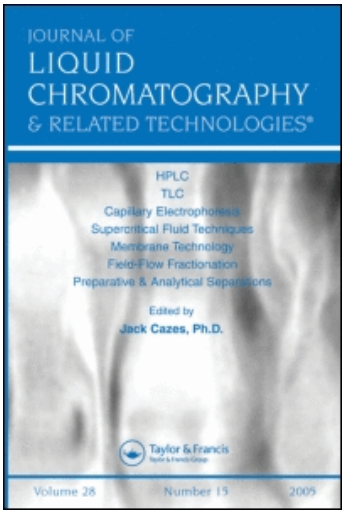

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USE OF RP-HPLC FOR PURIFICATION OF A CCK-39 RADIO-LIGAND AND OF ITS PHOTOACTIVABLE DERIVATIVE. CONTROLS OF DEGRADATIONS

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ABSTRACT

In order to characterize CCK receptors on guinea-pig pancreatic acini we developped a method of purification and control of degradation of CCK-39 radio-ligands and of their photoactivable derivatives. CCK-39 was radio-labeled with N-hydroxysuccinimide 3-(4-hydroxy 5 [^{125}I] phenyl) propionate at pH = 8.5. Mono-labeled CCK-39 was separated from di- and tri-labeled CCK-39 on a C-18 column and coupled with N-hydroxysuccinimidyl-4-azido-benzoate. The resulting photoactivable radio-ligand ^{125}I-BH-[4-azidobenzoyl]-CCK-39 specifically bound to pancreatic membranes. After photolysis under UV irradiation and SDS-PAGE a major labeled protein of Mr 90 000 could be identified. Furthermore, RP-HPLC permitted the control of degradation of each ligand. Presence of endo-peptidasic and aminopeptidasic activities sensitive to EDTA and bacitracin were demonstrated.

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INTRODUCTION

Cholecystokinin (CCK) is the major hormonal stimulant of the exocrine pancreas "in vivo" and "in vitro". Although the CCK receptors have been studied extensively using isolated pancreatic acini from several mammals (1-4), the receptors proteins have not yet been purified. Studies involving affinity labeling procedures followed by covalent cross-linking using UV irradiation or bifunctional reagent have permitted SDS-PAGE identification of Mr 76-80 000 proteins containing CCK-sites (5-7). Recently, the solubilization of a high molecular weight CCK receptor complex including guanine nucleotide regulatory protein was described (8). Moreover, solubilization of active CCK receptors from mouse pancreas was recently achieved by using digitonin (9). In light to the results that we obtained on dog pancreatic acini indicating a specificity among CCK/gastrin receptors (4), we plan the solubilisation and biochemical characterization of CCK receptors in superior mammals, especially Dog. As such a very important quantity of pancreatic membranes is required for such a study, we developped a rapid method for the preparation of crude membranes. Moreover, we investigated a methodological approach for preparation and purification of CCK-39 radio-ligands suitable for binding and covalent labeling of CCK receptors. The preliminary studies have been

done on guinea pig pancreas in order to set up the methodology on a convenient and cheeper model.

MATERIALS AND METHODS

Chemicals

^{125}I-Bolton-Hunter reagent [N-Succinimidyl 3(4-hydroxy, 5-^{125}I-iodophenyl) propionate] with a specific radio-activity of 1800-2000 Ci/mMol was obtained from Amersham France. N-hydroxysuccinimidyl 4-azidobenzoate (HSAB) was purchased from pierce, acetonitrile from fluka Lab. and CCK-39 from G.I.H. Research Unit, Karolinska Institute, Stockholm, Sweeden.

Preparation of radio-ligands

Coupling of the Bolton-Hunter reagent with CCK-39 was performed as in (10). Purification and degradation analysis of labeled peptides were achieved by reverse-phase HPLC (waters apparatus) on a C-18 column in a triethyl ammonium phosphate buffer (TEAP) 0.25 N, pH = 3,5 + acetonitrile (CH_3CN). A gradient of acetonitrile from 27 to 50 % was used. The photoactivable iodinated CCK-39 was obtained by coupling at 4°C for 15 h, 20 picomoles of mono-iodinated CCK-39 (^{125}I-BH-CCK-39) with an excess of HSAB (2 µmoles) in a solution of 0.2 M sodium Borate pH = 8,5 + dimethyl forma-

mid (0.5 ml/0.5 ml). The reaction was quenched by adding 100 µl of glycine 0.2 M.

Preparation of crude membranes

Guinea-pig were decapited and the pancreas homogeneized at 0-4°C in 50 mM HEPES buffer pH = 10,0, (1g in 40 ml) containing 0.2 % bovine serum albumin, 5 mM $MgCl_2$, 0.03 % soybean trypsin inhibitor, 0.1 % bacitracin, 0.1 mM phenyl methyl sufonylfluoride (PMSF), 1 mM EGTA, by using a polytron homogenizer. The homogenate was centrifuged at 27 000 xg for 20 min and the pellet resuspended in the same volume of 50 mM HEPES homogeneisation medium at pH = 7,0.

