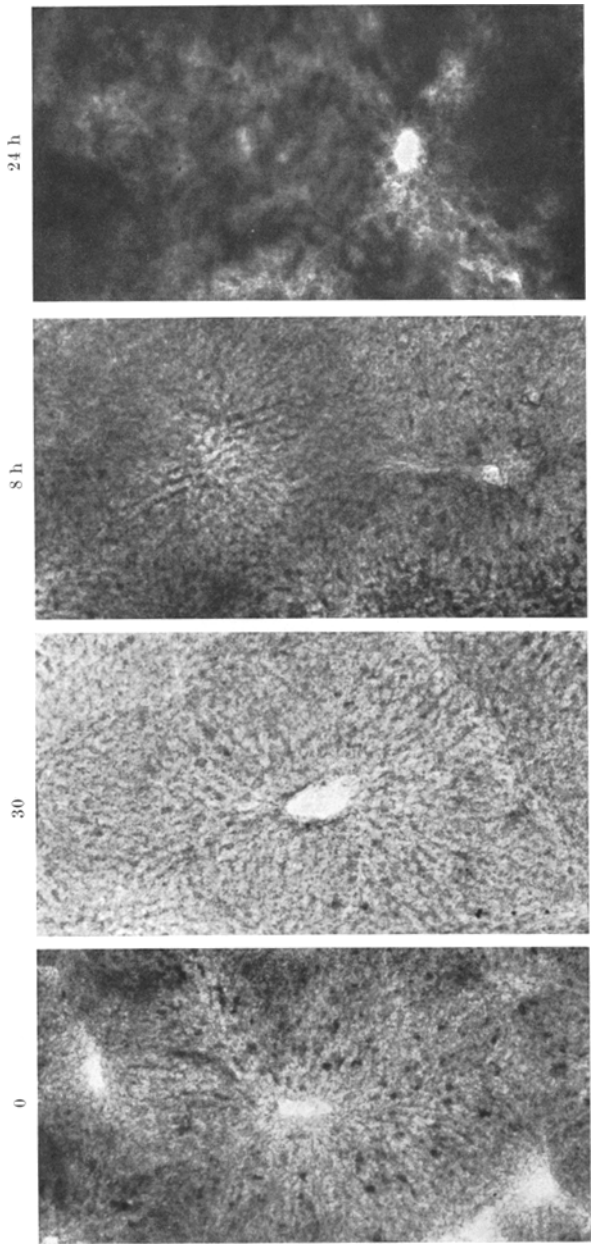



Fig. 15. Optical density of liver implants at various stages. Unfixed, unstained slices 0,2 mm. in thickness viewed in transmitted light (150×). The liver used as a control (left) was also used for preparing the 30′ implant. Note that the optical density is decreased at 30 minutes, increased thereafter. Compare with the quantitative measurements (Fig. 14). (The photographs were taken under identical conditions, and the negatives were processed simultaneously; a strip was cut from each of these negatives, the strips were taped together and printed as a unit)

that large fragments of tissue, introduced into the peritoneal cavity, underwent gross and microscopic changes similar to those of white infarcts and could,

therefore, be used as models of "necrosis" (for earlier literature see Wells 1906, Cameron and Oakley 1934; also Cain 1943, Groll 1949, Berenbom et al. 1955a and b). Like most of our predecessors, we chose implants of liver tissue, on account of its relatively uniform structure. While it is true that about one-third of the cells in a normal liver are not parenchymal cells (Yokoyama et al. 1953) the property relevant for our experiments is not so much a pure cell population, as one which is identical in all samples of the tissue.

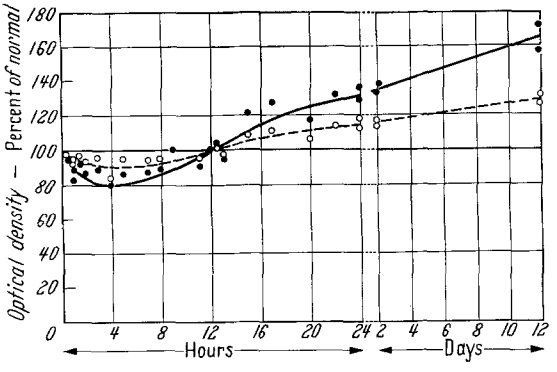



Fig. 16. Changes in optical density of unfixed liver tissue while incubated *in vitro* at 37° C in a humid atmosphere (measurements with ● and without ○ red filter). The shape of the curve is similar to that obtained from liver implants (Fig. 14) but the changes are somewhat slower

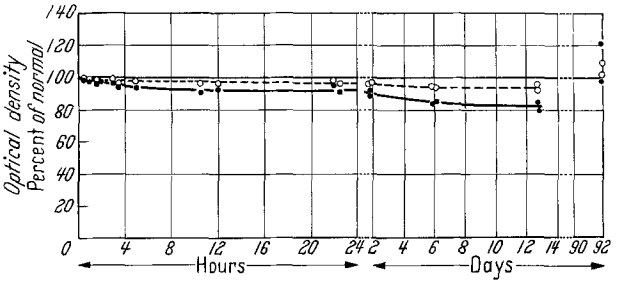



Fig. 17. Changes in optical density of unfixed liver tissue, maintained in the refrigerator at +4° C (measurements with ● and without ○ red filter). The overall trend is similar to that observed at 37° C (Fig. 16), but the changes are much slower. At the 92-day stage the cut surface showed two different zones, hence each of these was assayed separately: a brownish outer zone, corresponding to the part exposed to air (two lower points) and a deeper zone, whitish and more opaque in aspect (two upper points: the histologic aspect of these two zones is shown in Fig. 9a and b)

Fragments of an organ, isolated either *in vivo* or *in vitro*, can be assumed to die, fundamentally, of ischemia. As stated earlier in this paper, it should be possible to identify four steps in the course of ischemic damage: *reversible changes*, *cellular death* (which we identified with the point of no return), a *prenecrotic period*, and *necrosis*. Because our experiments cover this entire span, it becomes important to assign time limits, however tentative, to each of these periods, particularly to the time of cellular death.

From isolated tissues, of course, there is no way of establishing the point of no return. On the other hand, experiments with transient vascular occlusion *in vivo* (Baker 1956) have shown that the limit of tolerance of rat liver lobes with respect to total ischemia lies between 30 and 45 minutes: a figure which may be somewhat conservative, because it is apparent from the same work that 4 out of 10 livers subsequently developed very little necrosis even after 45 minutes of ischemia. Our own time-curves on liver implants should therefore be interpreted with these facts in mind: that the changes observed up to about 45 minutes are reversible, whereas the cells die, according to our definition, towards the end of the first hour.

*Morphologic aspects of cellular death and necrosis.* The cytologic findings at 1 hour are remarkably unimpressive; in fact almost nil in formol-fixed material.

