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Some Cytologic Changes of Rat Liver in Experimental Porphyria.* (30143)

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Experimental porphyria refers to a condition in which one of the most prominent metabolic aberrations is an over-production of porphyrins in the liver. This condition was first induced experimentally with allylisopropylacetylcarbamide (Sedormid), but in recent years a wide variety of compounds has been found to induce the same metabolic changes. Most investigated among these has been the increased formation and accumulation of porphyrins and their precursors in the liver as well as their excretion in the urine(1,2). Not only is the entire heme biosynthetic pathway accelerated in this condition(3), but the effects of porphyria-inducing drugs are now known to encompass several areas of metabolism. In lieu of a thorough literature review suffice it to say that a great variety of enzyme activities, metabolic pathways, and physiological values have been reported to be abnormal in experimental porphyria as well as in the naturally occurring porphyrias.

The observation that livers of porphyric animals are enlarged has been known for several years. Yet, relatively little investigation has been directed toward this effect of the drugs. The present observations on cytologic changes occurring in liver cells were made to obtain a more thorough understanding of the nature of experimental porphyria.

Materials and methods. Sprague-Dawley female rats weighing 180-200 g were divided into 2 groups of 4 animals each. To induce porphyria 26 mg of allylisopropylacetamide (AIA) in a solution of 0.45% NaCl (20 mg AIA/ml) were injected subcutaneously twice daily for 3 consecutive days in the one group. The other group received saline injections. The rats were then sacrificed by decapitation and the livers immediately removed for study. To aid in consistent porphyria induction all animals were starved beginning one day prior to the first injection and throughout the experiment. They were given water *ad libitum*.

Liver volume was determined by water displacement. A portion of each liver was fixed in neutral formalin and stained with hematoxylin and eosin. The number of cells per unit volume of liver tissue was determined by counting the nuclei in the mounted and stained tissue sections using oil immersion (970 ×) in the following manner: The microscope ocular contained a calibrated grid 1 cm × 1 cm and subdivided into 0.1 cm units. The grid was placed over an area chosen at random in the tissue section. All of the nuclei within the perimeter of the grid and in the entire thickness of the section were counted. The thickness of the section was estimated using the fine focus adjustment on the microscope. This ranged from 4 to 7 μ. Thus, knowing the size of the grid,

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magnification of the objective (97 ×), and thickness of the section, it was possible to calculate the number of cells, assuming one nucleus per cell. This counting procedure was repeated on randomly chosen fields until 28 grids had been counted on each slide. One slide was used for each animal. The total number of cells per liver was calculated from the liver volume and the number of cells per unit volume. Additional liver sections were stained with Periodic Acid Schiff Reagent (P.A.S.), Oil Red O, Prussian Blue, and Van Gieson's stains.

The analysis of liver composision was carried out using the following modifications (Benditt, E. P., personal communication) of the method described by Benditt *et al*(4): Approximately one gram of each liver was minced and placed in a 20 ml test tube. The tubes were kept at 70°C for 5 days and the dry weight of the tissue was then determined. The tubes were filled with ethyl alcohol and allowed to stand for 24 hours. The alcohol was decanted and fresh alcohol added. After the third 24-hour alcohol extraction the tubes were dried at 70°C for 3 days and weighed. This procedure was repeated using first ethyl ether and then water as extracting solvents. Finally, the tubes were dried for 5 days at 70°C and the residue weighed. Nucleic acid determinations of liver tissue were performed by Schneider's method(5).

Results. The total liver weight and volume of animals receiving AIA was 100% greater than the controls (Table I). The enlarged livers of the porphyric rats had 27% increase in the total number of cells per liver. Calculating from the number of cells per unit volume and the liver volume, one finds that the total volume per cell was increased by 64% as a result of AIA treatment.

TABLE I. Effect of AIA on Livers of Starved Rats. Physical measurements.

