Biochemical Pharmacology, Vol. 16, pp. 1099–1111. Pergamon Press Ltd. 1967. Printed in Great Britain

THE EFFECTS OF ANTI-INFLAMMATORY DRUGS UPON THE CHEMISTRY AND ENZYMOLOGY OF RAT SKIN*

J. C. HOUCK, Y. M. PATEL and J. GLADNER

Biochemical Research Laboratory, Children's Hospital, Washington, D.C.,
Department of Biochemistry, Georgetown University Medical School, Washington, D.C.,
and National Institute of Arthritis and Metabolic Diseases, Bethesda, Md., U.S.A.

(*Received* 11 *August* 1966; *accepted* 18 *November* 1966)

Abstract Normal rats contain in the extracellular extrafibrillar compartment of their skin no proteolytic or "free" collagenolytic activities. Within 26 hr after administration of cortisol, prednisolone, indomethacin, or oxyphenylbutazone, this cutaneous compartment demonstrated both a proteolytic and a free collagenolytic activity. Salicylate administration to rats did not produce these results. Associated with the appearance of these two enzymatic activities in the extracellular compartment of the skin were supernormal amounts of RNA and normal or subnormal amounts of β-glucuronidase activity. For these and other reasons it was felt that neither the proteolytic activity (maximal at pH 7·5) nor the collagneolytic activity (maximal at pH 5·5) was derived from the breakdown of intracellular lysosomes. Finally, paralleling the appearance of cutaneous, extracellular, free, collagenolytic activity was the loss of as much as 25–30 per cent of the insoluble collagen from the skin of these drug-treated rats.

The extracellular, cutaneous proteolytic activity described above could digest hemoglobin and fibrin clots at pH 7·5 and convert fibrinogen to fibrin at the same pH. This enzyme activity could not attack the synthetic substrate appropriate to trypsin or thrombin but was active upon the substrate for chymotrypsin. Both the proteolytic and clotting activities were inhibited equally by *p*-chloromercurobenzoate, salicylate, and soybean trypsin inhibitor. Therefore this clotting activity was not due to thrombin but represents a hitherto unknown clotting activity found *only* in the skin of drug-treated rats.

The free collagenolytic activity found extracellularly in the skin of drug-treated rats had properties identical with those demonstrated by trypsin-activated extracts from normal rat skin. Apparently this collagenolytic activity is also released from cells by drug administration into the extracellular compartment of the skin where it effects the enzymatic digestion of cutaneous collagen until it complexes with an inhibitor pre-existing in this compartment.

RELATIVELY small doses of cortisol in young animals have been claimed to decrease significantly the concentration of insoluble collagen within the skin.[1, 2] This loss of cutaneous collagen with cortisol administration has also been demonstrated histochemically[3] and chemically.[4–7] Kuhn *et al.* however, were unable to demonstrate any loss of collagen from the skin of prednisolone-treated rats.[8] More recently Kivirriko *et al.*[9] found a decrease in the amount of urinary hydroxyproline excreted by rats receiving cortisone. This last finding also suggests that rat collagen is not being catabolized subsequent to the administration of corticosteroid.

* Supported in part by Grant AM-8168 from the National Institutes of Health and NR 105-325 from the Office of Naval Research.

We have found that the extracellular, extrafibrillar compartment of rat skin (0·15 M NaCl-extractable material) normally does not demonstrate collagenolytic activity. After pretreatment of this extract of normal rat skin with a small amount of trypsin, however, incubation of the extract with insoluble collagen could effect the release of dialyzable peptide-bound hydroxyproline at pH 5·5 in the presence of an excess of soybean trypsin inhibitors.[10] After the administration of two daily doses of cortisol (3 mg/kg), the extracellular, extrafibrillar compartment of rat skin demonstrated a similar collagenolytic activity without prior pretreatment of the extract with trypsin.[11] The appearance of free collagenolytic activity in this compartment of rat skin after steroid administration was associated with significant losses of chemically determined insoluble collagen from the abdominal skin of these animals and with the appearance of a cutaneous, neutral-pH-optimal proteolytic activity.[11]

This paper reports the results of our studies of the effects of the administration of five anti-inflammatory drugs upon both the chemistry and the enzyme activities found in the skin of rats.