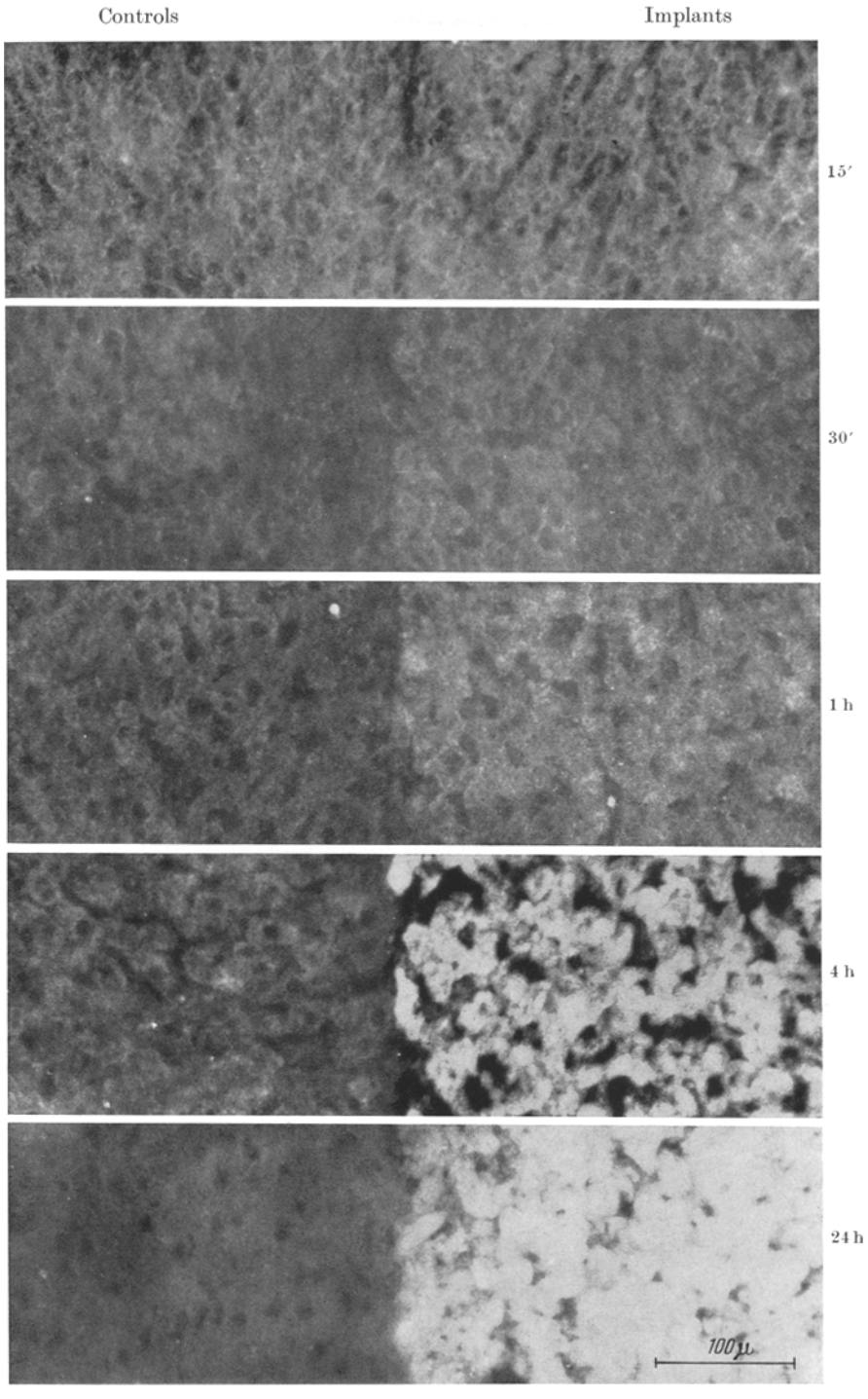



Fig. 18. Dark-field observations on liver tissue. The figure represents fresh, unfixed sections, cut in the cryostat at 10 micra, mounted in glycerin buffer, and viewed in the dark field at 200 ×. Each microphotograph shows equal portions of two adjacent blocks of tissue, simultaneously cut: *at the left*, normal rat liver (controls); *at the right*, liver tissue which has been isolated in the peritoneum for varying periods, as indicated. Note the increasing brightness of the pathologic livers, already apparent at the 30 minute stage; the change is interpreted as indicative of protein denaturation. Compare with the following figure. (The fuzzy aspect of this and particularly of the following figure is largely due to the Brownian motion during the long exposure: 15 seconds and 30 minutes, respectively)

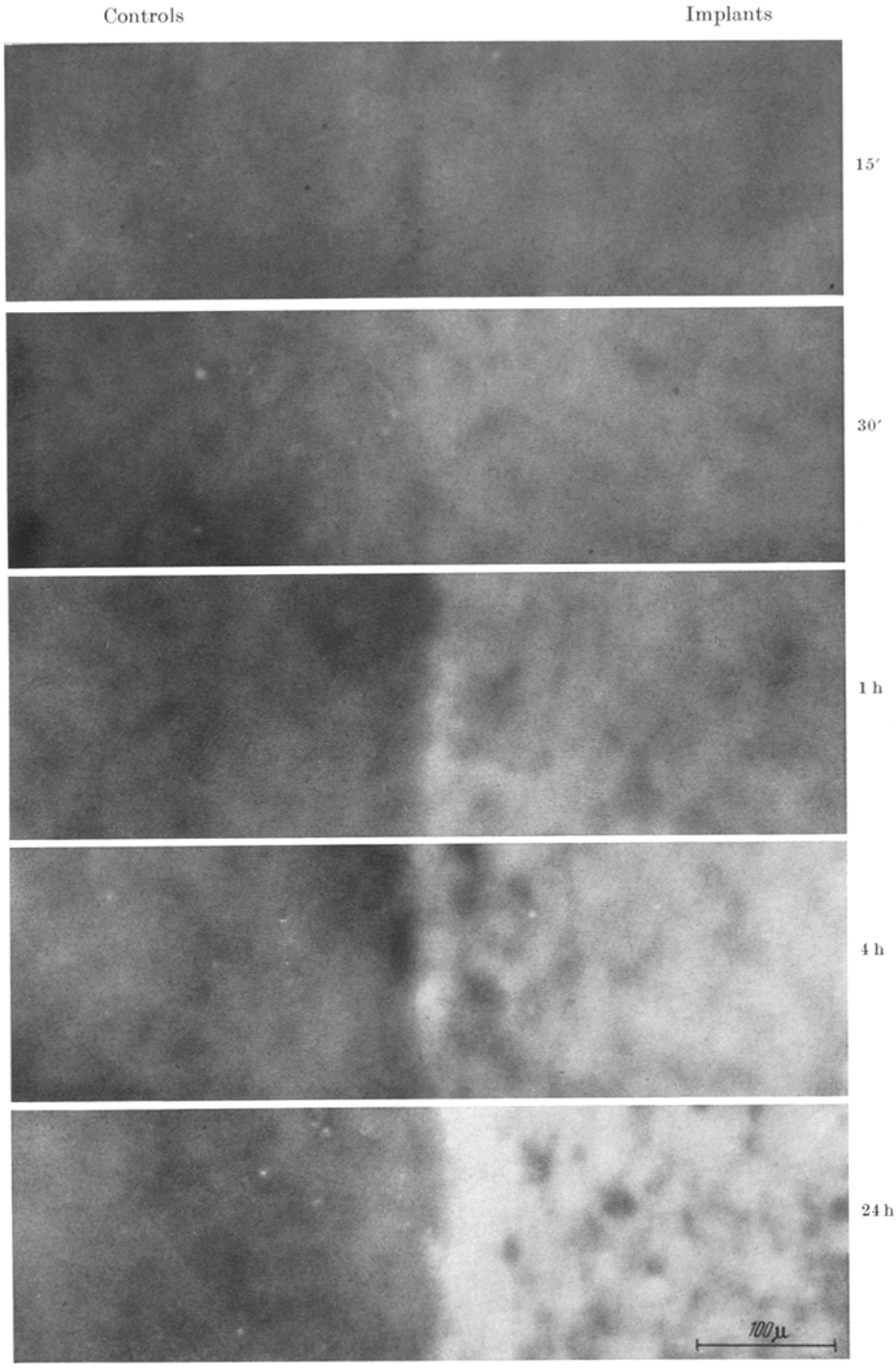



Fig. 19. The same material as in the preceding figure, photographed in ultraviolet light (the microscopic fields are not the same as in Fig. 18). The *control livers (left)* show a very faint autofluorescence, not noticeable without the aid of photography. The *pathologic livers (right)* show a progressive increase in autofluorescence, indicative of protein denaturation. The change is first apparent at the 30 minute stage, and this coincides with the appearance of abnormal "brightness" in the dark field (compare with Fig. 18)

After fixation in Helly's fluid, a good cytoplasmic fixative (Baker 1951) in contrast to formol (Stowell 1941), the picture is only slightly more informative. The great bulk of the specimen, the central portion, appears unchanged; the cytoplasm has the same clumped aspect as the controls (Fig. 2). A hint of damage is provided only by the glycogen stain: not only has this material disappeared from the cells, but some of it is spilled into the extracellular spaces, which may be taken as evidence of damage to the cellular membrane (Fig. 5). The cells in the superficial portion of the specimen show a distinctive change, which would be ordinarily considered as mild: the clumped aspect of normal liver cytoplasm

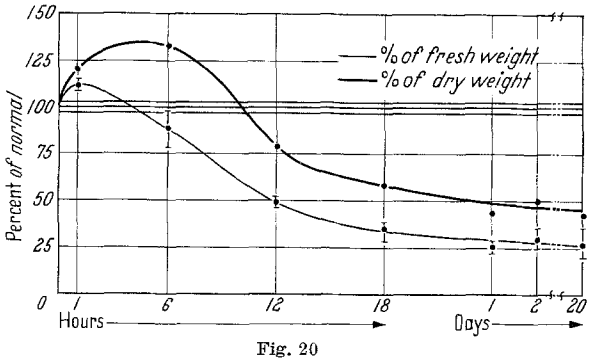



Fig. 20

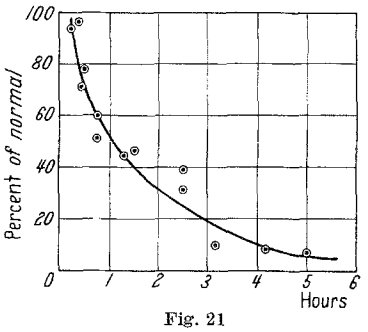



Fig. 21

Fig. 20. Progressive changes in concentration of "peptide bond", measured with a microbiuret method in the extracts of liver implants. Results are expressed as a per cent of the amount extractable from normal liver, considered as *fresh tissue (lower curve)* or as *dry weight (upper curve)*. On either side of the 100 per cent line is indicated the spread of 80 per cent of the control values

Fig. 21. Oxygen uptake of slices taken from liver fragments which had been isolated in the peritoneum for periods ranging from 20 minutes to 5 hours. Medium: Krebs-Ringer-phosphate with glucose. Results were calculated in microliters of oxygen per 100 mg. fresh weight, and expressed as a percentage of the value for normal liver slices

has disappeared, and the whole cell is uniformly filled with semitransparent granules, best described by the comparison with a mass of caviar (Figs. 2, and 3b). Most of these granules undoubtedly arise through swelling of mitochondria: a process which has been described as "enspherulation" and "vesiculation" (Duthie 1935) or "cavulation" (Bessis 1957). Larger vacuoles may arise through the fusion of dilated mitochondria (Gansler and Rouiller 1956). On the whole, there is nothing in the histological aspect of this liver, after 1 hour of total ischemia, to suggest irreversible damage.

The morphologic change which should be the easiest to perceive, the swelling, proved to be particularly deceptive. In the first place, the gross specimen continued to increase in volume for 6 hours. This means that the swelling, in our experiments at least, is not indicative of a mild cellular injury; *for the greater part it occurs when the cell has already been irreversibly damaged.* Furthermore, the increase in volume which is so obvious from the measurements of weight escapes detection almost completely in tissue sections of embedded material. After formol fixation swollen cells could not be found, either by inspection or by planimetric measurement. After fixation with Helly, some evidence of swelling could be found, but only in the peripheral portion of the specimen, and then not beyond the second hour.

At the 2-hour point, planimetric measurements (Fig. 6) showed that the diameter of a few cells was sufficiently increased—up to about 30%—to be recognized as such without

the aid of measurement. These cells must have enlarged at a faster rate, and to a greater extent, than the specimen as a whole: the specimen has gained 12—13% in weight (Fig. 10), whereas these swollen cells, judging from their diameter, must have about doubled their volume.

