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Electrical Sizing of Liver Cell Nuclei by the Particle beam Method. Mean Volume, Volume Distribution and Electrical Resistance

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Summary

Electrical sizing of liver cell nuclei gives similar results as histological techniques for the mean volume, the volume distribution and the proportions of the different nuclear volume populations. The electrical method has, compared to histological technics, however, the advantage of higher rapidity and accuracy. Due to the good volume resolution of the improved electrical sizing method two populations of nuclei with different mean volumes can be distinguished in rat liver tissue besides di-, tetra- and octoploid cell nuclei. In addition to the volume distribution curves, the mean electrical resistance of isolated liver nuclei can be determined by the electrical sizing method. A newly developed micromethod provides enough cell nuclei for electrical sizing from only 10 mg liver tissue in a few minutes. This should enable the measurement of nuclear volume distribution curves of human biopsy material from the liver and gastrointestinal tract for diagnostic purposes on a larger scale.

Zusammenfassung

Die elektrische Größenbestimmung von Leberzellkernen ergibt ähnliche Resultate wie histologische Techniken für das mittlere Volumen, die Volumenverteilung und den Anteil der verschiedenen Zellkernvolumenpopulationen. Die elektrische Methode hat gegenüber der histologischer Auswertung den Vorteil größerer Schnelligkeit und Genauigkeit. Auf Grund der guten Volumenauflösung der verbesserten elektrischen Größenbestimmungsmethode lassen sich im Rattenlebergewebe neben di-, tetra-, und oktoploiden Kernen zwei weitere Zellkernklassen mit unterschiedlichem mittlerem Volumen abgrenzen. Eine neu entwickelte Mikromethode erlaubt die Präparation ausreichender Zellkernmengen für die elektrische Grössenbestimmung aus nur 10 mg Lebergewebe in wenigen Minuten. Dadurch sollte die Bestimmung von Zellkernverteilungskurven aus menschlichem Biopsimaterial von Leber- sowie Magen- und Darmtrakt im Sinne verbesserter Diagnostik in größerem Maße möglich sein.

Key Words: Electrical sizing – Volume – Liver – Cell nuclei – Rat – Biopsy

Introduction

Di-, tetra- and octoploid liver cell nuclei can be distinguished on the basis of DNA content and size (4). The proportions of these different nuclear populations change during development (14) pharmacological treatment (18), after partial hepatectomy (8), hypophysectomy (23) and possibly other influences (15). Such changes are usually measured by histological technique which are time consuming and of limited accuracy.

As an alternative method we have sized liver cell nuclei electrically with an improved Coulter method (9). Thereby a rapid and accurate information on the proportions of the different nuclear populations, their volume distribution and their electrical resistance was obtained. Since very little tissue is needed this technique should be applicable to human biopsy material

Methods

Preparation of liver cell nuclei

Specific pathogen free (SPF) male and female Sprague-Dawley rats (Gassner, München-Ottobrunn) were anesthetized with ether and bleeded through the abdominal aorta until respiratory arrest. The livers were excised, cooled to $0^{\circ}C$ and citrate and sucrose liver cell nuclei prepared as described (18,22). The nuclei were resuspended in 1% citric acid or 0.3 M sucrose solution, 3mM $MgCl_2$ respectively. The temperature of the nuclei was mantained during the preparation at $0\text{-}4^{\circ}C$ until the volume measurement.

A rapid micromethod was performed as follows: 10 mg liver tissue were homogenized with 0.5 ml 1% citric acid in a Potter-Elvehjem 5 ml glass homogeniser with teflon pistle. The homogenate was centrifuged for 30 sec. at 12000 rpm (8000 g) in an Eppendorf centrifuge model 3200 (Eppendorf, Hamburg, Germany). The supernatant was discarded and the sediment resuspended in 0.2 ml 1% citric acid followed by 0.8 ml

2.5 M sucrose solution with 1% citric acid. After mixing, the suspension was centrifuged for 5 min at 8000 g. The supernatant was carefully removed with a syringe. The nuclear pellet on the bottom and outer side of the centrifugation vial was resuspended in 0.1 ml Tris buffered 0.2 M KCl solution (see below) and transfered to a new tube. For the better adaption of the electrical conductivity the nuclei were washed once with 1 ml KCl buffer, centrifuged for 30 sec at 8000 g and resuspended in 0.1 ml of the same buffer.

