TABLE III. Comparison of Effect on Permeability Activity of Heating Serum Before and After Addition of C'1 Esterase.

| C'1 esterase* (units/ml) | Permeability activity (diameter of lesion in mm) in mixtures containing: Unheated serum | Heated serum† | Heated (enzyme + serum)‡ | No serum |
|---|---|---|---|---|
| 4 | 7.3 | 2.7 | 7.3 | 4.4 |
| 2 | 6.3 | 3.3 | 4.5 | 3.1 |
| 1 | 5.5 | 2.2 | 3.0 | 1.9 |
| 0 | 3.4 | 2.8 | 3.0 | 2.7 |

* Final concentration in reaction mixtures.
† Serum heated at 56°C for 30 min before addition of C'1 esterase.
‡ Mixture of C'1 esterase and normal serum heated at 56°C for 30 min.

guinea pig serum *in vitro*. The action of this factor was blocked by treatment of test animals with an anti-histaminic drug, tripolidine. Generation of the factor from serum was prevented by inactivation of the enzymatic activity of C'1 esterase with DFP or human serum inhibitor of C'1 esterase. SBTI, without effect on C'1 esterase, did not inhibit generation. Concentrations of C'1 esterase which elaborated permeability activity in guinea pig serum also produced marked depression of the hemolytic complement titer of the serum. A relationship was also observed between the residual complement titers of heated sera and their capacity to produce a permeability factor upon mixture with C'1 esterase. The data are consistent with, but do not prove, the hypothesis that C'1 esterase mediates increased vascular permeability by interaction with the complement system.

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## Effects of Bromobenzene on Mouse Tissue Sulfhydryl and Insulin-I$^{131}$ Metabolism.* (29227)

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Enzyme studies(1,2) indicate that reduced glutathione (GSH) acts as hydrogen donor in the first step in insulin degradation, its reductive cleavage into the glycyl and phenylalanine chains. This raises the question whether the rate of insulin degradation varies in any degree as result of physiological or even unphysiological variations in intracellu-

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lar GSH concentration. Marked decreases in rodent liver GSH concentration may be obtained by use of mercapturic acid precursors (3,4,5). This paper describes, for livers and kidneys of intact mice, effects on both GSH concentration and insulin-$I^{131}$ degradation of overnight fast plus bromobenzene or benzyl chloride administration. Increase in solubility of $I^{131}$ in TCA (5% trichloracetic acid) has been used as main criterion of tissue activity in degrading labeled insulin, in accordance with findings and considerations which have been summarized by Lee(6).

*Methods.* Swiss-Webster strain male mice, 10-20 weeks old and weighing 25-40 g, were used. In a given experiment mice were selected to give groups of nearly identical average weight and weight range. About 5 P.M., 16-18 hours before sacrifice, each mouse was caged alone with water but no food. Each mouse was also injected IM with sesame oil (controls) or drug in oil (test mice), .005 ml/g. Drug in oil mixtures consisted of 1 volume of bromobenzene, or benzyl chloride, plus 9 volumes sesame oil. In the bromobenzene experiments the IM injections were made 12-14 hours before sacrifice; in the benzyl chloride experiment these injections were made 2 hours before sacrifice. Each mouse was also injected by tail vein, 5 or 25 minutes before sacrifice, with .005 ml/g of insulin-$I^{131}$, prepared to contain per ml: human serum albumin, 60 mg; beef insulin, 2 $\mu$C and 1, 10 or 50 $\mu$g. Radioactivity was supplied by use of Abbott Co. $I^{131}$ labeled insulin, originally about 4 mC/mg, the bulk of the insulin by use of Eli Lilly insulin low in glucagon. Except when stated otherwise, dry ice frozen liver and kidney samples were obtained from each mouse at sacrifice. For each tissue sample, $t$ (total CPM/g), $s$ (TCA soluble $I^{131}$, CPM/g), $i$ (TCA insoluble $I^{131}$, $= t - s$) and *% $I^{131}$ TCA soluble* ($= 100\ s/t$) were estimated. Counting mixture for estimation of $t$ was prepared by homogenizing the tissue sample in .3 M sucrose, 9.3 ml/g, dissolving .5 ml of this homogenate in 1 ml of hyamine with heat (62°, 1-3 hours), then adding .5 ml 5% TCA and 8 ml of counting solution P611(7). Counting mixture for estimation of $s$ was prepared by centrifuging a 1:1 mixture of sucrose tissue homogenate and 10% TCA, and placing 1 ml of supernatant in a counting vial together with 1 ml hyamine and 8 ml P611. Values for $t_o$ (total CPM inj/g mouse) and $s_o$ (TCA soluble $I^{131}$, CPM inj/g mouse) were taken as equal to CPM, total and TCA soluble respectively, present per .005 ml of the Insulin-$I^{131}$ injected into the mice. Concentration ratios $T$, $S$ and $I$ were obtained for each tissue sample by dividing $t$, $s$ and $i$ respectively by $t_o$. The $T$, $S$, $I$ and *% $I^{131}$ TCA soluble* values of the Tables are Group means. The Abbott $I^{131}$ labeled preparations used in the preparations contained only about 3 to 4% of their $I^{131}$ in a TCA soluble form (100% $(s_o/t_o) = 3$ to 4%). Count rates were measured using an automatic TRI-CARB liquid scintillation spectrometer, voltage 1050, pulse discriminator window range 10-100, and corrected for decay.

