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Interaction of Insulin Analogs, Glucagon, Growth Hormone, Vasopressin, Oxytocin, and Scrambled Forms of Ribonuclease and Lysozyme with Glutathione–Insulin Transhydrogenase (Thiol:Protein-Disulfide Oxidoreductase): Dependence upon Conformation†

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ABSTRACT: Interactions of several proteins with glutathione–insulin transhydrogenase (GIT) have been investigated by determining their ability to inhibit degradation of ^{125}I-labeled insulin catalyzed by GIT. The inhibition by every insulin analog (des-Asn-des-Ala-pork insulin, desoctapeptide-pork insulin, des-Ala-pork insulin, pork insulin, proinsulin, and guinea pig insulin) was competitive vs. insulin indicating that they function as alternate substrates. The insulin analogs with the least hormonal activity showed the highest potency as inhibitors of insulin degradation. Whereas native ribonuclease and lysozyme showed little or no inhibition, their scrambled forms (i.e., reduced and randomly reoxidized) showed competitive inhibition with a potency greater than that of insulin. These results suggest that the conformation of the substrate or inhibitor is probably the major factor in determining the specificity for (or binding to) the enzyme. Studies with other peptide hormones showed competitive inhibition with vasopressin and oxytocin and noncompetitive inhibition with glucagon. The inhibition with growth hormone could be either competitive or noncompetitive. The inhibition by glucagon and growth hormone (physiologic antagonists of insulin) could serve as a control mechanism to modulate the activity of enzyme. The following showed very little or no inhibition: the native and scrambled form of pepsinogen, trypsin inhibitor of beef pancreas and of lima bean, C-peptide of pork proinsulin, and heptapeptide (B_{23}–B_{29}) of insulin.

In a companion publication we reported (Chandler and Varandani, 1975) on the kinetic mechanism of insulin degradation by glutathione–insulin transhydrogenase (GIT,[1] thiol:protein-disulfide oxidoreductase, EC 1.8.4.2). In addition to degrading insulin, GIT under appropriate in vitro conditions is capable of performing other activities; i.e., the inactivation of proinsulin (Varandani and Nafz, 1970), the reactivation of reduced (Katzen and Tietze, 1966) and scrambled (i.e., reduced and randomly reoxidized) (Ansorge et al., 1973; Varandani, 1974b) ribonuclease, the reactivation of scrambled proinsulin (Varandani and Nafz, 1970), the reactivation of reduced insulin (Katzen et al., 1963), the regeneration of insulin from reduced A and B chains (Varandani, 1967), and the reduction of insulin derivatives (Katzen and Tietze, 1966) and of oxytocin and vasopressin (Katzen and Stetten, 1962).

In the present paper, inhibition of GIT-catalyzed insulin degradation by several proteins, which are insulin analogs

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[1] Abbreviation used is: GIT, glutathione–insulin transhydrogenase. As noted in the accompanying paper, we have retained the designation glutathione–insulin transhydrogenase for consistency with previous publications.

with variable hormonal activities, other polypeptide hormones, or other disulfide-containing proteins was studied under identical experimental conditions. The objectives were to determine (a) whether the enzyme has greater specificity for any of the protein substrates, (b) whether the ability of GIT to degrade insulin derivatives correlates with the derivative's hormonal activity, and (c) whether other polypeptide hormones are able to modulate the activity of GIT.

Materials and Methods

The preparation of beef pancreatic GIT and biologically active [^{125}I]insulin (containing <1 atom of iodine/mole) has been described (Chandler and Varandani, 1975). Ribonuclease (3100 units/mg), lysozyme (LY633 two times crystallized), pepsinogen (3800 units/mg), beef pancreatic, and lima bean trypsin inhibitors (1 mg inhibits 1.5 mg of trypsin) were purchased from Worthington; synthetic oxytocin (16 USP Units/mg) was from Sigma; synthetic lysine-vasopressin (119 U/mg) was from Sigma and vasopressin (beef-pork mixture) (100 U/mg) was from Mann Schwarz. The following proteins were gifts: proinsulin, insulin, des-Ala insulin, and glucagon from Drs. Ronald Chance and William Bromer of the Lilly Company; des-Asn-des-Ala-pork insulin and desoctapeptide-pork insulin (Brugman and Arquilla, 1973) from Drs. Tom Brugman and Edward R. Arquilla; guinea pig insulin (Yip, 1972) from Dr. C. C. Yip; synthetic heptapeptide (B_{23}–B_{29}) of insulin from Dr. Fred Carpenter; C-peptide of pork proinsulin from Dr. J. Schlichtkrul of Novo Company; and bovine growth hormone (NIH-GH-B1003A DEAE-cellulose chromatographically pure, 1.3 IU/mg) from Dr. A. E. Wilhelmi through the NIAMDD Hormone Distribution Program.

