ESTERASES OF TESTIS AND OTHER TISSUES*

By CHARLES HUGGINS, M.D., AND STANLEY H. MOULTON,‡ M.D.

(From the Department of Surgery, The University of Chicago, Chicago)

(Received for publication, April 9, 1948)

This paper is concerned with some physiologic relationships of non-specific esterases of tissues of the rat, dog, and man which were investigated by a colorimetric technique. Two patterns of hydrolysis were revealed that were characteristic of several tissues. It was found also that the esterase of rat testis is produced in the interstitial cells, that its content is under control of the hypophysis, and that its concentration is related to the production of androgen by the testis.

The colorimetric method employed chromogenic substrates, colorless compounds that liberate color on hydrolysis. The color value is read directly, the depth of color produced by enzymatic hydrolysis being directly related to activity of the enzyme. The chromogenic substrate techniques of enzymatic investigation combine accuracy, delicacy, and simplicity. All the chromogenic substrates, beginning with the first use of this technique by Ohmori (1), have been compounds of phenolic acid-base indicators esterified with phosphoric (1, 2), glucuronic (3), sulfuric (4), or fatty acids (5). In the present studies the substrates were the acyl esters of *p*-nitrophenol previously synthesized in this laboratory.

Three principal esterase activities in animal tissues can be differentiated according to their distribution, their efficiency against various substrates, and the effect of inhibitors (5, 24) upon them. An enzyme cannot be characterized with certainty until it has been isolated in a pure state and this has not been achieved with any of the esterases. However, from indirect evidence esterolysis seems to be brought about by three separate types of enzymes; in this paper the effects will be designated according to conventional usage as cholinesterase, non-specific esterase, and lipase. No doubt there is some overlapping of their activities and, as will be brought out, the chief differentiating characteristics are quantitative rather than qualitative.

The choline esters are sufficiently soluble in water to serve as satisfactory substrates *in vitro*. Cholinesterase has an entirely different distribution than the other esterases. Marnay and Nachmansohn (6) found that the cerebral cortex and uterus of guinea pig hydrolyzed acetylcholine more effectively than extracts of kidney or liver did. In swine (7) acetylcholine is hydrolyzed more rapidly by certain structures which, in descending order of magnitude, are the parotid, lachrymal and sublingual glands, Fallopian tube, jejunal and gastric mucosa, and the medulla oblongata. Mendel,

* This work was aided by grants from the American Cancer Society recommended by the Committee on Growth of the National Research Council, the Committee for Research in Problems of Sex of the National Research Council, and Mr. Ben May, Mobile.

‡ United States Public Health Service.

Mundell, and Rudney (8) further differentiate two types of cholinesterase activity by their effect on various choline esters—true and pseudocholinesterase. According to their findings, both enzymes hydrolyze acetylcholine; acetyl-β-methylcholine is hydrolyzed by true but not by pseudocholinesterase while benzoylcholine is hydrolyzed by pseudo- but not by true cholinesterase. Sawyer and Everett (9) found that the tissues of the rat with the greatest hydrolytic activity against benzoylcholine were the salivary and Harderian glands, brown fat, ovary, uterus, and liver; acetyl-β-methylcholine was hydrolyzed best by the brain stem, red bone marrow, spleen, thymus, lymph node, adrenal cortex, skeletal muscle, and peripheral nerve. Other choline esters have also been used as substrates. Stedman, Stedman, and White (10) observed that butyrylcholine is hydrolyzed by serum of various animals about twice as rapidly as acetylcholine. Nachmansohn and Rothenberg (11) found that the brain of rodents, ox, and cat likewise showed hydrolytic patterns; no choline substrate was hydrolyzed by these tissues more rapidly than acetylcholine; propionylcholine was hydrolyzed equally well or less than acetylcholine while butyrylcholine was split much less. Extracts of the electric organ of the eel (5) were found to be 600,000 times more effective in splitting acetylcholine compared to the hydrolysis of *p*-nitrophenyl propionate. This is evidence that the hydrolysis of these acyl esters of *p*-nitrophenol is performed by enzymes other than cholinesterase.