While engaged in the present experiments we found(8) that if the nitroprusside method of Thompson and Watson(9) were applied to 5% TCA solutions of L-cysteine and/or GSH, the color developed by L-cysteine faded so rapidly that by delaying readings until 3 minutes after mixing it was possible to obtain optical density increases due entirely to GSH. The "GSH" values of the Tables have been obtained by this variation in the method of Thompson and Watson. It should be noted that acetoacetate shows slow color development in all nitroprusside methods supposedly specific for -SH compounds, and may be expected to be present in measurable amounts in tissues of fasted animals. Hence the "GSH" values of the Tables can be only approximations of true tissue GSH. However, use of this method has presented us with assurance that in the L-cysteine experiments of this paper (Table II) the repletion of tissue NPSH which we obtained in bromobenzene treated mice by use of L-cysteine was due to resynthesis of GSH (cf. also 3, 4), rather than to accumulation of L-cysteine in these tissues.

Values for total NPSH of the Tables were obtained by use of the nitroprusside method of Grunert and Phillips(10), in which both GSH and L-cysteine gave rapid develop-

TABLE I. Effects of Bromobenzene Pretreatment on Mouse Tissue Non-protein Sulfhydryl (NPSH), and Insulin-$I^{131}$ Fixation and Degradation.

| Exp* | Group of tissues analyzed† | No. of mice in group | Mean values ± standard deviations‡ NPSH as meq S/kg | $I^{131}$ concentration ratios *T* | *I* | % $I^{131}$ TCA soluble |
|---|---|---|---|---|---|---|
| 1 | LC5 | 7 | 5.24 ± .42 | 4.72 ± .69 | 4.24 ± .63 | 10.4 ± 1.0 |
| | LB5 | 5 | .62 ± .16§ | 3.06 ± .27§ | 2.84 ± .25§ | 7.4 ± 3.6 |
| | LC25 | 5 | 4.81 ± .88 | 1.53 ± .42 | 1.13 ± .32 | 27.8 ± 3.2 |
| | LB25 | 11 | 1.17 ± .65§ | 2.25 ± .47§ | 1.76 ± .55§ | 22.6 ± 5.0§ |
| | KC5 | 7 | 4.19 ± .29 | 6.21 ± .87 | 5.69 ± .76 | 8.3 ± 2.5 |
| | KB5 | 5 | .55 ± .22§ | 6.19 ±1.00 | 5.84 ±1.02 | 5.6 ± 2.0 |
| | KC25 | 5 | 3.77 ± .44 | 4.18 ±1.19 | 2.96 ± .93 | 29.7 ± 3.7 |
| | KB25 | 11 | 2.24 ±1.19§ | 7.40 ±2.79§ | 6.06 ±3.02§ | 20.8 ± 7.7§ |
| 2 | LC5 | 7 | 3.94 ± .68 | 4.56 ± .79 | 3.76 ± .74 | 17.7 ± 6.2 |
| | LB5 | 6 | 1.17 ± .80§ | 5.16 ±1.52 | 4.71 ±1.66§ | 10.5 ± 8.4§ |
| | LC25 | 7 | 4.17 ± .42 | 1.38 ± .20 | .89 ± .16 | 35.7 ± 2.5 |
| | LB25 | 5 | .75 ± .29 | 3.40 ± .96§ | 2.73 ± .80§ | 30.2 ± 4.4§ |
| | KC5 | 7 | 3.51 ± .52 | 8.52 ±3.15 | 7.55 ±2.93 | 11.5 ± 3.2 |
| | KB5 | 6 | 1.50 ±1.20§ | 6.30 ±4.44 | 5.90 ±4.41 | 10.4 ±14.7 |
| | KC25 | 7 | 3.65 ± .16 | 4.57 ± .77 | 2.97 ± .66 | 35.7 ± 3.7 |
| | KB25 | 5 | 1.59 ± .65§ | 8.28 ±3.50§ | 6.88 ±3.22§ | 18.0 ± 5.1§ |

