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DEGRADATION OF RADIOIODINATED PORCINE AND HUMAN SYNTHETIC CALCITONINS BY SERA OF DIFFERENT SPECIES

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Abstract—The *in vitro* degradation of porcine and human synthetic calcitonin was measured in the presence of sera of different species (rat, pig, human) using a chromato-electrophoresis method. Degradation is greater when heterologous sera are used. The degradation was not prevented by enzyme inhibition. Damage was greatest at acid pH and was not removed by heating of the serum. Ammonium sulfate precipitations and euglobulin separation of serum proteins did not result in the isolation of the factor responsible for the degradation of calcitonin. Mercaptoethanol prevented the damage from appearing which suggested that this damage was in part due to breakage of the disulfide bond.

BIOLOGICAL inactivation of porcine thyrocalcitonin was observed after *in vitro* incubation in the presence of sera[1,2] and organs.[3] Degradation of radioiodinated calcitonin has already been evaluated in immunoassays.[4] In physiological experiments using labelled calcitonin, it was important to measure the damage caused *in vitro* by homologous or heterologous sera. The fate of labelled synthetic human and porcine-labelled calcitonin was studied in the presence of different sera. Isolation of the (possibly enzymatic) factor was attempted.

MATERIAL AND METHODS

Synthetic porcine calcitonin and human calcitonin were kindly supplied by Sandoz Ltd (Basel) and Ciba Ltd (Basel). Iodination was performed with ^{125}iodine (Amersham Radiochemical Center) according to Hunter and Greenwood's method[5] with minor modifications. Separation of iodide and damaged components from pure [^{125}I]calcitonin was done by QuSo G32 microfine granules of precipitated silicates.† Elution of undamaged [^{125}I]calcitonin was performed with 1 ml of acetone (40%) in 1% acetic acid. After elution, acetone was evaporated at 4° by a nitrogen gas flow.[6] Efficiency of iodination and specific activity of [^{125}I]calcitonin were calculated from chromatoelectrophoresis strips according to the method of Berson *et al.*[7] Specific radioactivities were from 25 to 228 μc/μg.

Rat, pig, rabbit and human normal sera were kept at −20°. Heating of the serum was carried out at 56° for 4 hr. Sera were used at final dilutions of 1:2, 1:10, 1:100. EDTA was added to one series of test tubes to a final concentration of 0·01 M.

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A proteolytic enzyme inhibitor similar to Trasylol (Cy 66, Laboratoires Choay) was used at a final concentration of 5·000 U/ml, and ϵ aminocaproïc acid at a concentration of 10 mg/ml. Hydrochloric acid (0·1 N) was added to 0·5 ml of serum in order to obtain a pH of 4·5. 2-Mercaptoethanol was used at a final dilution of 10 mg/ml.

Rat serum protein fractions were obtained from 1 ml of serum by precipitation using equal volumes of 50% (w/v) and 80% (w/v) ammonium sulfate. Precipitates were dialyzed against phosphate 0·02 M sodium chloride 0·15 M pH 7·2 buffer. Euglobulins and the acid-soluble fraction of rat serum proteins were separated by dialysis of serum against phosphate 0·01 M, pH 5·2 buffer. All these operations were performed at 4°. Amounts of dissolved fractions of rat serum added to the incubation tubes were adjusted so that they contained the amounts of proteins corresponding to those present in 0·1 ml of rat serum.

All the incubation tests were performed in a final volume of 1 ml, in phosphate 0·01 M, NaCl 0·15 M, bovine serum albumin (0·5 mg/ml), pH 7·4 buffer. 10–50 μl of labelled calcitonin (200,000 counts/min) were added to duplicate test tubes. 100 μl aliquots were applied to duplicate paper strips at 2, 3, 4, 5, 6 and 24 hr, at 4° or 37°.

Separations of [^{125}I]calcitonin from damaged products and iodide was performed on Whatman 3 MC strips (46 × 2·5 cm) in veronal buffer 0·05 M, pH 8·6. Pure [^{125}I]calcitonin remained at the origin, damaged products moved toward the albumin zone and iodide migrated ahead of the albumin spot. After the separation conditions had been established, strips obtained from the initial samples, before the commencement of incubation, and from the final samples were cut every 1 cm in order to obtain a more complete plot of the radioactivity curve versus the length of the strips. A good separation of radioactive calcitonin was obtained at 8 cm from the origin.