Binding and degradation studies of CCK-39-ligands

Crude membranes at 2.5 mg/ml of proteins were incubated with 0.1 nM of CCK-39 radio-ligands alone or with increasing concentrations of CCK-39 at 37° C for the times required. Non-specific binding was determined in presence of 1 µM CCK-39.

Degradation of iodinated CCK-39 was studied in the incubation medium and at the level of receptors. At the end of the incubation period an aliquot of the suspension was transferred to plastic microtubes containing 0.5 ml of cold medium and was centrifuged at 10 000 x g for 10 min. The supernatant was removed for RP-HPLC analysis of free radio-

activity. The radioactivity bound to the pellet was measured.Then, the membranes were resuspended in 0.5 ml of HPLC buffer and centrifuged again. The specifically bound radioactivity was totally dissociated by this washing and analysed by RP-HPLC on a C-18 column.

Photo-affinity labeling and gel electrophoresis

1 ml of membranes were incubated with ^{125}I-BH-(azidobenzoyl)-CCK-39 at 5.10^{-10}M in the presence or absence of CCK-39 10^{-6}M at 37°C for 15 min. The membranes were washed twice in Hepes buffer and photolysed 15 min at 4°C with a mercury lamp (30 watts) at a distance of 20 cm. The membranes proteins were solubilized in 2 % S.D.S. at 80°C for 5 min and samples were analysed by SDS polyacrylamide gel electrophoresis (11). Linear gradient gel (5-15 % acrylamide, 1 mm thick, 16 cm length) were prepared as described by Bio-Rad (Richmond, California). After staining with cromassie bleue (G-250) and destaining, the gels were dried and autoradiographed. The films were exposed for 2-5 days at - 70°C. The Mr values of the standards used were :myosine (Mr:200 000) ; ß galactosidase (Mr : 116 250) ; phosphorylase B(Mr : 92 500) ; bovine serum albumin (Mr : 66 200) ; ovalbumin (Mr : 45 000) ;carbonic anhydrase (Mr : 31 000) ; soybean trypsin inhibitor (Mr : 21 500) ; lysozyme (Mr :14 400).

RESULTS

HPLC Purification of CCK-39 radio-ligands

In a first approach (12) applied to the purification of iodinated (Thr,Nle)-CCK-9 (10) we claimed that a step gradient of 27 to 50 % acetonitrile was required both for the obtention of a good chromatographic yield and for the elution of the iodinated peptide in a small volume. Nevertheless, CCK-39 possesses three free amino groups (the amine terminal and two $\mathcal{E}$-NH_2 from lysine intrachains). Several labeled species could be generated during the labeling with the Bolton-Hunter reagent. A step gradient procedure as described in (12) is no resolutive enought to separate all the possible products of the coupling in distinct peaks. Consequently, we investigated a chromatographic procedure of purification which led to separation of mono-iodinated CCK-39 from poly-iodinated CCK-39.

Fig. 1 illustrates a chromatographic run of a labeling mixture. The two first radio-active peaks corresponds to ^{125}I-BH-gly and to the hydrolysis product of ^{125}I-BH, respectively (12). The peak eluted at 20 min in isocratic conditions (27 % CH_3CN) and the larg peak eluted between 35 and 55 min contained molecules which specifically bound to pancreatic membranes. The radioactivity contained in the fractions corresponding to the last peaks represented 2,5 % and

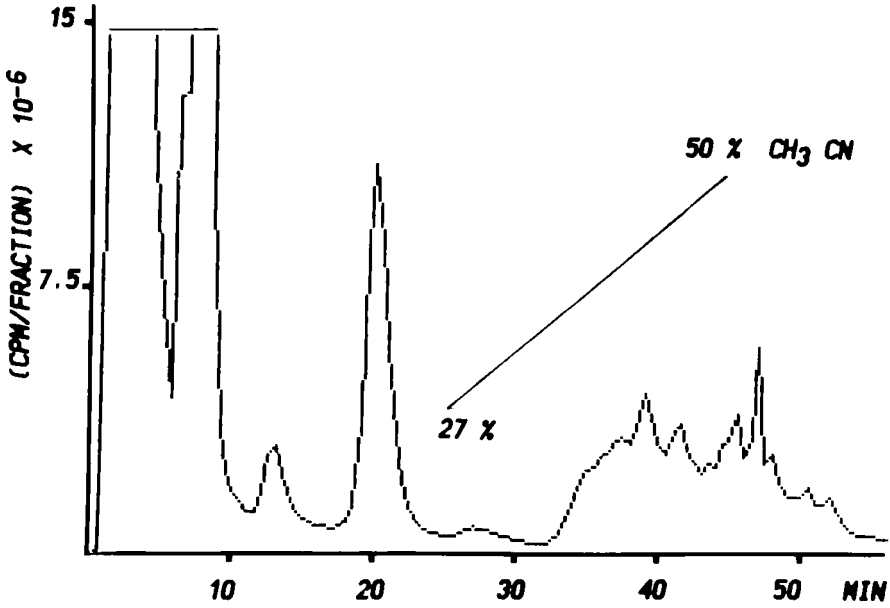

Fig. 1 : Radioactive profile of the eluted fractions from RP-HPLC of CCK-39 labeling medium (1 mCi).
Column : Bondapak C-18 (3,9 x 300 mm).
Mobil phase : TEAP 0,25 N, pH = 3,5 + CH_3CN.
Flow rate : 2 ml/min ; Back pressure : 2000 PSI.
Fraction volume : 1 ml.