MATERIALS AND METHODS

Six groups of 12 male Sprague–Dawley rats (190–205 g) each were employed. Each group of rats received one of the following drugs via initially a cutaneous and 24 hr later an i.p. route: (1) saline control; (2) 3mg cortisol/kg (Cortril, C, Pfizer); (3) 3mg prednisolone/kg; (4) 3mg oxyphenylbutazone/kg; (5) 3 mg indomethacin/kg and (6) 100 mg sodium salicylate/kg. Two hr after the second dose of drug i.p., these rats were sacrificed, shaven, and the entire skin removed. The indvidual skins of each rat were then minced with scissors and extracted sequentially in the cold in a Serval Omni-mixer (10,000 rpm) with 10 mg of 0·15 M NaCl/g for 45 min. After standing in the cold overnight, the supernatant was removed by centrifugation for 2 hr at 25,000 *g*. The residue was re-extracted with 10 ml of 0·5 M NaCl/g in a similar manner and the residue from this extraction was further re-extracted with 20 ml of 0·5 M citrate buffer (pH 3·6) per g. The final residue obtained after collecting the citrate supernatant by centrifugation was washed with dilute alkali and then autoclaved for 2 hr in 20 ml fresh citrate buffer/g. In this fashion the extracellular, extrafibrillar fraction, the salt-extractable collagen fraction, the acid-extractable collagen fraction and the insoluble collagen fraction may be quantitatively prepared.[12, 13] Aliquots of all four fractions representing one rat skin each were hydrolyzed in 4 N HCl for 9 hr at 100° in sealed tubes and analyzed in triplicate for hydroxyproline,[14] the signal imino acid of collagen. The results were calculated as the mean number of micromoles of hydroxyproline in each fraction per rat skin and tabulated as the mean per group of 12 animals. Since 1 mg collagen contains about 1 μmole hydroxyproline,[15] the final results were expressed as the mean concentration of collagen per rat representing 12 rats each per experimental group of animals. The standard deviation of these means was always less than 6 per cent of the mean itself, and means that differed by more than two standard deviations (more than 11 %)were found to be significantly different by the *t* test ($P < 0{\cdot}05$).

The isotonic saline extract of rat skin (S_1), which has been shown morphologically to represent the extracellular, extrafibrillar compartment of the skin,[12, 13, 16] was collected and dialyzed in the cold for 24 hr with stirring against 1000 vol. of

distilled water. After removing the globulin precipitate (including collagen) by centrifugation, the clear supernatant fraction was lyophilized. These preparations were analyzed for their contents of (a) β-glucuronidase.[17] the primary marker for lysosomal enzymes;[18] (b) ribonucleic acid;[19, 20] (c) neutral pH 7·5 optimal protease on denatured hemoglobin by a minor modification[11] of the procedure of Anson;[21] and (d) free collagenolytic activity of pH 5·5 by the ability of 5 mg S_1/ml to solubilize peptide-bound, dialyzable hydroxyproline from native, purified, and insoluble collagen.[10, 11]

Insoluble collagen was prepared from the skin of normal rats after the sequential extraction with salt and citrate buffer described above. The residue from the citrate extraction was washed free of acid with water and then air-dried at 20°. The insoluble. dried substrate was found to contain, after acid hydrolysis, about 96% collagen (by hydroxyproline analysis), slightly over 2% salt, and *less* than 2% noncollagenous protein (from nitrogen analysis).

Denatured hemoglobin, bovine fibrinogen and thrombin, tosylarginine methyl ester (TAMe) and acetyl tyrosine ethyl ester (ATEe), ε-amino acid (EACA), soybean trypsin inhibitor (SBTI), and *p*-chloromercurobenzoate (pCMB) were all obtained from Calbiochem. Highly purified trypsin, papain, and chymotrypsin were obtained from Worthington.

The hydrolysis of the synthetic substrates for trypsin (TAMe) and chymotrypsin (ATEe) was determined in a Radiometer pH stat at 20° and pH 7·0 in the usual manner. Fibrinolytic activity was determined on both heated and unheated fibrin plates according to the method of Astrup and Mullertz.[22]

RESULTS

The effects of the administration of 3 mg/kg, in two daily doses, of various anti-inflammatory drugs upon the total cutaneous content of saline and acid-extractable and insoluble collagens in the rat were determined as described above. The results are presented in Table 1 and indicate that, with the singular exception of salicylate, the

TABLE 1. THE CONCENTRATIONS (MG) OF THE VARIOUS SOLUBLE AND INSOLUBLE CUTANEOUS COLLAGENS PER WHOLE RAT 26 HOURS AFTER DRUG ADMINISTRATION

	Saline-extractable		Citrate	Insoluble
Drugs	0·15 M	0·50 M		
Control	51	150	150	1479
Cortisol	56	*204	*107	*1058
Prednisolone	55	*180	*76	*1013
Oxyphenylbutazone	*74	*223	*90	*1180
Indomethacin	48	*225	*58	*1118
Salicylate	46	146	141	1590

* Significantly different from the control ($P > 0·05$).

administration of both steroidal and nonsteroidal drugs to these animals resulted in a loss of insoluble collagen from their skin which represents about 30 per cent of the normal collagen content of the skin per rat. This loss occurred about 26 hr after the initiation of drug administration and was associated with an increase in the cutaneous

content of 0·5 M NaCl-extractable collagen and a marked reduction in the content of acid-extractable collagen. These latter results, with respect to the changes in the content of extractable collagens obtained per whole skin, differ from those found after cortisol administration, on the basis of the analysis of abdominal skin alone.[1,2]

