Specific Calcitonin-Binding Proteins in Man

The interaction of human calcitonin (H-CT) with serum proteins was investigated in order to detect and identify possible specific binding proteins, using equilibrium dialysis and two dimensional radioimmunoelectrophoresis.

Material and method. Synthetic H-CT was labelled with ^{125}I using a modified Greenwood Hunter technique[1]. Human synthetic H-CT was dissolved in phosphate buffer 0.1 *M* pH 7.4. Normal human plasma was stored at −20 °C.

1. *Equilibrium dialysis:* 2 ml plasma, 8 ml saline and 10,000 U crist. penicilin were introduced into a dialysis bag Nojax 23 Visking. 3 or 300 ng labelled H-CT were added to the dialysis bath. The stopped flasks were shaken for 72 h at 37 °C or 25 °C. In a few instances 25, 50, 75 and 100 µg cold H-CT were added to the dialysis bath. At the end of the experimental period, H-CT present in the bag was precipitated by TCA (10% final concentration). H-CT remaining in the bath was precipitated after addition of plasma (20% final concentration). The binding of H-CT to serum proteins was calculated using:

$$\text{Binding } (\%) = 100\,[1 - A_e \cdot V_i / A_i \cdot V_e]^{2,3}$$

where A_i = radioactivity in the bag; A_e = radioactivity present in the bath; V_i = volume inside the bag; V_e = volume of the bath.

Precipitable radioactivity was calculated as the difference between total and supernatant radioactivity after centrifugation of the TCA-precipitate.

2. *Two-dimensional radioimmunoelectrophoresis:* Labelled H-CT (1 µg or 250 ng) was added to 1 ml plasma and the mixture shaken for 1 h at 25 °C. The plasma proteins were separated using the LAURELL technique[4] modified by REBEYROTTE[5]. 5 µl were introduced in the reservoir of a 12 × 9 cm agar plate (1%) and the first electrophoretic separation performed (250 V, 10 mA, veronal buffer pH 7.6, $\mu = 0.03$, 2 h). Then an agar slice containing the separated proteins was cut, transfered to another glass slide and 2 ml antihuman goat immunoserum in agar poured on both sides of the slice. After gelification, the second electrophoresis was performed orthogonally (100 V, 3 mA, same buffer, 20 h). The plaque was washed for 2 days in saline (0.15 *M*), then in disstilled water. After radioautography of the dried plaque (Kodak – no screen film, 4 weeks exposure), the proteins were stained with

[1] F. C. GREENWOOD, W. M. HUNTER and J. S. GLOVER, Biochem. J. *89*, 114 (1963).
[2] W. R. SLAUNWHITE and A. A. SANDBERG, J. clin. Invest. *38*, 384 (1959).
[3] A. A. SANDBERG and W. R. SLAUNWHITE, J. clin. Invest. *38*, 1290 (1959).
[4] C. B. LAURELL, Ann. Biochem. *10*, 358 (1965).
[5] P. REBEYROTTE, A. KOUTSOUKOS and J. P. LABBE, C. r. Acad. Sci. Paris, série D, *269*, 531 (1969).