These results can be reconciled with the determination of water contents (Figs. 10 and 11) only by admitting that the embedding procedures tend to reverse the swelling due to the "waterlogging" of the cells. Paraffin embedding of formol-fixed tissues brings about considerable shrinkage (STOWELL 1941, ROBINS et al. 1956, MAJNO and KARNOVSKY 1958a), indeed an unfavorable preliminary to the study of cellular swelling.

It is noteworthy that the cells which are the farthest removed from the circulation, at the center of the specimen, die almost unaltered; whereas the cells close to the surface, while also dying, develop characteristic changes. The superficial layer enjoys a relative proximity to the blood supply and better conditions for exchange by diffusion; in fact, a few of the cells here located may even survive (CAMERON and OAKLEY 1934, GUILLERY 1939a). This raises the possibility that in this particular layer cellular death may be somewhat delayed, and that up to a point the "foamy" change might be reversible. While it is likely that some degree of mitochondrial swelling should be reversible *in vivo* as it is *in vitro*, the "foamy" change of the cytoplasm here described cannot be considered as a sign of reversible injury; it is found also in cells which are clearly damaged beyond repair, as indicated by severe shrinkage and distortion of the nucleus.

In summary, we find that the morphology of cellular death varies with the fixative employed, and with the environment in which the cell is dying: some cells die without alterations demonstrable in sections stained with H. and E., others develop changes which by current diagnostic standards would be considered as mild.

The histological diagnosis of *necrosis*, that is, of secondary changes in dead cells, was not yet warranted at the 8th hour in formol-fixed material (Fig. 4); with Helly's fluid the paler nuclear staining and sharper cytoplasmic detail allowed to recognize early signs of necrosis at the 8th hour, the same time-interval mentioned by HIMSWORTH (1950). With the naked eye, on the other hand, the tissue can be recognized as necrotic some 3—4 hours earlier than with the microscope. This may seem paradoxical, but it is easily understood if it is considered that we identify the tissue as necrotic principally by its optical properties: it becomes whitish and opaque. Both changes depend upon the denaturation of cellular proteins, as will be discussed below. The very purpose of fixation is to denature the cellular proteins; hence there is little hope to recognize the alteration in ordinary tissue sections, except in very advanced stages, when the cells will be recognized as dead by other criteria, such as the loss of the nucleus.

*Changes in wet and dry weight.* Among the early changes in the liver implants, the simplest to observe was the increase in *wet weight.* After the point of maximum swelling, which occurs at about 6 hours, the implants tend to lose some of the added weight (Fig. 10, top). An absolute loss of solids also occurs, as can be seen by comparing the changes in wet and dry weight (Fig. 11).

At 18 hours, for instance, the wet weight has risen by 25%, the dry residue has dropped by 38% (of the original dry weight); hence about 18% of the solids have been lost. Among the diffusible products which may account for this loss, those arising through the breakdown

of glycogen are probably foremost. Glycogen accounts for about 12% of the dry weight of the liver of normal, fed rats (see GUEST 1941); very little was stainable after 1 hour of implantation, and only traces were left after 4 hours (Fig. 5). A loss of protein may also occur (KING et al. 1959a).

During the phase of swelling, all the implants behaved in a very consistent fashion (Fig. 10, top). The subsequent shrinkage was less predictable, probably because during this period the implants became adherent at different sites, and the surrounding inflammatory reaction introduced another variable. This complicating factor could be eliminated by using aseptic implants, and by preventing their attachment to the peritoneum as described under Results. In this manner the decline in weight after 6 hours was brought out quite strikingly (Fig. 10, bottom). In fact, the samples continued to shrink even after having returned to the original weight, and the trend continued for several days. Whether this shrinkage represented continued autolysis, heterolysis, dehydration, or a combination of these processes, was not determined.

In summary, if our results are interpreted in terms of cellular changes, it would appear that the liver cells first swell, then shrink. This recalls to mind some experiments of LUCKÉ and MCCUTCHEON (1932) who followed the changes in diameter of injured and dying sea-urchin eggs; there was an initial steep rise, followed by a decline. The secondary shrinkage has been attributed to "bursting" of the cells (KING et al. 1959a) and to a loss of osmotically active material (LUCKÉ and MCCUTCHEON 1932).

It is beyond the purpose of this paper to discuss the mechanism of cellular swelling (see LEAF 1956). However, we would like to emphasize that the absorbed water may be unequally disributed among several distinct compartments. The *cytoplasm* proper (hyaloplasm) is probably capable of becoming more hydrated. Highly suggestive evidence has been obtained by ZOLLINGER (1948a and c) who studied the formation of "blisters" in free-floating cells, and by BESSIS, who studied dying mammalian blood cells with the aid of the phase contrast microscope combined with time-lapse cinematography (1954, 1956, 1957, 1958). The *nucleus*, according to the same author, shrinks somewhat while the cytoplasm is expanding; in our material swelling and shrinkage could be observed, but without consistent pattern, in agreement with the data of KING et al. (1959c). On the other hand, a large proportion of fluid can be taken up by the *mitochondria*, and occasional observations have indicated that the *endoplasmic reticulum* may behave in a similar fashion by breaking up into numerous dilated vesicules (PORTER 1953; see also SJØSTRAND and HANZON 1954, MOORE et al. 1956, BESSIS 1957, CAULFIELD and KLIONSKY 1959) even independently of the mitochondria (OBERLING and ROUILLER 1956). Also the vesicles of the *Golgi apparatus* have been seen to swell (OBERLING and ROUILLER 1956, CAULFIELD and KLIONSKY 1959). Hence the measurement of the overall cellular enlargement, or of the overall water uptake, is a relatively gross approach, which merely indicates the sum of changes occurring in different cellular compartments.

*Changes in $p_H$ "in vivo" and "in vitro".* It is well known since the extensive studies of BRADLEY and collaborators that the $p_H$ of liver brei, normally neutral or faintly alkaline, becomes acid with "almost explosive rapidity" (SEVRINGHAUS et al. 1923) after interruption of the circulation (BRADLEY 1922a and b, STIEGLITZ 1924, ROUS 1925a and b). Fig. 13 shows the rapid development of this acidification; the curve is very similar to that obtained by EMMEL (1940) from the totally ischemic kidney of the rat. In tissue preserved *in vitro* the acidity became somewhat greater at 4° C than at 37° C (Fig. 13), possibly because metabolism was more rapidly inhibited by enzyme denaturation at the higher temperature (see further). *In vivo* the drop in $p_H$ was transient: acids (SEVRINGHAUS

1923a and b) accumulated for about an hour, but thereafter the $p_H$ of the implant rose until the level finally reached at 8 hours was identical with that of blood taken from the heart of ether-anaesthetised rats ($p_H$ 7,6). It seems obvious that the aqueous phase of the dead tissue tends to equilibrate with the extracellular body fluids.

Previous workers found a neutral or slightly alkaline $p_H$ in the great majority of necrotic tissues of various kinds (Groll 1949) including "failed" skin grafts (Rous 1926), tuberculous necrosis (Graeff and Rappoport 1937, Weiss et al. 1954) and experimental liver implants (Bayerle and Borger 1939). The $p_H$ of brei from malignant tumors was found to be increasingly alkaline with increasing incidence of necrosis (Goldfeder 1929). Also a plug soaked with acid and implanted into the peritoneum contained a slightly alkaline fluid 70 hours later (Bayerle and Borger 1939).

Occasional statements to the effect that the $p_H$ of necrotic tissue is more alkaline than normal (Borger et al. 1933, Koller and Leuthardt 1934, Borger, Bayerle et al. 1935) are not supported by available evidence.

*Changes in optical density "in vivo" and "in vitro".* The study of optical density, despite its extreme simplicity, was among the most informative of all our measurements. It was suggested by two considerations. The first was that by means of such figures it might be possible to make more tangible the pathologic "cloudiness" described by Virchow. A second reason for using densitometry had reference to "coagulation necrosis". If coagulation of proteins does occur, it should be interesting to follow its time course through changes in light absorption. To our knowledge, no such measurements are available in the literature.

It seemed obvious that unfixed tissues were to be used for this purpose (Virchow's descriptions, incidentally, were derived almost exclusively from fresh, unfixed material).

As described under "Methods", two sets of measurements were made: with, and without a red filter, for the purpose of eliminating a possible error due to varying amounts of hemoglobin. The red filter had the effect of somewhat flattening all the curves (dotted lines on Figs. 14, 16 and 17), but on the whole it confirmed the histological impression that not enough blood was present to cause interference.

The results were unexpected: immediately after implantation, the optical density of the tissue begins to drop (Fig. 14); then, after two hours—while the cells are still swelling—it rises steadily to values above normal. When viewed in the unfixed tissue under a microscope, these changes are quite striking (Fig. 15).

A posteriori, if one lays aside the traditional expectation of an increased opacity, then there is no difficulty in admitting that liver cells, deprived of circulation, should at first become clearer. In normal rat liver, the refractive index of the mitochondrion is greater than that of the cytoplasm (Harman 1958); hence the optical density of the tissue as a whole can be assumed to depend, in part, on the difference in refractive index of these two components. In this respect, a slice of liver may be compared to a suspension of rat liver mitochondria, in which the optical density also depends upon the difference in refractive index of the particles and the suspending medium (Tedeschi and Harris 1955): if the mitochondria swell, the optical density of the suspension drops (Cleland 1952, Raaflaub 1953, Price and Davies 1954, Beyer et al. 1955, Tedeschi and Harris 1955, Witter and Cottone 1956, Recknagel and Malamed 1958, Lehninger 1959). When mitochondria are made to swell by anoxia, as in our

liver samples, they should at first approximate the density of the cytoplasm, and the overall refractive index should drop (BARER et al. 1953). That this actually occurs can be demonstrated very effectively with the phase contrast microscope (HARMAN 1958): fresh liver cells are crammed with dense, bright mitochondria, which fade into the background as soon as the cell is allowed to swell in de-ionized water.