Sizing of the nuclei

The volume of the liver cell nuclei was measured by an improved Coulter method developed in our laboratory (9,10,16). The orifice had 70 μm diameter and approximately 35 μm length, and was modified by a particle beam attachment (9). Thus all particles were sized under the same electrical and hydrodynamical conditions and methodologically right skewed curves of unmodified Coulter orifices were avoided (9). Fewer material was needed and no contact of the cell nuclei with products of electrolysis from the electrodes occurred. All measurements were performed at 25°C in 0.2 M KCl, 5 mM Tris/HCl pH 7.4 buffer with a specific electrical resistance of 41Ωcm. The orifice current was 0.52 mA. Between 150-250 particles/sec were measured at a suction of 0.1 kg/cm^2.The volume impulses were amplified and classified according to their maximal height by a multi channel analyzer with a maximal range of 60 classes. 1.0-1.5x10^4 particles were registered per volume distribution curve. For analysis the volume distribution curves were approximated by a computer with linear or logarithmic Gaussian normal distributions (6) with the method of least squares. From the analysed curves the modus, the mean value and the standard error were calculated for all populations of nuclei.

Electrical resistance of the cell nuclei

The cell nuclei were suspended in a 0.3 M sucrose solution to which increasing amounts of NaCl were added. The suspension was measured in the sizing apparatus with a 70 μm orifice at a current of 60 μA. The polarity of the impulses was observed on an oscilloscope. If the particle resistance was higher than the resistance of the suspending medium all impulses were positive, if it was approximatelly equal the impulses became small and an equal number of positive and negative impulses were seen on the screen and if it was inferior all impulses became negative. The specific electrical resistance of the sucrose/NaCl solution was determined when an equal number of positive and negative impulses were observed. This value was taken as the mean specific resistance of the cell nuclei in Ωcm.

Form factor

In some experiments the cell nuclei were photographed (11) on their way through the orifice to determine their form under the conditions of the volume measurement. The long and short axis of the nuclei was measured in the photographs and the form factor was calculated (7).

Volume calibration of the sizing apparatus

The absolute volume of the cell nuclei was calculated after calibration of the apparatus with latex particles of known volume (13) (Particle Information Service, Los Altos, Cal.) and with rat erythrocytes whose mean volume (MCV) had been determined from microhematocrit and cell concentration and by an electrical calibration method (13). All calibration methods agreed closely if form factors of 1.5 for spherical latex particles (5,7) and of 1.05 for rat erythrocytes (10) were used for the volume calculation (13).

Microscopic examination

For light microscopy the liver cell nuclei were suspended in 0.2 M KCl with 5 mM Tris/HCl pH 7.4 and phase contrast photographs were taken. For calibration purposes a micrometer slide with 10 μm divisions was photographed under the same conditions. The photographic negatives were projected 10 times enlarged on a screen and the long (a) and short (b) axis of the nuclei was measured. The volume of each nucleus was calculated assuming a rotational ellipsoid shape (volume = $4/3\pi$ ab^2 (μm^3)). 100 nuclei were examined per preparation.

For the scanning electron microscope (SEM) the nuclei were fixed in suspension with 3% glutaraldehyde in 0.1 M phosphate buffer pH 7.5 at 4°C for 2h. They were then dehydrated in ethanol and dried on small glass slides by critical point evaporation and finally shadowed by carbon and gold evaporation.

Results

Morphology

The morphological aspect of isolated citrate liver cell nuclei in phase contrast and SEM is shown in Fig. 1. Sucrose and citric acid nuclei had a similar appearence in both techniques except for more adhering material on the nuclear surface and a higher degree of aggregation in preparations of sucrose nuclei.

