TABLE I. Influence of Age and Testosterone Propionate (1 mg) on Reproductive Performance of Prepuberal Mice.

Treatment	No. mice	No. preg.	Days in breeding to 1st litter, avg (range)	Avg No./litter	Avg* weaning wt (g)
Control	14	14	31.6 (22-40)	6.6	5.9
T.p. at 5 days	14	0	Infertile	—	—
Control	13	13	33.3 (22-46)	6.5	10.0
T.p. at 10 days	12	8	37.0 (28-55)	7.0	9.7
Control	14	14	39.4 (22-60)	7.1	11.7
T.p. at 20 days	16	15	35.7 (22-71)	7.8	10.9

* Per individual at 22 days.

and the prepuberal ages appear to be the more sensitive periods(5,6).

Summary. Mice 5, 10 and 20 days of age were given a single 1 mg injection of testosterone propionate. Fertility was tested when these mice were 3 months old and compared with littermate controls. No litters were obtained with mice treated at 5 days of age despite 100 days in breeding. Fertility of mice treated when 20 days old, however, was not decreased whereas mice treated at 10 days of age assumed an intermediate position.

1. Shay, H., Gershon-Cohen, J., Paschkis, K. E., and Fels, S. S., *Endocrinology,* 1939, v25, 933.
2. Bradbury, J. T., *ibid.,* 1941, v28, 191.
3. Huffman, J. W., *ibid.,* 1941, v29, 77.
4. Barraclough, C. A., *Anat. Rec.,* 1954, v118, 281.
5. Emmens, C. W., *J. Endocrinol.,* 1953, v9, 204.
6. Merklin, R. J., *Endocrinol.,* 1953, v53, 342.

Received April 2, 1954. P.S.E.B.M., 1954, v85.

Distribution of Succinic Dehydrogenase and Hyaluronidase in Adult Rat Testis.* (20990)

ROGER L. GREIF. (Introduced by R. F. Pitts.)

From the Hospital of the Rockefeller Institute for Medical Research, New York City, and the Department of Physiology, Cornell University Medical College, New York City.

A study of testicular hyaluronidase has revealed, in confirmation of the work of Perlman *et al.*(1) that freezing and thawing and sterile autolysis of homogenates of testicular tissue make possible the extraction of large amounts of this enzyme. These steps were therefore incorporated into a reproducible method for the measurement of the hyaluronidase content of rat testes(2). The relatively low activity found in extracts of fresh unfrozen and unautolyzed tissue suggested that hyaluronidase might be localized within a structure which was altered by freezing and autolysis.

Arnesen, Buxton, and Dulaney(3) concluded from differential centrifugation experiments with homogenates of mouse testis suspended in hypertonic sucrose solution (.88 Molar) that hyaluronidase activity was localized in the so-called "soluble" fraction. The fractions obtained by these workers were tested neither for activity of an enzyme of known localization, nor for nitrogen content. In the present investigation the determination of succinic dehydrogenase activity and of nitrogen content have been employed as biochemical indications of the effectiveness of the separation procedure(4). Optimum conditions for the measurement of the hyaluronidase content of testicular fractions are still being sought, hence the results to be reported are of a preliminary nature.

Methods. Adult albino rats were killed by means of a blow on the head, the testes were

* This investigation was supported in part by the Helen Hay Whitney Foundation.

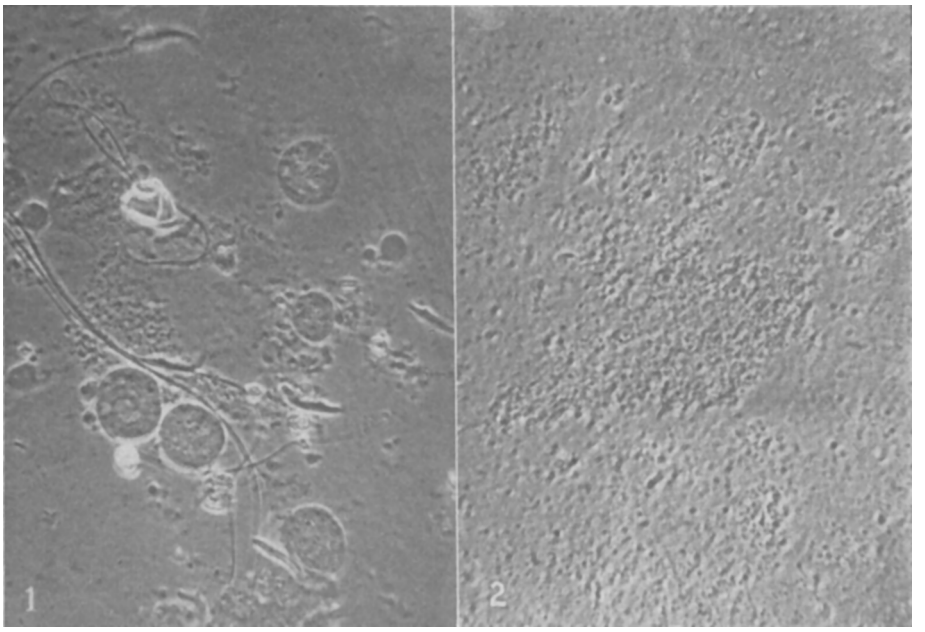

FIG. 1. Phase microscopic appearance of suspension of testicular homogenate in .25 M sucrose ("Total" fraction) × 1200.