In summary, then, we have described an experimental model which should provide a typical instance of "cloudy" swelling, but the measurements indicate that the actual change is in the direction of "clearing". The relevance of this finding will be presented in the last paragraph of this discussion.

In later stages, when the cells can be assumed to be dead, the optical density reverses its trend and becomes higher than normal (Fig. 14). For this phenomenon we can see but one explanation: that is, a progressive coagulation[1] or denaturation of cellular protein, with formation of larger and larger aggregates. True, it could also be conceived that the tissue may become more dense by dehydration (VIRCHOW 1867) or by addition of inward-diffusing material: however, either possibility is ruled out by the fact that the water content, both relative and absolute, is rising (Figs. 10 and 11), and by the fact that increased opacity occurs also *in vitro*, in an atmosphere of air and vapor (Fig. 16).

If it is true that the cellular proteins are denatured, it should be interesting to know whether this occurs in the dead cell or earlier, as a "denaturation *in vivo*". From the curve of optical density shown in Fig. 14, the onset of denaturation can be traced back at least as early as $1^1/_2$—2 hours: that is, to the point where the curve begins to swing upwards. Now, if at this stage the coagulating proteins form clumps large enough to interfere with transmitted light, there should be an earlier stage at which the clumps are too small to be visible in transmitted light, but large enough to be visible in the dark field. This is the case, as will be discussed below.

*Observations in the dark field and by ultraviolet light.* When viewed in the dark field, the dying liver cells began to appear more brilliant than the controls somewhere between 15 and 30 minutes after the onset of the experiment (Fig. 18). Therefore, the coagulation of protoplasm, determined in this manner, begins to occur after a period of total ischemia of less than 30 minutes; it is likely that by finer techniques the limit could be displaced even farther backwards.

Dark-field microscopy is a very valuable tool in the study of intracellular coagulation. We could find no reference to previous use of this method for the study of cellular death in tissue sections. On the other hand, observations similar to ours have been made ever since the early days of dark-field microscopy, shortly after the latter had been introduced by SIEDENTOPF and ZSIGMONDY (1903).

[1] The term "coagulation", applied to injured cytoplasm, has been used with different meanings (see WELLS 1925). For some it indicates a sol-gel transformation (GAIDUKOV 1910), for others a more profound change which is not wholly reversible (LEPESCHKIN 1924). HEILBRUNN (1956) has emphasized a reversible "coagulation" or clotting of the cytoplasm in injured cells, a change which represents a basic property of living matter; he views this phenomenon as reversible and very similar to that of blood clotting. The coagulation with which we are dealing here is intended as an *irreversible denaturation with formation of large insoluble aggregates*, probably quite unrelated to the phenomenon described by HEILBRUNN.

Gaidukov (1910) described living and dead cells as viewed in the ultramicroscope, and concluded that the brightness of dead cells indicated a sol-gel transformation. Russo's description of dying cells, both animal and vegetable (1910), applies almost literally to ours, and others used the dark field to compare the effect of different fixatives on living cells (Price 1914, Strangeways and Canti 1923). These early observations seem to have been all but forgotten in the medical literature, whereas they survived in the fields of biology and botany (W. Lewis 1923, M. Lewis 1923, Lepeschkin 1926, Guillermond 1932, Lepeschkin 1937). W. Lewis (1923) studied cultures of various chick embryo tissues with the dark field, and found that during cellular death the nucleus and then the cytoplasm became filled with "very small white granules" which he called *d-granules*, or *death-granules*. Guillermond (1932) studied dead yeast cells and mushroom filaments; his description of dead protoplasm in the dark field as "snow-white" is most appropriate (see Fig. 18).

There is no doubt, then, that very fine granules appear in the protoplasm of dying cells. This phenomenon is undoubtedly correlated with the gross aspect of necrotic tissue: the characteristic "whiteness" may be attributed to the scattering of light by the same submicroscopic particles which appear as "snow-white" in the dark field.

An obvious comparison suggests itself: when eggwhite is denatured by heat, it becomes firm, white and opaque; properties which are also characteristic of necrotic tissue.

One may still legitimately wonder whether these granules really represent denatured protein. The size of the particles involved, which can be as small as 0,004 micra (Lepeschkin 1924) precludes, of course, to distinguish their nature by direct visualization. However, we succeeded in obtaining evidence that the bright material represents denatured protein, by applying the principle that denatured proteins become autofluorescent when exposed to ultraviolet light. Most fresh normal cells are not fluorescent under ultraviolet light, but they become fluorescent if they are fixed (Hamperl 1934), boiled, or necrotic (Fahr 1943a and b).

As to the mechanism whereby proteins may exhibit fluorescence on denaturation, we will refer to recent studies by Teale and Weber (1959). In earlier work these authors (1957) showed that tyrosine, tryptophane and phenylalanine have characteristic fluorescence in water solution. It might be expected that proteins containing these amino acids would also exhibit characteristic fluorescence. However, in general such fluorescence in proteins appears to be much reduced or absent entirely. This observation may be explained on the basis of specific internal quenching within the protein molecule due to the nature of the immediate environment about the residues of these three amino acids. If this explanation is correct, then the unfolding of polypeptide chains associated with protein denaturation might be expected to result in an increased fluorescence. Teale and Weber have shown that exposure of trypsin and chymotrypsin to 8 *M* urea does result in an increase of fluorescence. The opposite effect, however, is observed with certain albumins. In the case of five haem proteins characteristic fluorescence could be observed only after removal of the haem group from the protein. It is reasonable then to assume that the ultraviolet fluorescence observable in dying cells is due, in part at least, to protein denaturation. The presence of aromatic amino-acids liberated by proteolysis might also play a role; however, it seems unlikely that these compounds should maintain an intracellular location in dying cells, particularly in thin tissue sections covered with an aqueous medium.

It is interesting to notice that there is an empirical method, in legal medicine, which appears to be a practical application of the phenomenon discussed above, whereby denatured proteins fluoresce in ultraviolet light: in searching for traces of dried semen, the suspect clothes are examined by ultraviolet light. Dried spots of other biological fluids also fluoresce (Moritz 1954).

By comparing Figs. 18 and 19 it is obvious that the appearance of intracellular bright material in the dark field goes in parallel with the appearance of autofluorescence: hence it is legitimate to conclude that the intracellular granules represent, in all likelihood, denatured protein.

An apparent contradiction arises at this point: the aggregates of denatured protein, demonstrable by the dark field and by UV light, should result in an increasing tissue optical density; however, they begin to form at a time when the optical density of the tissue is in fact dropping (Fig. 14). These observations may be reconciled in the following manner: In the early stages of the process it is the mitochondrial swelling which is responsible for the fall in the optical density. Little contribution of an opposite sign is made by the aggregates. In later stages, however, while the swelling subsides, the increase in number and size of the aggregates is the predominant process and results in the increasing optical density of the tissue.

This interpretation also explains a curious phenomenon which can be observed in unfixed slices of liver implants. If two slices—one from a fresh, normal liver, one from a one-hour-implant—are juxtaposed and examined by transmitted light, the pathologic slice appears definitely more transparent; however, if examined at an oblique angle by reflected light, it is also definitely "milky", and in this sense more turbid than the control.

It has often been stated that the liver, kidney and heart in certain acute diseases develop a "scalded" or "parboiled" appearance, *unrelated to the histological appearance of the tissue.* In fact, this has been one of the mainstays of the "cloudy swelling" concept ever since its origin (see COHNHEIM 1889). The paleness implied in the terms *scalded* and *parboiled* is probably explained, in some cases, by anemia or fatty change; however, it seems possible that protein denaturation *in vivo*, as described above, may also be involved.

In concluding this discussion of intracellular coagulation, we will recall that VIRCHOW, who did not accept WEIGERT's concept of coagulation necrosis, interpreted the gross changes occurring in dead tissues as the result of *inspissation*, or dehydration (VIRCHOW 1867). Whether dehydration may really occur in late stages of necrosis, we do not know; caseous material from tuberculous lymph nodes contains about 75% of water (CALDWELL 1919). It should be pointed out, however, that while necrotic tissue may appear to be "dry", its moisture content may be normal or even higher than normal (Fig. 11). The "dryness" is dependent more upon the state of the proteins than on the water content. The situation may be again compared to that of fresh versus boiled eggwhite, both of which contain essentially the same of water.

*Changes in "extractable peptide bond".* This set of measurements was made with the purpose of obtaining, indirectly, some evidence concerning the denaturation of the cellular proteins. The amount of extractable protein should be expected to decrease as the proteins are denatured.

During the first 4—6 hours, however, there was an *increase* in extractable peptide bond (Fig. 20). Since this can obviously not represent an increase in synthesis, the rise must indicate either solubilization of previously bound proteins (see VAN LANCKER and HOLTZER 1959), or breakdown of proteins into peptides, or both; phenomena fundamentally similar to those of autolysis *in vitro*. On the basis of our data we cannot speak of an increase in the *rate* of protein breakdown

with respect to the normal, because the results could also indicate a local accumulation of products which have not yet had the time to diffuse outwards.

However, there are good reasons for believing that an increase in proteolysis does actually occur.

The recent work of DE DUVE and collaborators should be mentioned at this point, because it offers an entirely new view of the process of autolysis. DE DUVE's studies point to the existence of a new subcellular particle, the lysosome, containing several hydrolytic enzymes (including cathepsins) with a $p_H$ of optimum activity in the acid range. Anoxia causes the lysosomes to rupture and to liberate their contents into the cell (DE DUVE 1959).