10 % of the total amount injected respectively. In a second experiment we chromatographied an aliquot of labeling mixture under isocratic conditions (27 % CH_3CN) until the elution of peak 3, followed by a step gradient (fig. 2). Radioacivity corresponding to peak 4 in fig. 1 was eluted in a single peak. When an aliquot of the labeled peptide eluted by the step gradient was analysed under a linear gradient from 30 to 50 % (fig. 3), the radio-activity was eluted in a major

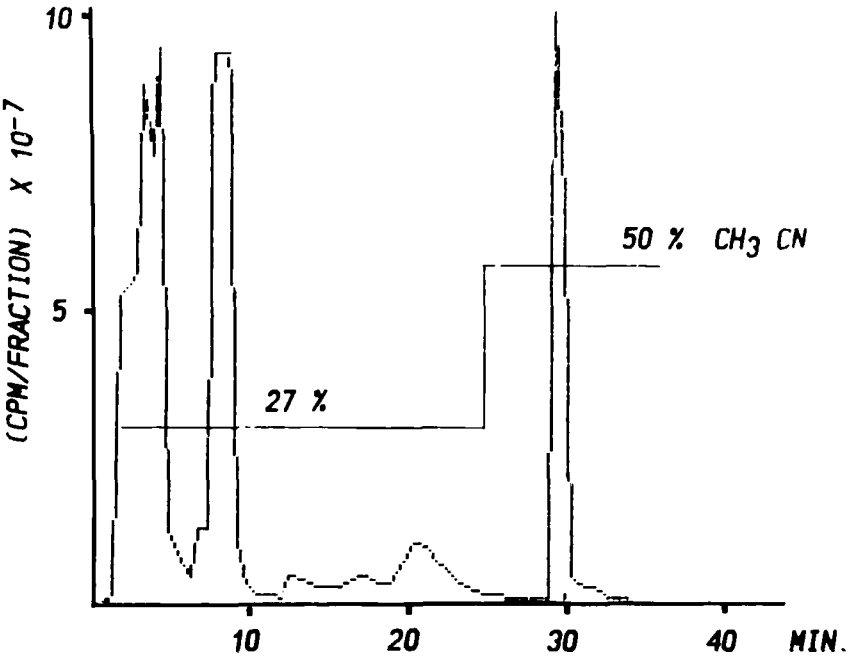

Fig. 2 : RP-HPLC of CCK-39 labeling medium (1 mCi). Conditions as in fig. 1 excepted the gradient of acetonitrile.

peak at 11 min followed by two minor peaks. Such results seems to indicated that several labeled CCK-39 derivatives were generated during the labeling. One of them, the less hydrophobe (peak 3, fig. 1), which probably corresponds to a mono-iodinated CCK-39, can be easily purified. A second group of labeled components exhibited strong hydrophobic interactions with the C-18 column. Its most likely corresponds to di-iodinated CCK-39. In fact, assuming that at the pH of labeling (pH 8,5), the N-terminal amino-group is the most

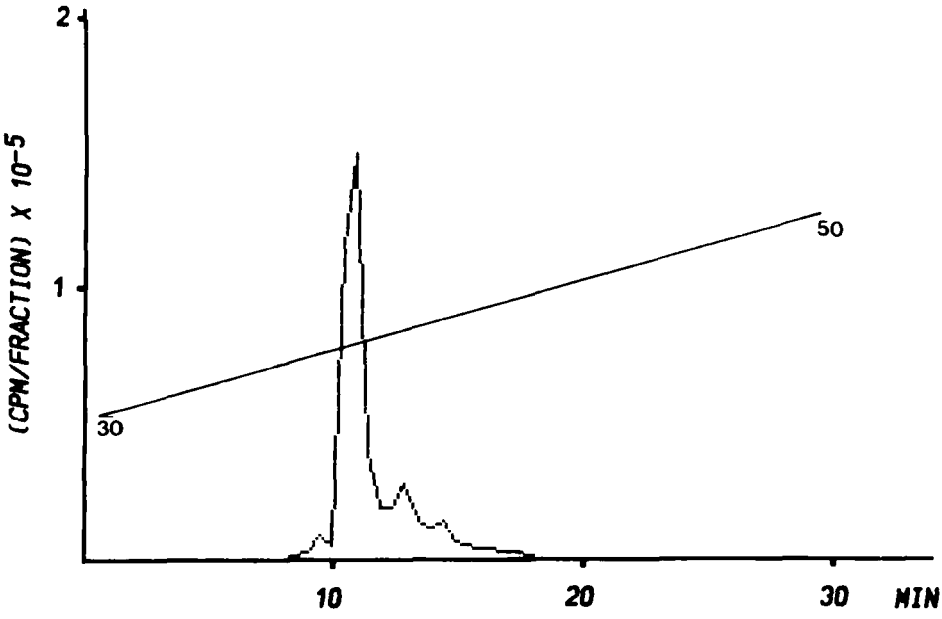

Fig. 3 : RP-HPLC of an aliquot of iodinated CCK-39 eluted at 30 min in TEAP/CH_3CN (50/50) buffer as in fig. 2.

