3. Vogt, M., *J. Physiol.*, 1954, v123, 451.
4. Shore, P. A., Olen, J. S., *J. Pharm. Exp. Therap.*, 1958, v122, 295.
5. Von Euler, U. S., Floding, I., *Acta Physiol. Scand.*, 1955, Suppl. 33, v118, 45.
6. Bogdanski, D. F., Pletscher, A., Brodie, B. B., Udenfriend, S., *J. Pharm. Exp. Therap.*, 1956, v117, 82.
7. Schayer, R. W., *J. Biol. Chem.*, 1951, v189, 301.
8. Grout, J. R., Creveling, C. R., Calon, D., *Fed. Proc.*, 1960, v19, 297.
9. Brodie, B. B., Shore, P. A., *Ann. N. Y. Acad. Sci.*, 1957, v66, 631.

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Biological Assay of Proteolytic Enzymes Capable of Debriding Third Degree Burn Eschars. (26541)

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In attempting to identify the proteolytic enzyme or enzymes in *Clostridium histolyticum* culture filtrates which were responsible for the ability of these preparations to debride third degree burn eschars, it was found that adequate methods of bioassay were lacking. Although in recent years a variety of enzymes have been proposed as agents for removal of burn slough, methods for comparing the biological potency of these enzymes have not been available. Mention has been made of the use of laboratory animals such as the guinea pig(1,2), dog(3,4) or rabbit (5,6) but no attempt has been made to develop a quantitative procedure.

The present paper describes a method for biological assay of proteolytic enzymes capable of debriding third degree burn eschars. A suitable bandaging technic for maintaining the enzyme solution in contact with the burned surface was developed and a scale for assessing the effectiveness of debridement was chosen. In addition, 2 examples of the use of this assay are given; 1) evaluation of debriding activities of various enzyme preparations, and 2) determination of the effect on debriding activity of pre-treatment of the burn eschar with pHisoHex and with petrolatum.

Materials and methods. Four proteolytic enzymes were used. Two of the enzyme preparations, designated as 0-22% enzymes and 30-35% enzymes, were obtained from *C. histolyticum*, H-4, filtrates by a modification of the procedure developed by deBellis *et al.* (7). The mold proteinase from culture X-108 (Lot No. 7-1162D-12) was kindly supplied by Frank B. Ablondi, Biochemical Research Section, Research Division, American Cyanamid Co. Purified crystalline Trypsin (approximately 50% $MgSO_4$, Lot TP11) was purchased from Worthington Biochemical Laboratory. All enzyme preparations were dissolved in 0.1 M tris (hydroxymethyl) aminomethane buffer, pH 7.5, containing 0.01 M $CaCl_2$ and 0.5% proteose peptone (Difco), except the mold enzyme which was dissolved in 5% proteose peptone, pH 7.5. In addition, 1000 units of potassium penicillin G and 500 μg of streptomycin were added for each ml of test solution to inhibit bacterial contamination.

Hartley strain guinea pigs (350-800 g) were anesthetized with sodium pentobarbital and the entire dorsal area was clipped as closely as possible. Four full thickness third degree burns with a diameter of approxi-