The distribution of esterases active on non-choline esters has not been systematically investigated. Moreover, earlier methods have presented technical difficulties since the fats and esters of fatty acids have only slight solubility in water. Except for the lipase method of Archibald (12) and our chromogenic technique, all previous quantitative studies have been done on suspensions or emulsions in which, to a large extent, the enzyme and the substrate have been in different phases. In comparative studies (13) employing different substrates the possibility has existed that the "results merely portray the degree of emulsification attained." In the earlier literature the terms lipase and esterase often have been used interchangeably although, as will be brought out, the enzymes are apparently separate entities.

In the activity of non-specific esterase and lipase the character of the fatty acid has far greater influence on the reaction than the hydroxyl-bearing group. Kastle (14) observed that the alkyl groups methyl, ethyl, iso-butyl, allyl, and benzyl exert nearly the same influence on the hydrolysis of esters by aqueous extracts of liver. Balls and Matlack (13) tested the effect of pancreatic extracts on fatty acids of various alcohols. The configuration of the alcohol was without effect on the enzyme except in so far as the hydroxyl-bearing carbon was concerned; esters of primary alcohols were split effectively but secondary and tertiary alcoholic esters were attacked slowly.

Most of the work done on non-specific esterase of cells has concerned liver and pancreas, tissues which differ widely from the esterase standpoint. Kastle and Loevenhart (15) observed that ethyl butyrate is hydrolyzed by extracts of liver, pancreas, kidney, and submaxillary gland; pancreatic extracts hydrolyzed in decreasing order ethyl esters of butyric, propionic, and acetic acids. Kastle (14) reported a pattern similar to that of pancreas for liver extracts, a finding in conflict with our observations using *p*-nitrophenyl esters. Loevenhart (16) found that liver extracts were several times as active as pancreas on esters of the lower fatty acids while the reverse was true with higher fatty acids. Balls and Matlack (13) found that pancreatic extracts hydro-

lyzed not only glycerides of fatty acids but esters of monatomic alcohols as well and at approximately the same rate as the saturated fats. Pancreatic extracts hydrolyzed many esters of stearic acid but extracts of horse liver, while rich in esterase (ethyl butyrate–splitting), had small or no activity against stearate esters. Both liver and pancreas split the lower esters but only the pancreas hydrolyzed esters of long chain acids efficiently. That esters of long chain fatty acids can be split by tissues other than pancreas was demonstrated by Gomori (17, 18) in his histochemical demonstration of lipase. Using polyglycol esters of palmitic and stearic acids he obtained intensely positive reactions in the liver of all species and the interstitial cells of the rat testis as well as in the zymogen granules of pancreatic cells. Each of these tissues, therefore, had some capacity to hydrolyze long chain esters.

Falk, Noyes, and Sugiura (19) employed miscellaneous esters of acetic, butyric, and benzoic acids in testing esterase activity of rat tissues and a transplantable carcinoma. They found that the esterase content of the tumor was small while the greatest activity was in kidney and liver, with testis following closely and then spleen and lung; the activity curves show characteristic "pictures" for the ester-hydrolyzing actions of the tissues.

Methods

All the rats utilized were exsanguinated by cardiac puncture and samples of their tissues (about 100 to 150 mg.) were then weighed on a torsion balance and homogenized in an all-glass grinder in 5 ml. of ice-cold water. Tissues obtained from two dogs killed by electrocution were treated similarly. Certain fresh human tissues were obtained directly from an operating room. The homogenates were centrifuged and aliquots of the supernatant fluid were diluted with water in volumetric flasks.

The esterase technique was that described previously (5) except that it was carried out at a temperature of 30°; each analysis was done in triplicate. One unit of esterase activity is defined as that amount of enzyme liberating one micromole of *p*-nitrophenol in 20 minutes at 30° and pH 7 in phosphate buffer when the substrate is at a concentration of 0.666 micromole per 10 ml., provided that not more than 40 per cent of the substrate is hydrolyzed; the units are expressed per 1 gm. of tissue or 1 ml. of fluid.

