It may be seen that the spread of amino acid values for these five homologous tuberculins is in every case considerably greater than the corresponding spread of values for the authors' uniform preparations from six different strains, representing four mycobacterial species.

The extracellular proteins prepared by the authors' procedure are apparently quite different in amino acid composition from the dried, defatted cells of the strains from which they were derived. The arginine content, in particular, is much higher in the cells than in the extracellular material. Histidine, likewise, is appreciably higher in the cells. Glutamic acid, aspartic acid, leucine, valine, isoleucine, phenylalanine, tyrosine and methionine, on the other hand, are of significantly greater abundance in the extracellular proteins than in the corresponding preparations of cellular material.

Summary. Extracellular protein has been obtained from 6 strains of mycobacteria (4 species) by a uniform procedure. The amino acid composition of the protein preparations is presented.

1. Ginsberg, B., Lovett, S. L., Dunn, M. S., *Arch. Biochem. Biophys.*, 1956, v60, 164.

2. Steenken, W., Jr., Smith, M. N., *Am. Rev. Tuberc.*, 1938, v38, 514.

3. Long, E. R., Seibert, F. B., *ibid.*, 1926, v13, 393.

4. Sauberlich, H. E., Baumann, C. A., *J. Biol., Chem.*, 1949, v177, 545.

5. Dunn, M. S., Camien, M. N., Shankman, S., Block, H., *ibid.*, 1946, v163, 577.

6. Dunn, M. A., Camien, M. N., Malin, R. B., Murphy, E. A., Reiner, P. J., *Univ. Calif. Pub. Physiol.*, 1949, v8, 293.

7. Jones, M. A., Larsen, A. B., Vardaman, T. H., Baisden, L. A., *Am. Rev. Tuberc.*, 1953, v68, 425.

8. Seibert, F. B., *Faraday Soc. Discussions*, 1953, v13, 251.

9. Nishihara, H., *J. Biochem.* (Japan), 1953, v40, 641.

Received November 22, 1957. P.S.E.B.M., 1958, v97.

Effect of Modification of Insulin on Its Degradation and Biological Activity.* (23704)

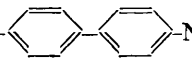
M. A. KARASEK,† BARBARA BRITTON, ARNE N. WICK
Scripps Clinic and Research Foundation, La Jolla, Calif.

Studies on the degradation of insulin labeled with I^{131} have shown that it is rapidly attacked by an enzyme or enzymes present in slices and homogenates of liver, kidney, pancreas, spleen, adipose tissue, and muscle(1-3). A large number of inorganic and organic compounds have been shown to inhibit insulin-I^{131} degradation in extracts of these tissues (4-7). The unusually high rate of insulin-I^{131} degradation observed after its injection into intact animals(8) poses many interesting questions for speculation, such as: Is the degradation linked in any way to the biological activity of insulin or are the two processes, degradation and biological action, independent of one another? In an attempt to gain information on this aspect of the problem, we have carried out a series of experiments in which the insulin-I^{131} molecule was modified by reagents which react with one or more groups of the insulin molecule. The modified insulins were injected into eviscerated-nephrectomized rabbits and their rate of degradation was measured. The biological activity of the injected materials was determined simultaneously.

Procedure. General treatment of animals and determination of presence of biological activity in modified insulins. Adult female rabbits were eviscerated-nephrectomized and maintained as previously described(9). The plasma sugar was kept at a normal concen-

* This investigation was supported in part by research grant from U. S. Public Health Service. The crystalline zinc insulin was contributed by Lilly Research Labs.