The remaining strips were divided into 2 parts from 0 to 8 cm, and from 8 to 25 cm. Radioactivity was measured in a NaI/well-crystal-gamma-counter (Saip-Mecaserto).

RESULTS

Estimated damage of pure radioiodinated synthetic porcine or human calcitonin was never above 11 per cent, before any of the incubation tests. The small amount of damage still present after elution may be the effect of the eluant, although acetone had been evaporated. The labelled hormone can be kept at −20° for 6 weeks without further damage. Damaged hormone products migrate with albumin, while pure calcitonin remains at the origin when the amount of serum proteins applied to the chromatoelectrophoresis does not exceed 7 mg, i.e. about 0·1 ml of serum. Above 7 mg, there is an unspecific drift of labelled calcitonin to the damaged protein zone. Amounts of serum to be run may vary from 0·01 to 0·1 ml. However, when the serum content of the incubation test aliquot was less than 0·1 ml, corrections were made just prior to chromatoelectrophoresis. After an incubation period of 24 hr the amount of free iodide never exceeds 15 per cent of total radioactivity.

Radioiodinated synthetic porcine calcitonin was extensively damaged when incubated *in vitro* with rat serum (Fig. 1). Heat markedly increased the damage. The addition of heated rat serum, or of EDTA which chelates serum calcium did not prevent the damage of the labelled hormone. Cy 66 or ϵ aminocaproïc acid, alone or added simultaneously to the incubation medium did not affect the degradation (Fig. 2). Lowering the pH to 4·5 increased the damage to iodinated calcitonin. 2-Mercapto-

TABLE 1. PERCENTAGE OF DAMAGED ^{125}I PORCINE CALCITONIN DURING INCUBATION AT 4° WITH BUFFER ALONE (CONTROL), WITH NORMAL RAT SERUM (NRS 1:2) DILUTED 1:2, AND WITH NORMAL RAT SERUM (DILUTED 1:2) WITH MERCAPTOETHANOL (NRS 1:2MPE)

Time (hr)	0	1	2	3	4	5	6	24
Control	10·5	15·5	16·5	17	19·5	20	21	20·5
NRS 1:2		18·5	21·5	19·5	25·5	35	29·5	29
NRS 1:2 MPE (10 mg/ml)		12·5	14·5	15·5	17	20	17·5	17

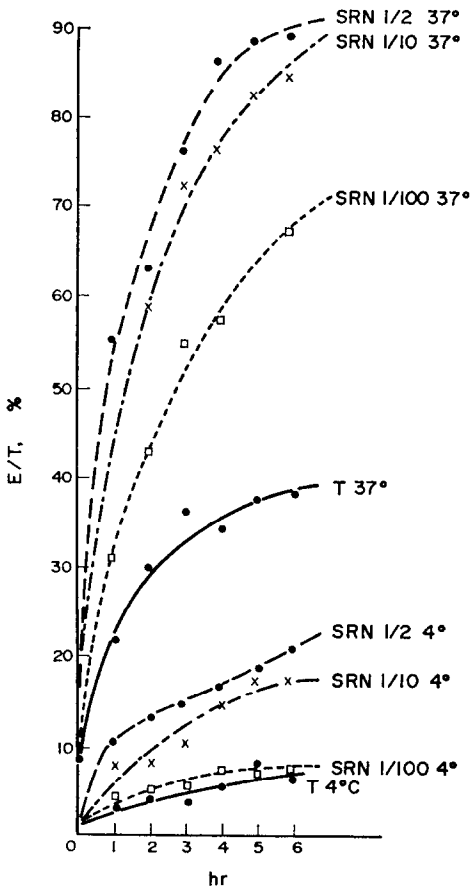

FIG. 1. Damage of ^{125}iodine porcine synthetic thyrocalcitonin (a) in buffer alone (T). (b) In presence of normal rat serum (SRN) at three different dilutions 1:2, 1:10, 1:100, incubated at 4 or 37° for 6 hr. Percentage of damage (E) over total radioactivity (E/T%) is expressed in the ordinate.

ethanol had a completely inhibitory effect on the damage of porcine calcitonin, at 4 and 37° (Table 1).