Since an increase in extractable peptide bond occurred consistently within one hour of implantation, it follows that a certain degree of autolysis—as defined above—does occur in the cells while they are still alive, without causing irreversible damage. This concept was also expressed by SEVRINGHAUS et al. (1923), who stated that autolysis can be assumed to start almost instantly upon interruption of the blood supply. Further support for the notion of "autolysis in vivo" arises from the above-mentioned word of DE DUVE. If the vascular pedicle of a rat liver lobe is ligated, a release of cathepsins and other lysosomal enzymes is detectable as early as 30 minutes after the operation, hence at a time when the cells should not yet be irreversibly damaged.

The expected *decrease* in extractable peptide bond was finally observed, but as late as the 10th—12th hour (Fig. 20): that is, with considerable delay with regard to the onset of protein denaturation. By 24 hours only one-third of the normal amount was extractable. This fits very well with the data of CALDWELL (1919) pertaining to caseous material from bovine tuberculous lymph glands and livers: the amount of protein which could be extracted was only $^1/_5$ to $^1/_3$ of that obtained from the corresponding normal tissues.

In summary, the rise and fall of the "extractable peptide bond" may be interpreted as follows. Two processes are at play in the destruction of the cellular proteins: autolysis and denaturation (both terms have been defined in earlier paragraphs). Autolysis predominates during the earlier phase, denaturation in the later.

It seems appropriate to mention here another milestone in the history of "cloudy swelling"; the contribution of HOPPE-SEYLER, who proposed the new name of *albuminous swelling* still found in current literature. The German chemist is usually quoted (WELLS 1925, FONNESU and SEVERI 1954) as having said that the protein content of cells increases as a result of "cloudy swelling", thus confirming Virchow's concept. When we found that the "extractable peptide bond" increased in our liver implants, in a fashion somewhat reminiscent of "albuminous swelling", we consulted HOPPE-SEYLER's original papers: only to find that the concept of albuminous swelling, and its implications, are devoid of any foundation. HOPPE-SEYLER analyzed the protein content of human livers which had been diagnosed by a pathologist as affected by "cloudy swelling" (1921, 1923, 1928). It is obvious from the figures that these were, essentially, large livers of various sorts. At any rate, HOPPE-SEYLER found that the *relative* protein content was the same as in the controls; however, assuming that the livers had enlarged by "Schwellung" of the cells, *without increase in their number*, he concluded that the total protein content of each cell had increased. Thus the essence of this work can be expressed by a graph showing that there is, not too surprisingly, a linear relationship between the weight of the liver and its total protein content. Similar comments apply to a paper on the kidney (1927). On the strength of these analyses HOPPE-SEYLER proposed the term of *albuminous swelling* as more exact than cloudy swelling. Ironically, this term probably survived by being misinterpreted. Later authors seem to have assumed that HOPPE-SEYLER had found what would have been more interesting: a *relative* increase, or accumulation, of protein.

*Coagulation of the protoplasm and cellular death.* The crux of our studies with transmitted light, dark field and autofluorescence is in the fact that they allow one to follow, more closely than has been possible heretofore, the denaturation of proteins in relation to cellular death. It is only fair to add, however, that our results are in line with one of the most ancient traditions of Pathology. MALPIGHI may well have been the first to notice a similarity between caseous necrosis of the lymph nodes and denatured protein. He thought that the caseous matter in tuberculous mesenteric nodes might be lymph coagulated locally by some acid[1], and he even tested his hypothesis with an experiment *in vitro:* by mixing in a flask some lymph together with an acid, he obtained a precipitate (MALPIGHI 1700). Almost two centuries later, WEIGERT laid a cornerstone of General Pathology by establishing the concept of "Koagulationsnekrose" (1880)[2].

Weigert based this concept on the observation that certain kinds of necrotic tissue were very similar, in their gross aspect, to the fibrinous portion of blood clots; cellular death, he observed, occurred in both situations, and late calcification was another common feature. He concluded that cellular death involved "clotting" of the protoplasm. This bold synthesis anticipates modern views, which hold that blood and cytoplasm contain clotting systems very similar in their properties (HEILBRUNN 1956).

Though Weigert's notion of coagulation necrosis became the focus of an abundant literature (see FRICKE et al. 1929, GROLL 1949, MUELLER 1955), very few firm facts have been added to support it since 1880, beyond the confirmation that tissues affected by "Koagulationsnekrose" contain a high proportion of insoluble protein (SCHMOLL 1904). Further progress was prevented by two major difficulties: a) The lack of a satisfactory method for recognizing (let alone measuring) the coagulation. Actually, the independent observations of biologists and botanists already quoted had demonstrated the value of dark-field microscopy for the study of protoplasmic coagulation, but apparently the method was never applied to animal tissues. b) The variety of meanings given to the very term of coagulation (WELLS 1925), such as formation of fibrin, sol-gel transformation, or irreversible denaturation.

GROLL and his associates (see GROLL 1949) attempted to reproduce a model of coagulation necrosis *in vitro*, by incubating tissue extracts and evaluating their clotting time by gross inspection. That the clotting of these extracts really duplicates the change of coagulation necrosis is debatable; at any rate, the phenomenon was not dependent upon the presence of fibrinogen. MOEGEN (1940) found that if slices of organs containing a white infarct (that is, a coagulated portion) were incubated with trypsin or papain, the normal area was attacked faster than the infarct. By applying this observation, the onset of coagulation was set at $4^1/_2$ hours after interruption of the blood supply. FAHR noticed that several kinds of infarcts became autofluorescent in ultraviolet light, similarly to boiled tissues (1943a). This was the most important single step in demonstrating that necrotic tissue contains denatured protein.

[1] "... *Ubi igitur vitriolatae particulae a sanguine derivatae in glandulis luxuriant, lympha stagnans concrescere potest, ...ibique subsistens, substantiae modum, et colorem glandulae variare...*". [For the interpretation of this passage see KLEMPERER, P.: The rise of pathologic anatomy in Italy. Arch. De Vecchi (Firenze) **31**, 241 (1960)].

[2] As a footnote to his paper "On the pathologic processes involving coagulation" (Über die pathologischen Gerinnungsvorgänge) (1880) Weigert makes the following comment in relation to "coagulation necrosis": "To this form of cellular death, which I was the first to mention, I earlier referred to with a circumlocution: transformation of the cells into a mass similar to coagulated fibrin. Professor COHNHEIM then suggested the above-mentioned, very appropriate name" (p. 94).

One point which received relatively little attention was the *time of onset of protein denaturation* with respect to cellular death. In our experiments, protein denaturation is shown to begin earlier than 30 minutes after interruption of the blood supply, and therefore at a stage at which the liver damage is presumably still reversible. In other words, the denaturation *per se* may be irreversible, but the cell is not necessarily doomed. This concept finds support in LEPESCHKIN's studies on Spirogyra (LEPESCHKIN 1924, 1937): mild injury brought about a partial coagulation of the protoplasm, but the alga survived and the granules finally disappeared.

The traditional view is that denaturation occurs after the cell is dead, through some action of the surrounding tissues, possibly as a protective mechanism against autolyzing tissues (necrosis *with* coagulation) (BORGER and MAYR 1935, BORGER, BAYERLE et al. 1935, BAUER 1943, KING et al. 1959d). The facts rather indicate that denaturation plays a role in killing the cell (necrosis *by* coagulation)[1].

Why should the cellular proteins undergo denaturation after interruption of the circulation? The presence of bacteria is obviously not necessary. We made an attempt to answer this question by studying whether denaturation would occur *in vitro*. In the sterile livers which were incubated aseptically at 37° C, denaturation did occur, though somewhat more slowly than *in vivo* (Fig. 16). In the livers which were kept at 4° C, denaturation again occurred, but after months (Fig. 17) and only in the portion which was not exposed to air. It may be concluded, then, that denaturation of the tissue proteins is accelerated if the ischemic liver is surrounded by normal tissue, but can be brought about by conditions which develop within the tissue itself.

This disposes of another erroneous belief, that coagulation necrosis can only occur within the body, autolysis being typical of isolated tissues (BECHHOLD 1919, BORGER, BAYERLE et al. 1935, BAUER 1943, FAHR 1943b). The experiments which were supposed to prove this point were not properly conceived, because the changes occurring in infarcts were compared with those of tissues left at room temperature (FAHR) or even in the icebox (BORGER et al.). Fig. 9c shows conclusively that the histological picture of tissues preserved at 37° C for 3 days is identical, in its salient characteristics, with that of a white infarct, and the gross aspect is also very similar (Fig. 7). On the other hand, after death the whole body does not undergo a massive and grossly visible denaturation, because the temperature drops. In fact, the well-known effect of cooling in prolonging the life-span of tissues deprived of circulation is probably dependent not only on a general slowing of metabolic processes, but also on the retardation of protein denaturation demonstrated in Fig. 17.

[1] This dualism—necrosis *with* or *by* coagulation—is implicit in the very name of *Koagulationsnekrose*, which may be interpreted in either way. This ambiguity was cause of much concern, among others (GROLL 1949), to VIRCHOW himself, who did not have much belief in the concept ("... Etwas derartiges möge vorkommen, sei aber nicht gewöhnlich"). In WEIGERT's original communication to the International Congress of Medicine at Copenhagen in 1886 the English translation of the title was "Necrosis *by* coagulation"; this was declared by WEIGERT to be a misunderstanding. In order to make it clear that necrosis came first, *then* coagulation, VIRCHOW went so far as suggesting another name as more appropriate: *Mortifikationskoagulation* (for this incident see ISRAEL 1891). Our findings support the "misunderstanding" of WEIGERT's concept.