† Charles Willard Stimson Postdoctoral Fellow in Biochemistry.

tration by the constant injection of glucose. Rate of glucose injection was guided by frequent determination of the plasma sugar concentration. Glucose utilization was calculated from the quantity of glucose infused. Non-insulinized eviscerated - nephrectomized rabbits require from 140 to 210 mg/kg/hour of glucose to maintain a normal blood sugar level. When insulin is given in excess of 0.2 unit/kg the glucose utilization increases to 350 to 500 mg/kg/hour, a maximal rate for this preparation. In these experiments no attempt was made to evaluate the presence of insulin in amounts less than the equivalent of 0.2 unit/kg. *Insulin I^{131} degradation assay.* At standard time intervals after injection of the insulin-I^{131} derivatives, 2.5 ml blood samples were removed in oxalated syringes, centrifuged, and 0.5 ml aliquots of plasma precipitated with 10% trichloroacetic acid (TCA). The radioactivity in an aliquot of the plasma and in TCA supernatant was measured with an Atomic Instrument Co. Well-type scintillation counter of 35% efficiency, and the degradation was expressed as the per cent of total I^{131} soluble in 10% TCA. *Preparation of insulin derivatives.* Insulin-I^{131} was purchased from the Abbott Laboratories and contained one atom of I^{131} per mole of insulin. In the preparation of all insulin derivatives, 10 mg of crystalline zinc insulin (250 units) was mixed with approximately 0.025 mg of insulin-I^{131}. This quantity of insulin-I^{131} was sufficient to give 30 to 50 million counts/minute in our well-type counter. Five % Na_2CO_3 was added until the crystalline zinc insulin was in solution, and the various insulin derivatives were prepared by treatment with the following reagents: *Diazobenzene Sulfonic Acid* was coupled to insulin by the procedure of Reiner and Lang(10). The diazotized product was pale yellow at pH 7.0, and was reprecipitated by lowering the pH to 5.5. A maximum of 3 radicals of diazotized sulfanilic acid was coupled to the insulin molecule. Aryldiazonium chlorides preferentially react with the aromatic ring of tyrosine and the heterocyclic ring of histidine(11). *Phenylisocyanate.* The urethan derivative of insulin was made according to the method of Fraenkel-Conrat and Fraenkel-Conrat(12). Phenylisocyanate reacts with amino-, imidazol-, and phenolic groups of insulin under the conditions used. *Methanol.* Carboxyl groups were esterified with acidic methanol by the method of Charles and Scott(13). Esterification of macromolecular polyacids at room temperature by mineral acid catalysis has been well demonstrated. *2,4-Dinitrofluorobenzene* was reacted with insulin under the conditions of Sanger(14). The insoluble yellow product was dissolved by shaking repeatedly with dilute alkali, pH 10, at 50°. Under the conditions used, 2,4-dinitrofluorobenzene reacts with amino-, phenolic-hydroxyl-, thio-, and possibly with imidazole groups. *Formaldehyde.* Insulin was treated with formaldehyde for 56 hours at room temperature and acid pH as described by Fraenkel-Conrat and Fraenkel-Conrat(12). The solution was dialyzed against distilled water at 0° for twenty-four hours, and the pH adjusted to 7.0 with dilute NaOH. *Bis-diazobenzidine.* The insulin solution was adjusted to pH 10 and chilled in an ice bath; 2.5 mg of bis-diazobenzidine was added slowly with stirring, and the resulting deep-red solution was dialyzed overnight against distilled water at 0-4°. *Bis-diazobenzidine-Albumin.* 10 mg of human serum albumin in saline was added to the insulin solution. The pH was adjusted to 8.0 with dilute NaOH and the solution chilled in an ice bath. Twenty mg of bis-diazobenzidine was added at a rate of 0.4 mg every 5 minutes. The albumin-insulin complex was dialyzed for 24 hours against distilled water. The precipitate which formed during dialysis was re-dissolved by raising the pH to 8.0. The solution was dark brown at this point. The formation of  linkages between proteins and cells constitutes the crosslinking bridge(15). *Sodium Hypochlorite.* Sodium hypochlorite containing 5% available chlorine was added to an alkaline solution of insulin, pH 10, in a ratio of 0.1 ml sodium hypochlorite per 10 mg insulin. Oxidation was complete in 3 minutes, and the material was dialyzed against distilled water at 0-4°. The solution was pale yellow at pH 7.0. This reaction has been

used to remove the guanidyl groups from collagen by Highberger and Salcedo(16). *Hydrogen peroxide.* Insulin was oxidized with 30% H_2O_2 by the procedure of Sanger and Thompson(17). The oxidized insulin was dialyzed for 24 hours at 0°-4°, to remove excess hydrogen peroxide and formic acid.