Preparation of "Scrambled" (Reduced and Randomly Reoxidized) Proteins. These were carried out essentially as previously described (Crestfield et al., 1963; Varandani, 1966b). Ribonuclease, lysozyme, and pepsinogen were reduced with 0.1 *M* mercaptoethanol or mercaptoethylamine in 8 *M* urea and then randomly reoxidized with dehydroascorbic acid (Givol et al., 1964). The reagents were removed by extensive dialysis. It was established by determining the sulfhydryl content (Ellman, 1959) that each protein was fully reduced prior to the addition of dehydroascorbic acid and was fully reoxidized subsequent to the addition.

Procedure for Inhibition of GIT Activity. This was measured by the conversion of [^{125}I]insulin to a form soluble in 5% trichloroacetic acid under conditions showing zero-order kinetics (cf. Chandler and Varandani, 1975). All studies were carried out in 0.1 *M* potassium phosphate–5 m*M* EDTA–0.3% bovine serum albumin (final pH 7.5) containing 1 m*M* GSH. A mixture of a constant tracer amount of [^{125}I]insulin, unlabeled insulin (seven or eight levels, giving final insulin concentrations from 0.021 to 33.3 μM), GIT (0.5 μg), and several fixed levels of test proteins was incubated for 5 min at 37° in a total volume of 0.45 ml of the buffer. The reaction was initiated by the addition of 0.5 μmol of GSH in 0.05 ml and was terminated after 5 min by the addition of 0.5 ml of 10% trichloroacetic acid. The precipitates were collected by centrifugation and washed once as previously described. All GIT activities reported have been corrected for the nonenzymatic insulin degradation which was determined by running matched control tubes with GIT omitted at every concentration of insulin. The nonenzymatic insulin degradation by GSH ranged between 0.3 and 0.5%/min; the addition of test proteins did not produce any detectable effect on the nonenzymatic insulin degradation.

Analysis of Kinetic Data. Velocities are expressed as picomoles of substrate (insulin) converted per minute per microgram of enzyme protein. Duplicate determinations were made for each experimental point. All experimental results shown in double reciprocal form were obtained by fitting the data with the relevant equation by a nonlinear least-squares method as described previously (Chandler and Varandani, 1975). The final values of the kinetic constants were obtained by fitting the data from each experiment to the appropriate rate equation. Data conforming to a competitive inhibition pattern were fit to both eq II and IV in order to distinguish competitive inhibition from a case of noncompetitive inhibition with a crossover point close to the vertical axis. None of the data conformed to an uncompetitive inhibition pattern by visual inspection; this was confirmed by fitting the data to eq III in order to distinguish uncompetitive inhibition from noncompetitive inhibition with a crossover point far to the left of the vertical axis. The fit yielding the lowest residual mean square error was chosen as the form best describing the inhibition data when the difference was significant at the $p < 0.05$ level by the F-test. In some cases the choice of the best model was determined by convergence of the solution to a very high value for one constant, i.e., convergence of eq IV to a high value for K_{ii} indicates that eq II is a better model. See Cleland (1963) for the definitions of types of inhibition and kinetic constants.

$$v = VS/(K + S) \quad \text{(I)}$$

$$v = \frac{VS}{K[1 + (I/K_{is})] + A} \quad \text{(II)}$$

$$v = \frac{VA}{K + [1 + (I/K_{ii})]A} \quad \text{(III)}$$

$$v = \frac{VA}{K[1 + (I/K_{is})] + [1 + (I/K_{ii})]A} \quad \text{(IV)}$$

Activation of Scrambled RNase. The scrambled RNase (2.9 μM) and 1 m*M* GSH in phoshate–EDTA–bovine serum albumin buffer were incubated at 37° in the presence and absence of GIT. The reaction was stopped by the addition of *N*-ethylmaleimide (10 m*M*, final concentration). RNase activity was assayed by measuring the rate of digestion of yeast RNA in 0.1 *M* acetate buffer (pH 5.0) (Anfinsen et al., 1954). Control experiments established that GIT itself is devoid of RNase activity and that the *N*-ethylmaleimide at the concentrations employed does not interfere with the RNase activity assay.

