ings suggest that part of the fed H^3-β-sitosterol may have undergone transformation in the intestinal wall or lumen so that it was no longer precipitated with digitonin and thus, would not have been detected in these experiments. Such changes occur with fed cholesterol-4-C^{14}(11). Another less probable alternative is that H^3-β-sitosterol was absorbed by some other route than through the lymphatic system. We are now using C^{14}-β-sitosterol to test these possibilities.

The present data also show that a considerable amount of H^3-β-sitosterol, like cholesterol(11), is taken up by the intestinal wall and is present almost entirely as free sterol. However, the amount taken up was about one-half that of cholesterol. Roth and Favarger (12) have also observed uptake of D-sitosterol by the intestinal wall. Thus, in this first step of the absorption process both sterols appear to be handled in a similar fashion, albeit, β-sitosterol is taken up to a lesser degree. After free cholesterol is transferred into the intestinal wall it is largely esterified and then transferred into the lymph as part of the chylomicron(11). It appears that β-sitosterol is not esterified in the intestinal wall or transferred to lymph in appreciable quantities. It should be emphasized that if the amount of H^3-β-sitosterol present in intestinal wall at end of 6 hours, had been transferred to the lymph during the next 18 hours, it could have been easily determined by the technics employed.

Summary. Administration of 44 mg of H^3-β-sitosterol to lymph fistula rats and analysis of feces, intestinal contents, intestine, and lymph gave the following results: (1) balance data indicated approximately 40% absorption in 48 hours, (2) there was increased fecal excretion of cholesterol and related sterols, (3) there was considerable uptake of H^3-β-sitosterol by the intestinal wall, but essentially no transfer into the lymph. Explanations for the apparent disappearance of H^3-β-sitosterol are discussed.

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Received November 5, 1958. P.S.E.B.M., 1959, v100.

Proteolytic Enzyme System of Skin. VI. Enzyme Patterns in Various Animal Species.*† (24553)

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Several enzymes which comprise, in part, the proteolytic enzyme system of rat skin have been described by Martin and Axelrod (1-5). In extracts prepared from rat skin acetone powder, 4 distinct enzymes, designated Proteinase A, Proteinase C, the A_1-esterase, and the A_2-esterase, have been characterized, partially purified, and in some cases separated from each other. With the exception of Proteinase C, synthetic sub-

strates are known for these enzymes. Proteinase A, in addition to its ability to hydrolyze a protein substrate will also hydrolyze acylated and non-acylated aromatic amino acid esters such as N-acetyl-L-tyrosine ethyl ester (ATEE)‡ and TEE(2,4). The specificity of the A_1- and the A_2-esterase is not as inclusive, each possessing one facet of the substrate specificity of Proteinase A. Thus, the A_1-esterase will hydrolyze only *acylated* aromatic amino acid esters, *e.g.*, ATEE, and conversely, only *non-acylated* aromatic amino acid esters, *e.g.*, TEE and PEE, are hydrolyzed by the A_2-esterase. The hydrolysis of TEE and PEE by A_2-esterase occurs at equal rates(5). Despite overlapping specificity patterns of Proteinase A, the A_1-esterase, and the A_2-esterase, the activity of each can be determined by a differential assay procedure using ATEE, TEE, and an inhibitor to Proteinase A (4,5). The Proteinase A inhibitor, AIn (isolated from sheep serum), is specific for Proteinase A in the sense that it does not inhibit activity of any of the other above mentioned enzymes. The distinguishing characteristic of Proteinase C in an initial skin extract is its ability to undergo reversible inactivation; a time-dependent process proceeding optimally in solutions of ionic strength 0.6 to 0.8(1). The reverse or activation reaction is essentially instantaneous upon exposure of the inactivated extract to ionic strength 1.4 or greater (1). This behavior is due to the ionic strength-dependent association and dissociation of Proteinase C with a skin inhibitor. Both components have been separated from each other and studied in some detail(3). Thus, with casein as test substrate, that portion of total proteolytic activity that can be *reversibly* inactivated by suitable changes in ionic strength can be ascribed to Proteinase C. Since we are interested in ascertaining the importance of skin proteolytic enzymes in the cellular disruptive processes occurring as sequelae to either a thermal insult or an antigen-antibody reaction and since use of animals other than rat can be envisioned in such studies, it was considered of importance to obtain information on the ubiquity of rat skin-type proteolytic enzymes in the skins of other animals. The case for proteolytic enzyme activation by such means has received support by various investigators(6-14). In the present paper, extraction technics and methods of assay developed by experience with enzyme activities in rat skin acetone powder have been applied to fresh skin preparations of the rat and other animals. A brief report of some of this material has been presented(15).