Results. In Table I are data showing rate of glucose utilization and the rate of appearance of I^{131} in the TCA soluble fraction after injection of the insulin derivatives. Derivatives prepared with diazobenzene sulfonic acid, formaldehyde, or methanol were biologically active; all other derivatives showed impairment of insulin activity. No alteration in rate of degradation measured over a 4-hour period was found with derivatives prepared by treatment with diazobenzene sulfonic acid, formaldehyde, hydrogen peroxide or methanol; all other derivatives show a decrease in the rate of degradation. A summary of the relationship between biological activity, rate of degradation, and the reactive groups of insulin is given in Fig. 1.

Discussion. The use of the eviscerated-nephrectomized animal to study the degradation of insulin and its derivatives has the advantage that this preparation represents predominantly muscle metabolism *in vivo*, which is an important site of insulin action. The rate of insulin-I^{131} degradation is also much slower in this preparation in comparison with intact animals(8), so that it is possible to correlate the rate of degradation with biological activity.

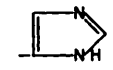
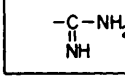
REAGENT NUMBER	GROUP ALTERED	ACTIVITY	DEGRADATION
1, 4, 5		+	+
1, 4, 5		+	+
2,4, 5	$-NH_2$	+	+
3	-COOH	+	+
9, 5	-S-S-	−	+
8		−	−

FIG. 1. Summary showing groups modified in the insulin molecule and its effect on biological activity and degradation. *Plus* indicates presence of biological activity in the derivative and no measurable decrease in its rate of degradation. Reagent numbers refer to the designations in Table I.

A fact to be considered in the study of protein derivatives is the lack of specificity of group reagents. Reagents once considered specific for a functional group can now be shown to react with more than one such group(18). Although the reagents used in this study were chosen to react mainly with one major group, the possibility of side reactions with other groups is present, and these studies, therefore, do not represent a specific relationship between a functional group, degradation, and activity.

The failure to observe biological activity with 6 derivatives of insulin (reagent Nos. 4-9 in Table I) is of greater significance than the 3 derivatives (Nos. 1, 2, 3) which showed no inhibition. This results from the fact that the presence of 0.2 unit of unreacted insulin from the initial 250 units of insulin employed for treatment with diazosulfanilic acid, formaldehyde, or methanol would produce the insulin-like glucose utilization values reported in Table I.

It has been shown that reagents which react mainly with tyrosine or histidine residues impair neither insulin degradation nor insulin activity. Similarly, mild non-oxidative iodination does not appear to alter either enzymatic degradation or biological activity(8). Replacement reactions on the tyrosine and histidine residues are possible without producing detectable changes in activity or degradation.

The importance of the configuration of the insulin molecule to its biological activity and the rate of degradation is shown with reagents which affect the size of the insulin molecule, *viz.*, bis-diazobenzidine and bis-diazobenzidine-albumin. Although these reagents react mainly with the histidine and tyrosine residues—ones which do not appear to be essential for either degradation or activity-polymerization and coupling with albumin destroyed insulin degradation and activity.

The guanidyl group of arginine appears to be linked with both insulin activity and insulin degradation. Removal of this group

TABLE I. Data Showing Rate of Glucose Utilization and % of Plasma-I^{131} Soluble in 10% Trichloracetic Acid (TCA) after Injection of Insulin Derivatives in Eviscerated-Nephrectomized Rabbits.