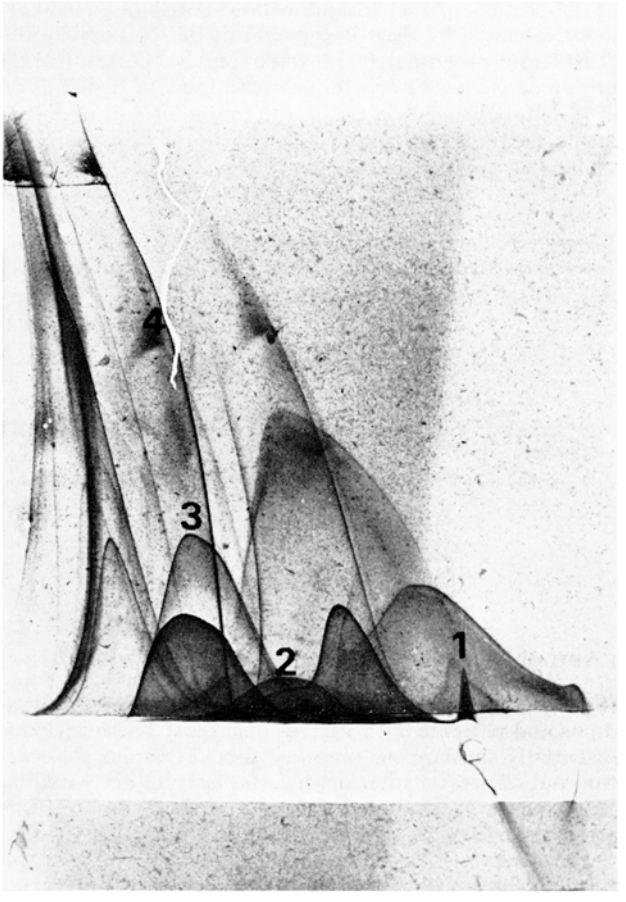

Fig. 1. Two-dimensional immunoelectrophoretic separation of normal human plasma. 1, IgM; 2, α_2-lipoprotein; 3, α_2-macroglobuline; 4, albumine.

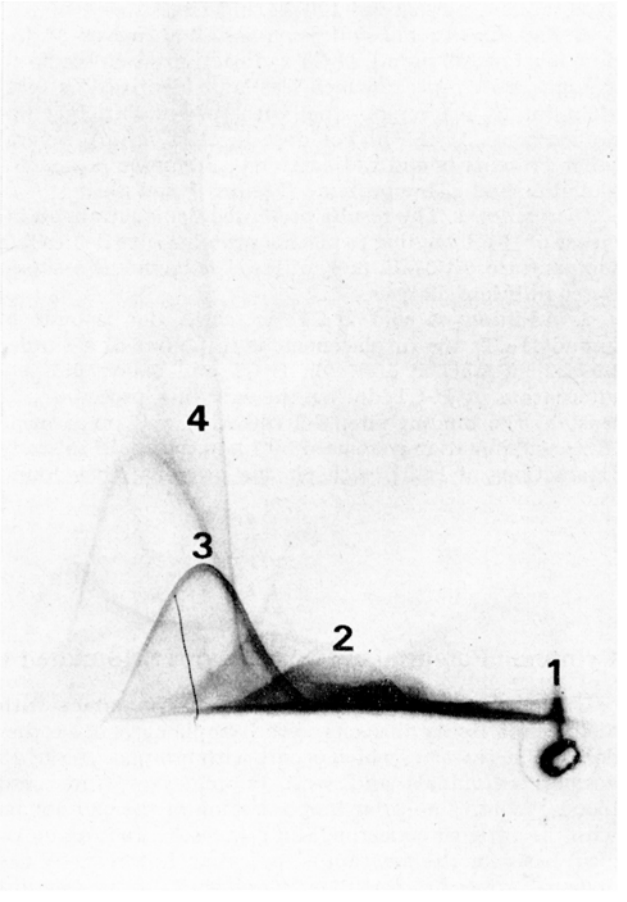

Fig. 2. Autoradiography of an-immunoelectrophoretic separation of TCT-I^{125} added to human plasma (1 µg/ml). 1, IgM; 2, α_2-lipoprotein; 3, α_2-macroglobuline; 4, albumine.

Table I

Experiment	Influence of temperature on TCT-binding by plasma-protein (%) 37 °C	25 °C
1	79	98
2	83	100
3	82	97
4	85	100

Table II

Amount of cold TCT added (μg)	TCT-I^{125} binding to plasma protein at 25 °C (%)
25	78
50	35
75	10
100	0

amidoblack to detect the binding proteins (Figures 1 and 2), with the fingerprint technique. Their identification was performed using monospecific immunosera, anti-α_1-antitrypsine, antitransferine, anti-α_1-lipoproteine, anti-α_2-macroglobuline, anti-IgA, anti-IgG anti-IgM. Furthermore plaques were stained after exposure to polyspecific immunosera anti-lipoproteins, -glycoproteins, -haptoglobuline and -hemopexine. Human γ-globuline, precipitated by addition of sodium sulfate, was used for two-dimensional electrophoretic separation.