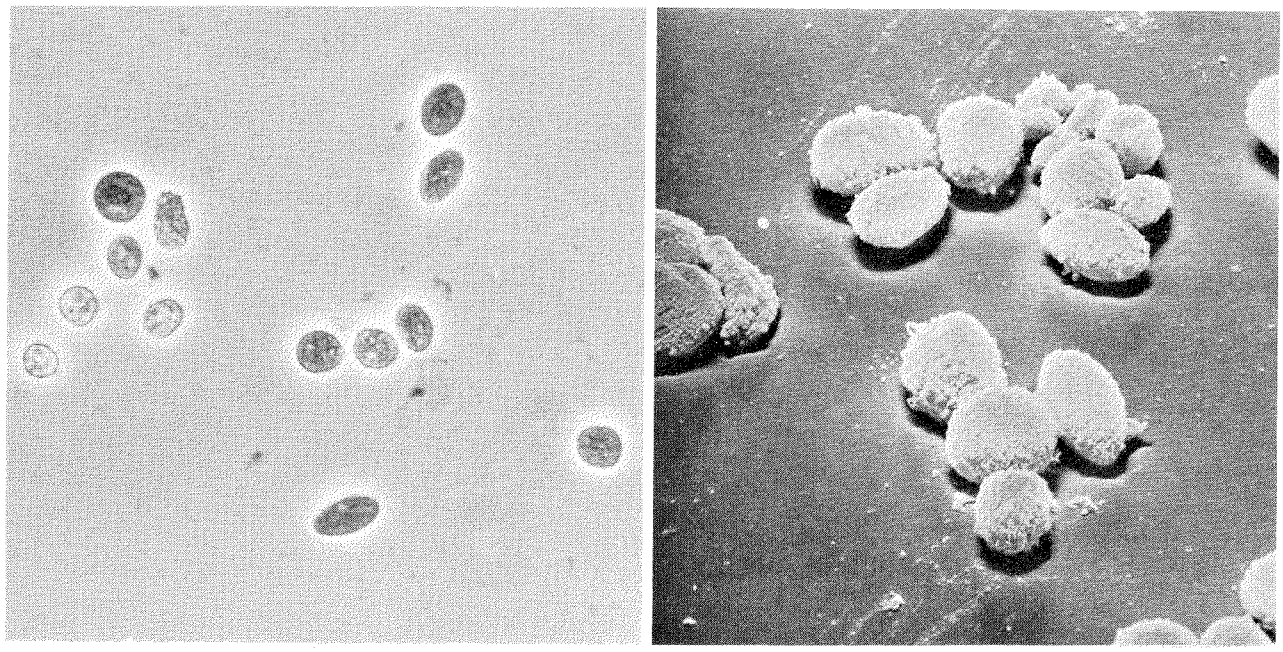

Fig. 1 Citric acid rat liver cell nuclei. a) phase contrast microscopy, magnification 820 fold. b) SEM, magnication 1370 fold.

Comparison between the volume distribution curves of sucrose and citric acid liver cell nuclei

The volume distribution curves of sucrose and citric acid liver cell nuclei of the same liver were similar (Fig. 2). Sucrose nuclei were 10-30% smaller than citric acid nuclei. Citric acid nuclei were better suited for sizing purposes since the curves had less background, deeper minima between the volume peaks and more constant mean volumes for the nuclear populations.

Form factor

Photographs of liver cell nuclei showed that no systematic deformation occurred in the orifice. The form factor could therefore be calculated (7) more easily from photographs of suspensions

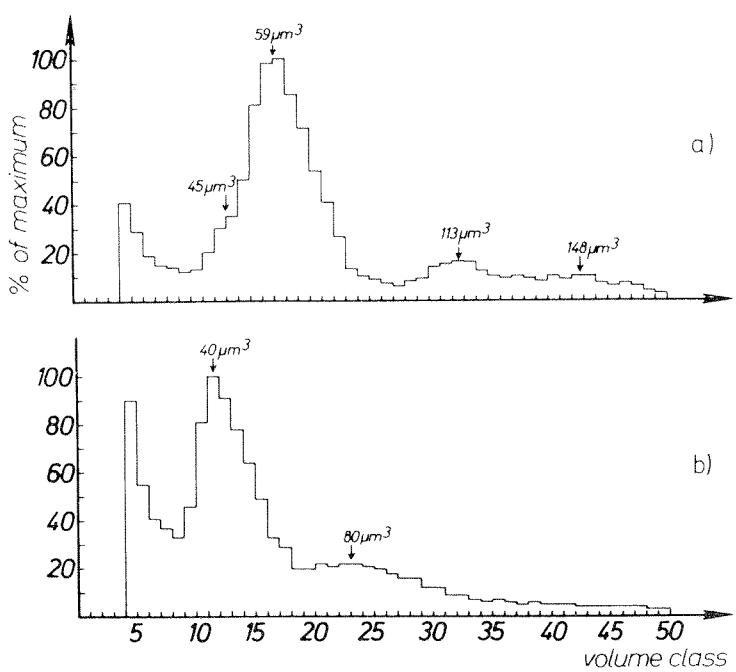

Fig. 2 Volume distribution curves of citric acid (a) and sucrose rat liver cell nuclei (b) from the same animal (130 g, ♀). One volume class corresponds to 3.46 μm^3. The volumes of the lower graph have to be multiplied with a correction factor (2) of 1.08 to obtain the absolute volume because sucrose liver cell nuclei have a low electrical resistance.

of liver cell nuclei. The form factors of 100 cell nuclei are shown in Fig. 3. The mean form factor of the distribution was 1.35 ±0.07. No apparent correlation between the form factor and the volume of liver cell nuclei was observed.