* The injected insulin-$I^{131}$ contained per ml 2 $\mu$C $I^{131}$ and total insulin as follows: Exp 1, 10 $\mu$g; Exp 2, 50 $\mu$g.

† L = liver, K = kidney; C = control, B = bromobenzene treated group; 5 & 25 = min between injection of insulin-$I^{131}$ and sacrifice.

‡ NPSH by method of Grunert & Phillips(10); *T* = $I^{131}$ found per g tissue/$I^{131}$ inj per g mouse; *I* = TCA insoluble $I^{131}$ found per g tissue/$I^{131}$ inj per g mouse; % $I^{131}$ TCA soluble = 100% (TCA soluble $I^{131}$/total $I^{131}$).

§ $P < .05$, difference between paired C and B groups.

ment of color and stoichiometrically equivalent optical density readings.

*Results.* I. *Fixation and TCA solubilization of $I^{131}$ of labeled insulin:* (a) Radioactivity was highly concentrated in livers and kidneys of mice sacrificed 5 minutes after injection of labeled insulin (cf. *T* values of Table I); most of this radioactivity was TCA insoluble (cf. *I* and *% $I^{131}$ TCA soluble* values of Tables), (b) by 25 minutes after injection of labeled insulin significant increases in tissue *% $I^{131}$ TCA soluble* values had occurred; in livers and kidneys of control mice there was also considerable decrease in total radioactivity present (cf. *T* values of Table I). All of these findings are similar to those which had previously been reported for livers and kidneys of rats by others(11,12).

II. *Bromobenzene effects using intact mice:* A. *NPSH* and "GSH": From the data of the Tables it is apparent that bromobenzene pretreatment induced appreciable and statistically significant decreases in NPSH and "GSH," not only of liver but also of kidney. (B) *Metabolic handling of Insulin-$I^{131}$:* (a) For mice sacrificed 25 minutes after injection of labeled insulin, it was found that the mean *% $I^{131}$ TCA soluble* value obtained for any particular control group of tissues, *e.g.*, LC25 or KC25 of Table I, was invariably significantly greater than that obtained for the corresponding test group of tissues, *e.g.*, LB25 or KB25 of Table I. It is therefore concluded that bromobenzene treatment results in impaired ability of both mouse liver and kidney to transform $I^{131}$ of labeled insulin from a TCA insoluble into TCA soluble form. (b) Over the period of time 5-25 minutes after injection of labeled insulin, mean *T* and *I* values were, in bromobenzene injected mice, moderately decreased in the liver, actually increased in the kidney. In control mice, the mean *T* and *I* values decreased significantly in kidney, markedly in liver. To explain these findings, we assume that radioactivity of insulin is more likely to remain in a given tissue if the $I^{131}$ is not changed into TCA soluble form, and that if a given tissue, *e.g.*, the

TABLE II. L-Cysteine Against Bromobenzene, *re* Tissue NPSH and Insulin-$I^{131}$ Metabolism.

