curonide which has been isolated and characterized as its triacetyl methyl ester. *p*-Hydroxybenzylacetamide and its *O*-acetyl derivative have been prepared and described for the first time.

5. *p*-Hydroxybenzaldehyde is largely converted into the glucuronide (33%) and sulphate (9%) of *p*-hydroxybenzoic acid. A small amount (2–3%) is, however, excreted as *p*-glucuronosidobenzaldehyde which has been isolated as a 2:4-dinitrophenylhydrazone.

6. *p*-Hydroxybenzoic acid is the least conjugated

of all the compounds studied here and forms only 18 % glucuronide and 9 % ethereal sulphate.

7. The results indicate that with phenolic amines of the type studied here the extent of glucuronic acid conjugation is inversely proportional to the rate of deamination, the conjugation being highest where no deamination takes place. The significance of this conclusion is discussed.

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Further Observations on the Proteolytic Enzymes in Rat Skin

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The object of this communication is to extend knowledge of the skin proteinase or 'dermoproteinase' previously described by Beloff & Peters (1945) and of its distinction from other proteolytic enzymes present in rat skin (see especially Fruton, 1946).

It was shown that the dermoproteinase will digest casein, serum globulin, serum albumin and myogen, and that it does not fall within the known groups of digestive proteinases by virtue of its failure to digest *N*-benzoyl-L-arginine amide and carbobenzyloxy-L-tyrosylglycine amide, the typical synthetic substrates for trypsin and chymotrypsin; Beloff & Peters (1945) also showed that this proteinase activity could be separated from other peptidases present by differential extraction of the skin.

The experimental results to be presented here will be considered under the separate headings (1) of the effect of methods of extraction upon the enzymes present, (2) of the character of the individual enzymes.

METHODS

Preparation of skin

(a) Acetone-dried rat skin was made as described previously (Beloff & Peters, 1945), 0.3 g. of dried skin being equivalent to 1.0 g. of fresh skin. Extracts were prepared so that 5 ml. of extract were equivalent to 0.3 g. of dried skin; either 5% (w/v) aqueous KCl or 5–6% (w/v) aqueous KNO₃ (see below) were used for the extraction of the proteinase.

(b) Fresh skin extracts were made by the technique of Fruton (1946). About 30 g. of skin (from two rats), carefully cleaned, were cut into small pieces and stirred with 100 ml. 2% (w/v) NaCl solution in the Waring Blendor for 8–10 min. A further 100 ml. of 2% NaCl were then added, and the whole stirred slowly for 3 hr. at room temperature. The pulpy mass of skin was strained off through muslin, and the extract filtered through a Whatman no. 41 filter paper. Certain comparisons between the extracts obtained by the two methods are given below.

Estimation of activity

The principle was followed throughout of mixing equal volumes of extract and substrate.

(a) The peptidase activity was estimated by the titration technique of Linderstrøm-Lang (1927) in 90% (v/v) acetone. That is to say, acetone was added to the aqueous solution to be titrated until the liquid in the titration flask contained 90% acetone, and the titration was then carried out with ethanolic HCl (a standard solution of HCl in acetone being impracticable owing to the volatility of acetone). The synthetic peptide substrates were made up in the required concentration in distilled water, or in veronal buffer of the required pH, according to the extract being used. A 2 hr. digestion period was normally allowed, duplicate 1 ml. samples being taken at the beginning and end of this time. The samples were run into a calculated quantity of 0.1N-ethanolic HCl, and the titration was completed with 0.01N-ethanolic HCl. A difference of 0.03 ml. or less (rarely 0.04 ml.) between titration of duplicates was observed.

The synthetic peptides used were supplied commercially by Hoffmann-La Roche and Co. Except in two experiments (Table 5) the racemic, DL peptides were used; a control experiment indicated that the presence of the D peptide exerted no inhibitory effect (see also Beloff & Peters, 1945, addendum).

(b) The proteinase activity was estimated by the Van Slyke method. An attempt was made to use the Linderstrøm-Lang titration for this, but it was found unsuitable for the following reasons. Casein, the substrate used, cannot be satisfactorily titrated, since it is precipitated on addition of acetone, the precipitation becoming heavier as the titration proceeds, and being apparently complete at the end point, thus rendering the colour change very difficult to observe. This was assumed to be due to the fact that the pH at the end point (c. 4) is approximately the same as at the isoelectric point of casein (4.7). β -Lactoglobulin and serum albumin were satisfactorily titrated, but it was discovered that concentration of salts also affects the accuracy of the titration. Phosphate in particular is precipitated very readily on addition of acetone, giving a rather cloudy effect, and causing a continuous shift of the end point. With 0.1M-phosphate buffer, titration was not complete for many hours. This effect varies markedly with the salts present, and a solution containing 1% chloride was readily titratable, test-tube experiments indicating that up to 5% chloride would probably be satisfactory. Solutions containing 6% KNO_3 were also satisfactorily titrated. The combination of the effects of protein precipitation and of salts made the titration too uncertain to be useful in this instance.