FIG. 2. Phase microscopic appearance of "granular" fraction × 1200.

removed immediately, stripped of *Tunicae albugineae,* and the remaining pulp weighed. All subsequent fractionation operations were carried out in a cold room at 0-4°C. The pulp was ground by hand in an all-glass tissue grinder, after which it was suspended in cold .25 M sucrose solution in a concentration of 0.2 g wet weight per ml of suspension. Fig. 1 is a phase contrast photomicrograph of such a preparation, and is seen to contain unbroken cells, nucleii, intact spermatozoa, sperm heads and tails, large cytoplasmic masses and granular material.† This uncentrifuged suspension will hereafter be referred to as "total". A portion of this suspension was centrifuged at 600 x g (International Centrifuge size 1) for 45 minutes. The sediment from this step, hereafter referred to as the "nuclear" fraction, on phase microscopic examination resembled Fig. 1. The supernatant fluid from the first centrifugation contained granular material. This fluid was subjected to centrifugation for one hour at 8500 x g (Servall S S angle centrifuge). A sediment was obtained which was light tan in color and had transparent edges. Fig. 2 illustrates the phase microscopic appearance of a suspension of this "granular" fraction. The supernatant fluid from the latter centrifugation appeared clear or slightly turbid, and on microscopic examination contained few visible structures other than fat globules. It will hereafter be called the "soluble" fraction, although the experience of others with differential centrifugation of liver homogenates suggests that microsomes are present in this preparation (4). To obtain a better yield of granular fraction the short procedure outlined above was modified as follows: the nuclear fraction was resuspended in fresh sucrose solution with the use of the tissue grinder, following which it was recentrifuged at 600 x g for 45 minutes. The supernatant liquids from 3 such nuclear washings were pooled with the supernatant fluid from the first centrifugation, and this pool was centrifuged at 8500 x g for one hour to obtain the granular fraction. Succinic dehydrogenase activity was measured on 2 different sized aliquots of the same specimen by

† To avoid blurring due to Brownian movement during exposure, the granular material in these phase photomicrographs was caused to aggregate by the addition of Janus Green B to the suspension.

TABLE I. Mean Succinic Dehydrogenase Activity and Nitrogen Content of Rat Testicular Fractions. (Seven separate fractionations in which the nuclear fraction is unwashed.)

Sample	O_2 uptake/ hr/g testis wet wt (μl)	mg N/g testis wet wt	μl O_2 taken up/hr/mg of N (specific activity)
Total	1948±235*	17.35± .68	112±12
Nucleii	1133±231	7.93± .98	143±24
Granules	687±121	2.53± .51	273±32
Soluble	<10	6.21±1.28	
% of total recovered in fractions	93.6±6.5	95.6 ±7.6	

* Stand. dev. about the mean.

the manometric technic of Schneider and Potter(5). The cytochrome C required for this method was obtained commercially.‡ Hyaluronidase and nitrogen determinations were made as previously described(2).

Results. Succinic dehydrogenase distribution. Table I shows the succinic dehydrogenase activity and the nitrogen content of testicular fractions obtained with the short procedure. Activities are referred to both wet weight of tissue and nitrogen content, and represent the mean value and standard deviation about the mean for 7 separate fractionations. It will be seen that the highest specific succinic dehydrogenase activity is present in the granular fraction, but that only 35% of the total amount of enzyme is present in that fraction.

The results obtained after use of the modified technic which involves 3 washings of the nuclear fraction are recorded in Table II. These data, compiled from 10 separate fractionations, show that the succinic dehydrogenase content of the nuclear fraction can be reduced by this washing procedure so that a larger proportion of the total amount of enzyme can be found in the granular fraction. Some loss of activity has resulted from the additional manipulations. The specific activity of the granular fraction remains higher than that of the nuclear and of the total fractions.

TABLE II. Mean Succinic Dehydrogenase Activity and Nitrogen Content of Rat Testicular Fractions. (Ten separate fractionations in which the nuclear fraction was washed 3 times.)

Sample	O_2 uptake/ hr/g testis wet wt (μl)	mg N/g testis wet wt	μl O_2 taken up/hr/mg of N (specific activity)
Total	1879±146*	17.75±.78	106± 8
Nucleii	564±174	6.05±.93	93±24
Granules	1025±197	3.39±.40	303±48
Soluble	<10	6.69±.76	
% of total recovered	84.7±5.8	91.6 ±5.1	

* Stand. dev. about the mean.

‡ Sigma Chemical Company, St. Louis, Mo.