easily acylable by the Bolton-Hunter reagent, the mono-iodinated CCK-39 most likely results from the labeling via the N-terminal part of CCK-39, while the most hydrophobic species results from the labeling via the N-terminal end as well as one $\mathcal{E}$-NH_2 of the lysines.

For synthesis of photoactivale CCK-radio-ligands, we incubated at 4°C for 15 h the mono- and di-iodinated CCK-39 with an exces of HSAB. Fig. 4 represents the radioactive profile obtained when an aliquot of the coupling medum of mono-iodinated CCK-39 with HSAB was injected on the C-18

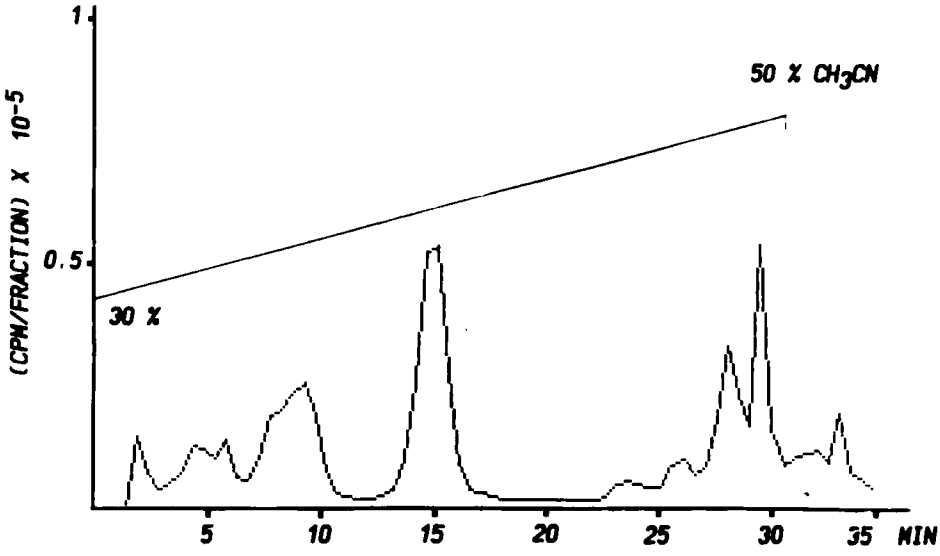

Fig. 4 : RP-HPLC of the coupling medium of mono-iodinated CCK-39 to N-hydroxysuccinimidyl, 4 azidobenzoate (HSAB).

colum. The radio-activity which was eluted as a single peak was strongly retained on the C-18 column after coupling to HSAB. Three main peaks were resolved indicating different sites and degrees of coupling with HSAB. Because of the good ability of binding to pancreatic membranes, we used the photoactive iodinated CCK-39 [^{125}I-BH-(acido-benzoyl)-CCK-39] without further purification steps.

Binding and degradation of iodinated CCK-39

On fig. 5 are represented kinetics of binding of labeled CCK-39 to pancreatic membranes. It appears that association of mono-iodinated CCK-39 is the most rapid but is not