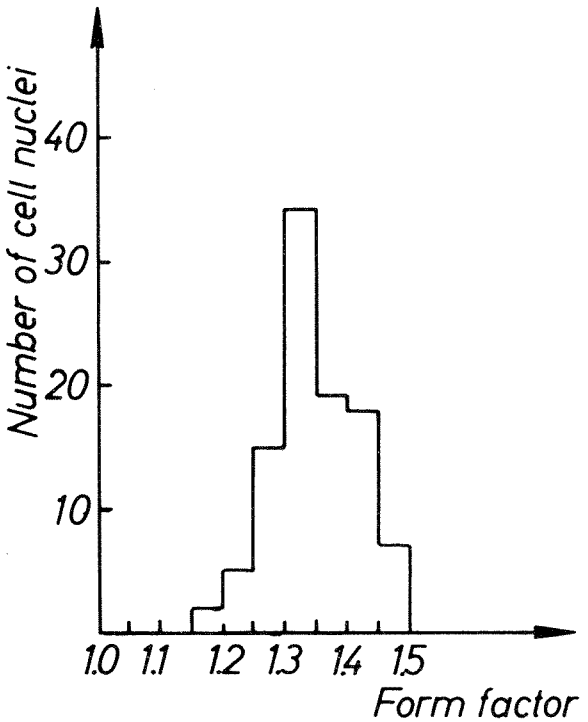

Fig. 3 Form factor distribution of 100 rat liver cell nuclei. The form factor was calculated (7) from the long and short axis of each nucleus. The mean form factor of 1.35±0.07 corresponds to an average axial ratio of 1.3:1.

Volume distribution curves of citric acid cell nuclei

Di-, tetra- and octoploid volume peaks B, C and E are distinguishable in the volume distribution curves of liver cell nuclei of rats of different age (Fig. 4). The ratio of their mean volumes is approximately 1:2:4 (Tab. 1). Besides the three population B, C and E small but reproducible proportions of two additional nuclear populations A and D are apparent. One was smaller (A) than diploid and the other (D) larger than tetraploid but smaller than octoploid nuclei. The proportions of the different cell nuclei changed with the age of the animals (Fig. 5). Young animals had essentially diploid cell nuclei, old animals had a higher proportion of tetraploid nuclei. Octoploid nuclei were only occasionally seen. The nuclear populations A and D accounted for 5-25% of the total nuclei. The mean volume of all populations remained unchanged throughout the life and was equal for male and female animals (Fig. 6). The computer analysis of the volume distribution curves showed that 2/3 of the curves were better approximated by logarithmic and 1/3 better by linear normal distributions.