Results

Inhibition by Insulin Derivatives. Structure–activity relationship studies have shown that the hormonal activity of insulin derivatives is related to conformational changes in the insulin molecule (see Blundell et al., 1971 for review). Therefore, the possibility that the ability of GIT to degrade an insulin derivative might be related to the derivative's hormonal activity was examined by determining the inhibition by various insulin and insulin analogs of the degradation of ^{125}I-labeled pork insulin catalyzed by GIT. The kinetics of inhibition with one insulin analog are shown for illustration in Figure 1; data obtained with all analogs tested are summarized in Table I. As expected for alternative substrates (Katzen and Tietze, 1966), the inhibition by every analog was competitive vs. insulin. Inspection of the inhibi-

Table I: Inhibition Constants for Inhibitors of Insulin Degradation Catalyzed by GSH–Insulin Transhydrogenase.

Agent	K_{IS}[a] (μM)	K_{II}[a] (μM)	Type of Inhibition[a]
Pork insulin	8.24 ± 0.43		Competitive
Des-Ala-pork insulin	6.78 ± 0.38		Competitive
Des-Asn-des-Ala-pork insulin	3.62 ± 0.13		Competitive
Desoctapeptide-pork insulin	4.72 ± 0.22		Competitive
Guinea pig insulin	5.04 ± 0.21		Competitive
Beef proinsulin	6.01 ± 0.24		Competitive
Glucagon (pork)	14.0 ± 0.6	17.7 ± 3.7	Noncompetitive
Vasopressin (synthetic)	20.3 ± 1.2		Competitive
Vasopressin (pork + beef)	19.8 ± 1.2		Competitive
Oxytocin (synthetic)	19.9 ± 1.3		Competitive
Growth hormone (bovine)	7.2 ± 0.4		Competitive
	7.8 ± 0.4	14.4 ± 4.4	Noncompetitive
Scrambled ribonuclease	3.00 ± 0.16		Competitive
Scrambled lysozyme	2.42 ± 0.26		Competitive

[a] See Cleland (1963) for the definition of kinetic constants and types of inhibition.

tion constant values shows that the analogs which have the least biological activity have the highest potency (i.e., the lowest K_I values) as inhibitors of [^{125}I]insulin degradation. If the degree of inhibition corresponds to the relative rate of degradation of the analogs, these results could be interpreted to indicate that insulin derivatives show lower hormonal activities because of more rapid inactivation.

The heptapeptide (B_{23}–B_{29}) at the highest concentration tested (74 μM) produced slight inhibition; this was evident only at an insulin concentration of 10^{-8} M. The C-peptide (48 μM) caused no inhibition at any insulin concentration tested.

Inhibition by Other Polypeptide Hormones. The kinetics of inhibition of GIT by five other polypeptide hormones was studied. Both pork glucagon and beef growth hormones, which are known to modulate the physiological action of insulin, inhibited the GIT activity appreciably, although as seen from the relative K_I values (Table I) the potency of each of these hormones as an inhibitor was less than that shown by the insulin analogs. Inhibition data obtained with pork glucagon are shown in Figure 2; it is of interest to note that this hormone, which contains no disulfide bonds, showed a noncompetitive type of inhibition. The inhibition data for growth hormone, which contains two disulfide bonds, could be fitted equally well by the competitive and noncompetitive equations; the data failed to distinguish clearly between the two mechanisms and, thus, it might be a "mixed" type inhibition.

Experiments with ACTH showed that this hormone, which lacks a disulfide bond, also inhibited insulin degradation by GIT. However, after the completion of experiments it was learned from the supplier that the preparation used was not pure. Thus, the results obtained with this hormone should be regarded as tentative.

Vasopressin and oxytocin, each of which contains one disulfide bond and an eight amino acid ring similar to that present in the A chain of insulin, were competitive inhibitors with similar K_I values; they showed the lowest potency of the five hormones tested (Table I). The competitive type of inhibition indicates that these two hormones probably function as alternative substrates, in keeping with the findings of Katzen and Stetten (1962).

