J. Lab. and Clin. Med., 1946, v31, 394.

13. Rapp, H., Dissertation submitted to The Johns Hopkins School of Hygiene and Public Health in partial fulfillment of the requirement for the Degree of Master of Science in Hygiene, 1953.

14. Halpern, B. N., and Pacaud, A. P., *C. R. Soc. Biol., Paris*, 1951, v145, 1465.

15. Rowley, D. A., *J. Immunol.*, 1950, v64, 289.

16. Buxton, A., *J. Gen. Microbiol.*, 1954, v10, 398.

17. Ambrus, J. L., *et. al., Transplant. Bull.*, 1955, v2, 50.

18. Coons, A. H., and Kaplan, M. H., *J. Exp. Med.*, 1950, v91, 1.

19. Felton, L. D., *J. Immunol.*, 1949, v61, 107.

20. Stark, O. K., *ibid.*, 1955, v74, 126.

Received June 7, 1956. P.S.E.B.M., 1956, v92.

Sulfhydryl Factors in Degradation of Insulin-I^{131} by Liver Extracts.* (22591)

HIROMICHI T. NARAHARA,† HENRY H. TOMIZAWA, AND ROBERT H. WILLIAMS.

Department of Medicine, University of Washington School of Medicine, Seattle.

The degradation of insulin-I^{131} by both a heat stable and a heat labile component of rat liver homogenate has been reported by Mirsky *et al.*(1). Lehmann and Schlossmann(2) had previously observed that insulin could be inactivated by a dialyzable heat stable factor in rabbit muscle extracts. This factor was able to reduce the disulfide linkages of insulin, and the authors concluded that it was a sulfhydryl compound such as glutathione (GSH) or cysteine. As liver has a relatively high content of GSH, it was of interest to find whether this compound could produce changes in the trichloroacetic acid (TCA) solubility of insulin-I^{131}. The present studies on the degradation of insulin-I^{131} in the presence of GSH were carried out in the hope of acquiring better understanding of the processes that might be involved in the inactivation of insulin by rat liver extracts.

Materials and methods. Crystalline zinc insulin, highly purified amorphous insulin, and crystalline glucagon were gifts of Eli Lilly and Co. The crystalline insulin was iodinated by Abbott Laboratories, and glucagon was kindly iodinated by Dr. Alfred Staub. Crystalline bovine plasma albumin and iodinated human serum albumin were obtained from the Armour Laboratories. Reagent grade GSH was a product of the Fisher Scientific Co., and oxidized glutathione (GSSG) was purchased from the Nutritional Biochemicals Corp. *p*-Chloromercuribenzoic acid (PCMB) was obtained from the Bios Labs, Inc. *Preparation of liver extracts.* Livers of decapitated 3-month-old Sprague-Dawley rats were perfused *in situ* with ice cold isotonic saline. The livers were then homogenized in 1.15% KCl with a glass homogenizer. The homogenate was diluted to a 10% tissue concentration and centrifuged for 25 minutes at 110,000 x *g*. The resultant supernatant fraction was used. For certain experiments, an acetone powder was prepared from the supernatant fraction. Prior to the assay procedure each 50 mg of powder was extracted with 1 ml of cold distilled water and material which had not dissolved at the end of 1 hour was removed by centrifugation. An aliquot of supernatant fraction from rat liver was placed in a boiling water bath for 10 minutes in order to prepare the heat stable factor. It was found in preliminary experiments that heating for 2 minutes was sufficient to produce maximum inactivation of the heat labile system which degraded insulin-I^{131}. *Measurement of insulin-I^{131} degradation.* 1 ml of liver extract or GSH solution that had been adjusted to pH 7.5 was preincubated for 5 minutes at 37°C in a 12 ml centrifuge tube. Then 1 ml of 0.067 M potassium phosphate buffer of pH 7.5, containing a trace quantity (0.1-2 μg) of dialyzed insulin-I^{131}, 1 mg of bovine plasma albumin,

* This work was supported in part by grants from U.S.P.H.S., the Atomic Energy Commission, and Burroughs Wellcome and Co.

