mation is provided by the results of chromatographic experiments. In these, proctolin-like bioactivity, without exception, elutes with authentic or [^{3}H]proctolin. Molecular sieving (Fig. 2B) shows that the bioactive component has a molecular size indistinguishable from that of proctolin (648.8 daltons). Proctolin-like bioactivity and authentic proctolin could not be separated in thin-layer chromatography with four different solvent systems (Table 1). Moreover, in reverse-phase (C_{18}) HPLC, extract bioactivity, [^{3}H]proctolin, and authentic proctolin were not separated (Fig. 2C).

The LW cell is the first to be associated with proctolin. In view of their accessibility for intracellular electrophysiological studies, the LW neuron and other neurons in the insect central nervous system may provide a simple model in which to investigate the precise sites and modes of action of a putative peptide neurotransmitter. Furthermore, the highly selective staining with neutral red suggests the presence of a biogenic amine (*5*). The LW neuron may, therefore, also provide a preparation in which functional and anatomical implications of amine and peptide co-localization (*15*) can be studied in a well-characterized cellular system.

MICHAEL O'SHEA
Department of Pharmacological and Physiological Sciences, University of Chicago, Chicago, Illinois 60637
MICHAEL E. ADAMS
Max Planck Institut für Verhaltensphysiologie, Abteilung Huber, D–8131 Seewiesen, Federal Republic of Germany

References and Notes

1. T. Hökfelt, O. Johansson, A. Ljungdahl, J. M. Lundberg, M. Schultzberg, *Nature (London)* **284**, 515 (1980); S. H. Snyder, *Science* **209**, 976 (1980); P. Emson, *Prog. Neurobiol.* **13**, 61 (1979).
2. S. W. Kuffler and J. G. Nicholls, *From Neuron to Brain* (Sinauer, Sunderland, Mass., 1976); E. R. Kandel, *Cellular Basis of Behavior* (Freeman, San Francisco, 1976); G. Hoyle, Ed., *Identified Neurons and Behavior of Arthropods* (Plenum, New York, 1977); D. S. Faber and H. Korn, Eds., *Neurobiology of the Mauthner Cell* (Raven, New York, 1978).
3. Abbreviations: Arg, arginine; Tyr, tyrosine; Leu, leucine; Pro, proline; and Thr, threonine. B. E. Brown, *Life Sci.* **17**, 1241 (1975); A. N. Starratt and B. E. Brown, *ibid.*, p. 1253; B. E. Brown, *J. Insect Physiol.* **23**, 861 (1977).
4. In orthopterous insects, the anterior abdominal ganglia may be fused with the most posterior thoracic ganglion (metathoracic ganglion). The number of fused ganglia depends on the species. Our investigation has focused on the LW neurons of the unfused ganglia only. In the cockroach, *P. americana*, the LW cells are conspicuous only in the three most anterior unfused abdominal ganglia, whereas in the cricket, *G. bimaculatas*, they are seen in all unfused abdominal ganglia.
5. A. E. Stuart, A. S. Hudspeth, Z. W. Hall, *Cell Tissue Res.* **153**, 55 (1974); P. D. Evans and M. O'Shea, *Nature (London)* **270**, 257 (1977).
6. M. Partanen, A. Hervonen, A Vaalasti, L. Kanerva, H. Harvonen, *Cell Tissue Res.* **199**, 373 (1979); M. E. Adams and M. O'Shea, in preparation.
7. Distinctive anatomical and electrophysiological features include axon projections to the cardiac nervous system and body wall musculature, resting potentials of 60 to 80 mV, input resistance of about 80 megohms, and action potentials of 70 to 90 mV. The LW neurons receive a common pattern of excitatory synaptic input, suggesting normal recruitment as a single functional unit. (M. O'Shea and M. E. Adams, in preparation.)