Materials and methods. Preparation of extracts. Abdominal skin samples of various animals were freed of hair and underlying superficial fascia. Each skin was then scissor-minced and passed through a motorized Latapie Tissue Grinder§ in cold room to give a skin mince of approximately the same particle size for all skins used. After extraction of the mince for 18 hours at 1°C in 1.6 M KCl (4 ml/g), the suspension was centrifuged at 25,-

* This investigation was supported, in part, by research grants from the Nat. Inst. of Arthritis and Metabolic Dis., and Nat. Inst. of Allergy and Infect. Dis., N.I.H., U.S.P.H.S.

† We are indebted to Armour and Co., for generous samples of sheep, cow, and hog abdominal skins from freshly slaughtered livestock; to Dr. H. B. Lewis, for Rhesus monkey skin; to Drs. B. Fisher, and R. S. Totten, for surgical samples of human skin; and to Mr. E. M. Perkins, for the design and construction of the modified Latapie Tissue Grinder.

‡ TEE, L-tyrosine ethyl ester; PEE, L-phenylalanine ethyl ester.

§ A Latapie Tissue Grinder, Arthur H. Thomas Co., Philadelphia, Pa., was modified and motorized as follows: a) the plunger crank was replaced with a 3 inch knurled wheel to give greater control over plunger displacement; b) the shaft attached to revolving plate was strengthened by increasing its outer diameter to approximately 5/16 inch; c) the mount was discarded and a new mount, incorporating a set screw partly into the chamber housing, was devised to prevent rotation about the long axis when the revolving plate shaft was attached to the motor; d) attachment to the motor was effected through a flexible coupling and both the motor and tissue grinder were rigidly mounted on an aluminum plate; and e) the series wound motor was obtained from Wilfred R. Uffelman, Bodine Electric Co., Cleveland, O. The torque rating was 19 inch-lb at 250 rpm. We found that even with this powerful motor, an appreciable slowing-down occurs upon passage of fresh scissor-minced skin through the grinder.

TABLE I. Esterase and Proteinase Activity of Skin Extracts.

Animal	mg protein per ml extract	$[k_0]^{ATEE}$		$[k_0]^{TEE}$	$[k_0]^{PEE*}/[k_0]^{TEE*}$	Proteinase activity ($\times 10^3$)	
		Total (I)	With AIn (II)	(III)	(IV)	Activation conditions (V)	Inactivation conditions (VI)
Rat	10.6 ± .6	1.71 ± .08	.83 ± .00	.32 ± .02	1.1 †	60 ± 10	23 ± 3
Guinea pig	7.3 ± 1.4	.45 ± .04	.46 ± .04	.18 ± .00	.67	9 ± 1	6 ± 1
Rabbit	8.9 ± 2.5	.0	.0	.28 ± .00	3.8	0	0
Hog	3.5 ± .8	.0	.0	.15 ± .05	2.7	0	0
Sheep	6.3 ± .8	.23 ± .05	.31 ± .04	.37 ± .08	1.9	0	0
Cow	1.9 ± .3	.0	.0	.0		0	0
Chicken	4.2 ± 1.2	.0	.0	.0		0	0
Monkey	4.7 ± .1	.35 ± .04	.35 ± .06	.11 ± .03	.68	18	11
						28	14
						41	12

* Activity determinations performed on pooled samples of skin extracts.
† Corrected for contribution of Proteinase A to hydrolysis of TEE and PEE(4).