Human and rabbit normal sera are as effective as rat serum in damaging porcine calcitonin at 4 and 37° (Fig. 3). However, in the presence of normal pig serum, damage of radioiodinated porcine calcitonin is identical to that observed in the buffer alone (Fig. 4).

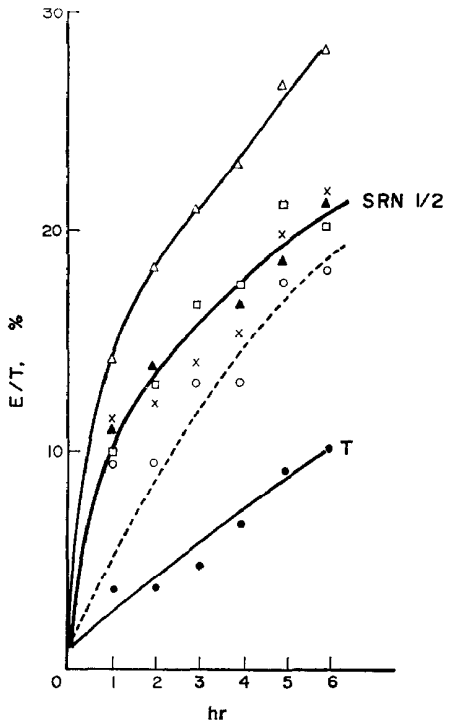

FIG. 2. Damage (E/T%) of ^{125}iodine porcine synthetic thyrocalcitonin incubated in buffer alone (T), in presence of normal rat serum diluted 1:2 alone (SRN 1/2 □——□), at pH 5 (△——△); or with Cy 66 (5000 U/ml ○- - - - - - - ○), with ε aminocaproïc acid (10 mg/ml ▲——▲) or with both Cy 66 and ε aminocaproïc acid (×——×).

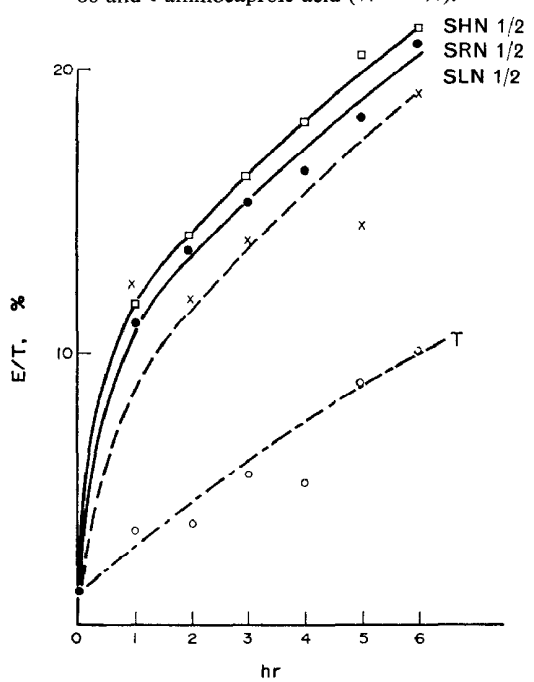

FIG. 3. Damage (E/T%) of ^{125}iodine porcine synthetic thyrocalcitonin incubated in buffer alone (T) with normal rat serum (SRN), human serum (SHN) and rabbit serum (SLN) diluted 1:2 for 6 hr.