Inhibition by Scrambled and Native Forms of Ribonuclease, Lysozyme, and Pepsinogen. Previously, Katzen and Tietze (1966) have shown that GIT of beef liver catalyzes the reactivation of reduced RNase; more recently Ansorge et al. (1973) reported the reactivation of scrambled RNase by rat liver GIT. We have previously (Varandani, 1973c) noted similar results using beef pancreatic GIT and scrambled RNase. Using the same experimental conditions, all our attempts to demonstrate the inactivation of native RNase by GIT yielded negative results.

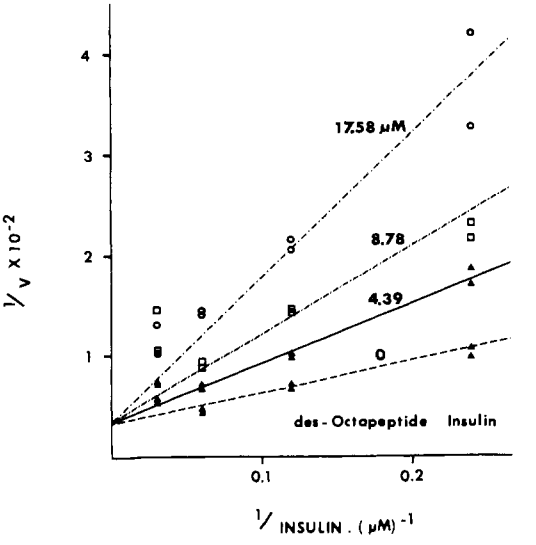

FIGURE 1: Inhibition by desoctapeptide pork insulin of GIT-catalyzed [^{125}I]insulin degradation. The ratio of the amount of insulin degraded enzymatically to that degraded nonenzymatically remained reasonably constant between 3.02 and 4.11 in the absence of the inhibitor. As noted in the text, the insulin concentration was varied from 0.021 to 33.3 μM. When the data for the complete range are shown, the effect of the inhibitor on the intercept at the y axis cannot be seen because of scale problems. Therefore, only a portion of the insulin concentrations used are shown in the figure.

The kinetics of inhibition of GIT-catalyzed insulin degradation by scrambled RNase (Table I) showed that scrambled RNase acted as a competitive inhibitor of insulin degradation with a K_I value much lower than that obtained with insulin. The competitive nature of the inhibition indicates that both insulin and scrambled RNase probably interact with the same binding site(s) on the enzyme.

As noted above, GIT was unable to inactivate native RNase as determined by measurement of RNase activity. Consistent with these findings, native RNase showed almost no inhibition of insulin degradation by GIT. Slight inhibition of insulin degradation by native RNase (highest