| Group of tissues analyzed* | No. of mice in group | Mean values ± standard deviations† NPSH (meq S/kg) "GSH" | Total NPSH | $I^{131}$ concentration ratios *T* | *I* | % $I^{131}$ TCA soluble |
|---|---|---|---|---|---|---|
| LC | 11 | 4.39 ± .43 | 4.76 ± .63 | .91 ± .15 | .44 ± .07 | 51.6 ± 4.3 |
| LC–SH | 11 | 3.97 ± .47 | 4.29 ± .63 | 1.04 ± .32 | .56 ± .32 | 47.9 ± 6.8 |
| LB | 12 | 1.95 ± .94‡ | 1.97 ±1.21‡ | 1.36 ± .36‡ | .88 ± .32‡ | 37.4 ± 8.2‡ |
| LB–SH | 12 | 4.64 ±1.60 | 5.59 ±1.58 | 1.50 ± .32‡ | .98 ± .32‡ | 36.1 ± 7.9‡ |
| KC | 11 | .96 ± .22 | 3.45 ± .57 | 4.32 ± .88 | 2.72 ± .86 | 38.4 ± 7.4 |
| KC–SH | 11 | .90 ± .26 | 3.28 ± .52 | 4.19 ± .73 | 2.85 ± .72 | 32.5 ± 5.9 |
| KB | 12 | .84 ± .42 | 2.72 ± .82‡ | 6.35 ±1.30‡ | 4.64 ±1.25‡ | 26.6 ± 4.8‡ |
| KB–SH | 12 | 1.38 ± .58 | 3.93 ± .93 | 6.22 ±1.64‡ | 4.64 ±1.66‡ | 26.8 ± 6.9‡ |

* Mice were injected at times before sacrifice as follows: (a) at 12-15 hr, IM, sesame oil into C and C–SH mice; bromobenzene in sesame oil into B and B–SH mice; (b) at 85 min, IP, 1 mmol/kg, L-alanine into C and B mice; L-cysteine into C–SH and B–SH mice; (c) at 25 min, IV, 10 $\mu$C and 50 $\mu$g per kg, insulin-$I^{131}$, ALL mice. L = liver, K = kidney.

† "GSH" by modified method of Thompson and Watson (see text); total NPSH by (10). For *T*, *I* and % $I^{131}$ TCA soluble see Table I footnotes.

‡ Where $P < .05$, difference between paired B and C, or B–SH and C–SH groups.

kidney, of the bromobenzene treated mouse, changes $I^{131}$ of labeled insulin into TCA soluble form at a slow rate, and good blood flow brings additional labeled insulin to this organ, actual increases in total and TCA insoluble radioactivity may occur, at least for a time.

III. *Effects of L-cysteine administration:* If bromobenzene impairment of the ability of mouse liver and kidney to make $I^{131}$ of labeled insulin TCA soluble were due primarily to decrease in GSH concentration, then tissues of mice with GSH previously depleted by use of bromobenzene, but with GSH repleted by use of L-cysteine, should NOT exhibit impairment of their ability to convert $I^{131}$ into TCA soluble form. An experiment was therefore performed which gave the data of Table II. Four groups of mice were deprived of food and injected IM at 9 PM. Control Groups C and C-SH received sesame oil, test groups B and B-SH received bromobenzene in oil. The next morning, 85 minutes before sacrifice, mice of Groups C and B were injected IP with L-alanine, $10^{-3}$ moles/kg, while mice of Groups C-SH and B-SH were injected IP with L-cysteine, $10^{-3}$ moles/kg. Twenty-five minutes before sacrifice, each mouse of all 4 groups was injected by tail vein with Insulin-$I^{131}$.

Data obtained are shown in Table II. From intercomparisons of the NPSH data of this Table it may be concluded that: (a) bromobenzene pretreatment resulted in marked depletion of liver "GSH" and total NPSH, moderate depletion of kidney "GSH" and total NPSH, (b) NPSH depletions induced in liver and kidney by use of bromobenzene plus fasting were more than nullified by administration of L-cysteine, $10^{-3}$ moles/kg, 85 minutes before sacrifice, (c) as expected from the literature, practically all of the liver NPSH consisted of "GSH," and (d) "GSH" constituted less than half of kidney NPSH. In other experiments we have found that mouse and rat kidneys contain sufficient Sullivan reacting material (L-cysteine, L-cysteinylglycine and their disulfides(13)) to more than account for the non-"GSH" of mouse tissue NPSH.

From intercomparison of Table II data on *T* and *I* concentration ratios, and *% $I^{131}$ TCA soluble values,* it may be concluded that: (a) as in the experiments of Table I, bromobenzene treatment resulted in impairment of ability of mouse liver and kidney to convert $I^{131}$ of labeled insulin into TCA soluble form, (b) this impairment was not reversed by administration of L-cysteine in amount sufficient to bring about an actual overrepletion of the previously depleted "GSH" of these 2 tissues.