000 x g for 30 minutes at the same temperature. Supernatant solutions were collected and kept at either 1°C. if the extract was to be used for immediate assay, or stored at −25°C. Storage at −25°C for several weeks had no effect on measured enzyme activity. *Esterase Assays.* Esterase activity was determined at 30°C in reaction solutions of ionic strength 0.6 using ATEE, TEE, and PEE as substrates(2,4,5). Hydrolysis of the non-acylated compounds was measured at pH 6.5; ATEE hydrolysis was measured at pH 8.0. Incubation of enzyme extracts with AIn preparation to inhibit Proteinase A or possible Proteinase A-type activity was executed under standardized conditions(2). Esterase activity was expressed as zero order reaction constant, k_o, defined as μmoles substrate hydrolyzed/minute/ml extract. *Proteinase assays.* Hydrolysis of alkali-denatured casein was determined after exposure of enzyme extracts to conditions which, in rat skin extracts, permit either activation or inactivation of Proteinase C(1-3). When *activation conditions* of assay were used, the extract was exposed to ionic strength 1.6 and 1°C before addition to substrate. After such treatment, Proteinase C will exhibit maximal activity. When *inactivation conditions* of assay were used, the enzyme solution was exposed to ionic strength 0.8 for approximately 6 hours at 1°C before assay. This type of enzyme pre-treatment permits association of Proteinase C with its inhibitor and is reflected by decrease in proteolytic activity. In both assay procedures, the enzyme-substrate reaction solution was at pH 7.5 and ionic strength 0.8. The temperature was 35°C. Activity was expressed as optical density change at 280 mμ/minute/ml of extract as determined under standard conditions(1,2). Protein was determined by the method of Lowry *et al.*(16), using commercial preparation‖ as the protein standard. All data obtained, except where indicated, represent mean value of 3 different assays with the attendant standard deviation, each assay being performed on the extract derived from the skin of a separate animal.

Results. The esterase and proteolytic activity of extracts from various animal skins are given in the Table. Activity towards ATEE was present in only rat, guinea pig, sheep, and monkey skin extracts (*cf.* Column I). Incubation of extracts possessing ATEEase activity with the AIn preparation produced inhibition of such activity in only rat skin extracts (compare Columns I and II). A slight potentiation of sheep ATEEase activity was noted by such treatment. Since the Proteinase A of rat skin extracts can be inhibited by AIn preparation, these results indicate that comparable activity is absent in skins of other animals tested. The AIn non-inhibitable ATEEase activity (*cf.* Column II) simulates activity of the A_1-esterase of rat skin extracts. The non-detection of a Pro-

‖ Protein Standard, Ampagent, Aloe Scientific.

teinase A-type enzyme in extracts other than those derived from the rat may be due either to its absence or to its existence in an inactive state. Evidence that Proteinase A exists partly in an inactive state in extracts of rat skin acetone powder(5) and in fresh rat skin extracts (unpublished results) has been obtained and such may be the case in other skin extracts.

Enzyme activity capable of TEE hydrolysis was somewhat more prevalent with only extracts of cow and chicken skin being devoid of activity toward this substrate (*cf.* Column III). However, hydrolysis of TEE by enzyme preparations apparently lacking an enzyme comparable to Proteinase A does not necessarily imply the existence of a rat skin-type A_2-esterase in such extracts. As mentioned above, the A_2-esterase hydrolyzes TEE and PEE at equal rates. Examination of the Table (Column IV) shows that the ratio of activity toward PEE and TEE is essentially unity only for the rat skin extract. In all other cases, TEE and PEE are hydrolyzed at different rates. In an attempt to determine if a single enzyme catalyzes hydrolysis of both TEE and PEE, as is true with the A_2-esterase in rat skin extracts, the enzyme preparations were stored at −25°C for 2½ months. PEEase to TEEase activity ratios remained constant. Likewise, heating for 10 minutes at 55°C, although producing a decrease in activity toward these 2 substrates, left the activity ratio unaltered. Although such evidence is equivocal, the data are not inconsistent with the viewpoint that a single enzyme in each extract catalyzes the hydrolysis of both TEE and PEE. The structural similarity of these 2 substrates strengthens this possibility. Therefore, despite the difference in TEE and PEE hydrolysis rates by extracts of skin other than the rat, the data suggest that an enzyme with a substrate specificity similar to the A_2-esterase is present in guinea pig, rabbit, hog, sheep, and monkey skin extracts. It should be mentioned that in 2 samples of human skin tested (data not given in the Table), one from abdominal area of a negro adult female and the other from the breast of a white adult female, only the extract from the former contained ATEEase activity and it was not inhibitable by the AIn preparation. Both samples were devoid of activity towards TEE.

