Effects of Hypophysectomy and Growth Hormone on Ploidy Distribution and Mitotic Activity of Rat Liver.* (22553)

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In a recent cytophotometric study of the effects of growth hormone on rat liver protein and nucleic acids, Di Stefano, *et al.*(1) reported that, within a given class of ploidy, the deoxyribose nucleic acid (DNA) content of liver nuclei was not affected either by hypophysectomy or by the administration of a purified growth hormone preparation. However, differences were noted in ratios of diploid and tetraploid nuclei in livers of hypophysectomized rats as compared with hypophysectomized rats treated with growth hormone and intact control animals. The diploid/tetraploid ratios observed in the hypophysectomized animals were found to be consistent with the pattern seen in younger animals, while the pattern in hypophysectomized animals treated with growth hormone was characteristic of that found in older intact animals.

In an attempt to arrive at a better understanding of the mechanisms responsible for these differences in ploidy patterns, it seemed pertinent to investigate the ploidy ratios in livers of hypophysectomized rats during the first 8 days postoperatively, and to correlate these with some index of mitotic activity in the same livers.

Methods. Hypophysectomized and intact male Sprague-Dawley rats were purchased from the Hormone Assay Laboratory, Chicago, Ill., separated into groups containing 10 animals per group and maintained on a diet of canned dog food which was fed *ad libitum*. These groups were treated as follows: 1. One group of intact control animals (average body weight on arrival, approximately 75 g) was sacrificed when their average body weight reached that of the hypophysectomized animals. This group of animals (average body weight, 93.7 ± 1.2 g at the time of sacrifice) was comparable to the hypophysectomized groups on the day of the operation and therefore constitutes what might be called the initial intact control group. 2. Five groups of hypophysectomized animals were sacrificed 1, 2, 4, 6 and 8 days postoperatively. The average weights of animals in these groups were 93.2 ± 2.2, 93.0 ± 1.6, 91.1 ± 1.6, 91.0 ± 2.4, and 95.9 ± 2.5 g respectively. 3. One group of hypophysectomized rats (8 days postoperatively) weighing approximately 92.7 ± 1.6 g, were injected with 0.25 mg of a purified growth hormone preparation (Armour & Co., Lot. No. R491017)† twice daily by intramuscular injection for 7 days and sacrificed on the following day. At the time of sacrifice the average body weight of animals in this group was 137.0 ± 2.3 g. 4. One group of intact control animals weighing approximately 91.6 ± 0.6 g, were given 0.25 ml of 0.85% saline as before twice daily for 7 days and sacrificed on the following day. The average body weight of the animals in this group at the time of sacrifice was 142 ± 1.6 g. All animals were sacrificed by decapitation, and the livers removed rapidly and weighed. Small pieces were fixed immediately in 25% neutral formalin, washed in running tapwater for 48 hours, and embedded in paraffin. Measurements of relative amounts of DNA were made using the same procedures and apparatus described in a previous publication(1). Absorption measurements of Feulgen stained DNA were made at 560 millimicra, isolated from a tungsten source by means of a 250 mm Bausch and Lomb grating monochromator. Counts of mitotic figures per field of liver cells were also made and expressed as number of mitotic figures per 100 cells counted. The values are expressed as averages for 100 fields of liver per group of animals (10 fields of

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† The purified growth hormone was generously supplied by Mr. Irby Bunding of Endocrine Research Division of Armour & Co.

TABLE I. Effects of Hypophysectomy and Treatment with Growth Hormone on the Mitotic Activity of Rat Liver.

Animal group (10 rats/group)	Mitotic activity (mean/100 cells counted ± S.E.)
Initial intact controls	1.94 ± .12
Hypophysectomized, 1 day	.18 ± .02
" , 2 days	.13 ± .02
" , 4 "	.14 ± .02
" , 6 "	.09 ± .02
" , 8 "	.08 ± .02
Final (8 day) intact controls	.48 ± .06
Hypophysectomized, 8 days; growth hormone, 7 days	.43 ± .04

liver per animal). Histograms showing the frequency distribution of relative DNA content of rat liver nuclei were constructed. Arbitrary units of DNA were calculated according to the method of Swift(2), for 25 liver nuclei in each of 10 rats per group. Since no DNA values below 5 were seen, an initial class interval of 5 to 5.5 was chosen. Each successive class interval was then increased 10% over the preceding interval. This method of presentation was selected because it was found that, if the class interval is kept constant, the representation of distribution in the 2 classes of nuclei (diploid and tetraploid) is not comparable. The method suggested‡ keeps the number of classes in the two ploidy ranges essentially the same.