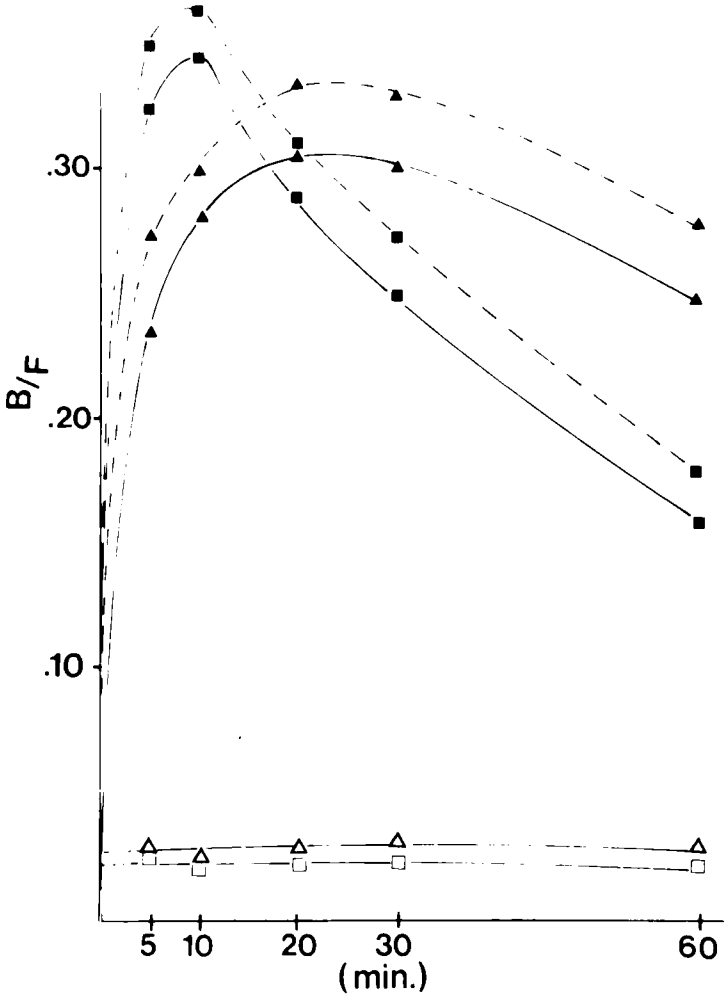

Fig. 5 : Kinetics of association of iodinated CCK-39 to pancreatic membranes at 37°C.

Mono-iodinated CCK-39
■- - -■ total binding
■——■ specific binding
□——□ non-specific binding

Di-iodinated CCK-39
▲- - -▲ total binding
▲——▲ specific binding
△——△ non-specific binding

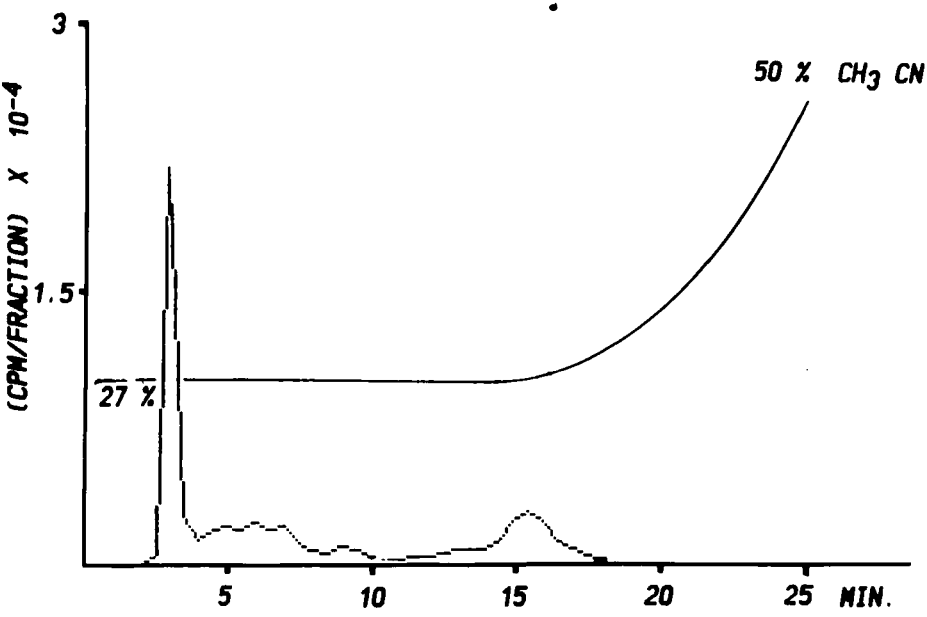

Fig. 6 : RP-HPLC analysis of mono-iodinated CCK-39 incubated30 min at 37°C in presence of pancreatic membranes.

stable as indicated by the rapide drop in the binding after 10 min of incubation. This result led us to check the degradation of labeled CCK-39 during incubation with pancreatic membranes.

Fig. 6 illustrates HPLC profile of mono-iodinated CCK-39 contained in the supernatant i.e. free radio-ligand of a suspension of membranes incubated 30 min at 37°C. The comparaison of this profile with that of fresh radio-ligand showed that the major part of the radio-ligand eluted in fractions 30 was eluted in fraction 6 after incubation. In-

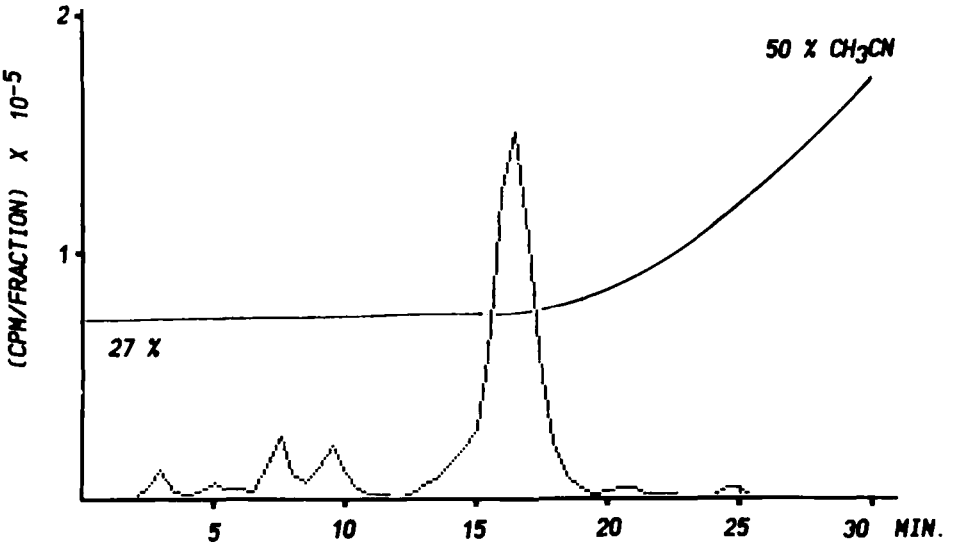

Fig. 7 : RP-HPLC analysis of membrane-bound radio-activity after 30 min of incubation at 37°C.