The following esters of *p*-nitrophenol were used: acetate, propionate (PNPP), *n*-butyrate (PNPB), *n*-valerate. In all cases the same molarity of substrate was employed. In preparing aqueous solutions of *p*-nitrophenyl-*n*-valerate precipitation frequently occurred; the precipitation was removed by filtering several times through a No. 40 Whatman filter paper Under ether anesthesia hypophysectomy was done in 42 rats using the parapharyngeal technique of Smith (20). Equine gonadotrophin[1] was freshly dissolved in saline before intramuscular injection and was administered after some days to 15 hypophysectomized rats.

RESULTS

Survey of Tissues Esterase.—In the rat the greatest concentration (Table I) of non-specific esterase (substrate: *p*-nitrophenyl propionate) was found in five tissues: liver, lung, pancreas, adult testis, and renal cortex. In the dog these same tissues were rich in esterase; in addition, the mucosa of the trachea

[1] The authors are indebted to the Upjohn Company, Kalamazoo, for the generous gift of this material, distributed by them under the trade name gonadogen.

and urinary bladder were exceptionally active and gastric mucosa had moderate activity. The lens of the dog had appreciable esterase activity. While all tissues examined had some esterase activity, thymus, spleen, bone marrow, and heart muscle were relatively inactive and skeletal muscle had very slight activity.

Esterase Patterns of Tissues.—The five tissues of the rat found to have the greatest esterase activity were tested simultaneoulsy against *p*-nitrophenyl

TABLE I

Esterase Content of Tissues of Rat and Dog

Substrate: *p*-nitrophenyl propionate.

	Rat	Dog
	Units per gm. or cc.	
Liver	823 -3460	1218 -2680
Lung	332 - 449	156 - 298
Tracheal mucosa	74 - 117	587 - 685
Pancreas	241 - 584	194 - 526
Testis	224 - 380	18 - 29
Kidney, cortex	205 - 368	181 - 332
Kidney, medulla	28 - 76	3 - 14
Bladder mucosa	17 - 48	507 -2640
Pituitary	63.8	123 - 174
Cerebrum	20 - 37	8 - 25
Cerebellum	13 - 20	4 - 20
Suprarenal	25 - 75	9 - 16
Thyroid	17 - 50	46 - 239
Gastric mucosa	19 - 30	228 - 252
Spleen	14 - 26	9 - 75
Prostate, ventral	33 - 138	57 - 230
Thymus	18 - 23	—
Heart	21 - 25	14 - 18
Skeletal muscle	3 - 6	10 - 34
Lens	6	26 - 46
Bone marrow	8 - 14	—
Blood serum	7 - 9	4.7 - 7

esters of straight chain fatty acids containing 2 to 5 carbon atoms in the chain. Definite hydrolytic patterns emerged which were of two types. In one pattern propionic acid esters (C_3) were split preferentially by liver, lung, testis, and renal cortex; acetate esters (C_2) lagged slightly, while butyrate (C_4) and valerate (C_5) esters were attacked (Table II) with decreasing effectiveness. Profiles of blood serum of the dog, rabbit, and man (5) were similar to those of these tissues.

The pancreas of dog and rat was quite different in its effects. The splitting

of acetate esters (C_2) was feeble (Fig. 1) but there was a progressive increase of activity with chain length until valerate esters (C_5) were reached.

In the tissues of 3 infant rats 5 days of age patterns of hydrolysis were obtained which were identical with those of the corresponding adult tissues.

Esterases in Postnatal Development.—The non-specific esterases of tissues of the newborn rat were low in amount. In rats at age 4 and 5 postnatal days

TABLE II

Patterns of Hydrolysis of Esters of p-Nitrophenol by Rat Tissues

Values expressed as per cent of the ester split maximally.

Determination No.	*p*-Nitrophenyl			
	acetate	propionate	*n*-butyrate	*n*-valerate
		Liver		
1	95	100	62.4	41
2	94	100	85	76
3	88	100	71	25
		Lung		
1	74	100	84	48
2	59	100	58	26
3	80	100	62	31
		Testis		
1	92	100	87	32
2	83	100	53	23
3	96	100	80	46
		Renal Cortex		
1	81	100	39	45
2	91	100	36	40
3	88	100	58	52
		Pancreas		
1	3.5	19	22	100
2	6.3	29	38	100
3	3.2	16	52	100

average esterase (*p*-nitrophenyl propionate) values were found as follows: lung 10 units; renal cortex 80 units; liver 160 units. Compared to these were the average findings in litter mates at 13 days: lung 304 units; renal cortex 206 units; and liver 725 units.