The RNA content of the 0·15 M NaCl-extractable, nondialyzable components of the skin of both treated and control rats was determined.[19,20] The u.v. extinction coefficient of the RNA was shifted in a manner typical of true RNA by the addition of highly purified RNAase.[20] These results were compared with the neutral pH optimal proteolytic and acid pH optimal collagenolytic activities of the extracellular, extrafibrillar compartment of these skins as well as with the β-glucuronidase activities of the same extracts. This latter enzyme is a measure of the lysosomally derived materials[21] contained in the extracellular, extrafibrillar compartment of rat skin.[12,13,16] The comparison of these results is presented in Table 2 and indicates that, again with the exception of salicylate, the administration of both steroidal and nonsteroidal anti-inflammatory drugs to rats resulted in the coincident appearance in the skin of (1) supernormal amounts of RNA, (2) and proteolytic and (3) collagenolytic activities; all without any significant increase in the β-glucuronidase activity in this cutaneous compartment. In fact, with cortisol there was a significant reduction below normal in the amount of β-glucuronidase activity in this extract.

TABLE 2. THE ENZYME ACTIVITIES OF THE NONDIALYZABLE, LYOPHILIZED ISOTONIC SALINE EXTRACTS OF THE SKIN OF RATS 26 HOURS AFTER DRUG ADMINISTRATION

Drugs	RNA (μg/10 mg)	Enzyme activity		
		Protease†	Collagenase‡	β-glucuronidase§
Control	5·8	0·	0	0·008
Cortisol	*8·9	*0·20	*5·8	0·006
Prednisolone	*8.8	*0·18	*5·1	0·007
Oxyphenylbutazone	*7·1	*0·15	*4·6	0·008
Indomethacin	*7·4	*0·11	*4·3	0·008
Salicylate	6·0	0	0	0·008

* Significantly different from the control ($P > 0·05$).
† Absorbency of TGA-soluble peptides released fron denatured hemoglobin by 5mg extract/ml.
‡ Per cent insoluble collagen substrate solubilized by 5mg extract/ml.
§ Micromoles substrate hydrolyzed/min/mg.

From the data of Table 2 it is apparent that two enzymatic entities, one proteolytic at pH 7·5 and the other collagenolytic at pH 5·5, appeared in the skin of rats treated with four of the five anti-inflammatory drugs used above. The general properties of these two enzyme activities were determined separately.

Proteolytic activity

The lyophilized, nondialyzable 0·15 M NaC. extract from the skins of cortisol-and indomethacin-treated rats was prepared as described above. The effects were determined of time, extract concentration, and pretreatment for 15 min at various temperatures upon the amount of TCA soluble peptides released from denatured hemoglobin after 16 hr at 36° and pH 7·5 by the cortisol-released protease preparation (CRPP).

The absorbency of the peptides released over that of the zero-time control for each reaction condition mentioned above is presented in Table 3.

These data suggest, first, that the rate of proteolysis is directly related to the time of incubation; second, that the amount of proteolysis is limited and reaches a maximum when 5mg CRPP extract/ml was used; and third, that the activity is significantly thermolabile above 45°.

TABLE 3. THE EFFECTS OF INCUBATION TIME, PREPARATION CONCENTRATION, AND PRETREATMENT AT VARIOUS TEMPERATURES FOR 15 MINUTES UPON THE ABSORBENCY (ΔO.D.) OF LOWRY-REACTING TRICHLOROACETIC ACID-SOLUBLE PEPTIDES RELEASED FROM DENATURED HEMOGLOBIN AFTER 16 HOURS INCUBATION AT 36° WITH CRPP

Effects of time		Extract concentration		Inactivation temp.	
(hr)	(ΔO.D.)	(mg/ml)	(ΔO.D.)	(°C)	(%inhibition)
1	0·025	1	0·030	40	0–5
2	0·050	2	0·065	45	17
4	0·060	4	0·120	50	42
6	0·080	5	0·200	55	68
8	0·110	6	0·200	60	84
16	0·200	10	0·210	65	97

After 16-hr incubation of CRPP with denatured hemoglobin, fresh CRPP was added to the reaction mixture, and this mixture was divided into two parts; one served as a zero-time control, the other was allowed to incubate for another 4-hr. An equivalent sample of fresh CRPP was also incubated alone without hemoglobin substrate for 4-hr. A small amount of TCA-soluble peptide was released by autodigestion of the CRPP alone, and when the mixture of hemoglobin, old CRPP, and fresh CRPP was corrected for autodigestion, it was found that addition of fresh CRPP was *without* further proteolytic effect upon predigested, denatured hemoglobin.

The effects of reaction pH upon the absorbency of the TCA-soluble peptides released from denatured hemoglobin by either CRPP or indomethacin-released protease preparation (IRPP), was determined by using 0·05 M phosphate buffers ranging in pH from 5·5 to 8·5. These results are presented in Fig. 1 and demonstrate that the pH optimum was 7·5 for the proteolytic activity found in rat skin extracts after administration of either cortisol or indomethacin. Similar extracts of normal skin from untreated rats demonstrated no significant amounts of proteolytic activity at any pH between 5·5 and 8·5 in phosphate buffer.