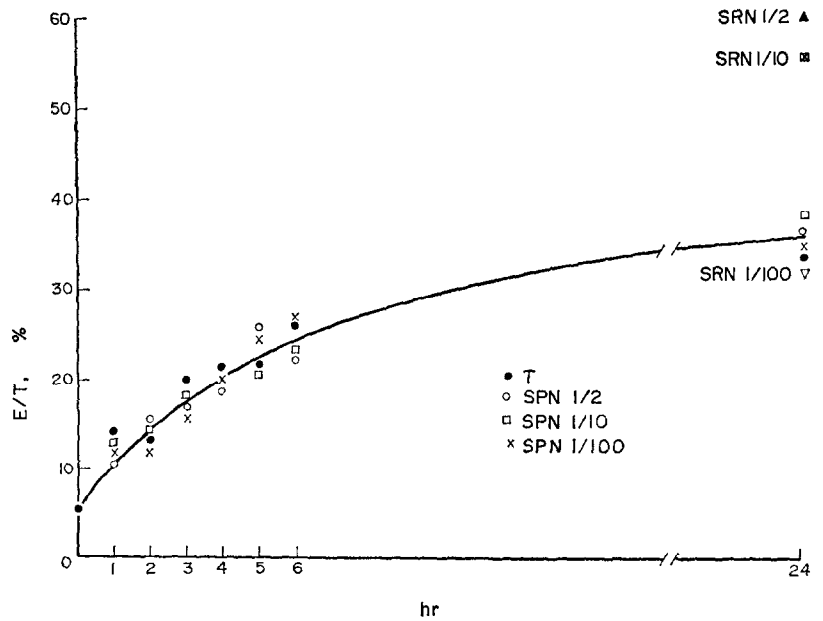

FIG. 4. Damage (E/T%) of ^{125}iodine porcine synthetic thyrocalcitonin incubated at 37° in buffer alone (T), in pig normal serum (SPN) diluted 1:2, 1:10, 1:100. Damage obtained in the same experiment after 24 hr incubation of labelled hormone with normal rat serum (SRN) diluted 1:2, 1:10, 1:100 have been plotted for comparison.

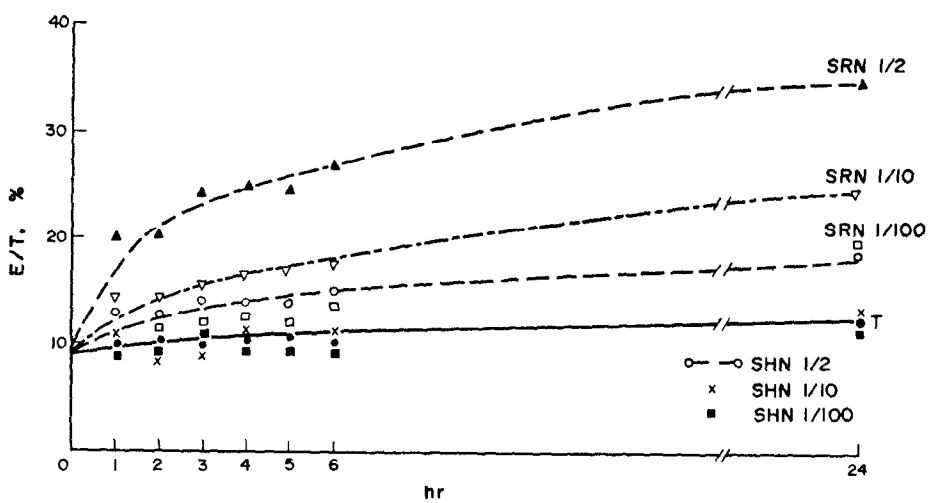

FIG. 5. Compared incubation damage (E/T%) during time of labelled human synthetic thyrocalcitonin with human normal serum (SHN) and normal rat serum (SRN) at three different dilutions (1:2, 1:10, 1:100) at 4° (T: ^{125}I hormone in buffer alone).