As for the mechanism of the pathologic denaturation, little can be said with certainty. It is conceivable that the drop in $p_H$ may play a direct role. However, if our measurements are an accurate representation of the intracellular changes, the lowest $p_H$ observed was 6,2—a relatively small variation [the nuclear proteins in liver homogenates begin to precipitate when the $p_H$ of the medium is 5,8—6 or less (PALADE 1952)]. Furthermore, the denaturation occurs more slowly *in vitro*, despite the fact that the acidity persists longer. GROLL and his associates have attempted to find whether the coagulation might be enzymatic in nature, similarly to that of milk or blood; their results are inconclusive. Autolysis may play a role in rendering the proteins less soluble (LUCK et al. 1949) or more susceptible to coagulating agents. This phase of the problem requires further work.

*The sequence of events in the course of cellular death and necrosis.* If our findings are combined with the information currently available, the sequence of events which takes place *in rat liver cells deprived of circulation* may be tentatively reconstructed as follows. As soon as the oxygen supply is curtailed, the cellular "sodium pump"—lacking energy—becomes ineffective (LEAF 1956, LEHNINGER 1956, PRICE et al. 1956) and sodium ions begin to diffuse in, while potassium leaks out (see ZOLLINGER 1948b, WHITTAM and DAVIES 1953, KING et al. 1959c). The cell's metabolic machinery limps along temporarily through energy provided by glycolysis; as the glycogen stores are drawn upon, acids rapidly accumulate, the intracellular $p_H$ drops, the lysosomal enzymes are freed and activated and the cell's own proteins begin to be hydrolyzed. Several factors contribute to increase the osmotic pressure within the cell: while autolysis increases the number of free molecules, synthetic processes come to a halt, and waste products are not removed. Within a matter of minutes, through hypertonicity and possibly also other mechanisms (see LEHNINGER 1956, 1959a and b) the cell and its various organelles begin to swell. Among the substances which accumulate is reduced glutathione, which is another cathepsin activator, and may also play a role through its capacity to induce mitochondrial swelling (LEHNINGER 1959b). While a portion of the living cell is thus digested by a process of true autolysis *in vivo*, the proteins also begin to precipitate, with increasing damage to the various cellular structures and enzymatic systems. Some enzymes, however, may experience a transient autolytic activation (POGELL 1952). By the end of the first hour the combined damage of metabolic breakdown (GALLAGHER, JUDAH and REES 1956), autolysis, and denaturation is such that the cell cannot recover. Swelling continues for a while, but the cellular membranes begin to give way, glycogen and other large molecules leak out, and in a few more hours the bulk of the cell is congealed into a lump of denatured protein from which all enzymatic activity is disappearing. Even autolysis is progressively inhibited: both the cathepsins and their substrates are denatured, and furthermore the $p_H$ is now shifting towards an unfavorable alkalinity. Towards the end of the fourth hour, protein denaturation is severe enough to be recognized by gross inspection, through a whitish color and a firmer consistency. Histologically the main outlines of the cell and its nucleus may be preserved, but no barriers are left, and the soluble materials of the cell and its environment slowly merge until equilibrium is reached. The cellular remains, barely an irritant to the surrounding tissues, will be slowly removed by "heterolysis" (JACOBY 1903).

While the description outlined above refers to ischemic liver tissue, we feel that it should be valid in its broad lines for other tissues as well. The drop in $p_H$, for instance, has been observed in a great variety of cells as a result of injury

(see Caldwell 1956) and the same is true for cellular swelling. As for the protein denaturation, comprehensive data such as those here presented for the liver are not available; however, we made a few tests with renal tissue implanted into the peritoneum, and the changes in optical density were even more pronounced than with liver tissue. Granules visible with the dark field appear in a variety of isolated cells as a result of injury or death, in a manner recalling the effect of histologic fixatives (Price 1914, W. Lewis 1923, Lepeschkin 1937, Schrek and Ott 1952). This is true not only for mammalian cells (Schrek and Ott 1952), but also for avian cells (W. Lewis 1923), various algae and fungi (Gaidukov 1910, Price 1914, Lepeschkin 1924, 1937) and yeasts (Guillermond 1932, Rahn 1934). Using the phase contrast microscope, Zollinger (1948a, d and e) described a reversible "hazy" and an irreversible "brilliant" change in the nuclei of various types of cell suspensions. These changes probably refer to the same phenomenon which is observed by dark field microscopy.

The relative importance of autolysis and denaturation should be expected to vary from one tissue to another, and possibly also within different parts of a given tissue. Implants of rat brain, for instance, disappeared very rapidly and left a large fluid-filled cavity, indicating that autolysis had liquefied the tissue before denaturation could "freeze" it. Under the conditions of spontaneous brain infarcts, however, some denaturation occurs, since amorphous masses of protein can remain *in situ* for months after the vascular accident (Dixon 1956; see also Dixon 1953).

This account would not be complete if it were not mentioned that all the changes here described in dying liver cells have also been described, at some time or another, with reference to the cataractous lens (see Duke-Elder 1941). The only major difference lies in the time factor, since the changes proceed far more slowly in the crystalline lens. Without indulging in a detailed parallel, suffice it to mention that the early stages of the cataract are often marked by a swelling of the lens, while the "bladder cells" appearing between the lens fibers are among the most striking examples of cellular swelling. The $p_H$ of the cataractous lens was found to be lowered in the early stages, while it rose again later; on the other hand, during autolysis of normal lens extracts *in vitro*, there was merely a slow drop to $p_H$ 6,2 (cf. our Fig. 13) (Krause 1933). Acids accumulating within the diseased lens activate specific proteolytic enzymes, and while part of the lens is thus autolyzed, a nucleus of lens material is often spared as a lump of denatured, "undigested" material (Morgagnian cataract). As opacification proceeds, respiratory activity drops, Na increases, K is lost, and Ca rises sharply. It seems likely that much of this detailed chemical knowledge could be applied to the study of ischemic tissues in general, since, in the words of Duke-Elder, "a cataractous lens is an asphyxiated lens". It should also be mentioned that Hamperl found an increased fluorescence in the lens of older people (1934).

Another pathologic condition to which some of our findings may apply is Wallerian degeneration. Axons severed from the cell body cannot survive; hence, when a nerve is transected, the early changes occurring in the distal stump should have much in common with the phenomena of cellular death described. In fact, the fibers swell, and the oxygen uptake of the nerve drops (Majno and Karnovsky 1958b).

*On some biological implications of autolysis and denaturation "in vivo".* Taking ischemic death as a prototype of cellular death, we have concluded above that two processes compete for the destruction of the cellular proteins: autolysis, and probably somewhat later, denaturation. It is interesting to speculate further on some biological implications of this sequence. Autolysis produces polypeptides, which (aside from their general effects) have a powerful local action as inflammatory agents (SPECTOR 1951). Coagulation reduces the rate of autolysis, and therefore should reduce the local and general damage resulting of an area of necrosis. If this were true, as a corollary, implants of tissue which were *already* necrotic should evoke little or no local reaction. This is the case: when sterile implants were transferred every few hours to the peritoneal cavity of a new recipient animal, the adhesions became milder and milder, until the fragments acquired the whitish, opaque aspect of necrotic tissue: at that point they remained free (Fig. 12a). A similar result can be obtained, as has been known for many years, with fragments of boiled liver (WELLS 1906, CORPER 1912, CAMERON and KARUNARATNE 1936). We repeated this experiment (Fig. 12b and c) and found that implants of this nature remained free in the peritoneum for as long as 3 weeks; their surface became colonized by a thin layer of connective tissue, invisible to the unaided eye. Clearly the denaturation of the proteins has a survival value; it deprives the necrotic, autolyzing mass of its major injurious properties, which are of a chemical nature, and transforms it into a relatively harmless material which will persist for a long time as a mild mechanical irritant.

Here too it is possible to find an analogy between the events in dying liver cells and in the cataractous lens: if the lens capsule is ruptured during the early phases of the development of a cataract, a lively reaction occurs in the neighboring vascularized tissues (iritis); in later stages the capsule can be opened with no danger of such effects.

These concepts find some application also in the pathogenesis of free bodies such as occasionally develop in the peritoneum or other cavities. Free bodies composed of fibrin, cartilage matrix, or ancient necrotic tissue, do not become anchored because they cannot elicit adhesions; for this they lack, or have long lost, the major source of chemical inflammatory agents: autolyzing cells. Our ordinary liver implants, weighing 500—700 mg., became adherent without exception at about 15 hours, even if sterile; on the other hand, smaller implants (50—80 mg.) often remained free and became coated with connective tissue. We interpret this as indicating that the small amount of irritants produced by these fragments was not sufficient to promote adhesions prior to the advent of denaturation.

It is interesting, in this respect, to note that in the experiments of BERENBOM et al. (1955b) intraperitoneal implants of mouse liver often remained free: the size of these implants was considerably smaller than in our experiments.

*Considerations on the meaning of "cloudy swelling".* Since our work has brought us to grapple with the concept of "cloudy swelling", it might be useful to review very briefly some of the past history of this term, and discuss its present meaning.

In Virchow's concept the most typical instance of Cloudy Swelling is that illustrated in Lecture XIV of his Cellular Pathology: that is, the cellular change found in the convoluted tubules in "Bright's Disease". Because "[he] convinced

[himself] that the changes characteristic of this condition take place inside the parenchymal cells" and that these changes represented a response to irritation, Virchow adopted the term *parenchymatous inflammation* as synonymous with *cloudy swelling* (1852). Virchow's own drawing, here reproduced (Fig. 22), shows a renal tubule; at one end, the cells are depicted as more opaque to transmitted light, and some are also larger. It should be emphasized that this illustration reproduces fresh, unfixed tissue, teased in water[1].

Virchow himself interpreted the granules in the renal epithelium as absorbed, but non-assimilated, nitrogen-containing material: which is quite acceptable by present concepts. From these facts, however, Virchow went on to build a broader generalization.