The nature of the substrate specificity of this proteolytic activity was explored first upon native and second upon synthetic substrates, as shown in Table 4. The extracts from the skins of normal, cortisol-, and indomethacin-treated rats were incubated at a concentration of 5 mg/ml in phosphate buffer (pH 7·5) with: (1) denatured hemoglobin; (2) purified bovine fibrinogen; (3) clotted fibrin plates; and (4) clotted fibrin plates which had been pretreated to destroy any contaminating thrombin or prothrombin. After 16 hr at 36°, the normal extract was without any effect upon these various protein substrates, while the extracts from the skins of rats which had received either drug-digested hemoglobin, clotted the fibrinogen and lysed both normal and preheated

fibrin plates. None of these extracts had any effect upon the synthetic substrate appropriate to trypsin or thrombin (tosylarginine methyl ester), while extracts of the skin from rats which had received either drug were capable of hydrolyzing 3–6% of the synthetic substrate appropriate to chymotrypsin (acetyl tyrosine methyl ester). Thus the extracellular compartment of the skin of rats receiving either a steroidal or a nonsteroidal anti-inflammatory drug contained a neutral pH-optimal protease which

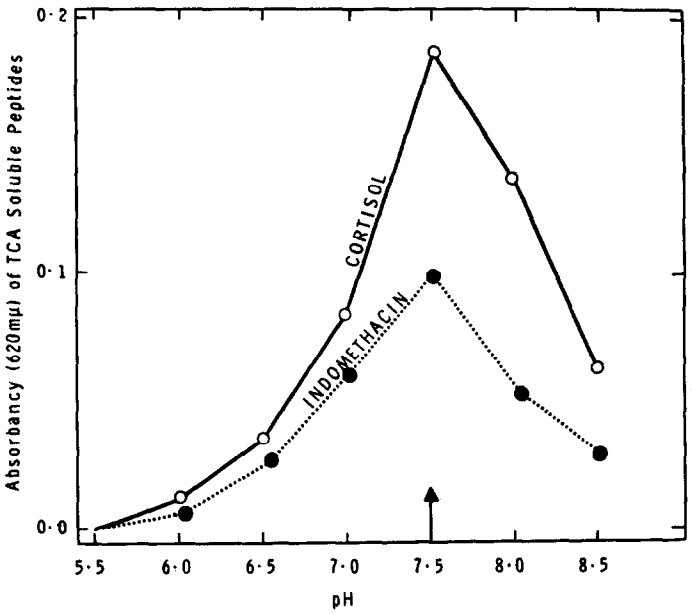

FIG. 1. The effect of pH upon the proteolytic activity of the 0·15 M NaCl extracts of rat skin from animals which had received cortisol (○) or indomethacin (●).

TABLE 4. THE SUBSTRATE FOR THE PROTEOLYTIC ACTIVITY FOUND IN THE NONDIALYZABLE, LYOPHILIZED ISOTONIC SALINE EXTRACTS OF THE SKIN OF RATS 26 HOURS AFTER DRUG ADMINISTRATION

Substrate	Extracts of rats treated with		
	Normal	Cortisol	Indomethacin
Hemoglobin*	0	0·20 (O.D.)	0·10 (O.D.)
Fibrinogen†	0	+	+
Fibrin‡			
Normal clot plate	0	205 sq. mm	110 sq mm
Heated clot plate	0	145 sq mm	75 sq mm
TAMe§	0	0	0
ATEe¶	0	6%	3%

* Absorbency of TCA-soluble peptides released fron denatured hemoglobin by 5mg extract/ml.
† Clotting of purified bovine fibrinogen within 120 min by 5mg extract/ml.
‡ Clot lysis in Astrup fibrin plate units (sq mm) per mg extract.
§ Per cent tosylarginine methyl ester hydrolyzed in 1 hr per mg extract.
¶ Per cent acetyl tyrosine ethyl ester hydrolyzed in 1 hr per mg extract.

could both clot fibrinogen (albeit very slowly) and lyse preformed clots. This proteolytic and clotting activity was associated only with activity upon the synthetic substrate for chymotrypsin and not with activity upon the trypsin or thrombin substrate.

Further experiments revealed that the ability of CRPP to lyse fibrin clots was not associated with the release of any trichloroacetic acid-soluble peptides from the fibrin. In addition, very little of the ability of CRPP to clot purified fibrinogen solutions could could be demonstrated at either pH 8 and above or at pH 7·0 and below. Finally, clotting was not produced by CRPP at the pH optimum for thrombin—pH 6·8.

The effects of various drugs such as *p*-chloromercurobenzoate (pCMB), Dilantin (5,5-biphenylhydantoin), ϵ-aminocaproic acid (EACA), or soybean trypsin inhibitor (SBTI) upon both the proteolytic and clotting activities of both CRPP and IRPP were compared. Clotting was determined on a "yes or no" basis after 3-hr incubation. Subsequent to this judgment for each enzyme–inhibitor–fibrinogen mixture, 20 μg of purified bovine thrombin was added to each tube. and the clotting of the solution determined after further incubation for 20 min. In this fashion the effects of the various. drugs upon thrombin activity in this system could be determined.