The presence of 2.5% KCl in the digestion mixture was found to have an unfavourable effect on the Van Slyke method, a grey scum forming in the chamber, and the mercury losing its characteristic physical properties, becoming dull and sticky. This phenomenon was known previously but became in these experiments too troublesome to manage. It was found to be due to a reaction which took place between chloride, nitrite, and mercury in the presence of acid (simplest conditions, HCl, nitrite and mercury). Bromide had a still more marked effect than chloride. The occurrence of the reaction was confirmed using the same amounts of the reagents (A.R.) as were present during our

estimation, with a specimen of chemically purified, vacuum-distilled mercury. This led to an attempt to use other salts for the extraction of the skin: a comparatively inactive extract was obtained with sodium acetate and K_2SO_4 , whereas 6% (w/v) KNO_3 extracted about two thirds as much activity as 5% KCl. Two experiments are given in Table 1 comparing the extracting activity for dermoproteinase of 5% KCl and 6% KNO_3 . In the later experiments 6% KNO_3 was used.

Table 1. Comparison of 5% KCl and 6% KNO_3 as extracting agents for dermoproteinase from skin

(Dried skin (0.3 g.) extracted for 30 min. at room temperature with 5 ml. extraction medium and extract mixed with an equal volume of substrate solution (0.5% casein). Digested at 38° for 2 hr. Proteinase activity estimated by Van Slyke method.)

Exp. no.	Agent	Amino N (mg./100 ml.)	
		Initial	Increase
1	KCl	0.813	0.379
	KNO_3	0.813	0.314
2	KCl	0.861	0.355
	KNO_3	0.888	0.281

Light white soluble casein (British Drug Houses Ltd.) was used; 1% solutions were made up directly in the Waring Blender with the addition of a little NaOH, the pH being adjusted to the required point with 0.1N-HCl.

The usual period of digestion of casein solutions and enzyme was 1 hr., unchanged protein being precipitated at the end of that time with 2 ml. 25% (w/v) trichloroacetic acid. The precipitates were washed with 2.5% trichloroacetic acid and the filtrates were made up to 25 ml., 5 ml. samples being estimated.

Control tubes, to which trichloroacetic acid was added immediately after mixing the casein solution and extract, were treated in exactly the same way.

RESULTS

(1) Effects of methods of extraction upon the enzymes present

(a) *Dried skin extracts.* Extracts of acetone-dried rat skin were previously shown to contain, in addition to the skin proteinase, some peptidase activity for leucylglycylglycine. The peptidase activities were extracted by a lower salt concentration. This was confirmed. Even 0.05M-phosphate extracted as much or slightly more peptidase than was present in a subsequent extraction of the same skin with addition of 6% nitrate; in contrast to this the higher salt concentration brought out much more proteinase (see Table 2). These extracts also contained some aminoxopeptidase activity, splitting leucylglycine with activation by manganese, though both peptidase activities were weak compared with those of Fruton-type extracts of fresh skin (see Table 3).

Table 2. *Influence of salt concentration upon the extraction of proteases from dried skin*

(Skin extracted as described in Table 1 and extract mixed with equal volume of substrate solution. Digested at 38° for 2 hr. Peptidase activity estimated by Linderstrøm-Lang titration; proteinase activity by Van Slyke method.)

Peptidase activity (substrate, DL-leucylglycylglycine)	
Extraction medium	Substrate split (mg./ml.)
1st extraction, 0.05 M-Ringer phosphate	0.17*
2nd extraction, 0.05 M-phosphate + 6% KNO ₃	0.11

Proteinase activity (substrate, 0.5% casein)		
Extraction medium	Amino N (mg./10 ml.)	
	Initial	Increase
1st extraction, 0.05 M-Ringer phosphate	1.671	0.102
2nd extraction, 0.05 M-phosphate + 6% KNO ₃	1.851	0.207

* The concentrations of substrate were not the same in these two experiments, and this figure has been corrected from the curve given (see Fig. 1) to be directly comparable with the other.