8. T. Piek and P. Mantel, *J. Insect Physiol.* **23**, 321 (1977); T. Miller, *Am. Zool.* **19**, 77 (1979); B. J. Cook and G. M. Holman, *Comp. Biochem. Physiol.* **64C**, 21 (1979); T. L. Schwartz *et al.*, *J. Neurobiol.*, in press.
9. The rhythm is generated myogenically by a small bundle of muscle fibers that form part of the main extensor muscle of the locust (*Schistocerca nitens*) hindleg. The movement was monitored photoelectrically, and 0.5- or 1-μl test samples were applied directly to the myogenic fibers, which were bathed in 10 μl of physiological saline. The response was quantified by dividing the intercontraction interval before application of test samples by the interval achieved at the peak of the response. Since the effect of proctolin is to increase the frequency, this ratio is always greater than 1 (see scale of bioassay response in Fig. 2). The assay is insensitive to micromolar concentrations of the neuropeptides bombesin, enkephalin, neurotensin, somatostatin, and vasoactive intestinal peptide. No other known compounds produce proctolin-like effects in this assay.
10. The isotonic physiological saline contained 140 m*M* NaCl, 5 m*M* KCl, 5 m*M* $CaCl_2$, 4 m*M* $NaHCO_3$, 1 m*M* $MgCl_2$, 5 m*M* trehalose, 5 m*M* trimethylaminoethane sulfonic acid, 100 m*M* sucrose, at *p*H 7.2.
11. Bio-Gel columns were eluted with 10 percent formic acid; thin-layer chromatography plates (cellulose) were divided into 1-cm fractions, scraped, and eluted with 2*N* acetic acid (solvent systems are given in the text). For reverse-phase HPLC a μ-Bondapack C_{18} column (Waters Associates) and a single M45 pump (Waters) were used. The liquid phase was 12 percent acetonitrile in 50 m*M* ammonium acetate, *p*H 4.5, pumped at 2500 pounds per square inch and 1.3 ml/min. Thirty-second fractions were collected.
12. Authentic proctolin was purchased from Sigma. [^{3}H]Proctolin was prepared by catalytic conversion (New England Nuclear) of an I_2-Tyr^2 analog of proctolin synthesized in Prof. T. Kaiser's laboratory in the Department of Chemistry, University of Chicago, by B. DeGrado.
13. This was estimated by comparing the response in the bioassay to known concentrations of authentic proctolin delivered to the same preparation in the same volume. It can be regarded as a low estimate because no account is made of the efficiency of the extraction procedure.
14. Samples were incubated for 2 hours at 37°C with leucine aminopeptidase (0.3 mg/ml). Trypsin was used at 1 mg/ml, and incubations up to 6 hours did not reduce the proctolin-like bioactivity. Enzymes were heat-inactivated before samples were tested in the bioassay.
15. V. Chan-Palay, G. Jonsson, S. L. Palay, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1582 (1978). Several other examples of co-localization exist and they are discussed in the references of (*1*). Little is known of the functional significance of co-localization.
16. We thank M. Schaffer and A. Meyer for technical assistance with some chromatography assays, C. Bishop, R. Dinerstein, and R. Miller for advice and critical reading of the manuscript, F. Huber for generous use of Max Planck facilities, and K. Agarwal and E. Schwartz for use of HPLC equipment. This work was supported by the University of Chicago and the Max Planck Society, by Deutsche Forschungsgemeinschaft grant HU 35/17 and NIH grant NS-16298 (to M.O.), and by a NATO postdoctoral fellowship and Max Planck stipendium (to M.E.A.).