After 6 washings, the specific succinic dehydrogenase activity of the nuclear fraction can be reduced to unmeasurable levels. Since this activity is based upon nitrogen content, the results of repeated washing cannot be explained on the basis of the large loss of nuclear material which occurs through fragmentation and distribution to other fractions.

The granular fraction probably includes mitochondria and microsomes as well as some nuclear fragments, and contains material which stains supravitally with Janus Green B. In 3 experiments this fraction was repeatedly resuspended in sucrose solution and centrifuged for 30 minutes at 25000 x g. In Table III will be found the specific succinic dehydrogenase activity of the fractions obtained as indicated in column 1. In contrast to the results of washing the nuclear fraction, the specific activity of the granular fraction remains constant or actually increases as inactive nitrogenous material is removed by washing.

Hyaluronidase distribution. Sterile autolysis at 37°C under toluene for 48 hours was required to obtain high hyaluronidase activity from the individual fractions. A large number of variables are introduced with this autolysis step, and to date it has not been possible to recover in the fractions all the activity present in the uncentrifuged preparation. The results of the hyaluronidase assay are therefore indicated in Table III in an approximate way only. It is evident that hyaluronidase activity persists in the washed granular fraction, and the data suggest also that the specific activity of the granular fraction is higher than that of the total preparation. The specific activity of the nuclear fraction can be reduced to zero

TABLE III. Effect of Repeated Washing with .25 M Sucrose upon Specific Succinic Dehydrogenase and Hyaluronidase Activity. (3 experiments arranged in columns a, b, and c respectively.)

Sample	Specific succinic dehydrogenase activity (μl O_2 uptake/mg N/hr) (a)	(b)	(c)	Estimated hyaluronidase activity/mg N (a)	(b)	(c)
Total	112	130	97	2+	2+	3+
Granules (unwashed)	306	368	144	—	3+	3+
Granules (washed 6×)	385	405	277	4+	4+	3+

by repeated washing. No more than traces of activity have been found in the soluble fraction.

Discussion. The large number of cell types present in the mature rat testis make this tissue far less satisfactory than liver for cell fractionation studies(6). The heterogeneous nature of the granular fraction isolated from liver homogenates(7) is even more striking in the case of the testis because the granules doubtless come from ruptured cells of the germinal epithelium, interstitial cells, and cells of supporting tissue. Thus far no attempt has been made to subdivide the granular fraction into mitochondria and microsomes. It seems evident, despite technical difficulties, that hyaluronidase activity can be found in the granular fraction of the rat testis and that it persists even after repeated washing. Should further experimentation confirm this observation, it is possible that the role of hyaluronidase, at present conjectural, in the fertilization process might be assigned to the midpiece of the spermatozoon, which has been observed to contain mitochondrial material and oxidase activity(8).

A number of mitochondria-linked enzymes, among them an acid phosphatase(9) and adenosine triphosphatase(10) have shown low activity in mitochondria immediately following their isolation from sucrose solution. The enzyme activity of these mitochondria increases following water or detergent extraction or simple aging. A similar process, presumably alteration of the mitochondrial membrane, may be responsible for the release of hyaluronidase from testicular homogenates after freezing and thawing and autolysis. Microscopic examination of tissue so treated reveals striking structural alteration. The physiological significance of enzymes which require drastic treatment for release from tissue remains obscure.

Summary. The distribution of succinic dehydrogenase in the testis of the adult albino rat has been studied by means of differential centrifugation in .25 M sucrose solution. The highest specific activity has been found in a granular fraction, and preliminary evidence is presented to indicate that hyaluronidase activity is also associated with this fraction.

The author is indebted to Dr. George Palade for assistance with cytological examinations and to Mrs. Elnora W. Van Winkle for technical assistance.

1. Perlman, P. L., Leonard, S. L., and Kurzrok, R., *Endocrinol.*, 1948, v42, 26.

2. Greif, R. L., *J. Biol. Chem.*, 1954, v206, 381.

3. Arnesen, K., Buxton, L., and Dulaney, A. D., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 264.

4. Schneider, W. C., *J. Biol. Chem.*, 1948, v176, 259.

5. Schneider, W. C., and Potter, V. R., *ibid.*, 1943, v149, 217.

6. Schneider, W. C., and Hogeboom, G. H., *Cancer Res.*, 1951, v11, 1.

7. Novikoff, A., Podber, E., Ryan, J., and Noe, E., *J. Hist. and Cytochem.*, 1953, v1, 27.

8. Elftman, H., in Engle, E. T., *Studies on Testis and Ovary Eggs and Sperm,* Charles C. Thomas Co., Springfield, Ill., 1952, p35.

9. Berthet, J., and de Duve, C., *Biochem. J.*, 1951, v50, 174.

10. Kielley, W. W., and Kielley, R. K., *J. Biol. Chem.*, 1951, v191, 485.

Received April 5, 1954. P.S.E.B.M., 1954, v85.