Reagent No.	Reagent used	No. of exp.*	Gluc. used, mg/k/hr	% plasma-I^{131} TCA soluble .5 hr	1 hr	2 hr	3 hr	4 hr
	Control, 250 u insulin-I^{131} only	8	390	12	24	36	45	60
1	Diazobenzene sulfonic acid	2	380	5	15	28	44	56
2	Formaldehyde	3	385	4	15	31	46	55
3	Methanol	1	355	6	14	32	51	61
4	Phenylisocyanate	2	210	4	7	7	22	46
5	2,4-dinitrofluorobenzene	1	193	.3	1	4	4	8
6	Bis-diazobenzidine	2	210	.9	2	11	10	
7	Bis-diazobenzidine-albumin	2	220	5	9	13	14	
8	Sodium hypochlorite	3	140	10	11	12	12	15
9	Hydrogen peroxide	2	211	5		30	43	60

* Avg values are shown when more than one exp. was carried out. All animals weighed approximately 1 kilo after evisceration.

with sodium hypochlorite markedly interfered with both activities. That this is not a result of oxidation of disulfide bonds is seen from a comparison of the results obtained with insulin-I^{131} treated with hydrogen peroxide. In this case biological activity was destroyed but insulin degradation was not altered.

It is apparent that reagents which interfere with insulin degradation also interfere with biological activity, and the chemical groups on the insulin molecule that are essential for increasing glucose utilization are similar to those necessary for the action of the destructive enzymes. However, the results with hydrogen peroxide in which insulin activity was destroyed but insulin degradation not altered, indicate that the degradation of insulin and the biological activity of insulin are not necessarily dependent on each other. These results thus support the contention of Elgee and Williams(19) that the degradation of insulin-I^{131} may occur independently of insulin action.

Summary. The biological activity and rate of degradation of 9 derivatives of insulin were studied in the eviscerated-nephrectomized rabbit. Derivatives of insulin made with methanol, diazotized sulfanilic acid, formaldehyde, or hydrogen peroxide, did not affect insulin degradation; those made with 2, 4 - dinitrofluorobenzene, bis - diazobenzidine, bis-diazobenzidine-albumin, and sodium hypochlorite impaired insulin degradation. Those reagents which interfered with the rate at which insulin was degraded also destroyed the biological activity of insulin. However, the reactive groups on the insulin molecule which were essential for biological activity were not necessarily centers of attack by the destructive enzymes. It is concluded that degradation of insulin-I^{131} observed in physiological systems is not necessarily a function of insulin action on target tissues.