Table 3. *Comparison of peptidase activity in different extracts of rat skin*

(Extracts of dried skin made as described in Table 1. Fruton-type extracts made as described under Methods, 1 (b). Digestion for 2 hr. at 38°. Estimations by Linderstrøm-Lang titration.)

Extraction medium	Substrate	Substrate concentration (mg./ml.)	pH of digest	Substrate split (mg./ml.)	
				0.001 M-MnSO ₄	No activator
Acetone-dried skin extracts					
2% NaCl	DL-Leucylglycine	9.4	7.9	0.61	0.26
	DL-Leucylglycine	9.4	7.9	0.33	0.22
	DL-Leucylglycine	9.4	7.9	0.88	0.29
	DL-Leucylglycylglycine	24.5	7.9	0.27	0.19
Fruton-type extracts					
2% NaCl	DL-Leucylglycine	9.4	7.9	2.73	0.56
	DL-Leucylglycylglycine	24.5	7.9	3.13	1.52

Table 4. *Test for presence of additional activator in skin*

(Three tubes containing equal amounts of DL-leucylglycine digested for 2 hr. each, (a) with a mixture of an acetone-dried skin extract and a fresh (Fruton-type) extract; (b) with the dried skin extract alone; and (c) with the fresh skin extract alone. The quantity of each extract separately was the same as that present in the mixture of the two, volumes being made up with distilled water.)

Extract	Substrate concentration (mg./ml.)	Leucylglycine split (mg./ml.)
Mixed	9.4	0.87
Fresh skin alone	9.4	0.56
Dried skin alone	9.4	0.35

It was thought possible that an additional activator, as well as the manganese, was present in the fresh skin extracts, but was partly destroyed by the acetone treatment; this was tested in two experiments. In the first, a normal extract of dried skin was made with 2% NaCl and portions of this extract were used for a digestion of DL-leucylglycine. To other portions were added small quantities of another very concentrated extract, which had previously been boiled to destroy the enzyme activity, and these were then used for similar digestions. The results were, within limits of experimental error, purely additive, and it was concluded that no heat-stable activator was present.

In the second experiment, samples of DL-leucylglycine were digested (1) with a mixture of the two extracts (dried skin extract and Fruton-type extract), (2) with the same volume of dried skin extract as used in the mixture, and (3) by the same volume of fresh skin extract as used in the mixture. Results are given in Table 4.

The effect, within the limits of experimental error, was again purely additive, and it was concluded that no additional activator was present, the difference between the two extracts being presumably due to the effect of the acetone treatment on the peptidase activity.

It is of interest in this connexion to note that Maschmann (1942), working on glycerol extracts of kidney, liver and other tissues, found that desiccation with acetone caused substantial losses in peptidase activity. The enzyme activity most affected was the cleavage of alanyl-glycylglycine and glycylglycylglycine, while that of leucylglycylglycine and leucylglycine was only slightly altered.

(b) *Fresh skin extracts.* Some of Fruton's results were confirmed. The crude extracts contained the aminoxopeptidase and dermo-peptidase (see Table 3). The former was found to be present in larger proportion, the latter in lower proportion than found by Fruton in rabbit skin. The two results are compared in Table 5.

Table 5. *Comparison of amounts of peptidase present in rat and rabbit skin*

(The activity of Fruton-type extracts of rat skin compared with Fruton's results on rabbit skin.)

Skin	Substrate	Concentration of L-substrate (mg./ml.)	pH	Time (hr.)	Percentage hydrolysis	
					No activator	0.001 M-MnSO ₄
Rat	DL-Leucylglycylglycine	12.25	7.9	2	12.4	25.6
	DL-Leucylglycylglycine	12.25	7.9	2	17.6	30.1
Rabbit (Fruton)	L-Leucylglycylglycine	12.25	7.9	2	24.0	38.0
Rat	L-Leucylglycine	9.4	7.9	2	19.5	78.5
	L-Leucylglycine	9.4	7.9	2	8.4	54.1
Rabbit (Fruton)	L-Leucylglycine	9.4	7.9	5	3.0	4.0

The proportionately greater effect of manganese in the case of the leucylglycylglycine-splitting activity of rat skin, as opposed to rabbit skin, is presumably due to the greater proportion of the leucine aminopeptidase present.