Results. 1. *Equilibrium dialysis:* TCA precipitated initially 80% of labelled H-CT and 25% of total radioactivity after dialysis (72 h). At 37 °C, 80 to 85% of H-CT were bound to plasma proteins; at 25 °C, the binding was nearly complete (Table I). The results were the same when amount of added H-CT changed from 300 to 3 ng. Addition of increasing amounts of cold H-CT to the plasma decreased the binding of labelled H-CT (Table II) which became undetectable in presence of 100 μg cold H-CT.

2. *Two-dimensional radioimmunoelectrophoresis:* At the dose level of 250 ng/ml, H-CT radioactivity was bound to a single protein peak, which was later identified as IgM-globuline (β_2-macroglobuline) with specific anti IgM immunoserum. At the higher dose level (1 μg/ml), several other proteins bound radioactivity: albumine, α_2-macroglobuline and α_2-lipoproteine (Figures 1 and 2).

Discussion. 1. The results presented demonstrate an increase of H-CT binding to plasma proteins with decreasing temperature. At 37 °C, 80% of H-CT is bound as assessed by equilibrium dialysis.

2. Addition of cold H-CT decreases the amount of bound H-CT: the displacement is in favour of a similar binding of labelled and cold H-CT and makes unlikely alternation of H-CT during the labelling procedure, at least at the binding site. Gel filtration and preparative ultracentrifugation were used by LEGGATE et al.[6] to study interactions of H-CT with plasma proteins; they found evidence for an interaction but did not identify specific binding proteins.

3. The amount of circulating CT in man is controversial. We have matched the concentrations of H-CT with the one detected in the hog. In most equilibrium dialysis experiments, H-CT was added in amounts equivalent to concentrations prevailing in thyroid venous plasma (150 ng/ml). In a few instances, 1.5 ng/ml were used in order to come closer to systemic circulating hormone levels. The ratio bound/free H-CT is identical in both cases, indicating that plasma proteins can display a binding capacity able to match the high hormone concentration of effluent thyroid blood[7].

Résumé. La protéine porteuse spécifique de la CT-H est l'IgM lorsque la teneur en hormone est relativement faible (jusqu'à 250 ng/ml de plasma). Lorsque la concentration augmente, la CT-H se lie aux α_2-macroglobulines, α_2-lipoprotéines et à l'albumine, dont l'affinité pour l'hormone est moindre mais la capacité de liaison importante. Il est logique d'admettre que des anomalies portant sur les protéines porteuses spécifiques modifient la distribution et le métabolisme de la CT, ce qui représenterait un mécanisme pathophysiologique nouveau de dyscalcitonie humaine.

G. MILHAUD and I. SZLAMKA

Laboratoire des Isotopes, Laboratoire associé au C.N.R.S., Faculté de Médecine Saint-Antoine, 27, rue Chaligny, F-75 Paris 12e (France), 1 June 1971.

[6] J. LEGGATE, A. D. CARE and S. C. FRASER, J. Endocr. *43*, 73 (1969).

[7] We thank Dr. H. HIRSCH-MARIE for his help during this work.

Cytotoxic Potential of Lymphocytes Stimulated with Autochthonous Lymphoid Cell Line Cells

Human lymphocytes are activated by contact with histocompatibility antigens on the lymphocytes of another donor. The reaction, which occurs with lymphocytes of all normal individuals and with lymphocytes from cord bloods, requires no prior immunisation of the cell donors with the antigens concerned (for refs. see[1]). An immunological basis for the reaction is indicated, however, by the demonstration, in rats, that lymphocytes from tolerant animals are specifically unresponsive to cells of the tolerance inducing strain[2,3]. Human lymphocytes are also activated by cells from human lymphoblastoid cell lines (LCLs) established from the blood cells of normal individuals and patients with various disorders. The reaction is essentially similar to a 'one-way' mixed lymphocyte reaction but of greater intensity[4]. Lymphocytes activated by culture with X-irradiated cells from a particular cell line are cytotoxic to these cells and the cells of other lines[5].

New surface antigens on the LCL cells as well as histocompatibility antigens must be capable of activating lymphocytes since cells from autochthonous LCLs are stimulatory[6-9], although if the conditions are carefully controlled, to a lesser degree than cells from a histoin-