IV. *In vitro experiments:* These experiments were carried out in the hope that by simplifying experimental conditions, it might be possible to obtain more clear cut conclu-

TABLE III. Bromobenzene (B) or Benzyl Chloride (X) Pretreatment on *in vitro* Tissue Degradation of Insulin-$I^{131}$.

| Exp* | Group of tissues analyzed† | No. of mice in group | Mean values ± standard deviations‡ NPSH (meq S/kg) "GSH" | NPSH (meq S/kg) Total NPSH | $I^{131}$ concentration ratios *T* | *I* | % $I^{131}$ TCA soluble |
|---|---|---|---|---|---|---|---|
| 1 | LC5–0 | 10 | 5.22 ±1.06 | 4.53 ± .79 | 5.43 ± .76 | 4.42 ± .68 | 20.7 ± 6.2 |
| | LC5–20 | " | 4.63 ±1.01 | 4.58 ± .65 | 5.48 ±2.16 | 1.77 ± .24 | 67.5 ± 3.0 |
| | LB5–0 | 10 | 2.02 ±1.13§ | 1.49 ±1.20§ | 5.19 ± .98 | 4.70 ± .93 | 9.6 ± 4.2§ |
| | LB5–20 | " | 1.71 ± .92§ | 1.46 ±1.20§ | 5.17 ± .94 | 2.38 ± .53 | 54.1 ± 5.2§ |
| | KC5–0 | 10 | 1.47 ± .25 | 3.28 ± .37 | 6.88 ±1.04 | 6.17 ± .89 | 10.2 ± .5 |
| | KC5–20 | " | 1.05 ± .21 | 3.28 ± .70 | 7.11 ±1.21 | 5.29 ± .98 | 25.5 ± 2.1 |
| | KB5–0 | 10 | 1.22 ± .60 | 3.17 ±1.44 | 7.75 ±2.07 | 7.28 ±2.07 | 6.8 ± 2.4§ |
| | KB5–20 | " | .64 ± .21§ | 2.21 ±1.29§ | 8.12 ±1.99 | 6.63 ±1.69 | 18.4 ± 3.2§ |
| 2 | LC2–0 | 11 | 4.01 ± .54 | | 5.45 ± .57 | 5.10 ± .53 | 6.4 ± 1.2 |
| | LC2–10 | " | 3.24 ± .53 | | 5.17 ± .56 | 3.60 ± .39 | 30.4 ± 3.5 |
| | LB2–0 | 9 | 1.62 ± .53§ | | 4.48 ± .45§ | 4.20 ± .43§ | 6.3 ± .8 |
| | LB2–10 | " | 1.22 ± .81§ | | 4.36 ± .45§ | 3.21 ± .35§ | 26.0 ± 5.9§ |
| 3 | LC2–0 | 7 | 4.83 ±1.50 | | 4.84 ± .92 | 4.56 ± .83 | 5.9 ± 1.4 |
| | LC2–10 | " | 4.07 ±1.57 | | 4.84 ± .92 | 3.28 ± .77 | 32.5 ± 5.7 |
| | LX2–0 | 8 | 2.48 ± .77§ | | 4.61 ±1.20 | 4.33 ±1.09 | 6.0 ± .9 |
| | LX2–10 | " | 1.78 ± .73§ | | 4.54 ±1.16 | 3.05 ± .90 | 32.3 ± 5.8 |

* Insulin-$I^{131}$ contained per ml 2 $\mu$C $I^{131}$ and total insulin: Exp 2, 1 $\mu$g; Exp 1 & 3, 10 $\mu$g.

† L = liver, K = kidney; C = control, B = bromobenzene, X = benzyl chloride injected mice; No. after C, B or X = min between insulin-$I^{131}$ injection and sacrifice; 0 after dash indicates tissue samples frozen immediately on sacrifice; 10 or 20 after dash indicates tissue samples incubated at 37°C for 10 or 20 min before freezing.

‡ See footnotes, Tables I and II.