† Research Fellow, U.S.P.H.S.

and varying amounts of amorphous insulin carrier, was added. Each ml of substrate contained approximately 0.5 μc of radioactivity. The albumin was included in order to help stabilize the radioactive insulin(3). In control mixtures liver extract and GSH were omitted. All incubations were conducted in duplicate. At appropriate intervals, 1 ml of 20% TCA was added to terminate the reaction. The extent of degradation of insulin-I^{131} was measured as described previously(4). Duplicate determinations generally agreed within 5% of each other. It has been demonstrated that changes in the TCA solubility of insulin-I^{131} in the presence of liver extract are correlated with the formation of non-protein nitrogen from insulin and biological inactivation of the latter(4,5). *Studies with sulfhydryl reagents.* 1 ml of a 4% heated or unheated liver extract containing PCMB in a concentration of 6×10^{-4} M (an amount just sufficient to abolish the nitroprusside reaction for sulfhydryl groups) or GSSG in a concentration of 3×10^{-2} M was preincubated in a Dubnoff shaker for 30 minutes, shaking at a rate of 80 cycles per minute. 1 ml of phosphate buffer containing a trace quantity of insulin-I^{131} without carrier insulin was then added and incubation was continued for another 15 minutes, at which time the reaction was stopped with TCA. *Electrophoretic studies.* Four different incubation mixtures (A, B, C and D) were prepared in duplicate in 0.5 ml of 0.033 M phosphate buffer of pH 7.5 with the following components: (A) 20 μg (37 μc) of dialyzed insulin-I^{131}, (B) 20 μg of dialyzed insulin-I^{131} plus 0.25 mg of GSH (final concentration 1.6×10^{-3} M), (C) 10 μc of carrier-free NaI^{131}, and (D) 10 μc of NaI^{131}, plus 0.25 mg of GSH. Incubation was carried out in stoppered test tubes at 37°C for 1 hour. The tubes were then chilled in ice, and 0.3 ml of a 2% human plasma solution, 0.1 ml of 10% potassium iodide carrier solution, and 0.3 ml of 20% TCA were added rapidly with mixing. The mixtures were centrifuged, and 10 μl of each TCA supernatant were applied at the origin (O) of a strip of Whatman No. 1 filter paper moistened with 0.47 M potassium phosphate buffer of pH 7.7. The electrophoresis apparatus was that described by Grassmann and Hannig(6); the strips were run simultaneously in the cold room for 1 hour at a potential of 200 volts. The strips were then air-dried and counted with an end-window Geiger-Mueller tube arranged for automatic scanning and recording.‡ In a separate control experiment, 10 μc of carrier-free NaI^{131} were incubated with 20 μg of unlabeled insulin with or without the addition of 0.25 mg of GSH for 1 hour and then subjected to electrophoresis under conditions similar to those just described.

TABLE I. Effect of GSH upon Insulin-I^{131} in Presence of Varying Amounts of Amorphous Insulin Carrier.

Amorphous insulin, mg	Final GSH concentration, M	% TCA supernatant radioactivity in 20 min.
.02	8×10^{-3}	9.5
"	4 "	8.8
"	2 "	6.2
"	1 "	2.9
2.0	2×10^{-3}	1.3
.2	"	4.6
.02	"	6.0
.00	"	6.4

Results. Effect of GSH upon insulin-I^{131}. GSH was incubated in varying concentrations with a trace quantity of insulin-I^{131} and different amounts of amorphous insulin carrier. The results are shown in Table I. It is evident that glutathione produced degradation of the labeled insulin and that the amount of degradation tended to increase as the GSH concentration was raised, especially at lower levels of GSH. The percentage degradation of insulin-I^{131} fell only slightly with the addition of carrier insulin until relatively large amounts had been added.

In contrast to the behavior of iodinated insulin, radio-iodinated human serum albumin and glucagon-I^{131} showed no degradation when incubated with GSH in a concentration of 0.004 M for 30 minutes. This suggests that the effect of GSH upon insulin-I^{131} may not be one of direct deiodination of the intact protein molecule.

‡ This instrument was kindly loaned by Dr. Donald J. Hanahan.