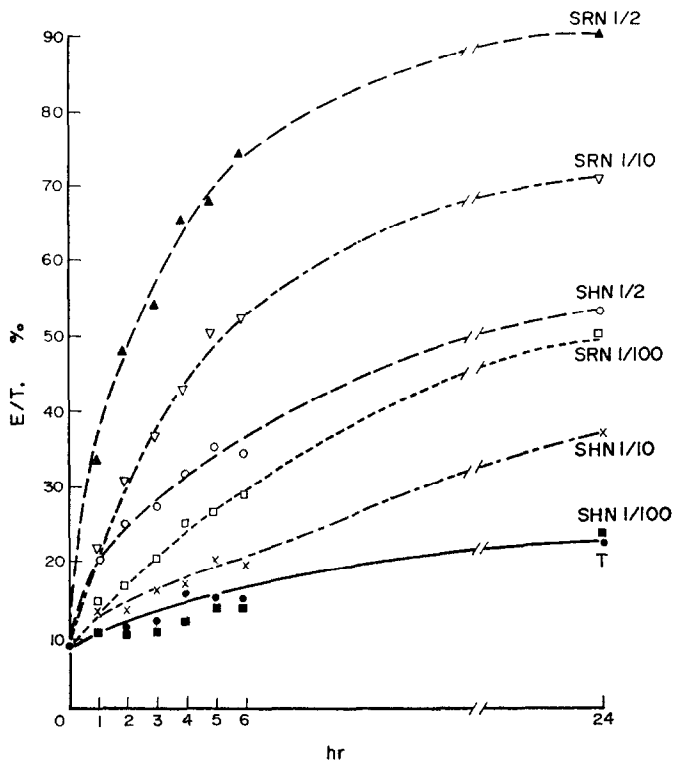

FIG. 6. Incubation damage of ^{125}I human synthetic thyrocalcitonin observed in the same conditions as on Fig. 5, at 37°.

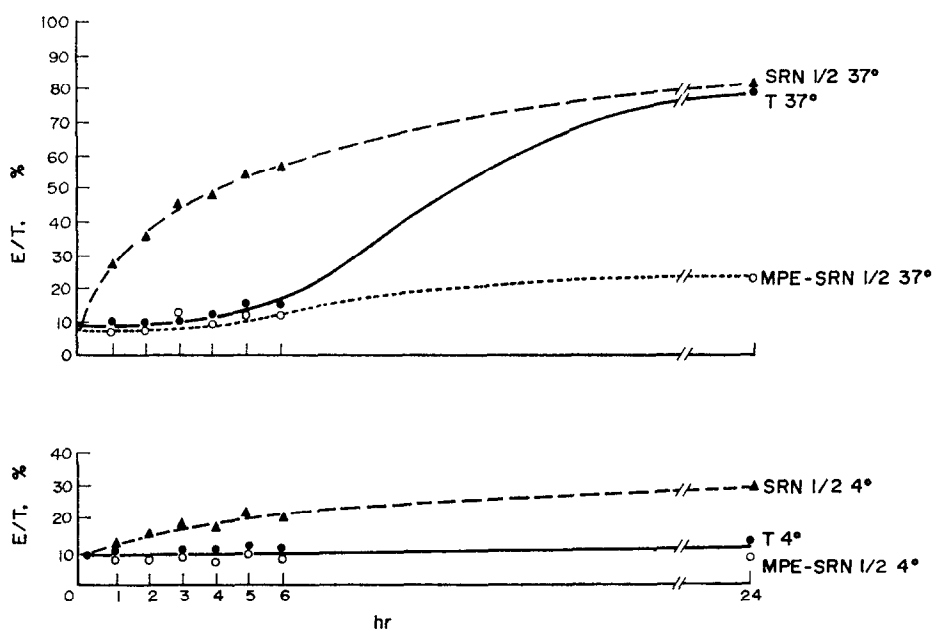

FIG. 7. Effects of mercaptoethanol on incubation damage (E/T%) of ^{125}I human synthetic thyrocalcitonin in presence of normal rat serum diluted 1:2 (MPE—SRN 1/2) at 4 or 37°. T: labelled hormone in buffer alone. SRN 1/2: labelled hormone with normal rat serum, diluted 1:2.