Results. Fig. 1 shows a comparison of the ploidy distributions in livers of hypophysectomized rats compared with the patterns seen in intact control animals of the same weight. In the latter group of animals a predominance of diploid over tetraploid nuclei is seen. This characteristic ratio persists in livers of hypophysectomized rats as late as 8 days postoperatively.

A comparison of liver ploidy patterns of hypophysectomized, growth hormone treated hypophysectomized, and intact control rats may be seen in Fig. 2. Here one can observe: first, the ploidy distribution profile in livers of intact control rats shifts from one favoring a predominance of the diploid class to one in which the tetraploid class becomes the predominant one; second, the ploidy profile of the younger, lighter, rats persists as such after the removal of the hypophysis; and third, that treatment of hypophysectomized rats with a purified growth hormone preparation results in a liver ploidy distribution pattern essentially identical with that of the older, heavier intact control animals.

‡ This method was suggested to us by Prof. C. L. Yntema.

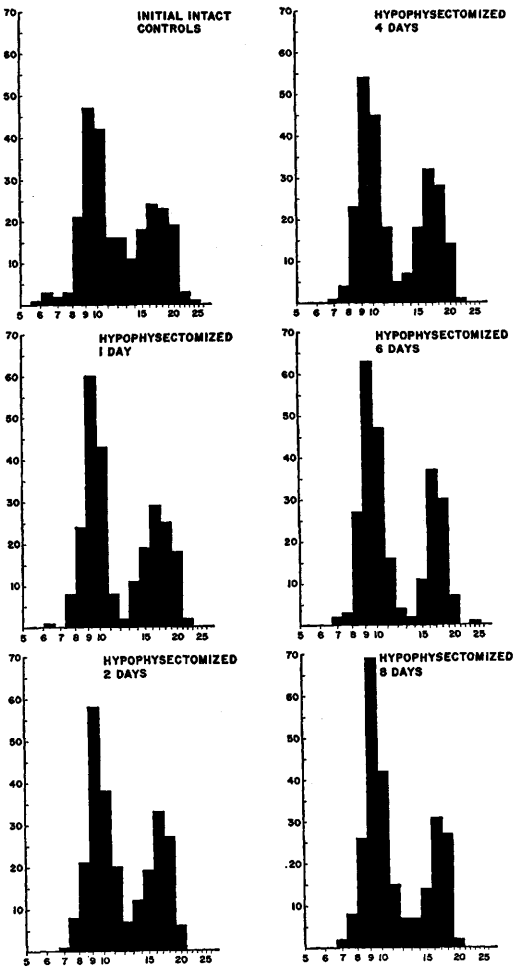

FIG. 1. Effect of hypophysectomy on ploidy pattern of rat liver. Ordinates represent frequency and abscissas arbitrary units of DNA.

The results of mitotic counts are summarized in Table I. Hypophysectomy results in a striking reduction of mitotic activity. As early as the first day after removal of the hypophysis the mitotic activity appears to be reduced by a factor of about 10. It may also be noted that the mitotic activity in livers of

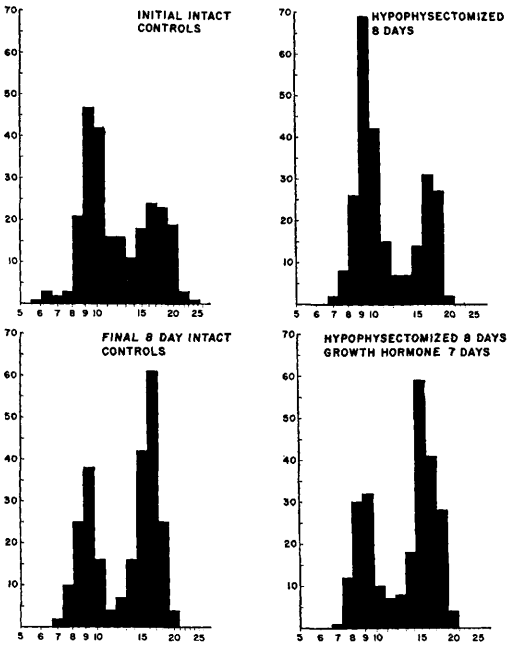

FIG. 2. Comparison of liver ploidy patterns of hypophysectomized, growth hormone treated hypophysectomized, and control rats. Ordinates represent frequency and abscissas arbitrary units of DNA.

heavier and older intact animals is normally lower than that of the initial intact controls and that growth hormone replacement in the hypophysectomized rat results in a mitotic activity consistent with the rate observed in intact control rats of comparable weight.