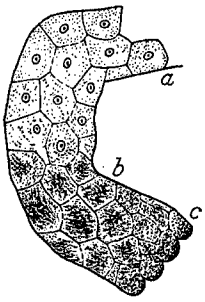



Fig. 22. Virchow's own illustration of "Cloudy Swelling", from Lecture XIV of his Cellular Pathology, entitled "Activity and Irritability of Cellular Elements; Different Forms of Irritation" (American Translation, 1860). Original legend: "Convoluted tubule from the cortex of the kidney in morbus Brightii. *a* Tolerably normal epithelium, *b* state of cloudy swelling, *c* commencing fatty metamorphosis and disintegration. At *b* and *c* increased breadth of the tubule. 300 diameters". The preparation is teased in water

The cells in the renal tubules showed two abnormalities: they were swollen, and they contained proteinaceous granules. He therefore grouped the renal lesion together with a number of other conditions in which the cells become larger or "granular". Hence the concept of hypertrophy came to overlap rather broadly with that of cloudy swelling, which Virchow defined as "hypertrophy with a tendency towards degeneration". On the other hand, cells with a "granular" cytoplasm are of common occurrence in pathologic tissues, and they were also included. When Virchow mentions the "granules" which appear in cells as the result of a variety of injuries (Lecture XIV, op. cit.) he is probably referring—in part at least—to swollen mitochondria; and from the studies of later authors, often based on liver cells, it is quite obvious that the material described as causing "cloudiness" consists of a variety of inclusions, normal and pathologic, such as basophilic material, mitochondria (LANDSTEINER 1903), denatured protein, and even fat droplets. It is not surprising that the concept of cloudy swelling soon became lost among this variety of "granules".

Virchow justified the generalization by considering the various cellular conditions as "responses to irritation", in a broad sense; and his concept certainly had the merit of focussing attention onto the early cellular responses to injury. Virchow's followers, however, became hopelessly entangled in the multiplicity of conditions designated by the same name. A number of authors studied "cloudy swelling" by producing compensatory renal hypertrophy (see BELL 1913); others claimed that swelling and cloudiness were not necessarily associated (FRICKE et al. 1929). As fixation and embedding procedures were introduced it was largely forgotten that Virchow's descriptions referred to fresh, unfixed tissue, examined in water, and could not be directly applied to paraffin sections. HOPPE-SEYLER contributed in no small measure to the confusion, as already mentioned, by lending Virchow's notion of protein accumulation the support of his analyses. At about the same time, in an effort to retain something of the concept of cloudy swelling, BELL introduced the use of the term for gross description *only*, thus divorcing it completely from the field of cellular pathology.

[1] We wish to thank Dr. W. ACKERKNECHT, author of a profound study on VIRCHOW (ACKERKNECHT 1953), for giving to this statement the support of his authority (personal communication).

It is interesting to notice that during a century of research on "cloudy swelling", the least attention was devoted to the "cloudiness" and to the "swelling" *per se*. Both properties were taken for granted. Among the plethora of papers on the subject, we found only one author who actually measured the diameters of liver cells which were supposedly the site of cloudy swelling: he found that the cells were sometimes bigger, sometimes smaller, and sometimes the same (UHER 1931). As for the *Trübung*, it was never measured; and many descriptions betray the effort to describe something which is not there. Only one author seems to have been intrigued by the fact that cells should take up water, and at the same time become more opaque. Rather than doubt the "Trübung", however, he developed a complex optical theory, whereby the protoplasmic colloids, by becoming more hydrated, should appear less transparent (UHER 1931, 1939).

At present, the term is in use, but it remains to be seen which condition it should represent. The renal tubular change which first impressed Virchow, the nephrotic tubule, is more appropriately designated as an example of *protein storage*. There is a tendency nowadays to label as cloudy swelling, in rather vague fashion, the early cellular changes which are observed, or presumed to occur as the result of quick-acting injury such as sudden ischemia. Here too it is necessary to separate fact and tradition. A rapid *swelling*, that is, a rapid uptake of water, certainly represents one of the basic responses of cells to injurious agents. It is easy to observe a rapid swelling by placing cells under conditions of anoxia. In fact, most if not all instances of rapid swelling can be related to some variety of anoxia, such as ischemic (e. g. see VAN HARREVELD 1957) or toxic. Hypotonic solutions will also induce a rapid swelling. It certainly seems justified to group together all the conditions, such as those just mentioned, in which the common denominator is a rapid uptake of water. There is no good reason, however, to designate this swelling as "cloudy", a term which was appropriate under entirely different conditions as descriptive of an increased optical density. We wish to propose a simpler substitute, perhaps the most adequate at the present state of knowledge: *cellular oedema*, defined as that *group of conditions in which the cell or a part of the cell swells by uptake of water*. The term implies a condition different from hypertrophy, in which protoplasm is added as well as water[1].

It should be expected that there be several varieties of cellular oedema, differing with regard to the intracellular compartments affected by water uptake, and with regard to accompanying biochemical disorders (such as a drop in intracellular $p_H$, protein denaturation, etc.).

[1] *Hydropic swelling* might also be appropriate. However, the term has been too much involved in the "cloudy swelling" debate to be now used in a different light. *Oncosis*, a name originally coined by von RECKLINGHAUSEN (1910) to indicate forms of "degenerative" cellular swelling, probably had much the same connotations as our "cellular oedema". The term was then used by v. RECKLINGHAUSEN himself, as it is now mainly by the school of E. RUTISHAUSER (see MAJNO and ROUILLER 1951), to indicate one specific instance of swelling, that of the osteocytes and their lacunae as a result of injury. *Parenchymatous degeneration* was used again recently in the sense of our cellular oedema (POPJÁK); this term is descriptively not very satisfying, since it implies a restriction to the cells of "parenchymatous organs". The term *cellular oedema* is being currently used by BESSIS (1957) and others, with essentially the same meaning as here defined.

There has been much debate as to the "progressive" or "regressive" nature of cloudy swelling (see POPJÁK 1948, GROLL 1949). The question is entirely unanswerable if it is applied, as it has been traditionally, to a heterogeneous group of cellular changes; on the other hand, if referred to our more limited notion of *cellular oedema*, it becomes quite relevant. We will assume that by "progressive" and "regressive" is meant, in a general way, the prevailing of anabolic and catabolic phenomena, respectively. A situation such as that of our liver implants, which were submitted to sudden, total ischemia, should provide a relatively simple example of purely regressive changes. On the other hand, when the cells are injured in a less drastic fashion—such as by partial ischemia, or by noxious agents present in the circulation—it is quite conceivable that reactive changes should also appear, either simultaneously or in a healing phase. It is thus that we interpret the findings of POPJÁK: two days after the injection of large doses of diphtheria toxin into rabbits, the livers were enlarged, the water content was increased, but there was also an increase in total liver mass. The cells were swollen and there was evidence of multiplication. Assuming that all the liver cells were affected (a point which is not specifically mentioned), it is obvious that the regressive cellular changes of cellular oedema were present simultaneously with others of a reparative nature. In other words, there is a blending of regressive changes with "irritation" and hypertrophy: a condition which is, perhaps, morphologically inextricable—but astonishingly close, after all, to Virchow's notion of "parenchymatous inflammation".

## Conclusions and Summary

1. The concepts of "cloudy swelling", "albuminous degeneration", "cellular death", "necrosis" and "coagulation necrosis" are briefly reviewed. The difference between "cellular death" and "necrosis" is emphasized.

2. When a tissue is submitted to sudden, complete ischemia the ensuing changes may be viewed in the following sequence: a) a period of *reversible changes*, b) a "*point of no return*" herein identified with the time of cellular death, c) a "*pre-necrotic period*", d) a period of changes recognizable histologically as *necrosis*.

3. The purpose of this work was to follow the sequence of events outlined above, under experimental conditions which would essentially preclude concomitant repair. Fragments of rat liver were implanted into the peritoneum of recipient rats, or preserved aseptically *in vitro* at 4° or 37° C. The samples were examined with respect to wet weight, dry weight, $p_H$, extractable protein as determined with a microbiuret method, and oxygen uptake. Protein denaturation was followed by the study of fresh, unfixed sections in the dark field, using visible as well as ultraviolet light; and by measurements of optical density in transmitted light. Morphological changes were studied at all stages, and time-curves were constructed for all variables.

4. Morphological changes in liver implants varied with the fixative used and with the depth of the cells in the specimen. *At the end of the first hour*, formalin-fixed material could not be recognized as pathologic on sections stained with hematoxylin and eosin. After fixation in Helly, the peripheral cells appeared filled with semitransparent "granules", and vacuoles also developed. The cells in the inner core were normal in aspect. The *histological diagnosis of necrosis*

could not be brought earlier than the 8th hour. It is recalled that the point of no return for ischemic rat liver lies towards the end of the first hour. Hence many cellular changes which would ordinarily be described as "mild" actually occur after the death of the cell. On the other hand, the *gross diagnosis of necrosis* could be established 3—4 hours earlier than with the microscope. The characteristic whiteness of necrotic tissue depends upon protein denaturation (below), which is more readily observed in the fresh state than in sections of embedded tissue.

5. Liver implants continued to swell for 6 hours, later they tended to decrease in weight. Only occasional cells could be recognized as swollen in paraffin sections. Planimetric measurements of cells up to the 8th hour failed to show significant changes. It is concluded that this acute type of "waterlogging" of the cells is almost completely reversed by paraffin embedding.

6. At the time when the cells should be undergoing "cloudy swelling", the optical density of the tissue actually drops, presumably as a consequence of mitochondrial swelling. Later the optical density rises, and this effect is attributed to protein denaturation (the latter is defined as irreversible precipitation of protein with formation of large insoluble aggregates).