It was confirmed that dialysis had the effect, reported by Fruton, of removing the leucine aminopeptidase activity, leaving the leucylglycylglycine-splitting activity which is not activated by manganese, though, in the present work, a longer period of dialysis was necessary. The dermopeptidase activity against leucylglycylglycine was also reduced, apparently to an extent greater than proportional to the dilution occurring during dialysis. A volume of 26 ml. of the crude extract was dialyzed for 3 days (Fruton's time) at about 1° against 1500 ml. of distilled water: 29 ml. of extract were

collected from this dialysis, and it was then found that the leucylglycylglycine-splitting activity had been reduced by half, the leucylglycine-splitting activity by only one third. The remaining extract was dialyzed against 4 l. distilled water for a further 12 hr., during which time no further change in volume occurred. It was then found that the leucylglycylglycine-splitting activity had remained constant, while the leucylglycine-splitting activity had fallen markedly, though it was still not entirely removed. The results are given in Table 6.

The fresh skin extract was found to contain the dermo-proteinase, though the amount was low compared with that in the dried skin extracts. Hence this enzyme is again proved to be distinct from the other two. Results are given in Table 7.

Table 6. *Effect of dialysis upon the peptidases of rat skin*

(Fruton-type extract dialyzed twice, once for 3 days, then again for 12 hr. (see text). Activities against leucylglycine and leucylglycylglycine are given after each dialysis for comparison with original values.)

Dialyzed	Substrate	Substrate concentration (mg./ml.)	pH	Time (hr.)	Substrate split (mg./ml.)	
					No activator	0.001 M-MnSO ₄
Not dialyzed	DL-Leucylglycine	9.4	7.9	2	0.56	2.73
Once	DL-Leucylglycine	9.4	7.9	2	0.38	—
Twice	DL-Leucylglycine	9.4	7.9	2	0.11	0.30
Not dialyzed	DL-Leucylglycylglycine	24.5	7.9	2	1.52	3.13
Once	DL-Leucylglycylglycine	24.5	7.9	2	0.74	—
Twice	DL-Leucylglycylglycine	24.5	7.9	2	0.76	0.76

Table 7. *Dermo-proteinase activity of Fruton-type extracts of fresh rat skin compared with that of dried skin extracts*

(Extracts (5 ml.) made as previously described, mixed in each case with 5 ml. of a 1% casein solution, and digestion allowed to proceed at 38° for 1 hr. Amino N determined by Van Slyke method.)

Extract	Skin preparation	Amino N (mg./10 ml.)		
		Before digestion	After digestion	Increase
Dried skin	—	—	—	0.329*
Fresh skin	1	0.844	0.927	0.083
Fresh skin	2	0.970	1.181	0.211
Fresh skin	3	0.463	0.603	0.139
Fresh skin	4	0.523	—	0.154
Extract of residual skin	4	0.262	—	0.254†

* Average of ten determinations.

† One determination.

In addition, the skin remaining after the Fruton-type extract had been made was re-extracted with 6% KNO_3 in the proportion 1 g. fresh skin (≈ 0.3 g. dried skin) to 5 ml. 6% KNO_3 and the peptidase and proteinase activities compared with the original extract. Tables 7 and 8 show that there was little peptidase, but good proteinase activity in the final KNO_3 extract.

In one experiment using DL-leucylglycine as substrate (9.4 mg./ml.) at pH 7.9 and a digestion period of 2 hr., an extract of rat skin gave a splitting of 1.12 mg./ml. without addition, which was increased to 1.5 mg./ml. by addition of 0.01M- MgSO_4 and to 2.17 mg./ml. by 0.002M- CoCl_2 . Berger & Johnson (1939) stated that the leucylpeptidase activities of their preparations were unaffected and slightly

Table 8. Comparison of peptidase activities of Fruton-type extract of rat skin, and of extract made of residual skin with 6% KNO_3

(Extracts made as described in Table 1.)

Extract	Substrate	Concentration of L-substrate (mg./ml.)	pH	Time (hr.)	Percentage hydrolysis	
					No activator	0.001M- MnSO_4
Fruton-type	DL-Leucylglycylglycine	12.25	7.9	2	17.6	30.1
Fruton-type	L-Leucylglycine	9.4	7.9	2	8.4	54.1
Residual skin	DL-Leucylglycylglycine	12.25	7.9	2	3.4	3.6
Residual skin	DL-Leucylglycine	9.4	7.9	2	2.5	3.1

(2) Some further details about the enzymes

Substrates. Precise data about the optimal concentration of substrates could not be found in the literature: the results of varying the amount of leucylglycylglycine are given in Fig. 1; these show that the concentration used by Fruton (1946) is approximately maximal.

Fig. 1. Effect of substrate concentration. pH 7.9, 38°. Acetone-dried skin extracted with 2% NaCl . Extract mixed with equal volume of DL-leucylglycylglycine solution and incubated for 2 hr. Estimations by titration.