Measurement	Control	AIA treated
Liver wt (g)	4.2 ± .2	8.3 ± .3
Liver vol (ml)	3.9 ± .1	7.8 ± .3
Cells/mm³	5.7 ± .4 × 10^5	3.6 ± .2 × 10^5
Cells/liver	2.2 ± .1 × 10^9	2.8 ± .3 × 10^9
Cell vol (mm³)	1.4 ± .1 × 10^{-6}	2.3 ± .2 × 10^{-6}

Each value represents average and standard deviation obtained from 4 rats in each group.

TABLE II. Effect of AIA on Livers of Starved Rats. Chemical analyses.

Measurement	Control	AIA treated
RNA/g wet tissue	9.1 ± .6 mg	8.5 ± .9 mg
DNA/g " "	2.8 ± .3 "	1.8 ± .2 "
RNA/liver	38.3 mg	70.7 mg
DNA/liver	11.8 "	15.0 "
RNA/cell	1.8 ± .2 × 10^{-8} mg	2.6 ± .3 × 10^{-8} mg
DNA/cell	5.2 ± .7 × 10^{-9} mg	5.2 ± .7 × 10^{-9} mg
Water	71.9 ±1.3%	71.2 ±1.1%
Alcohol soluble	2.9 ± .3%	5.5 ± .5%
Ether "	.7 ± .1%	1.5 ± .2%
Water "	2.3 ± .2%	2.5 ± .2%
Residue	22.2 ± .9%	19.3 ±1.1%

Each value represents average and standard deviation obtained from 4 rats in each group.

Chemical analyses have shown that RNA concentration was not significantly changed in the porphyric liver (Table II). However, a calculation of total RNA per liver shows that an increase of 85% occurred. DNA, on the other hand, was found to be decreased in concentration while the total DNA per liver was increased 27% following AIA treatment. When these results were converted to a cellular basis, the RNA per cell increased 44% in the porphyric liver while the DNA per cell remained unchanged.

Of the gross chemical separations which were performed, the only significant differences found in the porphyric livers were 100% increases in the alcohol and ether soluble fractions.

Liver tissue from the porphyric animals showed several mitotic figures and vacuoles in the hematoxylin and eosin stained sections. The tissue otherwise appeared normal. The Oil Red O stained sections showed an increase in lipids which corresponded to the vacuoles in the H and E sections. No additional differences between the groups were seen in the P.A.S., Prussian Blue, or Van Gieson stained sections.

Discussion. When data obtained for the liver weight, number of cells, and size of cells were subjected to the Student's "t" test, it was found that the probability was under 0.01 in each case. In calculating total number of cells per liver from number of cells per unit volume, no correction was

made for tissue shrinkage during preparation. Thus, the absolute figure for the total number of cells per liver in each case is too large by a factor, depending upon the amount of shrinkage, which may have been as high as 37%(6). The cell volume would then be proportionally smaller. Since all samples for one experiment had the same water content and were processed simultaneously, the assumption was made that shrinkage was uniform and therefore the figures would be valid for comparative purposes. It was further assumed, from careful scanning only, that there was one nucleus per cell.

The most prominent effect demonstrated was a cellular hypertrophy. Significantly, the RNA content per liver increased in direct proportion to the liver size. In addition, there also appeared to be a measurable degree of hyperplasia. It was perhaps beyond coincidence that the number of cells per liver and the DNA content per liver both increased to exactly the same extent. The net gain in liver nucleic acids resulting from AIA treatment was observed previously with the use of labeled precursors; Sloop(7) reported increased incorporation of both adenine-8-C^{14} and glycine-2-C^{14} into the total liver nucleic acids of porphyric rats. Although the livers of AIA-treated animals showed a 100% increase in the lipid fractions, the lipid compartment still made up a very small part of the liver. The total water content of the livers remained normal, thus ruling out massive lipid infiltration as an explanation of increase in organ size. No significant changes in connective tissue, glycogen, iron stores or total protein content of the livers could be demonstrated by staining and microscopic observation.