The esterase values of umbilical cord blood of children at birth also were very low; in 12 newborn babes the value ranged from 1.53 to 2.37 units per ml. while the blood of the mothers taken at the same time by venipuncture ranged from 4.12 to 8.15 units.

The esterase of rat testis between age 5 and 27 days (Fig. 2) varied between 5 and 14 units per gm.; between age 27 and 29 days the esterase doubled and

there was a sharp and progressive rise to 35 days, when adult levels were encountered. In albino rats raised in this laboratory, spermatozoa are found in the testis between age 34 and 37 days.

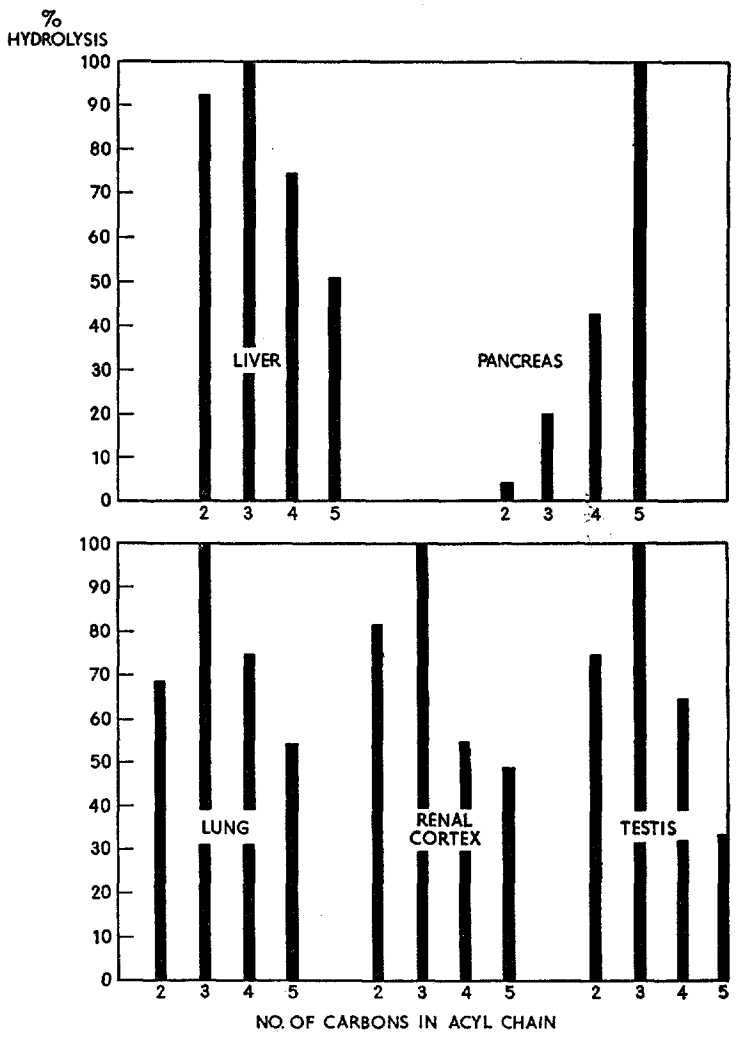

FIG. 1. Patterns of enzymatic hydrolysis of acyl esters of *p*-nitrophenol by tissues. Acetate (C_2), propionate (C_3), *n*-butyrate (C_4), and *n*-valerate (C_5) esters were used as substrates in equimolar concentration and were tested simultaneously. Average values are given and the results are expressed in percentage of that ester hydrolyzed at the fastest rate.

Some preliminary assays of human tissues may be noted for comparison. The esterase of the testes of 6 men between 55 and 70 years of age ranged from

2.06 to 7.61 units. A few isolated normal tissues were studied: renal cortex 44 units; bladder mucosa 29 units; colon mucosa 39 units.