terestly, the membrane bound radioactivity remained almost intact during the same period of incubation (fig. 7). Table 1 summarizes results concerning the degradation of the two radio-labeled CCK-39. The rapid degradation of mono-iodinated CCK-39 correlates well the decrease of the binding after 10 min of incubation at 37°C. The di-iodinated form was more stable (not shown). Effects of several enzymatic inhibitors were investigated. Increasing the concentrations of STI, PMSF did not decrease the degradation phenomenae. However, the addition of EDTA 5 mM and increasing the bacitracin concentration up to 5 mg/ml significantly changed the

TABLE 1 : RP-HPLC analysis of incubated ^{125}I-BH-CCK-39 in presence of pancreatic membranes.
C-18 column, TEAP/CH_3CN eluant. Gradient n°9, 27 %-> 50 % CH_3CN in 30 min. Flow-rate : 2 ml/min ; fraction volume : 1 ml. Standard medium was composed as indicated in the section materials and methods.

	Radio-activity recovered in peaks (% of total)				
Retention time(min)	3	6	8	9	19
^{125}I-BH-CCK-39	3	0	2	3,5	91,5
Standard medium(1)	21	8	-	-	60
+ STI (1 mg/ml)	23	9	-	-	56
+ PMSF (0,5 mM)	25	10	-	-	55
+ Bacitracin (5mg/ml)	12	8	6	13	54
+ EDTA (5 mM)	10	4	-	-	74

profile of degradations. (Table 1). Furthermore, chromatographic profiles of iodinated CCK-39 incubated in presence of EDTA 5 mM or bacitracin 5 mg/ml suggests that at least two enzymic activities are involved ; an endopeptidasic activity (EDTA-sensitive) and an amino-peptidasic activity (bacitracin-sensitive) which generated the less hydrophobic peptidic fragments.

Binding and covalent cross-linking of ^{125}I-BH-(Azidobenzoyl)-CCK-39 to the CCK receptors

Fig. 8 shows kinetics of binding of ^{125}I-BH-(Azidobenzoyl)-CCK-39 to pancreatic membranes. Compared to ^{125}I-BH-CCK-39 binding, the photoactivable ligand exhibited the same total binding with, however a higher non-specific binding. After photolysis and solubilisation of membranes proteins by SDS, both gel permeation analysis (fig. 9a) and gel electrophoresis (fig. 9b) revealed that a fraction of ^{125}I-BH-Azidobenzyol)-CCK-39 was covalently and specifically bound to membrane proteins. As indicated by the autoradiograph, the major component was labeled with a good yield. It had an apparent Mr of 85 000-89 000. Also seen were components with apparent Mr values of > 200 000, 125 000, 57 000, 37 000.- Performing the photolysis in presence of CCK-39 10^{-6} M abolished the labeling of component with Mr 125 000, 87 000 and 57 000 suggesting that the other band represented non-specific binding.

DISCUSSION

Biologically active CCK radio-ligands, in particular ^{125}I-BH-CCK-33 were synthetized few years ago (2) using ^{125}I-Bolton-Hunter reagent which upon reaction with the pep-