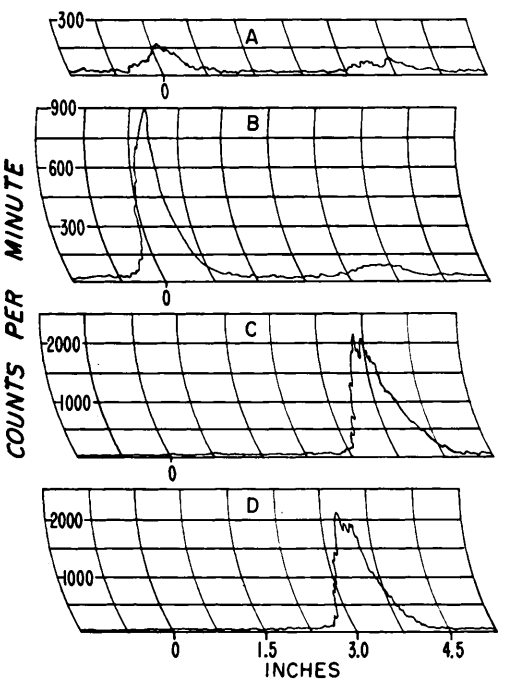

FIG. 1. Electrophoretic pattern of TCA soluble material resulting from action of GSH upon insulin-I^{131}.

Electrophoretic studies were conducted to test this possibility further. The results are shown in Fig. 1. Duplicate experiments yielded similar patterns. It is seen that incubation of insulin-I^{131} with GSH gave rise to a large amount of TCA soluble radioactivity, as shown in Strip B, which appeared as a band that migrated much more slowly than the iodide band of strip C. Strip D demonstrates that GSH itself does not produce such an alteration of the mobility of iodide. In a separate control experiment it was found that the presence of unlabeled crystalline zinc insulin, with or without the addition of GSH, similarly had no effect upon the migration of radioactive iodide. Therefore, the newly formed radioactive band near the origin probably represents an alteration of the insulin molecule rather than deiodination. Thus, the changes in TCA solubility of insulin-I^{131} produced by GSH may reflect reduction of the disulfide linkages of insulin, but activation of a proteolytic contaminant of the insulin cannot be ruled out.

Sulfhydryl nature of the heat stable and heat labile factors of liver. Mirsky and Broh-Kahn(7) and de Barbieri and Grassi (8) have found that liver homogenate treated with sulfhydryl inhibitors is less capable of inactivating insulin. It was of interest to see whether or not this inhibition extended to the heat stable component as well. PCMB and GSSG were chosen as reagents because both are among the more specific sulfhydryl inhibitors. The data of Table II show that PCMB in a concentration of 6×10^{-4} M completely inhibited the activity of boiled and unboiled liver extract. GSSG in a concentration of 3×10^{-2} was also able to inhibit the heat stable factor completely, but produced incomplete inhibition of the unheated extract. This suggests that the heat stable factor and heat labile factor both contain sulfhydryl groups which are essential for their activity, but that the sulfhydryl groups of the latter are less reactive towards GSSG. In control experiments the possibility that PCMB might cause precipitation of degradation products of insulin-I^{131} was ruled out.

As GSH could produce degradation of insulin-I^{131}, it was considered likely that the presence of this compound in liver extracts could account for the activity of the heat stable factor. However, it was found that when rat liver acetone powder extract was dialyzed against phosphate buffer of pH 6.2 for 48 hours and then heated in a boiling water bath for 10 minutes, it still retained considerable ability to degrade insulin-I^{131} (Table III). No GSH (TCA-soluble sulfhydryl material) could be detected in the dialyzed extract by the nitroprusside reaction under conditions which could detect GSH in a concentration of 5×10^{-5} M. Liver extract that had been precipitated and washed 3

TABLE II. Effect of Sulfhydryl Inhibitors upon Activity of Heat Stable and Heat Labile Factors of Liver.