Results similar to those described here in rat liver cells have been observed also in HeLa cells and in mouse fibroblast Strain L-929 cells grown in tissue culture(8). Chemical analyses of the cultured cells revealed that AIA increased the RNA per cell with no significant change in DNA. In addition increased protein content per cell was found. Cells treated with AIA were slightly larger in diameter and contained varying numbers and sizes of lipid vacuoles.

Herdson *et al*(9) have shown with rats that phenobarbital included in the diet in small amounts induced a liver enlargement through parenchymal cell hyperplasia. Since the level of drug administration was somewhat less than is ordinarily used for porphyria induction there is possibly a dual effect which can result from barbiturate administration. Thus, small doses of these drugs may produce metabolic changes resulting in cellular hyperplasia, while larger doses or a longer period of administration may produce metabolic changes resulting in cellular hypertrophy. The fact that experiments described here have yielded evidence of both types of changes occurring is significant in this regard.

Summary. The treatment of rats with allylisopropylacetamide resulted in a doubling of liver size within 3 days. Microscopic examination showed an increase in cell size and less increase in cell number per liver. By chemical analyses, there were found net gains in RNA and DNA per liver which paralleled the size and number of cells respectively. In per cent composition, lipids doubled while other components examined remained unchanged. It was concluded that the cytologic effect of the drug may be 2-fold, resulting primarily in cellular hypertrophy, but also in cellular hyperplasia.

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Buphthalmia in the Rabbit: A Test for Early Diagnosis.* (30144)

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Studies on buphthalmia in the rabbit indicate that a reliable technique for identification of the mutant genotype in early or prepathological stages is needed. Hanna *et al* (1) state that buphthalmia is due to a single recessive gene (*bu*) with incomplete penetrance. They also suggest that buphthalmia may be part of a primary systemic disorder. Kolker *et al*(2) report that at early ages the corneal diameter is not a reliable index of buphthalmia and that the intraocular pressures fluctuate considerably, particularly in the buphthalmic rabbits. The authors show a difference in the facility of outflow, as measured tonographically, for the two groups. They point out that their data are based only on small numbers of animals.

Unpublished observations in this laboratory on corneal diameters, and by Dr. L. B. Sheppard, Virginia Medical School, Richmond, on intraocular pressure corroborate the variability reported by Kolker *et al.* Marked strain differences among buphthalmics were also observed, strain AX having larger corneal diameters while those of strain III were considerably lower and unreliable. It was the experience of one of us (RRF) that the penetrance of buphthalmia appeared to be decreased by a dietary change made in 1959. We therefore surveyed clinical assays for nutritional deficiencies as possible diagnostic tests for buphthalmia.

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Huber and Smith(3) reported a technique for field diagnosis of bovine vit. A deficiency based on degree of cornification of the bulbar conjunctiva. Since the symptoms of vit. A deficiency in calves were similar to some of the observations made on buphthalmic rabbits it was felt that modification of this technique might provide a means for identification at preclinical stages. A procedure developed for this purpose is presented below.

Materials and methods. Animals of 3 types from The Jackson Laboratory's rabbit colony [*i.e.*, those which were (a) phenotypically buphthalmic (AX strain), (b) genetically but not phenotypically buphthalmic (III_{bu}, subline of strain III), and (c) known normals representing strain III_{mo} (another subline of strain III) and the small strains X, OS, AC, and ACEP] were used in this study. The rabbits' eyes were anesthetized with 2 drops 3% cocaine in normal saline solution (NSS) per eye. The eyelids were closed and massaged gently to insure a uniform distribution of liquid. The eyelids were held open and a probe (Fig. 1) moistened with 0.9% NaCl was placed lightly on the center of the cornea and rotated 180°. Subsequently the probe was placed lightly (*not streaked*) on a dry microscope slide that had just been removed from 95% ethyl alcohol. The slide was dried by a hot air blower for 3 minutes and stained with Giemsa (1 part stock : 20 parts distilled water) for 30 minutes, rinsed with distilled water, and dried with the blower for 20 minutes. The complete field of cells was first centered with a 3× objective and then observed under a 10× objective (Wild M-20 microscope with 15× wide field ocu-