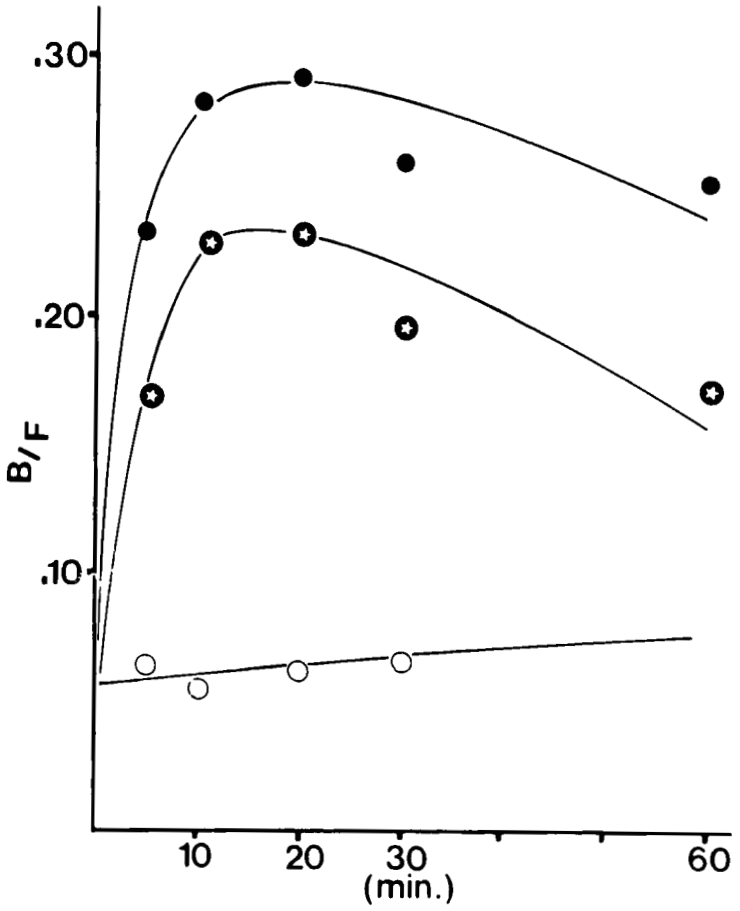

Fig. 8 : Kinetic of association of ^{125}I-BH-(Azidobenzoyl)CCK-39 to pancreatic membranes at 37°C.

● — ● Total binding
✪ — ✪ Specific binding
○ — ○ Non-specific binding determined in presence of 10^{-6}M CCK-39.

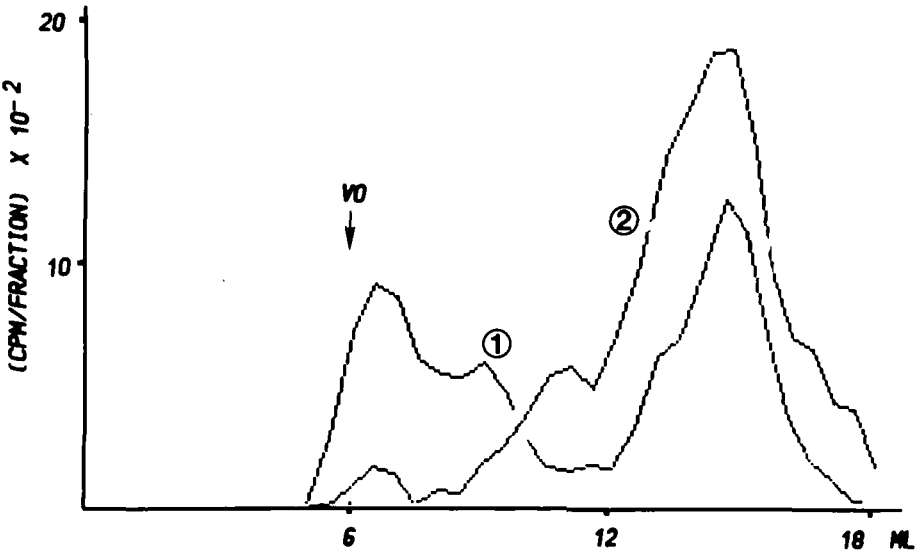

Fig. 9a : Gel permeation analysis of membranes proteins cross-linked with ^{125}I-BH-(Azidobenzoyl)-CCK-39. Membranes were incubated with ^{125}I-BH-(Azido-benzoyl)-CCK-39 at 5.10^{-10}M alone (profile 1) or in presence of CCK-39 10^{-6}M (profile 2) for 15 min at 37°C. After washing and photolysis at 4°C, the membrane proteins were solubilized in 2 % Triton X-100 at 20°C.
Column : I-125 (Waters). Buffer : Hepes 50 mM pH = 7,0, Urea 6 M, Triton X-100 0,02 %. Flow rate : 0,5 ml/min.
Back-pressure 800 PSI. Fraction volume : 0,5 ml.

tide acylates free amino-groups. As CCK-33, CCK-39 possesses three primary amino-groups located at position Tyr-1, lys-7 and lys-17. Recently, Rosenzweig et al (7)determined whether the labeled CCK-33 synthetized at pH = 10,0 and purified on a sephadex column still possessed a free-NH_2. They tested the ability of ^{125}I-BH-CCK-33 to be covalently linked to CCK directed antibodies in presence of dissuccinimidyl subera-

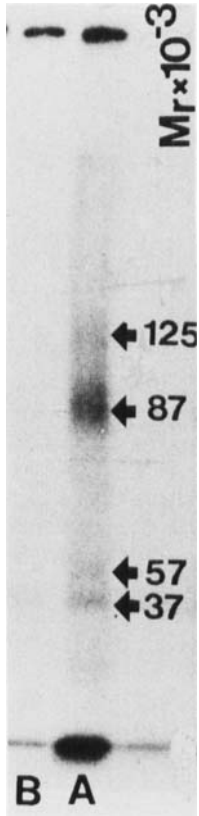

Fig. 9b : SDS-PAGE Analysis of membranes proteins cross-linked with ^{125}I-BH-(Azidobenzoyl)-CCK-39. conditions as described in fig. 1 and in roler experimental procedure. Incubation with ^{125}I-BH-(Azidobenzoyl)- CCK-39 alone, line A, with 10^{-6}M CCK-39, line B. (a representative exp. of 3 others.