Liver supernatant cell fraction	Inhibitor	TCA supernatant radioactivity, %
Unheated	None	73
	PCMB	0
	GSSG	39
Heated	None	6.2
	PCMB	0
	GSSG	0

TABLE III. Effect of Liver Acetone Powder Extracts Containing No Detectable GSH. .03 mg of carrier insulin added.

Liver preparation	Incubation, min.	TCA supernatant radioactivity, %
Dialyzed, unheated	10	10
	20	17
	30	23
	60	35
Dialyzed, heated	30	3.4
	60	6.7
TCA precipitated, heated	60	6.9

times with 5% TCA and then heated in a boiling water bath for 10 minutes was also able to degrade insulin-I^{131}. Thus, it is probable that protein-bound sulfhydryl groups contribute to the activity of the heat stable factor, but it is possible that some of these groups are less reactive in undenatured proteins.

Moreover, it was noted that GSH enhanced the degradation of insulin-I^{131} by liver extracts, and that this was more than a purely additive effect. This phenomenon (Table IV) would be compatible with an activation of a proteolytic enzyme system of liver by GSH. It is also possible that reduction of the insulin molecule may denature it and render it a better substrate for proteolytic enzyme action.

Reduction of the insulin molecule does not appear to be an absolute prerequisite for proteolytic degradation, however, because it was found that liver acetone powder extract after dialysis for 48 hours was still able to produce significant degradation of insulin-I^{131} (Table III). In this experiment 0.2 ml of dialyzed liver extract was incubated with insulin-I^{131} plus 0.03 mg of added carrier insulin. In another experiment, in which 0.3 ml of dialyzed extract was incubated with insulin-I^{131} in the absence of amorphous insulin, 73% of the radioactivity became TCA soluble in 10 minutes. Under conditions where insulin is completely inactivated(9), GSH was found to produce at most about a 25% increase in TCA supernatant radioactivity. Racker(10) has found that crude liver extracts can catalyze the reduction of insulin. Since the sites of cleavage of the insulin molecule are not known, and since GSH can affect the activity of the proteolytic enzyme system of liver which acts on insulin, it is difficult to distinguish the exact contributions of reductive and proteolytic processes when insulin-I^{131} is degraded by crude liver homogenates or by purified extracts in the presence of added GSH.

TABLE IV. Effect of GSH upon Degradation of Insulin-I^{131} by Liver Acetone Powder Extract. .03 mg of carrier insulin added.

Incubation time, min.	10	20	30	40	60
Reaction mixture additions	TCA supernatant radioactivity, %				
GSH	6	10	13	15	19
Liver extract	10	19	25	29	39
" " + GSH	16	30	43	53	69

Summary. 1. Glutathione degrades insulin-I^{131} and renders it more soluble in trichloroacetic acid. Evidence is presented that this is not the result of direct deiodination. 2. Rat liver extracts, even after exposure to a boiling water bath, can degrade insulin-I^{131}. This heat stable activity could be explained by the presence of sulfhydryl compounds in liver. 3. Reductive as well as proteolytic processes must be considered when analyzing the degradation of insulin by crude liver extracts.

The authors are indebted to Miss Ruth Miller for valuable technical assistance.

1. Mirsky, I. A., Perisutti, G., and Dixon, F. J., *J. Biol. Chem.*, 1955, v214, 397.

2. Lehmann, H., and Schlossmann, H., *J. Physiol.*, 1938, v94, 15P.

3. Narahara, H. T., Tomizawa, H. H., Miller, R., and Williams, R. H., *J. Biol. Chem.*, 1955, v217, 675.

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

5. Mirsky, I. A., Perisutti, G., and Dixon, F. J., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 228.

6. Grassmann, W., and Hannig, K., *Hoppe-Seyler's Z. physiol. Chem.*, 1952, v290, 1.

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

8. De Barbieri, A., and Grassi, M., *Farm. sci. e tec., Pavia*, 1951, v6, 170.

9. Du Vigneaud, V., Fitch, A., Pekarek, E., and Lockwood, W. W., *J. Biol. Chem.*, 1931-32, v94, 233.

10. Racker, E., *Fed. Proc.*, 1953, v12, 711.

Received June 7, 1956. P.S.E.B.M., 1956, v92.