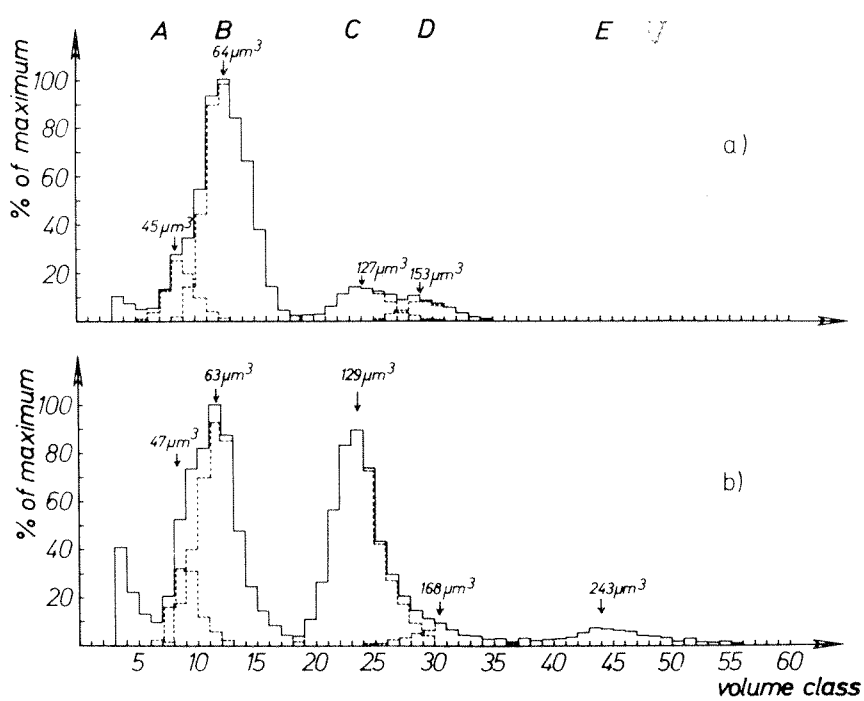

Fig. 4 Volume distribution curves of citric acid rat liver cell nuclei. Five populations of nuclei (A-E) with different modal volume can be distringuished. The female animal (a) weighed 199 g and the male animal 185 g (b). The absolute volume calibration was 5.12 μm^3 and 5.80μm^3 per volume class respectively.

Table 1 Volume und variation coefficients * of liver cell nuclei of the rat

Nuclear population	Volume of the peak (Modus) (μm^3)	Mean volume (μm^3)	Linear variation coefficient (%)
A	43.8 ± 0.9	45.2 ± 0.9	14.1 ± 0.7
B (diploid)	58.8 ± 0.8	60.7 ± 0.7	13.8 ± 0.7
C (tetraploid)	123 ± 1	125 ± 1	10.3 ± 0.7
D	155 ± 3	158 ± 4	10.4 ± 0.9
E (octoploid)	236 ± 2	250 ± 3	-

* ± standard error
The number of experiments was 27. The linear variation coefficient in % was calculated as the ratio of the linear standard deviation and mean volume of each nuclear population times 100.

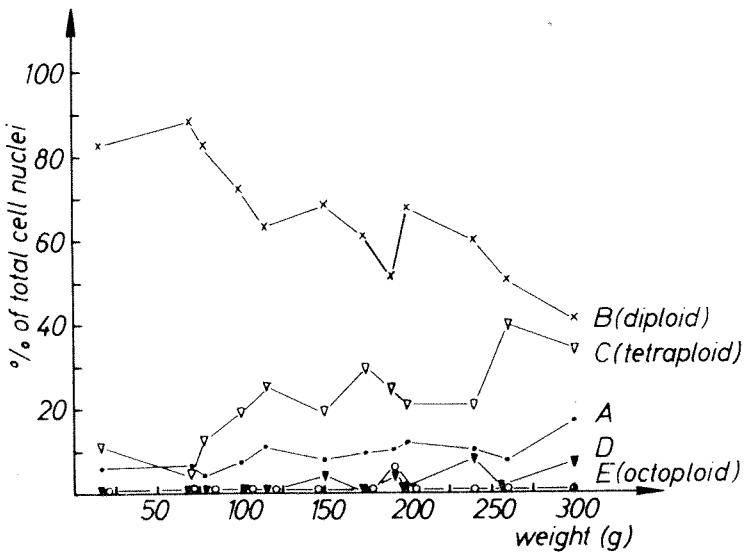

Fig. 5 Proportion of population A-E rat liver cell nuclei during the life of female animals.