te. The authors concluded that at least one free amino-group was available for reaction with IgG. In the work reported here, we carried out radio-iodination of CCK-39 at a lower pH than in Rosenzweig's study to limitate acylation of $-NH_2$ in the two lysine. Nevertheless, RP-HPLC analysis of labeling medium revealed that two major iodinated species exhibiting different hydrophobic properties were obtained. Assuming that the N (^{125}I-desaminotyrosyl) residues introduced in the peptide were responsible of the increasing of the hydrophobicity of the molecules, we can claim that the less retained molecules on the C-18 column, most likely corresponded to the mono-iodinated CCK-39 via the N-terminal end of CCK-39. While both of the two major labeled CCK-39 specifically bound to CCK receptors, we observed that in the presence of membranes, the mono-iodinated CCK-39 was more rapidly converted to polar labeled components than di-iodinated CCK-39. This observation suggests that mapping the $-NH_2$ with N [^{125}I-desaminotyrosyl) increased the resistance of CCK-39 to enzymatic cleavages and favours the hypothesis of three primary amino-groups located at position Tyr-1, lys-7 and lys-17. Recently, Rosenzweig et al (7)determined whether the labeled CCK-33 synthetized at pH = 10,0 and purified on a sephadex column still possessed a free-NH_2. They tested the ability of ^{125}I-BH-CCK-33 to be covalently linked to CCK

directed antibodies in presence of dissuccinimidyl suberate. The authors concluded that at least one free amino-group was available for reaction with IgG. In the work reported here, we carried out radio-iodination of CCK-39 at a lower pH than in Rosenzweig's study to limitate acylation of $-NH_2$ in the two lysine. Nevertheless, RP-HPLC analysis of labeling medium revealed that two major iodinated species exhibiting different hydrophobic properties were obtained. Assuming that the N (^{125}I-desaminotyrosyl) residues introduced in the peptide were responsible of the increasing of the hydrophobicity of the molecules, we can claim that the less retained molecules on the C-18 column, most likely corresponded to the mono-iodinated CCK-39 via the N-terminal end of CCK-39. While both of the two major labeled CCK-39 specifically bound to CCK receptors, we observed that in the presence of membranes, the mono-iodinated CCK-39 was more rapidly converted to polar labeled components than di-iodinated CCK-39. This observation suggests that mapping the $-NH_2$ with N [^{125}I-desaminotyrosyl) increased the resistance of CCK-39 to enzymatic cleavages and favours the hypothesis of involvement of trypsine-like enzymes sensitive to the presence of EDTA. Amino-peptidases which were strongly inhibited by bacitracin were also involved in degradation phenomenae.

We chose a rapid method suitable for the preparation of pancreatic membranes. The procedure can be scale for preparative purpose. This method offers the advantage of avoïding acini preparation step by collagenase digestion during which a fraction of membranes and their receptors is lost. Nevertheless, assuming that until now, CCK-receptors were only characterized on the surface area of the acinar cell plasmalemma and on beta and alpha cells in the islets (13), and that acinar plasma membranes represent at least 95 % of the total pancreatic plasma membranes, we can postulate that the labeled CCK receptors mainly originated from the exocrine tissue. The homogeneization at alcaline pH was introduced because at pH = 10 the CCK binding properties of the membranes were not affected while a part (20 %) of extrinsic proteins, especially enzymatic proteins could be solubilized. Residual enzymatic activities were still present after alcaline treatment. The exact nature of these enzymes still remains to be elucidated. Whatever, this study illustrates how RP-HPLC is a reliable method suitable to controle the radio-ligand degradations. The precipitation method by trichloroacetic acid (TCA) is based on the statement whereby in a TCA solution, the intact radio-ligand precipitates whereas the degradated forms are soluble (2). Such a method cannot report degradation phenomenae occuring in

the C-terminal part of the molecule which is the most essential for its bioactivity. An other method currently used consists in comparing kinetics of association of the radioligands previously submitted to incubation in presence of membranes with fresh radio-ligand (14). We elsewhere found that a 20 % degradation of ^{125}I-BH-(Thr,Nle)-CCK-9 in presence of guinea-pig pancreatic acini measured by using a CCK antibody did not significantly impairs kinetic of association (10). These data illustrate that among the different methods available up to now for tracer degradation studies, none is really satisfactory.

Since the first development in the field of affinity cross-linking of hormonal receptors, many cross-linking reagents were synthetized and tested. More and more, photoactivable reagents are used in replacement to homo-or bifunctional cross-linkers.

Several approach to cross-linked ^{125}I-CCK to its receptors including direct UV irradiation (5, 6), and chemicals regents able to linked the radio-ligand to its receptor via free amino-groups (6, 7) were recently reported. We present here a photo-activable CCK-radio-ligand which was successfuly cross-linked to CCK receptors on pancreatic membranes from guinea-pig.

The results obtained during the first step of our work show that the new photoactivable CCK radio-ligand would be a promising tool in the field of CCK-receptors characterization.

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