23 January 1981; revised 10 April 1981

Thyroidectomy Increases Rat Hepatic Ferritin Iron

Abstract. *Studies of the hepatic polysome profiles of hypothyroid rats demonstrated contamination of the free monosome peak with ferritin. This led to the serendipitous observation of a fivefold increase in ferritin iron in livers and kidneys of hypothyroid rats.*

Evidence suggests that thyroid hormone (T_3) exerts control over general and specific messenger RNA (mRNA) transcription in the nuclei of responsive cells, an effect mediated by the interaction of T_3 with nuclear receptor sites (*1*). Also, some data suggest that T_3, directly or indirectly, affects the translation of mRNA (*2–6*). To investigate this possibility we isolated and analyzed by sucrose gradient sedimentation free and membrane-bound polysomes from thyroidectomized and euthyroid rats. Our studies appeared to indicate the presence of a large monosome peak in the free ribosomal fraction; however, additional studies described herein indicated that this was an artifact due to contamination with ferritin.

Free and membrane-bound hepatic polysomes were isolated from the livers of Sprague-Dawley rats by the method of Ramsey and Steele (*7*). Fractions of liver homogenate containing either membrane-bound or free polysomes were centrifuged through discontinuous sucrose gradients 1.3*M* and 2.0*M* designed to retard monoribosomes, ribosomal subunits, and other small macromolecules while allowing the polysomes to form pellets (*8*). We then sedimented purified polysomes in linear sucrose density gradients; the polysomes were subsequently fractionated through a continuous ultraviolet (254 nm) absorbance monitor to generate polysome profiles. Representative profiles from the livers of ten thyroidectomized and ten euthyroid rats are shown in Fig. 1, A and D, respectively. Whereas membrane-bound polysomes (not shown) were essentially the same with respect to mean and median polysome size, the free polysomes from thyroidectomized rats showed a large absorbance peak in the 75*S* portion of the gradient; this peak was almost absent in the free polysomes from the euthyroid rats. Daily administration of intraperitoneal T_3 to thyroidectomized rats (20 μg of T_3 per 100 g for 6 days or 300 ng of T_3 per 100 g for 28 days) largely eradicated this abnormal peak (Fig. 1, B and C). Furthermore, whereas a 5-day fast did not produce a large 75*S* peak,

0036-8075/81/0731-0569$00.50/0

hypophysectomy produced results similar to those in thyroidectomized rats (Fig. 1, E and F, respectively).

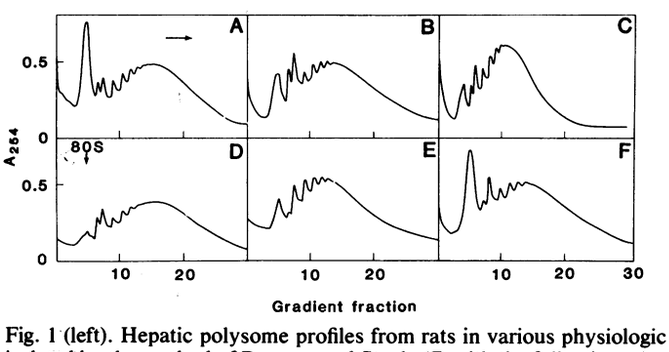
Several lines of evidence suggested that the substance responsible for the abnormal 75*S* absorbance peak in the free polysome profiles of thyroidectomized rats was not ribosomal material. Our suspicions were first aroused by the finding that, in contrast to the opalescent color of purified euthyroid polysome suspensions, free polysomes from thyroidectomized rats were invariably yellow-brown. Material of this color sedimented on linear sucrose density gradients in a narrow band corresponding precisely to the position of the abnormal peak.

Moreover, when RNA of liver from hypothyroid rats was labeled with [^{14}C]orotic acid (0.1 mCi of [^{14}C]orotic acid injected intraperitoneally 18 hours before the rats were killed), no [^{14}C]orotic acid was taken up by free polysomes in the 75*S* portion of the sucrose gradient. Although RNA was readily detectable in the gradient fractions from the middle of the polysome profiles either by the method of Fleck and Munro (*9*) or by the orcinol reaction (*10*), none was measured in the pooled 75*S* fractions of the free polysomes from thyroidectomized rats. These data indicated that a substance other than RNA was responsible for the abnormal 254 nm absorbance peak in the free polysome profiles of thyroidectomized rats.

We next considered the possibility that our results could be explained by contamination with ferritin in free polysomes from thyroidectomized rats. This hypothesis was prompted by a description (*11*) of ferritin as a contaminant of polysome preparations sedimenting at 65*S* on linear sucrose gradients; subsequent modifications (*7*) of these techniques were believed to have eliminated the problem. Nevertheless, the high molecular weight of ferritin (430,000 to 490,000 as apoferritin and 900,000 as fully iron-loaded holoferritin) and its ultraviolet absorbance spectra made it conceivable that some ferritin could cosediment with polysomes and cause a large ultraviolet absorbance peak on polysome profiles (*12*).

Although the 70*S* to 80*S* gradient fractions from free polysomes of thyroidectomized animals showed absorbance in the 250- to 300-nm wavelength region, no specific absorbance maxima characteristic of proteins or RNA-rich macromolecules were observed (Fig. 2A) (*13*). In addition, colorimetric reactions for iron (*14*) and protein (*15*) were strongly positive in the fractions containing the abnormal peak, whereas normal monosomes from euthyroid rats contained no iron and much less protein. When the 70*S* to 80*S* fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, a single protein band at a molecular weight of approximately 18,000 was observed, equal in size to the rat liver ferritin subunit (Fig. 2B) (*12*). On native gel electrophoresis the abnormal fraction migrated as a single major and one minor band, both of which stained for iron and protein (Fig. 2C). This pattern is characteristic for rat liver ferritin. Moreover, when polysomes were prepared from homogenates to which ^{125}I-labeled human ferritin had been added, 4.5 percent of the original radioactivity sedimented on linear sucrose gradients at 75*S*.