1. Mirsky, I. A., Broh-Kahn, R. H., *Arch. Biochem.*, 1949, v20, 1.

2. Elgee, N. J., Williams, R. H., Lee, N. D., Wong, T., Hogness, J. R., *Diabetes,* 1953, v2, 370.

3. Haugaard, N., Vaughan, M., Haugaard, E. S., Stadies, W., *J. Biol. Chem.*, 1954, v208, 549.

4. Tobizawa, H. H., Nutley, M. L., Narahara, H. T., Williams, R. H. *ibid.,* 1955, v214, 285.

5. Mirsky, I. A., *Ciba Foundation Colloquia on Endocrinology,* 1953, v6, 263.

6. Williams, R. H., *Metabolism,* 1956, v5, 128.

7. Berson, S. A., Yalow, R. S., Weisenfeld, S., Goldner, M. G., Volk, B. W., *Diabetes,* 1957, v6, 54.

8. Drury, D. R., Karasek, M. A., Britton, B., Wick, A. N., *Am. J. Physiol.*, in press.

9. Drury, D. R., *ibid.*, 1935, v111, 289.

10. Reiner, L., Lang, E. H., *J. Biol. Chem.*, 1941, v139, 641.

11. Landsteiner, K., *The Specificity of Serological Reactions,* Springfield, p100, 1936, Charles C. Thomas.

12. Fraenkel-Conrat, J., Fraenkel-Conrat, H., *Biochimica et Biophysica Acta,* 1950, v5, 89.

13. Charles, A. F., Scott, D. A., *J. Biol. Chem.*, 1931, v92, 289.

14. Sanger, F., *Biochem. J.*, 1945, v39, 507.

15. Arquila, E., Stavitsky, A. B., *J. Clin. Invest.*, 1956, v35, 458.

16. Highberger, J. H., Salcedo, J. S., *J. Am. Leather Assn.*, 1940, v35, 11.

17. Sanger, F., Thompson, E. O. P., *Biochem. J.*, 1953, v53, 353.

18. Howard, A. N., Wild, F., *ibid.*, 1957, v65, 651.

19. Elgee, N. J., Williams, R. H., *Am. J. Physiol.*, 1955, v180, 9.

Received October 4, 1957. P.S.E.B.M., 1958, v97.

Distribution of Sulfur in Regenerating Wound Tissue. (23705)

ROBERT C. NORDLIE* AND HERBERT J. FROMM (Introduced by W. E. Cornatzer)
Guy and Bertha Ireland Research Laboratory, Department of Biochemistry, University of N. Dakota Medical School, Grand Forks

Methionine and cystine supplementation have been shown to be equally effective in accelerating healing rate of wounds in rats fed low and high protein diets(1,2). Methionine and cystine + cysteine (hereafter referred to as cystine) content of wound tissue was reported to exceed that of corresponding unwounded skin tissue 4 to 31 days after injury (3). Attempts to detect sulfur-containing compounds other than protein-bound amino acids have revealed no non-protein-bound wound sulfur(3). It was tentatively concluded that increased demand for methionine or cystine following injury is due to requirement of regenerating tissue for these amino acids, *per se*, as structural units of proteins (or, in the case of methionine, also as precursor of cystine). Since regenerating wound tissue contains considerable quantity of sulfate-containing mucopolysaccharides(4,5) the sulfur of which may be a product of *in vivo* metabolism of methionine and cystine(6), the presence of mucopolysaccharide sulfate in wound tissue was studied. Glutathione has been increased at site of rapid cell division(7) such as accompanies wound tissue regeneration. Non-protein fractions of wound tissue were studied with alterations in content of the peptide. Finally, levels of free cystine and methionine, and amount of S^{35} recently injected as S^{35}-L-methionine present in all forms discussed was studied. Special micro-technics were employed to detect minute amounts of methionine, cystine, and sulfate in protein and non-protein fractions of wound tissue. Rate of S^{35} uptake following administration of S^{35}-L-methionine was also studied, radioactive tracers serving as the most sensitive available method for detection of the various compounds.

Methods. Sixteen individually caged, female, albino rats (180 ± 15 g) of Sprague-Dawley strain, receiving distilled water *ad lib.*, were maintained on a protein-free diet (3) throughout. After 5-day acclimation period, each animal received intraperitoneally 5.05×10^6 cpm of S^{35}-L-methionine (Abbott) in 0.25 ml saline. Two days later, they were anesthetized with pentobarbital and a standard 4 cm circular skin wound placed on the back directly below scapula(3). Wound tissues were removed from 8 animals on 4 and 6 days after injury, the animals then sacrified by decapitation. Tissues were carefully removed, weighed, and frozen at −20°. The frozen tissues were ground in mortar with sand and transferred to centrifuge tubes by washing with approximately 10 ml of saline. Ten ml of aqueous 10% trichloroacetic acid solution (TCA) was added to precipitate wound protein, the tubes then being centrifuged at 2000 rpm at 5° for 10 minutes. The precipitate was washed once with 5 ml of 5% TCA and supernatant fluids combined, filtered through Whatman No. 2 paper and diluted to 35 ml. The protein fraction was hydrolyzed 20 hours in 12 ml of 20% HCl - 50% HCOOH mixture(8), then diluted to 25 ml and filtered. S^{35} radioactivity present as mucopolysaccharide sulfate was determined by direct precipitation from acid hydrolyzate as $BaSO_4$, after addition of carrier sulfate. Sulfate-S^{35} in supernatant fluid was determined similarly. Total S^{35} radioactivity was determined on aliquots of supernatant fluid

* Part of thesis for degree of Master of Science.