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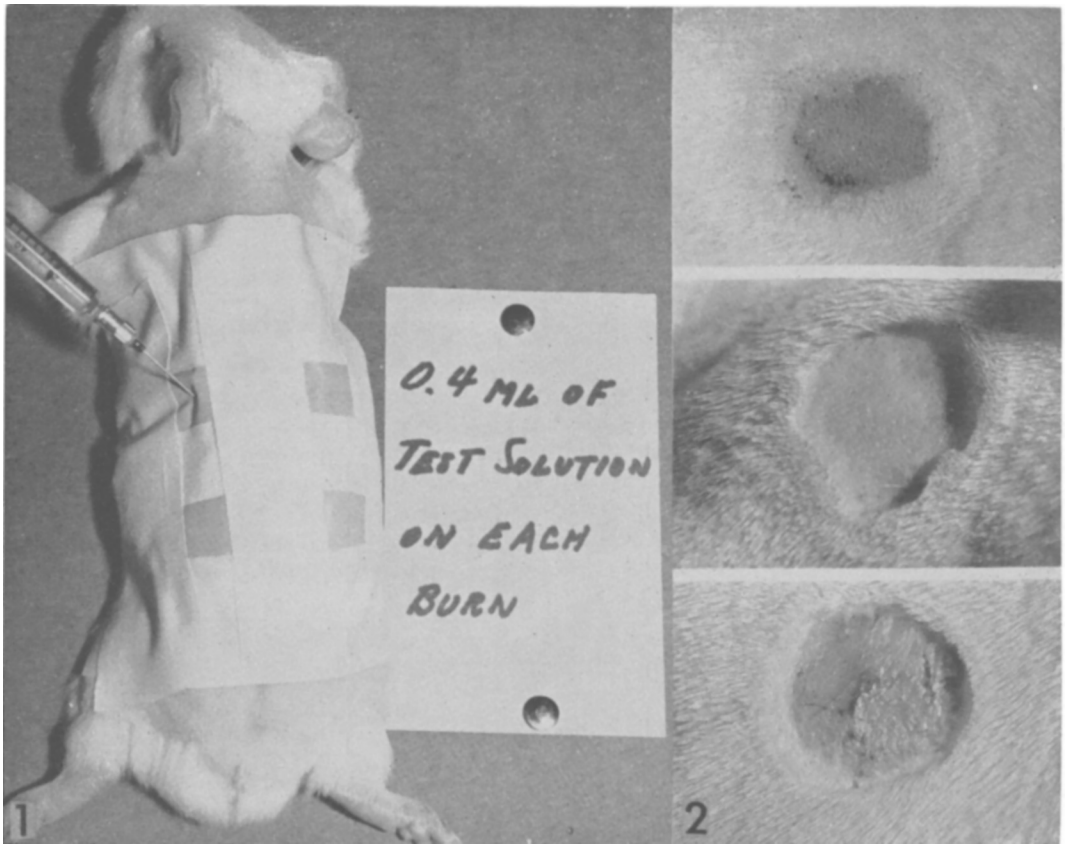

FIG. 1. Typical method of bandaging of guinea pigs for assay of ability of proteolytic enzymes to debride third degree burn eschars.

FIG. 2. Surface of burn eschar after application of test solutions. Top: Buffer, 0% debridement. Middle: 16 azocasein units 0-22% enzymes, 37.5% debridement. Bottom: 400 azocasein units 0-22% enzymes, 100% debridement.

mately 1 cm were produced on the shaved area with a Stanley No. 435 80 watt soldering iron, the tip of which had been filed down to give an oval surface approximately 10 × 6 mm. The hot iron (360-400°C) was applied for 15 seconds with sufficient pressure to insure good contact without making a depression in the skin of the guinea pig. Charred tissue was removed from the tip of the iron between each burn. Unventilated 1½″ × 1½″ plastic patches, kindly supplied by Johnson & Johnson Co., were carefully laid over each burn so that the small gauze pad would be directly over and in contact with the burn. The edges of the patches were sealed and reinforced with strips of adhesive tape. With a hypodermic syringe and needle, 0.4 ml of test solution was inoculated into each of the 4 plastic patches on a guinea pig, as shown in Fig. 1. The opening made by the needle was sealed with a small piece of plastic tape or with a drop of Duco cement.

After 18 hours the guinea pigs were reanesthetized and the bandages removed. All burns were examined to check whether the sites were moist and the assay results valid; occasionally a corner of the bandage became loosened permitting the site to dry, or the patch became elevated over the burn giving poor contact and consequently poor debridement. The digested tissue was removed by wiping the treated area with cotton balls held in forceps, rubbing with moderate pressure in all directions. The enzymes do not remove the burn tissue, but rather soften the eschar so that it can be removed by wiping. If the surface of the burn becomes dry after digestion by the enzymes, the slough hardens and

TABLE I. Influence of Weight of Guinea Pigs and Concentration of Enzymes on Debridement.

Conc. of 0-22% proteinases	Wt of guinea pigs in g		
	793 ± 26	589 ± 46	388 ± 33
Azocasein units	Avg % debridement		
160	42	55	69
80	41	45	63
40	28	31	56
Buffer	0	0	0

Weight investigated by joining of 3 Latin squares, each square representing 4 guinea pigs (columns) of same weight with 4 sites (rows) per guinea pig(8). Three dilutions of enzyme were tested together with a buffer control. Two males and 2 females were used to form each Latin square in lower weight groups. Experiment was replicated on the following day.

can no longer be removed. Therefore, it is necessary that the burn eschar be kept moist with the enzyme solution. When high concentrations of enzyme were applied to the burn sites in an attempt to obtain 100% debridement, leakage to adjacent control sites occurred; this could be eliminated by sealing the edges of the plastic patches with Duco cement. Comparison of values obtained which were below 75% suggested that the results with either method of sealing the bandage were similar, indicating that leakage had no appreciable effect when lower enzyme concentrations were used. It was also found that debridement greater than 75% was not necessarily proportional to enzyme concentration. Therefore, the rapid technic with adhesive tape alone was utilized in these studies. Biological activity was recorded on a scale ranging from 0 to 8 in the following manner: 0 (0%), no effect after rubbing 10-20 times; 1 (12.5%), slight break in eschar; 2 (25%), surface removal of eschar; 3 (37.5%), partial removal of eschar to corium; 4 (50%), complete removal of eschar to corium; 5 (62.5%), partial removal of eschar to panniculus carnosus; 6 (75%), complete removal of eschar to panniculus carnosus except at edges of burn; 7 (87.5%), partial removal of denatured tissue at edges of burn; 8 (100%), complete removal of all devitalized tissue (Fig. 2).