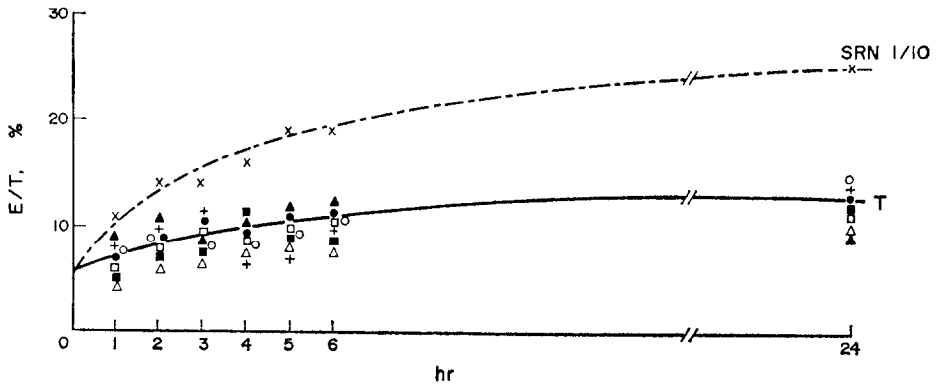

FIG. 8. Incubation of ^{125}iodine porcine synthetic thyrocalcitonin at 4°: (a) with buffer alone (T), (b) with normal rat serum diluted 1:10 (SRN 1/10), (c) with rat serum fractions. Obtained by 50% $(NH_4)SO_4$ precipitation: Dissolved precipitate ■——■; Supernatant □——□. Obtained by 80% $(NH_4)SO_4$ precipitation: Dissolved precipitate ▲——▲; Supernatant ○——○. Euglobulins fraction +——+. Acid soluble fraction △——△.

Iodine-labelled human synthetic calcitonin incubated at 4° with normal human serum diluted 1:2 showed a small incubation damage after 24 hr. The damage was more important with rat serum (Fig. 5). At 37°, damage was higher with rat and human serum as well (Fig. 6). Mercaptoethanol inhibited the degradation of 1:2 diluted rat serum at both 4 and 37° (Fig. 7).

Fractions of rat serum obtained by precipitations with ammonium sulfate and by dialysing against pH 5·2 phosphate buffer did not increase the damage of labelled porcine calcitonin (Fig. 8).

DISCUSSION AND CONCLUSION

Chromatoelectrophoresis is considered to be an adequate method for estimating pure and damaged hormone.[6] It has been shown for glucagon that damaged hormone might form clumps which remain on the paper at the site of application.[8] However, for several other protein hormones, damage estimated by chromatoelectrophoresis, dextran-coated charcoal, talc, QuSo, or in presence of antibody excess were similar.[9,10] In addition, chromatoelectrophoresis allows the measurement of free iodide released as part of the damage.

Biological inactivation by serum of several hormones such as glucagon, ACTH or pituitary growth hormone has been observed before.[11] The factor involved in ACTH inactivation experiments was thought to be plasminogen which is present in the euglobulin fraction of serum. Plasminogen is activated to plasmin by streptokinase. On the other hand, loss of biological activity and protein damage to thyrocalcitonin may be due either to breakage of the disulfide bond, or to a proteinase present in the serum. Furthermore, in our experience, oxidation of thyrocalcitonin with chloramine T did not destroy its biological activity. However, loss of biological activity of calcitonin was reported after oxidation by peroxide[12] and of ACTH after iodination.[13] These are conflicting results which cannot be explained at the present time.

Since biological inactivation of thyrocalcitonin in the presence of serum occurs, it was important to evaluate the potential damage of radioiodinated thyrocalcitonin, when used, in physiological experiments or radioimmunoassays.

The mechanism by which damage occurs to an iodine-labelled hormone still remains misunderstood.[14] Deiodination of the protein is probably due to a plasma deiodase which affects molecular conformation. Its importance can be measured by the amount of iodide present on the chromatoelectrophoresis strip. In addition to the deiodination mechanism, biochemical modification of the hormone occurs, as revealed by the damaged products peak apparent in the albumin zone. And last, radioactive decay of the isotope products instability of the protein molecule. In our case, the latter mechanism is probably slow, as the half life of ^{125}iodine is 60 days.

Damage of thyrocalcitonin has been noted in radioimmunoassays when serum dilutions were 1/5 or 2/5.[8] Damage was temperature-dependent.[4] Degradation was higher when porcine thyrocalcitonin was incubated with rat, rabbit or human serum. It was much lower in the presence of pig serum. All the serum samples from the same species do not possess the same degrading activity. At the same time, different preparations of labelled calcitonin are more or less sensitive to damage, in the presence of serum or buffer alone. The latter fact may account for the higher rate of degradation (Figs. 6 and 7) observed for human calcitonin in an experiment performed several times at 37°. Human radioiodinated thyrocalcitonin was less damaged when incubated with human serum than with rat serum. In radioimmunoassays, the phenomenon has to be checked when antithyrocalcitonin serum is used at low dilutions, or when heterologous serum or anti-serum is added in the double antibody system[15] or when unknown serum is tested at high concentration.[16] Incubation time has to be controlled in order to obtain minimum damage. In bioassays, biological inactivation of thyrocalcitonin by rat serum indicates that one has to be cautious about comparing biological activities of thyrocalcitonin of different species in the same animal. Determinations of half-lives and turn-over of radioiodinated human or porcine calcitonins in rats might not represent a true estimation of the fate of the hormone.