§ $P < .05$, difference between paired C and B, or C and X groups.

sions than can be obtained by use of whole animals. The general procedure was to inject a mouse, control or test, with Insulin-$I^{131}$, wait 2 or 5 minutes, sacrifice, rapidly excise the liver and kidneys, dry ice freeze half the liver and one kidney immediately, and incubate the other half of the liver and the other kidney on Ringer moistened filter paper inside a stoppered glass vial which was held in a shaking water bath at 37°C. After 10 or 20 minutes' incubation, the other half of the liver and the other kidney were frozen. Data obtained are summarized in Table III.

As was expected, *T* values obtained for each pair of liver halves, or for each pair of kidneys excised from a given mouse, were throughout the experiments reasonably close to each other. Hence, by use of an *in vitro* procedure, we have been able to eliminate the considerable changes in total $I^{131}$ concentrations which occur within particular tissues of whole animals with passage of time, and thus to focus on mean *% $I^{131}$ TCA soluble* values as criteria of possible effects of an experimental procedure on metabolic degradation of labeled insulin. The data obtained indicate clearly that the incubation conditions used did not prevent mouse kidney, and liver especially, from converting $I^{131}$ of labeled insulin into TCA soluble form rapidly.

In the bromobenzene experiments (1 and 2 of Table III), test mice exhibited decreased liver NPSH values and impaired ability in both liver and kidney to convert $I^{131}$ of labeled insulin into TCA soluble form. However, the data suggest that most of bromobenzene action on ability of liver and kidney to solubilize the $I^{131}$ may have been due to changes which occurred in these tissues prior to the incubation periods. In Exp. 2, in which mice were sacrificed only 2 minutes after i.v. injection of Insulin-$I^{131}$, appreciably lower *T* values were obtained for livers of test than of control mice, suggesting that bromobenzene treatment may result in slowed fixation of insulin, as well as decreased ability to convert $I^{131}$ of labeled insulin into TCA soluble form. In Exp. 3 of Table III, benzyl chloride, given 2 hours before sacrifice, was substituted for bromobenzene as the tissue

NPSH depleting agent. From the data obtained it is apparent that benzyl chloride as used induced significant decreases in mouse liver "GSH" but had no measurable effect on ability of liver to solubilize $I^{131}$ of labeled insulin.

*Discussion.* Stoichiometric considerations suggest that, no matter how essential GSH may be for *in vivo* degradation of insulin, physiologic alterations of insulin degradation rate are not likely to be due in any appreciable degree to alterations in intracellular GSH concentration. In the present experiments, wide departures from usual tissue insulin-GSH ratios were achieved by combination of high insulin dosage and marked lowering of intracellular GSH concentration, obtained by use of bromobenzene or benzyl chloride. With such high insulin dosage, and using increase in TCA solubility of $I^{131}$ of labeled insulin as criterion of insulin degradation(6), we found that mice pretreated with bromobenzene exhibited significant decreases in ability of liver and kidney to degrade insulin. However, other data indicate that this bromobenzene effect is due to some action of bromobenzene other than its action in lowering intracellular GSH concentration: (a) L-cysteine administration resulted in rapid repletion of the bromobenzene depleted GSH, but not in reversal of the bromobenzene induced inhibition of insulin degradation, and (b) benzyl chloride induced much more rapid depletion of GSH than can be obtained using bromobenzene, without measurably affecting insulin degradation. Hence it seems quite unlikely that physiologic variations in intracellular GSH concentration result in significant variations in insulin degradation rate.

*Summary.* 1) The radioactivity of $I^{131}$ labeled insulin injected by tail vein into mice was rapidly concentrated by liver and kidney, and converted by these organs from TCA insoluble into TCA soluble form. 2) Overnight fasting plus bromobenzene administration resulted in significant decreases in: (a) liver and kidney non-protein sulfhydryl (reduced glutathione) concentrations, and (b) abilities of these organs to convert radioactivity of administered labeled insulin from TCA insoluble into TCA soluble form. 3) Rapid repletion of liver and kidney GSH, previously depleted by use of bromobenzene, was obtained by use of L-cysteine. The accompanying bromobenzene induced impairment of mouse tissue abilities to solubilize radioactivity of labeled insulin was not restored by L-cysteine administration. 4) Benzyl chloride administration to overnight fasted mice resulted within 2 hours in marked decrease in liver GSH concentration, not accompanied by measurable change in insulin degradation. 5) It therefore appears unlikely that insulin degradation rate varies significantly as result of physiologic variations in intracellular GSH concentrations.

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