The results of this study, as shown in Table 5, indicated that the proteolytic and clotting activities of both CRPP and IRPP were similarly effected by all five agents employed. Further, when thrombin was added to the mixture of fibrinogen and inhibitor, the thrombin clotted the mixture and was obviously not inhibited by these

TABLE 5. THE INHIBITION OF THE PROTEOLYTIC ACTIVITY RELEASED INTO THE 0·15 M NaCl EXTRACT OF INTACT RAT SKIN FROM ANIMALS TREATED WITH EITHER CORTISOL OR INDOMETHACIN ASSAYED EITHER ON DENATURED HEMOGLOBIN (INCREASES IN ABSORBENCY OVER ZERO-TIME CONTROLS) OR FOR ABILITY TO CLOT FIBRINOGEN COMPARED WITH THROMBIN

Inhibitor	Cortisol protease		Indomethacin protease		Thrombin
	Hgb (ΔO.D.)	Fbg	Hgb (ΔO.D.)	Fgb	Fbg
None	0·20	clots	0·10	clots	clots
pCMB	0·00	no clots	0·00	no clots	clots
Dilantin	0·07	no clots	0·03	no clots	clots
Salicylate	0·01	no clots	0·02	no clots	fine clots
EACA	0·02	no clots	0·02	no clots	clots
SBTI	0·00	no clots	0·00	no clots	clots
(Normal skin) (Extract only)	0·00	no clots	0·00	no clots	clots

Hbb = Denatured hemoglobin; Fbg = fibrinogen.
pCMB = *p*-Chloromercurobenzoate (10^{-4} M); Dilantin = 5,5-biphenylhydantoin (1 mg/ml).
EACA = ϵ-Aminocaproic acid (1 mg/ml).
SBTI = Soybean trypsin inhibitor (1 mg/ml).

drugs. Finally, the extract of the normal skin from untreated rats could not effect the clotting of fibrinogen solutions.

Similar 0·15 M NaCl extracts were also made of the skeletal and the cardiac muscle of both normal and anti-inflammatory drug-treated animals. None of these extracts of tissues other than skin demonstrated any proteolytic or clotting activities.

Collagenolytic activity

Highly purified insoluble collagen was prepared from the skin of rats as described above. This substrate at a concentration of 10 mg ml was suspended in pH 7·0 buffer (0·1 M acetate) and incubated for 16 hr at 32° with 0·1 mg of trypsin, activated papain, or chymotrypsin per ml. The supernatant was collected by centrifugation at 2° and, after acid hydrolysis of an aliquot, was analyzed for hydroxyproline.[14] The remaining solution was dialyzed for 72 hr against 2 vol. of buffer in the cold with stirring. The hydroxyproline content of the fluid outside the dialysis bag was determined after being concentrated *in vacuo* to 20 per cent of its initial volume. Both papain and chymotrypsin were unable to solubilize any peptide-bound hydroxyproline from this substrate. Trypsin effected the solubilization of almost 1 per cent of the substrate hydroxyproline but none of this was dialyzable. When this insoluble collagen substrate was incubated with the extract of normal skin from untreated rats (5 mg/ml) at pH 5·5 for 16 hr at 32°, no solubilization of hydroxyproline took place. This extract of normal skin was also pretreated with crystalline trypsin, 5 μg/ml and 100 μg/ml, for 10 min at pH 7·0; subsequent to this period 1 mg of soybean trypsin inhibitor/ml was added to the solution and the pH readjusted to 5·5. These solutions had *no* proteolytic activity at either pH 7·0 or 5·5 upon denatured hemoglobin. The solution pretreated with 5 μg trypsin/ml, when incubated with insoluble collagen at pH 5·5 for 16 hr and 32°, effected the solubilization of about 10 per cent of the substrate, 60 per cent of which was dialyzable. But pretreatment of the extract with 100 μg trypsin/ml seemed to destroy completely this collagenolytic activity. Similar studies with activated papain, another enzyme like trypsin which attacks basic amino acid substrates, gave similar results except that soybean trypsin inhibitor did not inhibit the proteolytic activity of papain upon denatured hemoglobin. Various concentrations of chymotrypsin (1–1000 μg/ml) were found to be without effect in terms of "activating" the inert collagenolytic activity of normal rat skin. These results are presented in Table 6.

TABLE 6. THE PERCENTAGE OF INSOLUBLE COLLAGEN RENDERED SOLUBLE AND DIALYZABLE BY VARIOUS PROTEOLYTIC ENZYMES ALONE AND VARIOUSLY PRETREATED CUTANEOUS EXTRACTS AFTER INCUBATION FOR 16 HOURS AT 32° AND PH 5·5

	% solubilized	% dialyzable
Proteolytic enzymes alone at pH 7·0		
Trypsin, 0·1 mg/ml	1	0
Papain, 0·1 mg/ml	0	0
Chymotrypsin, 0·1 mg/ml	0	0
Cutaneous extracts at pH 5·5		
No pretreatment	0	0
Pretreatment with trypsin, 5μg/ml (pH 7.0)	10	60
Pretreatment with trypsin, 100 μg/ml (pH 7·0)	0	0
Pretreatment with papain, 5 μg/ml (pH 7·0)	8	50
Pretreatment with papain, 100 μg/ml (pH 7·0)	0	0
Pretreatment with chymotrypsin, 1, 5, 10, 50, 100 and 1000 μg/ml (pH 7·0)	0	0

Seventy-two male rats weighing 200–220 g were divided into six groups of twelve animals each and injected as described above with two doses of carrier solvent alone (control), and cortisol, prednisolone, indomethacin, oxyphenylbutazone, or salicylate (3mg/kg).