Table II contains a summary of the percentage distribution of diploid and tetraploid nuclei in all groups of rats (10 rats per group) represented in Fig. 1 and 2. It is clearly apparent that, within each class of ploidy, the distribution pattern of final intact controls and growth hormone-treated hypophysectomized rats are significantly different from all other groups at a confidence level of less than 1%. It is also evident that the distributions in hypophysectomized groups are not different from that of initial intact controls and that in hypophysectomized, growth-hormone-treated animals, the distribution does not differ from that of final intact controls.

TABLE II. Percentage Distribution of Diploid and Tetraploid Liver Nuclei of Control, Hypophysectomized, and Growth Hormone Treated Hypophysectomized Rats.

Animal group (10 rats/group)	Distribution of nuclei 2 N	4 N
Intact controls	58.7±1.3*	41.3±1.3*
Hypophysectomized, 1 day	60.0±1.1	40.0±1.1
" , 2 days	59.6± .9	40.4± .9
" , 4 "	59.2±1.0	40.8±1.0
" , 6 "	64.0±1.2	36.0±1.2
" , 8 "	63.6±1.3	36.4±1.3
Final intact controls	37.2±1.2†	62.8±1.2†
Hypophysectomized, growth hormone treated	40.0±1.1†	60.0±1.1†

* Mean ± S.E.

† Significantly different from all other values ($P<0.01$, Student's t test) but not significantly different from each other.

Discussion. In young rats the ploidy distribution profile of the liver shows a predominance of diploid over tetraploid nuclei. As the animal grows there is a reversal of this ratio brought about by a shift towards tetraploidy. The significance of the tetraploid shift that accompanies growth of the rat liver is not known. In any case, the present study reveals an easily detectable shift within the 8-day period of this experiment (Fig. 2).

The fact that the ploidy distribution pattern in the hypophysectomized rats resembles that seen in the initial intact controls strongly suggests that hypophysectomy results in an arrest of the tetraploid shift that occurs in the normal intact controls over a comparable period of time. In fact, observations made 21 days after hypophysectomy show a persistence of the more juvenile state of ploidy seen in the initial intact controls and in all untreated hypophysectomized groups. In light of these observations it is unnecessary to postulate (as was done in a previous publication) (1) a reversion to a lower state of ploidy following hypophysectomy. These data confirm and extend the results of previous studies (1) in which differences in ploidy profiles were found between hypophysectomized rats and larger intact controls of the same *age*. They are also consistent with observations on congenitally dwarfed mice made by Leuchtenberger, *et al.*(3).

The suggestion made by Bass, *et al.*(4) that there is an increase in diploid and octaploid nuclei must be considered in relation to the observations reported here. Their "increase" in the diploid population may be only

an apparent one, since they based their conclusions on a comparison of 141 g hypophysectomized rats with 226 g controls. The implication of a true increase cannot be accepted without question unless data on 141 g intact control animals are included for comparison. No "octaploid" shift of the sort described by Bass, *et al.*(4) was seen in this study, but this, too, may be attributed to the fact that the tissues of younger rats were examined. Most of the nuclei that contribute to the "octaploid" shift of Bass, *et al.*(4) are intermediate values between the tetraploid of about 17 and the octaploid of 34. In order to refer to this distribution as an octaploid shift it would be necessary to show that this pattern does not exist in the normal 141 g rat. In hypophysectomized animals, a similar appearance of large numbers of "intermediate" nuclei between the diploid and tetraploid groups was not seen in the present study. In fact, the intermediate values tended to diminish immediately after hypophysectomy; this finding is in agreement with the marked decrease in mitotic activity found at this time. Taken together, these data are not consistent with the construct that increased DNA synthesis (which is implicit in a shift towards octaploidy) is likely to occur after hypophysectomy.

The striking decrease in mitotic activity of the liver seen following hypophysectomy is consonant with the findings of Leblond and Carriere(5), who found a similar decrease in the mitotic activity of epithelium of crypts of Lieberkühn in the intestine of the rat after hypophysectomy. It should be noted that there was some mitotic activity after removal of the hypophysis in both liver and intestinal epithelium, suggesting that these tissues have an inherent mitotic activity which is independent of hypophyseal influences.