Finally, we measured total hepatic ferritin iron levels in normal and thyroidectomized rats using the heat coagulation method of Drysdale and Munro (*14*). The mean (± standard deviation) values obtained from four thyroidectomized and four euthyroid rats were 154 ± 42 μg and 27 ± 3 μg per gram of liver, respectively. A 4.3-fold difference in tissue ferritin iron levels was also observed in the kidney (39.9 ± 3.5 μg/g in thyroidectomized rats compared to 9.2 ± 0.9 μg/g in euthyroid rats), but no significant dif-



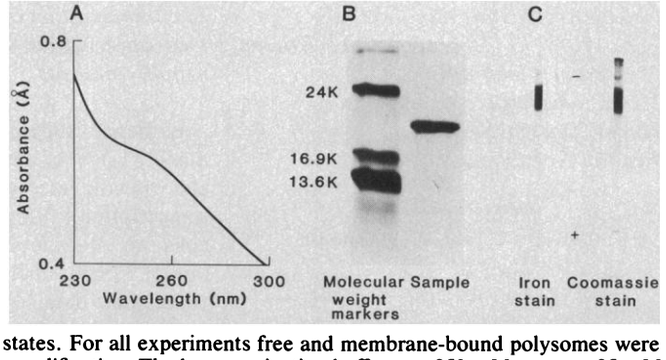


Fig. 1 (left). Hepatic polysome profiles from rats in various physiologic states. For all experiments free and membrane-bound polysomes were isolated by the method of Ramsey and Steele (*7*) with the following minor modification. The homogenization buffer was 250 m*M* sucrose, 25 m*M* Hepes, *p*H 7.4 at 4°C, 75 m*M* KCl, 5 m*M* $MgCl_2$, 3 m*M* glutathione. Membrane polysomes were released with a 1:13 dilution of 13 percent sodium deoxycholate. Portions of the supernatant-containing free polysomes and the supernatant-containing membrane-bound polysomes were layered over discontinuous sucrose gradients [3 ml each of 1.3*M* and 2.0*M* sucrose made by diluting 2.3*M* sucrose, 25 m*M* Hepes, *p*H 7.4 at 4°C, 250 m*M* KCl, 5 m*M* $MgCl_2$, and 3 m*M* glutathione (GSH) with 50 percent cell sap] and centrifuged for 20 hours at 174,000 *g*. The resulting polysome pellets were gently resuspended in 25 m*M* Hepes, *p*H 7.4 at 4°C, 75 m*M* KCl, 5 m*M* $MgCl_2$, and 3 m*M* GSH and either frozen at −80°C or analyzed immediately. Polysome profiles were obtained by layering isolated polysomes (10 absorbance units at 260 nm over 12.4-ml linear sucrose density gradients (0.5*M* to 1.5*M* sucrose) and centrifuging for 90 minutes at 200,000*g*. The gradients were fractionated with continuous ultraviolet (254 nm) absorbance monitoring. Variations in scale are the result of variable gradient fractionator speed. (A) Representative free polysome profile from ten pooled thyroidectomized rats. Thyroidectomized rats weighing 100 to 125 g were obtained commercially, and injected with 100 μCi of ^{131}I (Mallinckrodt) to ensure complete hypothyroidism. (B) and (C) Free polysome profiles from pooled livers of three hypothyroid rats injected daily with 20 μg of T_3 per 100 g of body weight for 6 days and 300 ng of T_3 per 100 g of body weight for 28 days, respectively. (D) Representative free polysome profile from ten euthyroid male rats. (E) Representative free polysome profile from three euthyroid rats fasted for 5 days. (F) Free polysome profile from pooled livers of three hypophysectomized rats purchased from Hormone Assay, Chicago, Illinois. Fig. 2 (right). Studies on purified 70*S* to 80*S* fraction from free hepatic polysomes of thyroidectomized rats. Fractions from several linear gradients were pooled and diluted in distilled water. After centrifugation of the material at 150,000*g* for 2 hours the resulting pellet was dissolved in 200 μl of distilled water. (A) Ultraviolet spectrum of the 70*S* to 80*S* absorbance peak. A small portion of the purified material was diluted in distilled water and its 300- to 240-nm spectrum determined. (B) Denaturing gel electrophoresis. The protein (100 μg) was electrophoresed in a 15 percent polyacrylamide gel by the method of Laemmli (*20*). Gels were stained for protein with 0.2 percent Coomassie blue (weight to volume) in 50 percent methanol (by volume), and 7 percent acetic acid (by volume). Molecular weight markers were chymotrypsinogen, cytochrome *c*, and myoglobin. (C) Native gel electrophoresis. Protein (100 μg) was electrophoresed in an identical manner to the denaturing system except that SDS was removed from the buffers and gel. Protein was stained as in (B), and iron was stained with 0.2 percent potassium ferrocyanide (weight to volume) in 10 percent trichloroacetic acid (weight to volume).