Isolation of the inactivating factor was unsuccessful. Proteolytic enzymes such as trypsin, chymotrypsin or pepsin destroy the biological activity of calcitonin completely.[17] Inhibitors of proteolysis (Cy 66) and of plasminogen activation (ε aminocaproïc acid) were ineffective in protecting calcitonin from damage. These data do not agree with the protecting action of both Trasylol and ε aminocaproïc acid.[8] Inactivation of thyrocalcitonin by serum might be due to pepsin or cathepsins which are more active at pH 4·5 and are poorly inhibited by the usual antiproteolytic enzymes. The effect of pH on labelled calcitonin's behaviour on chromatoelectrophoresis differs from its effect on the hormone's biological activity. According to Aldred and Schlueter,[2] at pH 7·5, the hypocalcemic activity of calcitonin is completely abolished in buffer alone. At the same pH, chromatoelectrophoretic damage is low. At pH 3·2, calcitonin is still biologically active. Dropping the pH to 4·5 increased the chromatoelectrophoretic damage. At pH over 5, the presence of rat or dog serum (1:100 to 1:1000 dilution) protects at 37°C the calcitonin from biological inactivation.[2] By chromatoelectrophoresis damage was increased with rat serum. These are conflicting reports which remain unexplained. Heating of serum was ineffective in destroying the damaging factor. This suggests an unknown non-enzymatic phenomenon. However, some iso-enzymes are not heat-labile.[18]

Reduced sulfhydryl groups such as 2-mercaptoethanol are supposed to break disulfide bridges. When mercaptoethanol has been added to thyrocalcitonin, biological inactivation has been observed.[19] This would seem to imply that the disulfide

bridge is necessary for biological activity. In the presence of serum and mercaptoethanol, thyrocalcitonin was protected from damage. This suggests that reduced sulfhydryl groups supplied by mercaptoethanol had blocked an enzymatic activity responsible for the breakage of disulfide bonds by shifting the reaction equilibrium. Optimal pH and temperature of such disulfide bridge enzymes are not well known. No specific anti-enzyme can be used.

Thioglycollate suppresses biological activity of oxytocin,[20] without affecting immunological activity. As the disulfide bridge is not necessary for biological activity of this hormone, thioglycollate cannot act merely by disrupting the disulfide bond. The presumed mechanism is a major alteration of the three-dimensional conformation of the hormone. However, if such a mechanism is involved in the case of thyrocalcitonin it does not affect the chromatoelectrophoretic pattern of the radioiodinated hormone. Whether it affects its immunoreactivity is not known.

Recently, an acid proteinase purified from pig thyroid gland has been shown to rapidly provoke degradation of porcine thyrocalcitonin labelled with radioiodine and destroy the biological activity of unlabelled calcitonin.[21] The proteinase was most active at a pH below 5·2 and inactive at pH 7·7. No inhibitor of the degradation was used in these experiments. It is difficult to believe that the serum damaging activity is similar to this acid proteinase.

Animal specificity of the damage might be due to "protein recognition" of thyrocalcitonin. This might be due to enzymatic activities or to binding proteins of serum, as shown for several protein hormones such as growth hormone[22] or as recently demonstrated by Leggate, Care and Frazer[23] to a reversible binding of HCT to a protein which would migrate with albumin. In this case, the radioactivity which has migrated away from the point of origin would not correspond to damaged hormone: whether a specific carrier protein exists remains to be proven. The mechanism by which a foreign protein might be inactivated *in vitro* by serum proteins is entirely hypothetical. The existence of a "thyrocalcitoninase" similar to oxytocinase[21] is unknown.

Practical importance has to be given to these inactivators or protectors in bioassays or immunoassays. Each one of the assays might measure degradation or modified products and give irrelevant concentrations of hormone in biological fluids.

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