Effect of Cryptorchism on Testicular Esterase.—In 18 rats the left testis was anchored through an abdominal incision by suture to the anterior surface of

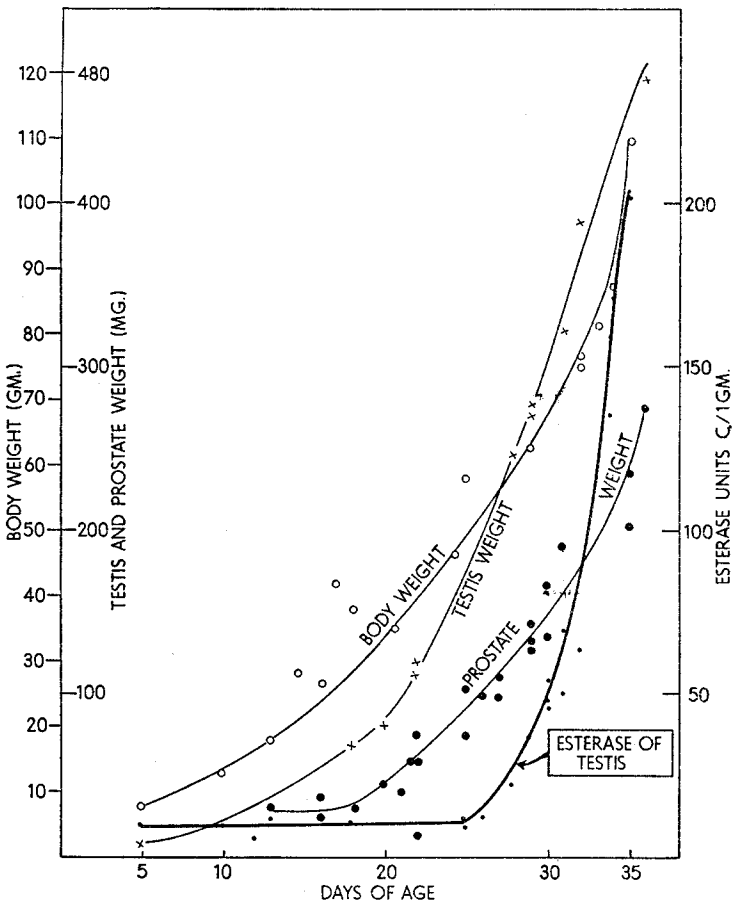

FIG. 2. Growth curves of development of testis, ventral prostate, and body weight of the rat. The concentration of non-specific esterase (substrate *p*-nitrophenyl propionate) is indicated in the curve on the right.

the peritoneal cavity; 6 to 8½ weeks later the cryptorchid testis and its normal mate were recovered for esterase determination. The concentration of esterase was increased in the abdominal testis (Table III) compared to the control which was not operated upon; the total amount in the cryptorchid testis progressively decreased with time from 89 to 50 per cent of the normal.

Testicular Esterases after Hypophysectomy and Gonadotrophin Injection.—Two weeks after hypophysectomy both the content and concentration of the testicular esterase were found to be markedly decreased (Table IV) as compared with litter mate control rats. It is perhaps significant that the low values of early puberty (before 27 days) were not reached, so from an enzymatic standpoint the testis of a hypophysectomized rat differs from the testis of the infant.

TABLE III

Esterase in Normal and Cryptorchid Testes

No.	Post-operative	Normal testis			Cryptorchid testis			Total esterase ratio $\frac{\text{Cryptorchid}}{\text{Normal}}$
		Weight	Esterase	Total esterase	Weight	Esterase	Total esterase	
	days	*gm.*	*units/gm.*	*units/gm.*	*gm.*	*units/gm.*	*units/gm.*	
1	42	1.444	144	208	0.643	288	185	0.89
2	56	1.367	533	729	0.680	636	432	0.59
3	60	1.836	575	1056	0.521	1021	532	0.50
4	60	1.373	409	562	0.411	753	309	0.55