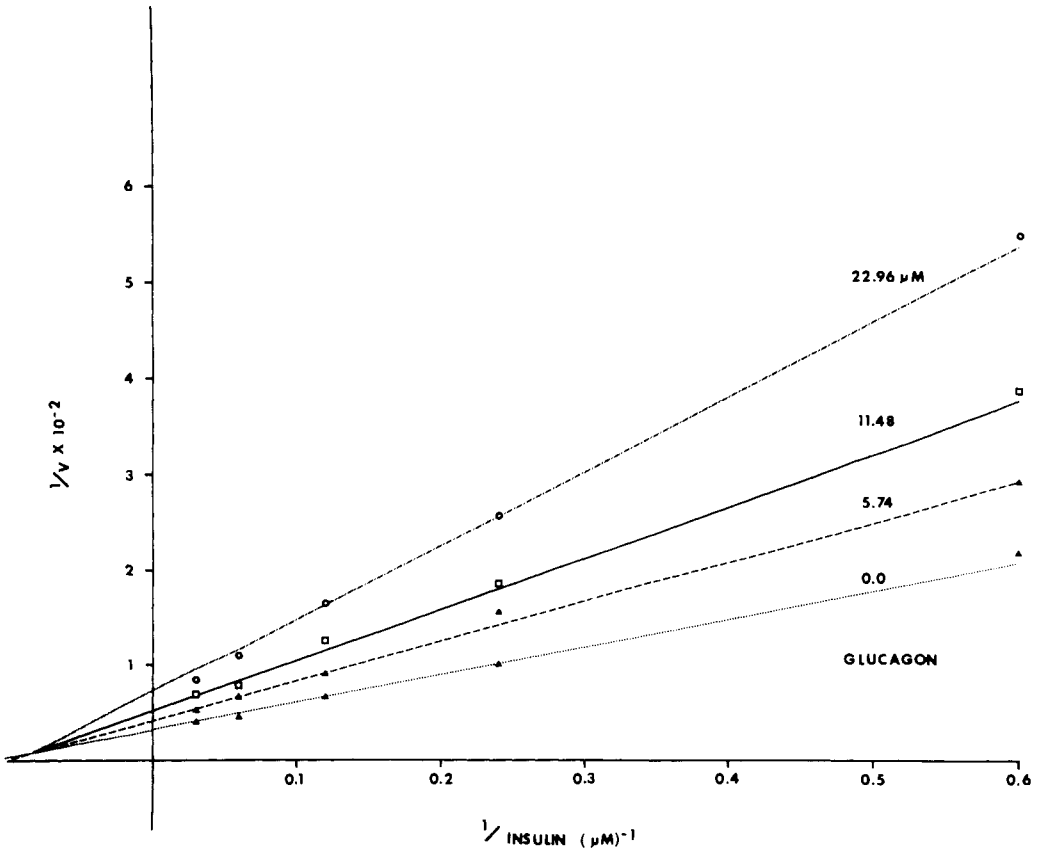

FIGURE 2: Inhibition by glucagon of GIT-catalyzed [^{125}I]insulin degradation. Only a portion of the insulin concentrations used are shown in the figure; see the legend to Figure 1 for explanation.

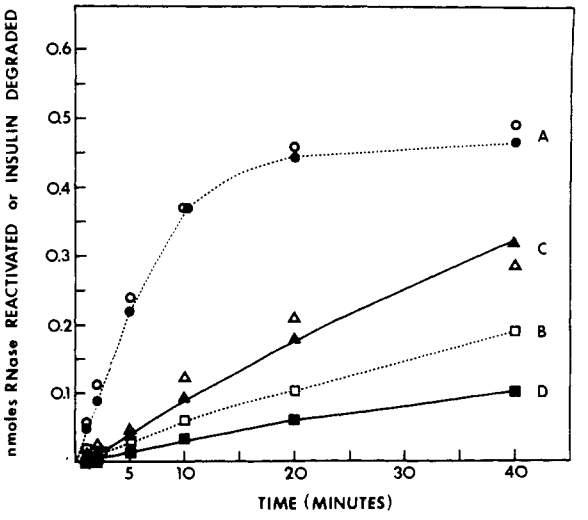

FIGURE 3: Rates of catalysis by GIT of activation of scrambled ribonuclease and degradation of insulin present in the same assay tubes. Scrambled ribonuclease and [^{125}I]insulin (2.9 μM of each) in the same tubes were incubated in 0.5 ml with 1 mM GSH and 2 μg of GIT at 37°. Aliquots were withdrawn at the indicated time periods and reaction was stopped by the addition of N-ethylmaleimide (10 mM, final concentration); ribonuclease activity and insulin degrading activity were assayed as described in the text. Matched control tubes with GIT omitted were run throughout for nonenzymatic reaction. Since neither scrambled RNase nor insulin influenced the nonenzymatic rates of insulin degradation and ribonuclease reactivation, respectively, nonenzymatic rate of each is represented by a single symbol. Insulin degradation: (A) insulin alone + GIT, (O- - -O); insulin + scrambled ribonuclease together + GIT (●- - -●); (B) nonenzymatic insulin degradation (□- - -□). Ribonuclease reactivation: (C) scrambled ribonuclease alone + GIT (△—△); scrambled ribonuclease + insulin together + GIT (▲—▲); (D) nonenzymatic ribonuclease reactivation (■—■).

concentration tested, 24 μM) could be observed only when concentrations of insulin used were 10^{-8} M.

Likewise, whereas the scrambled form of lysozyme was found to inhibit insulin degradation with a K_I value less than that obtained with insulin, native lysozyme (highest concentration tested, 46 μM) only slightly inhibited the activity of GIT. Again inhibition by native lysozyme could be observed only at insulin concentrations of 10^{-8} M.