ference was found in the spleen or the brain of these animals.

Although the conventional isolation techniques used in these studies (*7*) succeeded in removing detectable contaminating material from polysomes of euthyroid livers, our results show that free polysomes of thyroidectomized rats contain a large amount of nonpolysomal material which we have provisionally identified as ferritin. Furthermore, because this abnormality was mimicked by hypophysectomy and corrected in a dose-dependent fashion by exogenous T_3 administration, it is clearly a hormone-dependent phenomenon. Since the bound polysome profiles of thyroidectomized and euthyroid rats were identical, as were the free polysomes outside the 75*S* region, our studies call into question previous reports of differences in polysome structure and function in hypothyroidism (*3–6*).

To the best of our knowledge, this is the first report that concentrations of hepatic ferritin iron vary with thyroid hormone status. The mechanism by which this occurs is unclear. It is unlikely that existing ferritin apoprotein simply takes on more iron in hypothyroidism since the fivefold increase in ferritin iron observed would oversaturate the known ferritin apoprotein iron storage capacity (*16*). However, we cannot rigorously exclude this possibility because direct measurements of ferritin apoprotein were not made. A more probable explanation is that ferritin iron and apoprotein are elevated concomitantly, the result of either a primary increase in the synthesis or a primary decrease in the degradation of ferritin apoprotein. This phenomenon could be a direct effect of thyroid hormone or an indirect effect mediated by a primary increase in tissue iron in hypothyroidism (*17*).

However, it is interesting that ferritin iron increases in liver and kidney of hypothyroid animals but not in spleen and brain, an association that parallels the well-known thyroid hormone responsivity of these tissues as gauged by oxygen consumption (*18*). Of interest in this connection is the recent finding in our laboratory that thyroid hormone can inhibit the formation of specific mRNA species (*19*).

JOHN C. WINKELMANN
CARY N. MARIASH
HOWARD C. TOWLE
JACK H. OPPENHEIMER
Division of Endocrinology and Metabolism, Department of Medicine and *Department of Biochemistry, University of Minnesota, Minneapolis 55455*

References and Notes

1. J. H. Oppenheimer, *Science* **203**, 971 (1979).
2. R. W. Mathews, A. Oronsky, A. E. V. Haschmeyer, *J. Biol. Chem.* **248**, 1329 (1973).
3. L. D. Garren, A. P. Richardson, Jr., R. M. Crocco, *ibid.* **242**, 650 (1970).
4. J. R. Tata and C. C. Widnell, *Biochem. J.* **98**, 604 (1966).
5. C. L. Correze, P. Pinell, J. Nunez, *FEBS Lett.* **23**, 87 (1972).
6. C. L. Correze, A. Felous, P. Pinell, R. Lahalle, J. Nunez, *Biochim. Biophys. Acta* **272**, 596 (1972).
7. J. C. Ramsey and W. J. Steele, *Biochemistry* **15**, 1704 (1976).
8. F. O. Wettstein, T. Staehlin, H. Noll, *Nature (London)* **197**, 430 (1963).
9. A. Fleck and H. N. Munro, *Biochim. Biophys. Acta* **55**, 571 (1962).
10. G. Ceriotti, *J. Biol. Chem* **214**, 59 (1955).
11. R. J. Jackson, A. J. Munro, A. Korner, *Biochim. Biophys. Acta* **91**, 666 (1964).
12. H. N. Munro and M. C. Linder, *Physiol. Rev.* **58**, 317 (1978).
13. J. W. Drysdale and H. N. Munro, *Biochem. J.* **96**, 831 (1965).
14. ———, *ibid.* **95**, 851 (1965).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
16. R. B. Crichton, in *Proteins of Iron Storage and Transport in Biochemistry and Medicine*, R. R. Crichton, Ed. (American Elsevier, New York, 1975), pp. 183–184.
17. J. W. Drysdale and H. N. Munro, *J. Biol. Chem.* **241**, 3630 (1966).
18. S. B. Barker and H. M. Klitgaard, *Am. J. Physiol.* **170**, 81 (1952).
19. S. Seelig, C. Liaw, H. Towle, J. H. Oppenheimer, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
20. U. K. Laemmli, *Nature (London)* **222**, 680 (1970).
21. We thank C. Jahnel and N. Rutledge for their secretarial assistance. Supported in part by grants NIH AM19812 (J.H.O.), AM26919 (H.C.T.), clinical investigator award AM00800 (C.N.M.), and a student stipend grant from the Minnesota Medical Foundation (J.C.W.).