Administration of a purified pituitary growth hormone to a hypophysectomized rat can restore normal ploidy patterns, normal mitotic activity and normal concentrations of nuclear and cytoplasmic ribose nucleic acid and protein in liver cells(1,6,7). In addition the present observations on mitotic activity suggest that our previous finding of a significant increase in DNA per diploid nucleus after 2 days of growth hormone treatment (of 4 day hypophysectomized rats)(1), suggests that DNA synthesis may be among the consequences of growth hormone administration under these circumstances. Thus, growth hormone exerts an influence on the basic cellular biochemical machinery which is concerned with cell division and protein synthesis. The mechanisms by which this effect is exerted are quite unknown. In terms of reaction sequence, the biochemical locus of action of the hormone might be very remote from the phenomena described above. This is simply a reiteration of the view that the description of intracellular changes following the administration of a hormone does not necessarily signify that the hormone participated directly in effecting these changes.

Summary. 1) Ploidy distribution patterns and mitotic activity in livers of young rats have been studied at various times following hypophysectomy, and compared with distributions in livers of rats of comparable weight. 2) It was found, within the time limits of this experiment, that there is a shift of ploidy distribution from one in which the diploid class of nuclei predominates to one in which the tetraploid class predominates. During this period of time the mitotic activity in these same livers decreases. 3) Hypophysectomy results in an arrest of the ploidy distribution consistent with the ratios present at the time of hypophysectomy. This is accompanied by a marked reduction in mitotic activity. 4) Growth hormone replacement in the operated animals restores both ploidy distribution pattern and mitotic activity to that seen in intact controls of comparable weight.

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A Component of Substance P Active in Releasing ACTH.* (22554)

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Substance P is a potent smooth muscle stimulating factor first described by von Euler and Gaddum(1), and since studied by other investigators(2-7). The precise function of P is not clear, nor has the substance been well characterized chemically, although it is regarded by some as a complex polypeptide(1,5,8). The typical action of P upon smooth muscle, *e.g.*, the guinea pig ileum, is induction of a slow contraction starting after a short latent period. This effect is not abolished by atropine, antihistaminics or ganglionic blocking agents(4). Similarities in activity between the acid hydrolysate of P and that of vasopressin (Pitressin, Parke, Davis and Co.), now definitely known to contain a component which releases adrenocorticotropin, induced the writers to test P for comparable releasing activity.

Methods. Substance P,† prepared from the intestine of the horse, was tested for ACTH-releasing activity using the *in vitro* technic of Saffran *et al.*(9,10) and the Sayers adrenal ascorbic acid depletion test on 120-140 g male rats hypophysectomized by the parapharyngeal approach for 18-24 hours. Two minor modifications of the Saffran method were introduced in these experiments: (1) the rats from which the pituitaries were removed for incubation were gentled for several days before use and then quickly guillotined without anesthesia; (2) arterenol was not added to the incubation fluid since it served no useful purpose.

Results. Substance P was hydrolyzed by boiling 1.5 hours in 2.2 N HCl, using the method previously described for hydrolysis of vasopressin(11,12). The results of the tests, shown in Table IA, demonstrate that both hydrolyzed and nonhydrolyzed P exhibit marked ACTH-releasing activity amounting to -106 ± 11.5 and -110 ± 12.3 mg % adrenal ascorbic acid depletion. Thus acid hydrolysis does not seem to destroy the activity of P. Equivalent amounts of incubation fluid from the Warburg flasks containing control pituitary glands and given I.V. to 18 hypophysectomized rats induced negligible ascorbic acid decline. Nonhydrolyzed P was also tested for its *in vivo* ACTH-releasing action on intact rats whose ACTH output had been inhibited by subcutaneous injections of 10 mg DCA plus 10 mg of the free alcohol of hydrocortisone 18 hours previously. P was administered I.V., to 6 rats in a dose of 0.25 mg one hour before removal of the second adrenal for the ascorbic acid test. The results were essentially negative, but should be repeated when purer preparations are available. Since P is destroyed by strong acid at boiling temperature, whereas the hydrolysate retains its ACTH-releasing activity, it seems evident that the releasing agent is not the same substance designated as P by von Euler, Gaddum and others, but is quite a different entity.§ To avoid confusion in terminology the ACTH-releasing component of the P complex will be referred to hereafter simply as P_1 until it is more precisely characterized chemically.

* Part of the expenses of this investigation was defrayed by Sharp and Dohme, Division of Merck and Co., West Point, Pa., and Ciba Pharmaceutical Products Co., Summit, N. J.

† We are indebted to Drs. D. A. McGinty and J. J. Pfiffner of Parke, Davis and Co. for generous supplies of Pitressin and Substance P.

§ Chromatographic fractions (obtained from Merck & Co.) also show separation of releasing agent and P.