In contrast to ribonuclease and lysozyme, although native pepsinogen (30 μM) showed no inhibition of insulin degradation by GIT the scrambled form (17 μM) showed only slight inhibition at 10^{-8} M concentration of insulin. Neither trypsin inhibitor from beef pancreas (15 μM) nor from lima bean (16 μM) inhibited insulin degradation by GIT.

Thus, it would appear from the inhibition data that some altered proteins, i.e., insulin analogs, scrambled ribonuclease, and scrambled lysozyme, are preferred substrates compared to native insulin. However, insulin is the preferred substrate of the naturally occurring polypeptides, i.e., insulin, vasopressin, and oxytocin.

Since GIT is able to degrade insulin as well as reactivate inactive forms of RNase, it was of interest to determine whether GIT has greater specificity for the degradation of insulin or for the reactivation of scrambled RNase. Equimolar quantities of insulin and scrambled RNase contained in the same tube were incubated with GIT in the presence of 1 mM GSH, and the degradation of insulin and the reactivation of RNase were measured as a function of time (Figure 3). For these experiments it was necessary to increase the amount of GIT used to 4 μg/ml in order to obtain measurable rates of RNase reactivation. During the linear portion of the reaction period, net enzymatic insulin

degradation was from 3.4 to 6.4 times faster than net ribonuclease activation, indicating that this increased rate of insulin degradation is probably not because insulin contains fewer disulfide bonds (three as compared to four in ribonuclease), since this difference could account for only a 33% difference in rate. Thus while GIT promotes insulin degradation at a greater rate than the rate at which it promotes the reactivation of RNase, scrambled RNase was nonetheless a more potent inhibitor in insulin degradation than was additional insulin (used as an index of comparison). This difference might be because these experiments were performed under non-steady-state kinetic conditions (i.e., due to the use of relatively high concentration of enzyme), and/or possibly because of the involvement of multiple sites on the enzyme and a complex mechanism of action for GIT (Chandler and Varandani, 1975).

Discussion

The finding of a close inverse relation between the ability of insulin analogs to inhibit GIT activity and the hormonal activity of the analogs, together with the marked differences in inhibition between native and scrambled forms of several proteins, demonstrate that the conformation of the substrate or inhibitor is a major factor in determining the specificity or degree of binding to the enzyme. In other words, the accessibility of the enzyme to the disulfide bonds of the substrate may be a limiting factor in determining the specificity of substrate for GIT. In the case of scrambled proteins, which are in thermodynamic unstable forms, the enzyme apparently causes the random reshuffling of disulfide bonds either until they can no longer be reached because of steric factors or until no further decrease in the net conformational free energy can be brought about. In the case of proteins containing relatively unstable disulfide bonds such as insulin, insulin analogs, and proinsulin, GIT promotes the formation of chain fragments (e.g., A chain) and an inactive cross-linked chain polymer (e.g., B chain rich-A chain aggregate). Each product, including B chain rich-A chain aggregate, can be formed spontaneously; GIT accelerates this process in each case. Thus, the nature of the final product of the reshuffling of disulfide bonds is a characteristic of the substrate rather than of GIT. Some investigators feel that the enzyme promotes inactivation in the case of multichain proteins and activation in the case of single chain proteins. This is not strictly true, however, since the enzyme is able to inactivate a single chain molecule such as proinsulin.[2]