6 April 1981

Pineal *N*-Acetyltransferase Is Inactivated by Disulfide-Containing Peptides: Insulin Is the Most Potent

Abstract. *Pineal* N-*acetyltransferase can be inactivated in broken cell preparations by cystamine through a mechanism of thiol-disulfide exchange. Some, but not all, disulfide-containing peptides can inactivate this enzyme; the most potent inactivator is insulin. These findings suggest that a disulfide-containing peptide with high reactivity toward* N-*acetyltransferase may participate in the intracellular regulation of this enzyme.*

The activity of rat pineal *N*-acetyltransferase, the enzyme that controls large daily changes in melatonin production, is neurally stimulated 30- to 70-fold at night (*1, 2*). The stimulatory signals originate in another area of the brain, the suprachiasmatic nuclei. The pineal gland is stimulated for 10 to 14 hours every night through a neural circuit that includes central and peripheral structures. The suprachiasmatic nuclei appear to function as a biological clock. Light acting on this mechanism resets the clock every day, ensuring that it is entrained to environmental lighting, and it also prevents transmission of signals from the suprachiasmatic nucleus to the pineal gland. In the pineal gland, neural stimulation causes the release of norepinephrine, which elevates *N*-acetyltransferase activity through a β-adrenergic–cyclic AMP (adenosine 3′,5′-monophosphate) system; induction and activation mechanisms appear to be involved. When stimulation ceases, enzyme inactivation occurs and the activity of the enzyme decreases with a half-time ($t_{1/2}$) of about 3 to 4 minutes (*3, 4*).

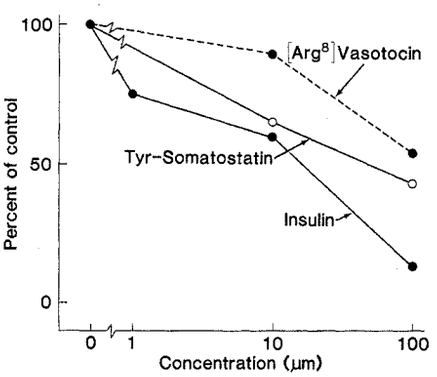


Fig. 1. Effects of disulfide-containing peptides on *N*-acetyltransferase activity. The peptides (*7, 8*) were dissolved in buffer (0.2*M* sodium citrate plus 0.02*M* barbital hydrochloride, *p*H 8.5) and 5-μl samples were added to 5 μl of a pineal gland supernatant. The solution was then incubated for 5 minutes. The enzyme activity was then measured in a total volume of 200 μl with acetyl coenzyme A (0.5 m*M*, 2 μCi/μmole) and tryptamine hydrochloride (10 m*M*) (*5*). Every value is based on duplicate runs, each of which was within 5 percent of the mean value. Insulin (100 μ*M*) did not go into solution completely at 0° to 2°C and was used as a suspension; lower concentrations were soluble.

The biochemical nature of this inactivation has been a mystery. We proposed that inactivation might occur by thiol-disulfide exchange (*5*)

$$\text{ENZ–SH}_{(\text{active})} + \text{X–S–S–X} \rightleftarrows \text{ENZ–S–S–X}_{(\text{inactive})} + \text{XSH}$$

This was primarily based on studies in which cystamine $[NH_2CH_2CH_2S–]_2$ was used (*5*). We now report that, of the nine disulfide-containing peptides we tested, the most potent inactivator was insulin. This is of some added interest because immunoreactive insulin reportedly oc-

0036-8075/81/0731-0571$00.50/0