Metals. The results of similar experiments on the variation of activity with varying concentration of manganese are shown in Table 9, and indicate an optimal concentration of 0.002M-Mn; it is to be noted that Fruton used 0.001M and Berger & Johnson (1939) found that the leucylpeptidase activity of their extracts of hog intestinal mucosa increased steadily as the concentration of Mn increased from 10^{-6} to 10^{-2} M.

inhibited by cobalt, whereas Maschmann (1940) found that the leucylpeptidase activities of sera were activated by cobalt though less so than by manganese, a result consistent with our experiment.

Table 9. Effect of manganese concentration on leucylglycine activity

(9.4 mg. DL-leucylglycine/ml.; 2 hr. incubation; pH 7.9.)

Exp. no.	MnSO_4 (M)	Leucylglycine split (mg./ml.)
1	Nil	0.37
	0.0002	0.80
	0.0006	1.19
	0.001	1.33
	0.002	1.45
	0.005	1.35
2	Nil	1.18
	0.001	4.02
	0.002	4.17

(Experiments 1 and 2 were made with different skin extracts.)

DISCUSSION

Comparison of these results upon rat skin with those of Fruton (1946) upon rabbit skin show that the skins differ in the proportions present of the two peptidase activities investigated.

Since the Fruton-type extracts contain the skin proteinase, though in weak concentration, it can be concluded from Fruton's negative results with carbobenzyloxy-L-glutamyl-L-tyrosine and carbobenzyloxyglycyl-L-phenylalanine, that the skin proteinase differs from pepsin and carboxypeptidase, and therefore from any of the hitherto recognized groups of proteolytic enzymes. Since, also, it has

recently been shown (Peters & Wakelin, 1948; Grob, 1946) that trypsin and chymotrypsin are inactivated by thiol compounds, whereas the skin proteinase is not, the conclusion stands firm at present that it is a proteinase of different type.

SUMMARY

1. The proteinase present in acetone-dried extracts and in Fruton-type extracts of fresh skin from the rat have been compared and further characterized in the light of Fruton's results.

2. The acetone-dried extracts have high pro-

teinase activity and low dermo- and amino-peptidase activity with only slight activation effects of manganese, whereas the fresh skin extracts have a high peptidase and low proteinase activity.

3. The skin proteinase differs from previously recognized enzymes.

4. Manganese activates the leucine aminopeptidase optimally at 0.002M; cobalt and magnesium also activate.

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Separation and Estimation of Saturated C₂-C₈ Fatty Acids by Buffered Partition Columns*

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The lack of adequate small-scale methods for the analysis of mixtures of fatty acids has long been felt. Following up an observation of Lester Smith (1942), Elsdén (1946*a*) has recently introduced an important new technique, based on partition chromatography, for the separation and estimation of the lower members of the saturated series of fatty acids. A wet silica gel was impregnated with bromocresol green, a small sample of the mixture of acids, dissolved in chloroform, was introduced at the top of the column and, after its penetration into the gel had taken place, a suitable mixture of butanol and chloroform was allowed to percolate through the column. By using the colour change of the indicator as a pointer to the positions occupied by the acids, Elsdén was able to estimate propionic and butyric acids. Under certain conditions valeric acid also could be estimated, but the precise behaviour of the gel is conditional upon its properties. Acetic acid could not be estimated except by difference. The method has been used by Elsdén (1946*b*) and by

Elsden, Hitchcock, Marshall & Phillipson (1946) with excellent results in the analysis of rumen contents, but it has certain disadvantages. Different batches of silica prepared in the same manner often differ considerably in their properties, and of several batches only one may be suitable for use. The range of the method is very limited: acetic acid can only be estimated by difference and the method cannot ordinarily be applied to acids higher than butyric; such higher acids fail to undergo separation and can only be determined collectively. Finally, no separation is possible between the isomers of any given acid.

We have now devised a procedure which obviates most of the disadvantages of Elsdén's method and are able to identify, separate and estimate most of the naturally occurring, steam-volatile members of the saturated fatty acid series with a high order of accuracy and recovery. The use of indicators on the column is dispensed with, acetic acid can be determined directly, small differences in the properties of the silica are of no consequence, and almost complete resolution of certain isomeric valeric acids has been achieved.

* A preliminary account of this work was communicated by one of us (R. S.) to the Biochemical Society on 31 October 1947 (Scarisbrick, Baldwin & Moyle, 1948).