Results. It was found (Table I) that differences in weight of the test guinea pigs greatly influenced the amount of enzyme required to accomplish debridement; 40 units on guinea pigs weighing 388 g gave more complete debridement than 160 units on guinea pigs weighing 793 g. Presumably older animals with thicker skins furnished an increased amount of substrate for the enzyme to digest. Two-fold enzyme concentrations did not always give significantly different results but 4-fold enzyme concentrations could readily be distinguished.

Degree of debridement was not influenced by sex of the animal, but was significantly affected by the location of skin area tested, greater debridement occurring at the 2 top sites than at the 2 bottom sites (Table II). Whether these differences are due to variation in skin thickness at these locations or to a greater leak rate occurring from the bandage at the lower sites is not known. Nonetheless, for accurate determination of enzymatic activities the effect of sites must be controlled in the experimental design.

On the basis of the results described above, a standard method was adopted for comparing the ability of various proteolytic enzyme preparations to digest third degree burn eschars. Experimental burns were produced, as previously described, on the backs of Hartley strain guinea pigs of similar weights. Appropriate dilutions of the enzyme preparations under investigation were applied under the plastic patches to the burn sites. Enough animals were used to permit testing of each preparation on each of the 4 available sites, a different location on each animal. The readings of degree of debridement at each of the treated sites were averaged.

With this method, a comparison was made

TABLE II. Influence of Sex and Sites on Debridement.

Variable		Avg % debridement
Sex	Female	41
	Male	39
Sites	Left top	39
	" bottom	33
	Right "	30
	" top	41

Data from experiment in Table I. Avg of responses furnished by 3 concentrations of enzyme.

TABLE III. Comparison of Ability of 4 Proteinases to Debride Burns.

Proteolytic enzymes	Concentration applied: Units*	Azocasein units*	Protein (mg)†	Avg % debridement
Mold	1,600	72	.007	62
0-22%	80	80	.027	61
Trypsin	1,600	760	.200	56
30-35%	30	6	.047	54

* Units applied are those of manufacturers. Substrate for mold enzyme was azocoll; for trypsin, N-benzoyl-L-arginine ethylester; for 0-22%, azocasein and for 30-35%, gelatin. Azocasein units furnish comparison of these proteinases on this substrate.

† Calculated from nitrogen determination.

Results obtained by joining of 2 Latin squares. Experiment was replicated on another day.

of the ability of 4 proteolytic enzyme preparations to debride third degree burns (Table III). On the basis of their protein content, the highly purified mold proteinase was a more effective debriding agent than the 0-22% and 30-35% proteinases from *C. histolyticum*. Trypsin, on the other hand, required much higher concentrations than the other enzymes to digest this substrate and it is apparent that the ability of these proteinases to attack azocasein does not parallel their ability to debride a third degree burn eschar.

To determine the effect of pre-treatment of the burn eschar on the debriding activity of *C. histolyticum* proteinases, 2 substances which are commonly used to treat burns in human patients were studied; pHisoHex, which is used to cleanse the burn, and petrolatum, which is used to protect the eschar from air. pHisoHex was a potent inhibitor of the debriding activity of the 0-22% proteinase (Table IV). Washing the burn to remove pHisoHex before application of the enzyme effectively removed the inhibitor. Petrolatum under these conditions did not inhibit enzymatic digestion.

Discussion. This study has demonstrated that a semi-quantitative *in vivo* assay for measuring debriding activity of various proteinase preparations is practical. Antibiotics or other bacteriostatic or bactericidal agents must be added to the enzyme solution under test to prevent contaminating bacteria from causing false-positive results. The need for this combination of enzymes and antibiotics in the human patient has been adequately discussed by Greuer and Hess(9). Removal of the bulk of the dead tissue by enzymatic debridement carried out in the presence of antibiotics may prove to be of value in reducing the late deaths in burns due to bacterial infection. The proteolytic enzymes used in these studies, when applied topically and in concentrations suitable for removal of the eschars, did not digest normal skin and apparently limited their activity to digestion of denatured tissue. No gross inflammatory changes beyond the margin of such burns have been observed.