The nondialyzable, lyophilized 0·15 M NaCl extracts of the skins of these animals were prepared and their free collagenolytic activity at pH 5·5 determined. The total collagenolytic activity of these extracts was also determined by pretreatment with trypsin at pH 5·5, as described previously.[10] Finally, the arithmetic difference between the total and free collagenolytic activities of these extracts was calculated as bound activity, and the results are presented in Table 7. The data of this table indicated that

TABLE 7. THE EFFECTS OF VARIOUS DRUGS UPON THE VARIOUS FORMS OF COLLAGENOLYTIC ACTIVITY OF THE NONDIALYZABLE, LYOPHILIZED 0·15 M NaCl EXTRACT OF RAT SKIN

Drugs	Forms of collagenolytic activity†		
	Free	Total	Bound
Control	0	10	10·0
Cortisol	*5·5	*16	10·5
Prednisolone	*4·5	*14	9·5
Oxyphenylbutazone	*5·0	*15	10·0
Indomethacin	*4·0	*14	10·0
Salicylate	0	10	10

* Significantly different from normal ($P < 0·05$).

† Per cent insoluble collagen substrate rendered soluble after 16-hr incubation at pH 5·5 (acetate buffer) and 36°.

regardless of the amount of free collagenolytic activity found in these cutaneous extracts, the amount of "bound" or "inert" activity was always, regardless of drug administration, the same as that demonstrated for the control rat skins.

The effects of reaction pH from 4·0 to 7·0 upon the collagenolytic activities of trypsin-activated rat skin extracts were compared with the effects of pH upon the free collagenolytic activity of the nontrypsin-treated cutaneous extract of rats which had received cortisol. These results are presented in Fig. 2 and indicate that both the free collagenolytic activity of the cutaneous extracts of cortisol-treated rats and the trypsin-activated collagenolytic activity of the cutaneous extracts of normal rat skin shared the same reasonablly sharp pH optimum at pH 5·5.

The inhibitory effects of various agents were explored upon both the free collagenolytic activity of the cutaneous extracts of cortisol-treated rats and the trypsin-activated collagenolytic activity of the cutaneous extracts of normal rats. Essentially similar results were obtained, as presented in Table 8, for both kinds of cutaneous collagenolytic activity with respect to the inhibition of these activities. Further, it is of interest that the 0·15 M NaCl extracts of normal rat skin could inhibit free collagenolytic activity.

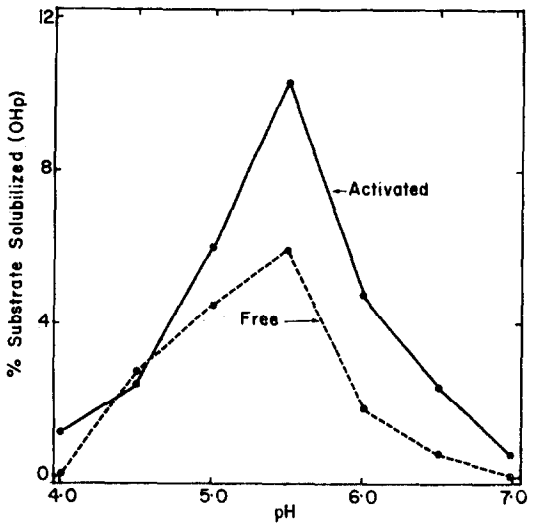

FIG. 2. The effect of pH upon the collagenolytic activity of 0·15 M NaCl extracts of rat skin from animals which had received cortisol and which had not been activated by preincubation with trypsin (free) or from animals receiving no steroid which had been activated by preincubation with trypsin (activated).

TABLE 8. THE PER CENT INHIBITION OF THE COLLAGENOLYTIC ACTIVITY OF THE NONDIALYZABLE, LYOPHILIZED ISOTONIC SALINE EXTRACTS OF THE SKIN OF NORMAL OR CORTISOL-TREATED RATS PRODUCED BY VARIOUS MATERIALS

Inhibitors	Free collagenase (cortisol)	Activated collagenase (normal)
pCMB	25	30
Salicylate	0	0
Dilantin	60	50
EACA	40	50
EDTA	50	60
SBTI	0	0
Phosphate buffer (0·05 M)	100	100
2·5 mg of normal extract	80	60

pCMB = *p*-Chloromercurobenzoate (10^{-4} M); Dilantin = 5,5-biphenylhydantoin (1 mg/ml). EACA = ε-Aminocaproic acid (1 mg/ml); EDTA = ethylenediamine tetraacetate (1 mg/ml). SBTI = Soybean trypsin inhibitor (1 mg/ml).