Surprisingly, only extracts of rat, guinea pig, and monkey skin contained proteinase activity, and in all cases, exposure of enzyme preparations to *inactivation conditions* prior to assay led to decreased activity (compare Columns V and VI). Admittedly, the decrease in activity of guinea pig extract was slight but, as with rat and monkey skin extracts, it was completely reactivated upon exposure to *activation conditions*. Thus, extracts of monkey skin and, to a slight extent, of guinea pig skin, contain proteolytic activity capable of ionic strength-dependent reversible inactivation, *i.e.*, activity comparable to Proteinase C. The data for all monkey skin proteinase determinations have been presented separately due to variation of results obtained with each animal.

Summary. Fresh skin extracts of various animals have been surveyed for ability to hydrolyze N-acetyl-L-tyrosine ethyl ester, L-tyrosine ethyl ester, L-phenylalanine ethyl ester, and casein. The data obtained have been interpreted in terms of conformity to results previously obtained with extracts of rat skin acetone powder.

ADDENDUM. Since the preparation of this manuscript, a comprehensive review by Ungar, G., and Hayashi, H. (*Ann. Allergy,* 1958, v16, 542) has appeared. This review summarizes the experimental evidence for the role of enzymatic mechanisms in antigen-antibcdy reactions.

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Received November 6, 1958. P.S.E.B.M., 1959, v100.

Intraocular Grafts of Embryonic Sexual Ducts of the Rat.* (24554)

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The guiding hypothesis behind much of the investigation pertaining to differentiation of sexual ducts in vertebrates calls for a response of the ducts to secretions provided by the developing gonads. Accordingly, developing testes provide materials which selectively promote elaboration of wolffian ducts while suppressing the oviducts; developing ovaries do the converse. Generally speaking, this 'modus operandi' appears to prevail, yet some workers(1,2,3) incline to the view that in mammals only the testes are concerned in duct differentiation while another(4,5) feels development of the reproductive tract is not controlled by the gonads at all. With these alternatives in mind, it is believed that a report of some observations on behavior of gonads and sexual ducts of the embryo rat interplanted to the anterior chamber of the eye may be of interest. The observations derive from 230 grafts prepared for earlier studies on gonads and rete tubules(8,9). The general procedures in preparing this material have been described. It should be added, however, that not all two hundred odd grafts included the primordial sex ducts; thus, this report is based on a lesser group of 67 relevant cases selected from the total pool. Specific details as to ages of donor embryos, constitution and survival of grafts and other pertinent data will be given as these cases are described. A complete series of "normal stages" of rat embryos has been available as standard of reference. A necessary first step towards interpretation of the grafts is a brief review of the time-table of normal embryogeny of the parts concerned (6,7). The wolffian ducts split directly off the top of the nephrogenic ridge, beginning 10th day postcoitum, then progressively project themselves caudad by free terminal growth. They contact the wall of the cloaca during 11th day and 1 day later open thereto. By the 19th day, in females, wolffian ducts have disappeared. The first mesonephric tubules appear early on 11th day. A maximum number of 15 to 18 pairs of tubules is reached near the end of 12th day. Of these, all disappear by the end of 16th day except the 3 most anterior pairs which are retained as epigonadal elements. In contrast to the early appearing wolffian ducts, the oviducts do not arise until the latter half of 13th day and do not open to cloaca until 2 days later. They disappear in males by the end of 19th day. As for the gonads, those destined to be testes begin to assume their characteristic structure towards the close of 13th day; prospective ovaries do not express themselves until the 16th day. It follows from this listing that embryos of 14 days, plus or minus a few hours, present prospective ducts and epigonadal elements for both sexes and gonads of one sexual prospect or the other. Moreover, the presence of the opposite elements in grafts of a given sex residing in the eye chamber for periods which would make them equivalent to term or postpartum ages is indicative of departure from their normal fate. The grafts to be considered all derived from embryos ranging in age from 13½ to 14¼ days postcoitum and are of 2 types. Fig. 1 is intended to portray these. In one series, the urogenital

* Contribution No. 661 from Zoological Labs., Indiana Univ.