7. In sections of unfixed liver implants viewed with the dark field, intracellular bright granules began to appear between the 15 and the 30 minute stages; later these granules became so numerous that the cell acquired a "snow-white" aspect. The granules are interpreted as aggregates of denatured protein.

8. When the same sections were examined in ultraviolet light, normal liver cells remained practically invisible, but in sections of implants autofluorescence began to develop between the 15 and the 30 minute stages, and increased thereafter. Inasmuch as autofluorescence is indicative of protein denaturation, this observation supports the view mentioned above, that intracellular protein denaturation is demonstrable between the 15th and the 30th minute. Contrary to previous statements, denaturation was found to occur also *in vitro*, though not as rapidly as *in vivo*, and with complete histological resemblance to "coagulation necrosis".

9. The proteinaceous material defined as "extractable peptide bond" increased in liver implants up to about 6 hours, indicative of an underlying process fundamentally similar to that of autolysis. Later the extractable peptide bond decreased, as may be expected from the occurrence of protein denaturation.

10. It is suggested that two processes compete for the destruction of the cellular proteins, *even before the cell is irreversibly damaged:* autolysis and denaturation.

11. On the basis of original results, as well as of data obtained from the literature, a tentative sequence of events is reconstructed, leading from the early reversible changes through cellular death and necrosis.

12. The validity of our data is discussed with reference to other tissues, and some implications of protein denaturation *in vivo* are discussed. Implants of fully necrotic liver tissue were found to evoke little or no local reaction, similar to fragments of boiled liver tissue.

13. The history and present meaning of the term "cloudy swelling" are reviewed. It is pointed out that this term was originally coined by Virchow to describe an alteration of the convoluted tubules of the kidney in "Bright's disease", whereby the cells, *viewed in the fresh state*, appeared more opaque (cloudy) than

normal. This condition is now better defined as protein storage. The present use of the term to indicate the earliest observable and presumably mild cellular alterations is inappropriate and misleading. *Cellular oedema* is suggested as an alternate, to comprise those conditions in which the cell, or parts of the cell, enlarge by uptake of water.

## Schlußfolgerungen und Zusammenfassung

1. In der vorliegenden Arbeit wird zuerst ein kurzer Überblick der Begriffe „trübe Schwellung", „albuminöse Schwellung", „Zelltod", „Nekrose" und „Koagulationsnekrose" gegeben. Der Unterschied zwischen Zelltod und Nekrose wird besonders hervorgehoben.

2. Die durch plötzliche, vollkommene Ischämie verursachten Gewebsveränderungen können in folgender Anordnung zusammengefaßt werden: a) Eine Zeitspanne reversibler Veränderungen, b) ein Schädigungsgrad ohne Wendepunkt ("point of no return"); diesen bezeichnen wir als die Zeit des Zelltodes. c) Eine pränekrotische Phase, und d) eine Zeitspanne von Veränderungen, welche histologisch als Nekrose erkennbar sind.

3. Es war die Absicht, in dieser Arbeit die oben geschilderte Reihenfolge zu erläutern, und zwar unter experimentellen Bedingungen, welche reparative Vorgänge praktisch ausschließen würden. Stücke von Rattenleber wurden entweder in der Bauchhöhle einer Empfängerratte isoliert, oder aseptisch *in vitro*, bei einer Temperatur von 4° oder 37° C aufbewahrt. Dieses Versuchsmaterial wurde in bezug auf Frischgewicht, Trockengewicht, $p_H$, lösliches Protein (mit Hilfe einer Mikrobiuretmethode bestimmt) und Atmungsfähigkeit untersucht. Die Proteindenaturierung wurde mit verschiedenen Methoden verfolgt: a) durch Untersuchung unfixierter Schnitte im Dunkelfeld bei Anwendung von sichtbarem wie auch ultraviolettem Licht, und b) durch Messungen optischer Dichte unfixierter Schnitte bei durchfallendem Licht. Die morphologischen Veränderungen wurden in jedem Stadium verfolgt, und für alle Variablen wurden Zeitkurven angefertigt.

4. Die morphologischen Veränderungen isolierter Leberstückchen variierten mit verschiedenen Fixierungen und mit verschiedener Dicke des Schnittes. Formol-fixiertes Material, Hämatoxylin-Eosin gefärbt, konnte *am Ende der ersten Stunde* nicht als pathologisch angesehen werden. Nach Fixierung mit Hellyscher Flüssigkeit wiesen die peripheren Zellen semitransparente „Körnchen" auf, sowie auch kleine Vacuolen. Die Zellen im Zentrum der Leberstückchen behielten dabei ein normales Aussehen. *Histologisch* konnte die Nekrose nicht vor der achten Stunde diagnostiziert werden. Es ist bekannt, daß Rattenleber nach einer Stunde Ischämie irreversibel geschädigt ist. Dementsprechend treten viele Zellveränderungen, welche gewöhnlicherweise als „geringfügig" bezeichnet wurden, erst nach dem Tode der Zelle ein. Dagegen konnte die *makroskopische Diagnose* der Nekrose 3—4 Std vor der mikroskopischen gestellt werden. Das typisch weiße Aussehen nekrotischen Gewebes entspricht einer Proteindenaturierung (s. unten), welche besser im frischen Zustande als in Schnitten eingebetteten Gewebes ersichtlich ist.

5. Während der ersten sechs Stunden schwollen die in der Bauchhöhle isolierten Leberstückchen allmählich an; später zeigten sie eine Gewichtsabnahme. In Paraffinschnitten konnten nur vereinzelte Zellen als geschwollen bezeichnet werden; auch planimetrische Messungen der Zellen, welche bis zum 8 Std-Stadium

gemacht wurden, wiesen keinerlei nennenswerte Schwellung auf. Daraus wird der Schluß gezogen, daß diese Art akuter „Wasserquellung" der Zellen durch Paraffineinbettung fast gänzlich verloren geht.

6. Zur Zeit, da eine „trübe" Schwellung der Zellen, im traditionellen Sinne aufgefaßt, zu erwarten wäre, ist die optische Dichte des frischen Gewebes in Wirklichkeit vermindert, möglicherweise als Folge einer Schwellung der Mitochondrien. Später nimmt die optische Dichte zu, ein Phänomen, welches der Proteindenaturierung zugeschrieben wird (letztere wird als irreversible Proteinausfällung mit Bildung großer, unlöslicher Aggregate definiert).

7. In Schnitten unfixierter Leberstückchen, die in der Bauchhöhle isoliert waren, wurden, wenn im Dunkelfeld betrachtet, zwischen dem 15 und 30 Minuten-Stadium intracelluläre helle Körnchen sichtbar; später wurden diese Körnchen so zahlreich, daß die Zelle einen „schneeweißen" Anblick darbot. Die Körnchen werden als Aggregate von denaturiertem Protein angesehen.

8. Wenn dieselben Schnitte in ultraviolettem Licht betrachtet wurden, blieben normale Leberzellen beinahe unsichtbar; in Schnitten der in der Bauchhöhle isolierten Leberstückchen dagegen, entwickelte sich eine Autofluorescenz, die zwischen dem 15 und 30 Minuten-Stadium zuerst sichtbar wurde, später aber anstieg. Diese Beobachtung, insofern die Autofluorescenz für eine Proteindenaturierung bezeichnend ist, unterstützt die oben erwähnte Ansicht, daß die intracelluläre Proteindenaturierung zwischen 15 und 30 Min. nach Isolierung des Gewebes nachweisbar ist. Früheren Aussagen widersprechend wurde festgestellt, daß die Denaturierung ebenfalls *in vitro* stattfinden kann, wenn auch langsamer als *in vivo*, und mit vollständiger histologischer Ähnlichkeit zur Koagulationsnekrose.

9. In Leberstückchen, welche in der Bauchhöhle isoliert wurden, stieg das proteinhaltige Material, definiert als „extrahierbare Peptidbindung", bis ungefähr zur sechsten Stunde an; ein Vorgang, welcher grundsätzlich dem der Autolyse ähnlich ist. Später, wie von dem Vorkommen der Proteindenaturierung zu erwarten wäre, nahm die „extrahierbare Peptidbindung" ab.

10. Es wird darauf hingewiesen, daß, schon bevor die Zelle irreversibel geschädigt ist, zwei Vorgänge um die Vernichtung des cellulären Proteins wetteifern: nämlich Autolyse und Denaturierung.

11. Auf Grund eigener Resultate, sowie Literaturangaben, wird eine tentative Sequenz von Ereignissen rekonstruiert, welche von frühen, reversiblen Veränderungen bis zu Zelltod und Nekrose führt.

12. Die Gültigkeit unserer Resultate wird mit Bezugnahme zu anderen Geweben besprochen, und gewisse Folgerungen der Proteindenaturierung *in vivo* werden in Betracht gezogen. Es wurde festgestellt, daß völlig nekrotische Leberstückchen wenig oder gar keine lokale Reaktion in der Bauchhöhle hervorriefen, ein Verhalten, das dem von gekochter Leber ähnlich ist.

13. Die Geschichte und gegenwärtige Bedeutung des Begriffes „trübe Schwellung" werden kritisch zusammengefaßt. Dabei wird betont, daß dieser Name ursprünglich von VIRCHOW zur Beschreibung der Tubuli contorti der Niere in der „Brightschen Krankheit" geprägt wurde, wobei die Zellen, *im frischen Zustand* betrachtet, undurchsichtiger (trüber) als normal erschienen. Dieser Zustand ist heute besser als Proteinspeicherung definiert. Die gegenwärtige Anwendung

dieses Ausdruckes zur Beschreibung der frühest beobachtbaren und vermutlich geringfügigen Zellveränderungen, ist unpassend und irreleitend. Als eine alternative Bezeichnung derjenigen Zustände, in denen die Zelle, oder gewisse Zellteile, sich durch Wasseraufnahme erweitern, wird *celluläres Ödem* vorgeschlagen.

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