TABLE IV

Effect of Hypophysectomy and Gonadotrophin on Esterase of Testis

Experiment No.	Length of time after hypophysectomy	Gonadotrophin	Body weight	Testis weight		Esterase		
						Prostate	Testis	Testis
	days	*units*	*gm.*	*mg.*	*mg.*	*units/gm.*	*units/gm.*	*units/gm.*
1	Unoperated control	None	178	1169	1194	33	258	332
2	Unoperated control	None	220	1210	1230	112	380	413
3	16	None	140	744	821	89	52	40
4	18	None	155	890	912	17	27	53
5	21	30	132	396	408	133	133	138
6	22	40	160	896	904	54	250	255

Equine gonadotrophin, 10 units daily for 3 and 4 days, caused the content and concentration of esterase to increase in the testis (Table IV), but neither the esterase values nor the weight of the testis approached the normal values.

DISCUSSION

The distribution of non-specific esterase was found to be quite different from that of cholinesterase. Cholinesterase in the rat has previously (9) been found to have its greatest concentration in certain small glands, the uterus, and the brain stem, while our observations show that non-specific esterase has its greatest accumulation in liver, lung, testis, renal cortex, and pancreas. These findings are interpreted as additional evidence for the dissimilarity of non-

specific esterase and cholinesterase. It is of interest that the lens, a tissue of sluggish metabolic activity, has an appreciable esterase content.

Two patterns emerge from differences in tissues in their hydrolysis of fatty acid esters of varying chain length. In one pattern, characteristic of lipase, there is increasing hydrolytic activity with increases of the acyl chain from 2 to 5 carbons. This pattern was found in the pancreas of several kinds of animals, namely, the dog, rabbit, and rat, and elsewhere only in rat serum. The serum of the rat is more effective in splitting C_2 and C_3 acid esters than is pancreas, no doubt because it contains appreciable quantities of non-specific esterase, but the predominant effect resembles pancreas more than liver.

In the other hydrolytic pattern the 3-carbon acyl ester (propionate) is split more effectively than the other members of the series. This pattern is exhibited in the rat by extracts of liver, lung, renal cortex, and testis and also by dog, human, and rabbit serum. This pattern we consider to be characteristic of non-specific esterase.

It is possible to predict the pattern of hydrolysis of acyl esters of *p*-nitrophenol by testing any extract against propionate (PNPP) and butyrate (PNPB) esters. If PNPP is split faster than PNPB, acetate is always split slower than propionate and valerate is hydrolyzed more slowly than butyrate. When PNPB is hydrolyzed faster than PNPP, acetate is split less effectively than propionate and valerate esters are split faster than butyrate. Acetate esters of *p*-nitrophenol are always hydrolyzed less effectively by non-specific esterase and lipase than propionate esters.

The patterns in infant tissues do not differ from those of the adult—merely quantitative differences occur. The tissues of the infant rat (4 to 5 days) are very low in non-specific esterase. Concerning enzymes of young animals, it is known that very young fetuses of the goat contain extraordinarily little carbonic anhydrase (21) and this enzyme in the blood of newborn infants (22) is less than half that found in the blood of adults. Esterase in rat tissues other than the testis accumulates rapidly so that at 12 to 18 days normal values are found. The testicular esterase remains at low and fairly regular levels until age 27 days when it increases markedly in content and concentration, reaching adult levels at puberty—about 35 days.

The pattern of testis resembles that of liver rather than of pancreas. The enzyme seems to be associated with interstitial cells as deduced from several indirect experiments. The elevated temperature of the abdomen (23) causes germinal epithelium of the testis to disappear. In cryptorchid testes the concentration of esterase is increased above normal and only after 6 weeks—long after the germinal epithelium has disappeared—does the total esterase content fall. Hypophysectomy causes a profound decrease of testicular esterase; the injection of gonadotrophin, which stimulated Leydig cells of the testis, caused an increase of esterase. Gomori (18) found that in the rat testis only the in-

terstitial cells split glycol esters of stearic acid, germinal epithelium being devoid of "lipase." It should be pointed out, however, that in this, the histochemical method, liver, lung, and renal cortex show intense staining so that this reaction is interpreted by us as lacking specificity for lipase; non-specific esterase exhibits this hydrolytic phenomenon as well.