DISCUSSION

From the preceeding data it is obvious that after the administration of two doses of various steroidal and nonsteroidal anti-inflammatory drugs, the total and insoluble collagen content of rat skin was significantly reduced by about 500 mg per rat. This loss of cutaneous collagen with drug administration was quantitatively about ten times greater than that appropriate to the normal catabolism of rat collagen during

the same time period.[23] Thus, while corticosteroids may inhibit collagen synthesis,[9] antianabolism alone is not enough to explain a loss of skin collagen of this magnitude. Therefore, in view of the very small turnover and long biological half-life of rat skin collagen,[23] large losses of cutaneous collagen (25–30%) within 26 hr after drug administration suggest strongly that these drugs (with the exception of salicylate) stimulate some system in the skin leading to collagen catabolism.

The appearance in the extracellular, extrafibrillar compartment of rat skin of a collagenolytic activity in those animals which had received four of the five drugs studied above was correlated with this loss of cutaneous collagen. The appearance of collagenolytic activity was also associated with the appearance of a proteolytic activity and supernormal amounts of RNA extracellularly. No changes in the trivial amounts of β-glucuronidase activity found in this cutaneous extracellular compartment were observed with the administration of any of these anti-inflammatory drugs. This latter finding suggests strongly that the extracellular appearance of the proteolytic and collagenolytic activities was not derived from the rupture of intracellular lysosomes.[24] In fact, steroids have been shown to stabilize lysosomes rather than bring about their rupture.[24] It would appear rather that the enzymes were derived from the cytoplasm of some type or types of skin cells which had been released extracellularly along with increased amounts of cytoplasmic RNA in response to drug administration.

The extracellular proteolytic activity was found to be time-dependent and thermolabile and to demonstrate a fairly sharp otptimum at pH 7·5. It would also appear that this proteolytic activity was attacking only a few bonds in hemoglobin, since further increases in extract concentration were without further effect in digesting this substrate. While normal skin extracts were found to be totally without either collagenolytic or proteolytic activity of any kind, the extracts of the skin of rats which had received either steroidal or nonsteroidal anti-inflammatory drugs could solubilize both hemoglobin and fibrin. Further, this proteolytic activity upon fibrin was not associated with the release of TCA-soluble peptides from the substrate and was decreased by only 25 per cent when the fibrin plates were preheated to destroy plasminogen.[22] Thus this fibrin digestion by incubation with CRPP may be due to a direct lytic activity upon the clot which releases macromolecular products.

None of the proteolytic activity of skin extracts from drug-treated rats was associated with any digestion of the synthetic substrate for trypsin or thrombin (TAMe). Rather, these extracts were found to be quite active upon the synthetic substrate appropriate to chymotrypsin (ATEe). Remarkably enough, despite the inability of CRPP or IRPP to hydrolyze the synthetic substrate for thrombin, these extracts were capable of slowly clotting solutions of purified fibrinogen.

Further evidence for (1) the essential identity of the proteolytic and clotting activities of these cutaneous preparations, (2) for the non-identity of the proteolytic and collagenolytic activities of these cutaneous preparations, and (3) for their non-identity with thrombin was produced by a study of the effects of various inhibitors upon these activities. The results, presented in Table 5, indicate that the proteolytic and clotting activities found extracellularly in the skin of rats receiving either steroidal or nonsteroidal anti-inflammatory drugs were equally inhibited by pCMB, Dilantin, salicylate, EACA, or SBTI, whereas thrombin was still able to clot fibrinogen in the presence of these agents. Further, after CRPP or IRPP clotting of fibrinogen was accomplished, the fibrinogen remaining in excess in solution could still be clotted by

thrombin. Therefore, the proteolytic activities of the extracts of the skins of drug-treated rats were limited in terms of bond specifically and did not digest in a nonspecific manner the excess fibrinogen. In view of the remarkable enzymatic specificity of the conversion of fibrinogen to fibrin, this finding of a clotting enzyme in skin, unrelated to thrombin, appears unique, since thrombin is the only mammalian clotting enzyme heretofore known.

Finally, even in drug-treated rats, no similar proteolytic activity could be demonstrated in tissues other than skin—even those which were rich in blood supply, such as skeletal and cardiac muscle.

The substrate used in the study of collagenolysis was totally insoluble dermal collagen which by hydroxyproline:nitrogen ratio was shown to be essentially pure. More importantly, trypsin, papain, and chymotrypsin were essentially without effect upon the substrate, thus suggesting that the insoluble collagen did not contain significant amounts of either noncollagenous protein or gelatin. The fact that most of the solubilized peptide-bound hydroxyproline released from this substrate by trypsin-pretreated cutaneous extracts (or by CRPP) was dialyzable, is strong evidence that the products released from the substrate were of a small molecular weight.