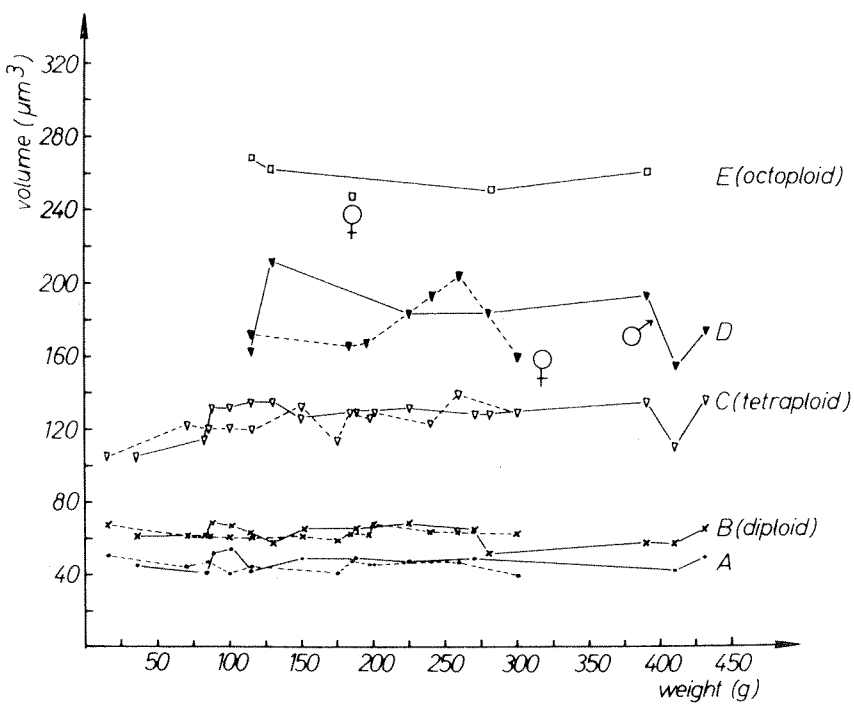

Fig. 6 Mean volume of the population A-E rat liver cell nuclei in male (solid lines) and female animals (broken lines) during the life. The data of Fig. 5 and 6 represent the results obtained from the volume distribution curve of liver cell nuclei of one animal at each weight point.

Absolute volumes

The absolute volumes of the cell nuclei were determined by electrical and microscopical sizing of six different preparations which contained approximately 80% diploid and 20% larger cell nuclei. A mean volume of 77 ± 3 μm^3 was determined from 60.000 electrically sized nuclei as compared to 93 ± 11 μm^3 for 600 microscopic determinations. The electrical volume was independent of the orifice current in the range 0.1-0.9 mA.

Electrical resistance

The specific electrical resistance in six preparations was 840 ± 44 Ωcm for sucrose and greater than 9600 Ωcm for citric acid cell nuclei.

Discussion

The mean volume of diploid citric acid liver cell nuclei by electrical sizing is approximately 20% smaller than the microscopic mean volume. Several possibilities have to be considered to explain this. In the microscopic measurements a systematic overestimation of the length of the nuclear axis could occur which would be potentiated through the volume calculation. It is however unlikely because care was taken to avoid this. In the electrical measurements, the volume of the nuclei might be understimated because of a low electrical resistance (18), of a transcellular ion flow (24) or of conductive channels through the matrix of the nuclei. The first possibility of a low electrical resistance is not the case in citric acid nuclei since their electrical resistance was 235 times higher than that of the suspending medium. The electrical volume of sucrose nuclei however has to be multiplied by a correction factor (2) of 1.08 to obtain the absolute volume because their electrical resistance is lower. The unequal resistance of both types of nuclei is a consequence of the preparation method which leads to a different biochemical constitution (1). A transnuclear ion flow (24) due to a dielectric breakdown of the nuclear membrane as the second possibility does not occur with the sizing conditions employed because the volume of the nuclei was independent of the electrical current between 0.1–0.9 mA. The third possibility of conductive channels across a non conductive matrix of nuclear material similar as in a sponge is the most likely. The volume of these channels would not be measurable by electrical sizing because the electrical current flows through these channels. The resistance between the outside and inside of the nuclei should then be low. In intact cells this was observed by micropuncture studies for the nuclei of oocytes but not of other cells (12). Considering however that during the preparative procedure an abrasion of the endoplasmatic reticulum from the nuclear surface occurs, it is possible that the nuclear membrane has discontinuities and that the non conductive DNA/protein matrix can be permeated by conductive channels. The degree of permation is then comparatively similar in all nuclear populations because the volume of tetra- and octoploid nuclei is two and four times higher than that of diploid cell nuclei as one would expect from their increasing DNA contents.

With additional parameters the electrical sizing method gave similar results as histological techniques. There was the characteristic increase of tetraploid nuclei with age (14) and a better approximation of the volume distribution curves by logarithmic than by linear normal distributions (17). The difference between both approximations is however not great since the mean and modal values for each population of nuclei are closely together (Tab. 1). The coefficient of variation of the nuclear populations was between 10-14% with a tendency to decrease in larger nuclei. It is even smaller in reality because the shape variations of the nuclei (Fig. 3). broaden the volume distribution of each nuclear population and thereby increase their coefficient of variation. The nuclear volume (Fig. 6) remains constant for all population of nuclei throughout life which one would expect from the fixed DNA content for each class of nuclei.