The evidence reveals further that the concentration of esterase in the testis of the rat is an indicator of the level of hormone production. The onset of pubertal growth of the prostate could be correlated (Fig. 2) with the increase of testicular esterase; the decrease of androgen production by the testis, as demonstrated by the weight of the ventral prostatic glands, was related to decreased testicular esterase, and its restoration by gonadotrophin caused an increase of esterase in the gonad.

SUMMARY

The tissues most effective in the enzymatic hydrolysis of acyl esters of *p*-nitrophenol by tissue extracts of the rat and dog were the liver, lung, pancreas, renal cortex, and testis; in the dog tracheal and vesical mucosae were also esterase-rich and the lens had appreciable activity. Esterase was at low concentration in the tissues of the rat for 4 and 5 days after birth, but an increase to adult levels soon took place except in the testis where the rise was delayed until puberty. The esterase values of the blood of newborn children were also low.

Two patterns of activity in tissues against these esters were found. A pattern in which propionate esters were hydrolyzed most rapidly was displayed regularly by liver, lung, renal cortex, and testis of the rat and also by dog, rabbit and human serum. A second pattern with progressive effectiveness in hydrolyzing fatty acid esters of 2-carbon to 5-carbon chain length was exhibited by the pancreas of the rat, rabbit, and dog and also by rat serum.

Esterase of the testis of the rat is located in the interstitial cells and its concentration seems to be directly related to androgenic hormone production. The increase of testicular esterase during puberty paralleled the increase of prostatic weight. Hypophysectomy caused a profound decrease of testicular esterase which was restored in part by gonadoptrophin. Artificial cryptorchism in the rat, causing elimination of germinal epithelium, resulted in an increase of esterase concentration although the total content of the testis slowly decreased.

BIBLIOGRAPHY

1. Ohmori, Y., *Enzymologia*, 1937, **4,** 217.
2. Huggins, C., and Talalay, P., *J. Biol. Chem.*, 1945, **159,** 399.
3. Talalay, P., Fishman, W. H., and Huggins, C., *J. Biol. Chem.*, 1946, **166,** 757.
4. Huggins, C., and Smith, D. R., *J. Biol. Chem.*, 1947, **170,** 391.
5. Huggins, C., and Lapides, J., *J. Biol. Chem.*, 1947, **170,** 467.
6. Marnay, A., and Nachmansohn, D., *J. Physiol.*, 1937, **89,** 359.
7. Glick, D., Lewin, A., and Antopol, W., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40,** 28.

8. Mendel, B., Mundell, D. B., and Rudney, H., *Biochem. J.*, 1943, **37,** 473.
9. Sawyer, C. H., and Everett, J. W., *Am. J. Physiol.*, 1947, **148,** 675.
10. Stedman, E., Stedman, E., and White, A. C., *Biochem. J.*, 1933, **27,** 1055.
11. Nachmansohn, D., and Rothenberg, M. A., *J. Biol. Chem.*, 1945, **158,** 653.
12. Archibald, R. M., *J. Biol. Chem.*, 1946, **165,** 443.
13. Balls, A. K., and Matlack, M. B., *J. Biol. Chem.*, 1938, **123,** 678.
14. Kastle, J. H., *Bull. Hyg. Lab., U. S. P. H. S.*, 1906, **26,** 43.
15. Kastle, J. H., and Loevenhart, A. S., *Am. Chem. J.*, 1900, **24,** 491.
16. Loevenhart, A. S., *J. Biol. Chem.*, 1907, **2,** 427.
17. Gomori, G., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58,** 362.
18. Gomori, G., *Arch. Path.*, 1946, **41,** 121.
19. Falk, K. G., Noyes, H. M. and Sugiura, K., *J. Biol. Chem.*, 1924, **50,** 183.
20. Smith, P. E., *Am. J. Anat.*, 1930, **45,** 205.
21. Meldrum, N. U., and Roughton, F. J. W., *J. Physiol.*, 1933, **80,** 113.
22. Stevenson, S. S., *J. Clin. Inv.*, 1943, **22,** 403.
23. Moore, C. R., *Am. J. Anat.*, 1924, **34,** 337.
24. Gomori, G., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67,** 4.