As seen from the data of Table 6, small amounts of either trypsin or papain could activate a collagenolytic activity in the cutaneous extracts of normal rats. Larger amounts of the enzymes which attack only TAMe, when preincubated with the cutaneous extract of normal rats, did not release or activate this collagenolytic activity, presumably because these concentrations of enzyme hydrolyzed the collagenolytic activity itself. Interestingly enough, no concentration of chymotrypsin could be found that could activate the collagenolytic activity of these normal cutaneous extracts. Further experiments have indicated that no conditions could be found with respect to concentration, pH, and incubation time in which the chymotrypsin-like protease containing cutaneous extracts of cortisol-treated rats could effect the release or activation of the collagenolytic activity of normal rat skin.

Therefore, it is difficult to support the previous proposal[11] that the protease found extracellularly in the skin of cortisol-treated rats was responsible for the limited proteolytic activation of the extracellular collagenolytic activity found in the skin of these animals. In fact, a careful study of the collagenolytic activity found in CRPP and IRPP indicated that the concentration of bound or inert collagenolytic activity remained constant despite the amount of free collagenolytic activity that could be determined in these preparations. Since the pH optimum for both the free and the bound collagenolytic activities was the same (pH 5·5), and the reactions of these two forms of collagenolytic activity to the presence of various inhibitors were the same, the possibility appears quite reasonable that the drug-induced or released free collagenolytic activity found extracellularly in the skin is a result of the release of active collagenolytic activity from the cell into the extracellular compartment of the tissue. Finally, the ability of similar extracts of normal rat skin to inhibit this free collagenolytic activity suggests that there exists in the extracellular compartment of rat skin an inhibitor of the colla genolytic activity described above.

Finally, the drug-released protease might be related to the dermoprotease described by Ungar and Damgaard.[25] These workers found that if they heated the skin to 60° prior to extraction with 0·15 M phosphate buffer, they obtained a neutral pH optimal protease which was inhibited by salicylate and soybean trypsin inhibitor. The

relationships between this protease[25] and that described by Wells and Babcock[26] to that described in extracts of burned skin by Beloff and Peters[27] remains unclear at present.

REFERENCES

1. P. Sethi, E. R. Ramey and J. C. Houck, *Proc. Soc. exp. Biol. Med.* **108**, 74 (1961).
2. J. C. Houck, *Am. J. Path.* **41**, 365 (1962).
3. R. Mancini, S. Stringa and L. Canepa, *J. invest. Derm.* **34**, 393 (1960).
4. R. Sakata, *Kumamoto, med. J.* **13**, 41 (1960).
5. N. C. Cadavid, B. Leiderman and R. Mancini, *Acta physiol. latinoam.* **14**, 366 (1964).
6. I. Ito, *Kumamoto med. J.* **17**, 79 (1964).
7. M. Natarajan and S. Bose, *Leather Sci.* **12**, 111 (1965).
8. K. Kuhn, P. Iwangoff, F. Hammerstein, K. Stecher, M. Durruti, H. Holzman and G. W. Korting, *Hoppe-Seyler's Z. physiol. Chem.* **337**, 249 (1964).
9. K. Kivirriko, O. Laitiner, J. Aer and J. Halme, *Biochem. Pharmac.* **14**, 1445 (1965).
10. E. R. Goldstein, Y. M. Patel and J. C. Houck, *Science* **146** 942 (1964).
11. J. C. Houck and Y. M. Patel, *Nature, Lond.* **206**, 158 (1965).
12. J. C. Houck and R. A. Jacob, *Proc. Soc. exp. Biol. Med.* **105**, 324 (1960).
13. J. C. Houck, *Ann. N.Y. Acad. Sci.* **105**, 765 (1963).
14. C. J. Martin and A. E. Axelrod, *Proc. Soc. exp. Biol. Med.* **83**, 46 (1963).
15. J. C. Houck and R. A. Jacob, *J. invest. Derm.* **36**, 451 (1961).
16. P. Sweeny, R. H. Pearce and H. Vance, *Can. J. Biochem. Physiol.* **41**, 2307 (1963).
17. W. Fishman, B. Springer and R. Brunetti, *J. biol. Chem.* **173**, 449 (1948).
18. C. DeDuve, in *Subcellular Particles*, Ed. T. Hayashi, p. 128, Ronald Press, New York (1959).
19. G. Schmit and S. J. Thannhauser, *J. biol. Chem.* **161**, 83 (1954).
20. A. H. Brown, *Archs Biochem. Biophys* **11**, 269 (1964).
21. M. L. Anson, *J. gen. Physiol.* **22**, 79 (1938).
22. T. Astrup and S. Mullertz, *Archs Biochem. Biophys* **140**, 346 (1952).
23. S. Lindstedt and D. J. Prockop, *J. biol. Chem.* **236**, 1399 (1961).
24. G. Weissman, *New Engl. J. Med.* **273**, 1084 (1965).
25. G. Ungar and N. Damgaard, *Proc. Soc. exp. Biol. Med.* **87**, 388 (1954).
26. G. Wells and C. Babcock, *J. invest. Derm.* **21**, 459 (1953).
27. A. Beloff and R. Peters, *J. Physiol. Lond.* **103**, 461 (1945).