The current studies indicate that insulin is probably the preferred substrate, at least of the naturally occurring substrates. The studies with insulinoma tissue (Varandani, 1974c) suggest that the function of GIT, whether to perform the degradation of insulin or the synthesis of proteins, may be dependent upon the relative amounts (in a given tissue) of GIT and the proteases capable of hydrolyzing further the product of GIT action. It has been demonstrated that hepatic GIT concentration in the rat is under feedback control by blood insulin (Varandani et al., 1972; Thomas and Wakefield, 1973; Varandani, 1974a); the adaptive changes in the amount of GIT protein brought about by changes in blood insulin in response to chronic conditions (such as prolonged starvation and overt diabetes) probably represent a "coarse control" with a long time constant. The inhibition of the activity of available GIT by glucagon and growth hormone (which are physiological antagonists of insulin) might represent a rapid, "fine control" mechanism during acute metabolic changes, since a regulatory mechanism might be expected to adjust the rate of insulin degradation to changing rates of insulin secretion and insulin needs. Utility of a decrease in insulin degradation brought about by hormones antagonist to insulin is apparent if GIT functions to promote the action of insulin as well as degradation (Chandler and Varandani, 1972, 1973); as another example, in a situation of starvation, where glucagon levels are known to be elevated, inhibition of GIT by glucagon would spare essential minimal levels of insulin needed for its action in protein synthesis and lipolysis. While the in vivo functionality and prevalence of the interactions noted here remains to be determined, such a modulation of GIT by glucagon is consistent with the recent finding that the insulin–glucagon molar ratio may be a critical factor in the regulation of glucose metabolism (Unger, 1971). In addition to the above, several other mechanisms that might possibly control the amount of functional GIT have been previously reported (Varandani, 1973c,d, 1974c). The finding of several controls over GIT activity is consistent with the necessary initial action of GIT in the pathway of insulin metabolism (Varandani, 1972, 1973a,b).

[2] In fact, part of the rationale which contributed to the discovery of proinsulin was that the single polypeptide chain of proinsulin could serve to facilitate the formation of the native structure of the two-chain insulin molecule by ensuring a correct and efficient pairing of cysteine residues during biosynthesis (Steiner, 1967; Steiner and Clark, 1968). It is, however, now recognized that this precursor processing of low molecular weight proteins is not restricted to disulfide-containing proteins, since molecules like glucagon and ACTH (which are devoid of disulfide bonds) also seem to be synthesized via large precursors (Rigopoulon et al., 1970; Yalow and Berson, 1971).

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Characterization by Mass Spectrometry of Blood Group A Active Glycolipids from Human and Dog Small Intestine†

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ABSTRACT: Glycolipids with blood group A activity isolated from human and dog small intestine have been characterized by mass spectrometry of intact lipid in methylated and in methylated and reduced ($LiAlH_4$) form. Without degradative studies the glycolipids were conclusively shown to be hexaglycosylceramides with phytosphingosine as the major long-chain base and hydroxypalmitic acid as the major fatty acid. The exact sugar ratio was hexose–hexosamine–deoxyhexose 3:2:1 and the sequence established as hexosamine–[deoxyhexose–]hexose–hexosamine–hexose–hexose–ceramide. Evidence is presented that mass spectrometry can differentiate between type 1 and type 2 saccharide chains.

A blood group A active hexaglycosylceramide was indicated several years ago in human erythrocytes (Hakomori and Strycharz, 1968), and the glycolipid nature of cellular ABH antigens has been known for a long time (see summary by Hakomori and Strycharz, 1968). Although the structure of the immunological determinants should bear resemblance to those of blood group active secreted glycoproteins (Watkins, 1972; Rovis et al., 1973), no conclusive structure of human A active glycolipids has so far been presented (Hakomori et al., 1972), and an A active glycolipid of hog gastric mucosa was proposed to be a heptaglycosylceramide (Slomiany and Horowitz, 1973; Slomiany et al., 1974). Recently, however, strong evidence for the structure of one of the basic H active structures of human erythrocytes has been presented as L-fucopyranosyl-α-(1→2)-galactopyranosyl-β-(1→4)-*N*-acetylglucosaminosyl-β-(1→3)-galactopyranosyl-β-(1→4)-glucopyranosyl-β-(1→1)-ceramide (Stellner et al., 1973; Kościelak et al., 1973). On the other hand, fucose attachment to glucosamine was reported for A active fucolipids from human erythrocytes (Kościelak et al., 1970).

In the present investigation blood group A active glycolipids isolated from human and dog small intestine have been characterized. By a novel mass spectrometric method for intact glycolipids (Karlsson, 1973) the carbohydrate composition and sequence of the lipids were conclusively established.

Materials and Methods

The methods of isolation and immunological characterization of the glycolipids have been described elsewhere (Vance et al., 1966; McKibbin, 1969; Smith and McKibbin, 1972; Hiramoto et al., 1973; Smith et al., 1973). The human glycolipid was identical with sample Hu-3F-1 of Hiramoto et al. (1973) and Smith et al. (1973), and the dog A active fractions were 15F-1, 20F-1, 26F-1, 29F-1, and

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