The use of statistical designs such as Latin squares, Youden squares, Lattice squares or other designs where the units per block are equal to 4 is especially valuable in this bioassay in controlling biological and spatial variation. A difficulty in application of statistics to biological results is the mathematical need for additivity of the data. Thus the natural desire of the biologist to use a buffer control as one of the treatments in the experimental design results in overestimating the significance of the statistical analyses. This may also occur when a given treatment (Table IV) causes complete inhibition of enzymatic activity resulting in zero variance. Nevertheless treatment means obtained by

TABLE IV. Effect of pHisoHex and Petrolatum on Debridement.

Treatment of burn	0-22% enzymes, applied units	Avg % debridement
None	200	72
"	80	63
pHisoHex	80	0
" *	80	63
Petrolatum	80	72
" *	80	72
None	Buffer	0

* After 24 hr pHisoHex and petrolatum were removed by washing with lukewarm water and 95% ethanol respectively.

Youden square statistical design. Seven treatments occurred once in each column (site) and each pair of treatments appeared together an equal number of times in the same block (guinea pig). Animals burned as usual and pHisoHex and petrolatum applied to appropriate site.

use of the statistical experimental design are unquestionably more accurate than if no design had been utilized.

In the bioassay procedure described a standard method of burning (contact) and a standard laboratory animal (guinea pig) have been chosen. Additional studies will be required to indicate whether the same burn on other animals requires the same enzymes or, conversely, whether different types of burns on the same laboratory animal are as readily debrided.

Summary. A semi-quantitative bioassay has been described for determining the ability of proteolytic enzymes to debride third degree burn eschars on guinea pigs. With the aid of statistical designs, body weight and site of application were established as the 2 most important experimental variables. Trypsin, a highly purified mold enzyme, and 2 proteinase preparations from *C. histolyticum* have been compared for their ability to debride contact burns. pHisoHex was found to inhibit the 0-22% proteinases.

1. Altemeier, W. A., Coith, R., Culbertson, W., Tytell, A., *Ann. Surgery,* 1951, v134, 581.
2. Connell, J. F., Rousselot, L. M., *S. Forum,* 1953, v4, 422.
3. May, S. C., Ziffren, S. E., Kallio, R. E., Larson, A. D., *ibid.,* 1953, (1952), 205.
4. Stucke, K., *Chirurg.,* 1954, v25, 289.
5. Howes, E. L., *N. Y. State J. Med.,* 1944, v44, 2006.
6. Howes, E. L., MacLennan, J. D., Mandl, I., DeBellis, R., *Bull. N. Y. Acad. Med.,* 1955, v31, 413.
7. DeBellis, R., Mandl, I., MacLennan, J. D., Howes, E. L., *Nature,* 1954, v174, 1191.
8. Cochran, W. G., Cox, G. M., *Experimental Designs,* John Wiley & Sons, Inc., New York, 1950.
9. Greuer, W., Hess, E., *Arzeneimihel-Forsch.,* 1954, v4, 432.

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Deposition of Cholesterol and Cholestanol in Experimental Rabbit Atherosclerosis.* (26542)

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When cholesterol-C^{14} (prepared biosynthetically from acetate-1-C^{14}) and cholesterol-H^3 (prepared by the Wilzbach method) were fed to rabbits(1), a striking discrepancy was observed in the counts of cholesterol samples isolated from tissues like aortae, livers or small intestines. Those isolated from cholesterol-H^3 fed animals showed a much lower radioactivity transfer than those from cholesterol-C^{14} fed rabbits. Since the C^{14} and H^3 cholesterols both appeared to be radiochemically homogeneous (having been subjected to bromination and various chromatographic procedures respectively) it was suggested that the difference in behavior between these 2 labelled cholesterols might have been the result of a peripheral oxidation of cholesterol during its stay in the body, eliminating H^3 atoms from the molecule. Shortly after these experiments were finished, a report of findings by Nystrom and Sunko(2) about the addition of tritium to the double bond of cholesterol when Wilzbach's method of tritiation(3) was used, raised the question whether cholestanol-H^3 was a contaminant in our previous cholesterol-H^3 sample, despite the purification technics we had used. Since cholestanol-H^3 might be metabolized differently from cholesterol-H^3 and might be responsible for the differences of radioactivity observed in earlier experiments, it was decided to repeat the earlier feeding experiments using cholesterol-H^3 purified in such a way as to eliminate all cholestanol-H^3 formed during tritiation. Special work showed that it is possible to eliminate completely cholestanol-H^3 by repeated bro-

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