The higher resolution of the improved electrical sizing method (10, 16, 20) allowed, in contrast

to earlier work with the Coulter counter (18,3) the distinction of two populations A and D of liver cell nuclei which do not fit into the di-, tetra- and octoploid pattern of parenchymal liver cell nuclei. The population A is similar to the stromal cell nuclei occasionally observed in preparative gradient sedimentation experiments (4). The population D cell nuclei derive probably from one of the endothelial, connective tissue, lymphoid or reticuloendothelial cell lines normally present in liver tissue.

The evaluation of the data shows that the electrical method can be used as a reliable sizing procedure for cell nuclei. It is not restricted to liver since cell nuclei of spleen, nervous tissue (21), gastrointestinal tract (19) and probably other tissues can also be sized. The rapidity of the measuring process allows the collection of large samples in a short time which facilitate consecutive mathematical and statistical analysis. Very little tissue is needed for the electric volume measurements because a total of approximately $5x10^4$ cell nuclei is sufficient to measure their volume distribution curve with a particle beam orifice. These amounts of nuclei can be prepared with the above described micromethod in a few minutes. The technique is therefore of interests for the measurement of nuclear size distribution in human biopsy material on a larger scale.

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Synthetic Capacity and Cell Metabolites of Bile Duct Obstructed Rat Livers. Effect of Free and Conjugated Dihydroxy Bile Acids

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Summary

Morphologic alterations in liver cells after bile duct ligation are well known and documented in numerous reports. Biochemical studies concerning metabolic changes in cholestatic liver are rare. Therefore, in this study, liver cell metabolites and the capacity of the perfused cholestatic rat liver to produce glucose, urea and ketone bodies were measured. In addition the influence of dihydroxy bile acids on normal and bile duct ligated rat livers was studied. Concentrations of adenine nucleotides, lactate, pyruvate, 3-hydroxybutyrate, acetoacetate, glucose and UDP-glucose were found to be identical in cholestatic and normal livers. Glycogen content, however, was significantly lowered in cholestatic livers. Gluconeogenesis from lactate and urea production from ammonium chloride were only slightly reduced in bile duct obstructed rat livers. Dihydroxy bile acids did not affect the metabolism of normal or cholestatic livers. Ketone body production from oleate was reduced to 66% in bile duct obstructed livers, taurochenodeoxycholate further reduced this value to 45% of the normal value.

In contrast to earlier reports (*Fisher* and co-workers, 1971 Lab. Invest. 21; 88-91; Gastroenterology 60: 742) chenodeoxycholate induced neither cholestasis nor a marked fall in ATP content of rat liver in our experiments with female Wistar rats.In conclusion,

Zusammenfassung

Es existieren wenig Untersuchungen über den Stoffwechsel der Leberzellen nach Gallenganstligatur, während die morphologischen Veränderungen bei der Cholestase sehr genau untersucht wurden. In der vorliegenden Arbeit wurden deshalb die Zellmetabolite sowie die Syntheseraten von Glucose, Harnstoff und Ketonkörpern in der isoliert perfundierten, cholestatischen Rattenleber gemessen. Der Gehalt der Leberzelle an Adeninnukleotiden, Laktat, Pyruvat, 3-Hydroxybutyrat, Acetacetat, Glucose und UDP-Glucose war nach Gallengangsverschluß nicht verändert. Der Glykogengehalt war jedoch signifikant erniedrigt. Die Glukoneogenese und Harnstoffbildung war nach Gallengangsverschluß leicht vermindert. Die Addition von Dihydroxygallensäuren zum Perfusionsmedium erzeugt keinen zusätzlichen hemmenden Effekt. Die Bildung von Ketonkörpern aus Oleat war jedoch auf 66% reduziert, dieser Wert wurde durch Addition von Taurochenodesoxycholsäure (0.25 M) weiter auf 45% erniedrigt.

Im Gegensatz zu Mitteilungen aus der Literatur (*Fischer* et al. (1971) Lab. Invest. 21: 88-91; Gastroenterology 60:742) konnte ein toxischer Einfluß von Chenodesoxycholsäure auf Gallefluß und ATP-Gehalt in Lebern weiblicher Wistar-Ratten nicht festgestellt werden.

Es folgt, daß Dihydroxygallensäuren in Kurzzeitexperi-