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Experiences Using Chloramine-T and 1, 3, 4, 6-Tetrachloro-3α, 6α-diphenylglycoluril (Iodogen®) for Radioiodination of Materials for Radioimmunoassay

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Dedicated to Prof. Dr. rer. nat. H. Holzer on the occasion of his 60th birthday

Summary: A comparison of labelling compounds with chloramine-T and with 1, 3, 4, 6-tetrachloro-3α, 6α-diphenylglycoluril (Iodogen®) has been carried out.

For human transferrin, human calcitonin, 1-84 bovine parathyrin, fibrinopeptide-A, human thyrotropin and F-CB3, a cyanogen bromide cleavage peptide of human fibrinogen, the quality of tracer produced by the Iodogen method was better. For rat lutropin, human growth hormone and human prolactin, labelling with Iodogen produced a tracer of unsatisfactory quality. For a further 13 peptides, the results from both methods were comparable.

Optimal reaction times using Iodogen were of the magnitude of two to three times longer than when using chloramine-T. Reduction of the volume of radioactive waste by up to 90% could be achieved when the Iodogen method was coupled with a short cation-exchange column to separate unreacted iodide from the labelled compound. Data is presented on the quality of tracer, expressed in terms of elution profiles and radioimmunoassay standard curves.

A novel "combi-method" of labelling proteins without tyrosine or histidine moieties is presented where N-succinimidyl-3-(4-hydroxyphenyl)-propionate is labelled at pH 7.5 using Iodogen to give "*Bolton-Hunter*" reagent, which is then transferred to a vessel containing the peptide to be labelled at pH 8.6.

Erfahrungen mit Chloramin-T und Iodogen® für die Iodierung von Peptiden für Radioimmunoassays

Zusammenfassung: Es wurde eine Vergleichsuntersuchung zweier Methoden zur Radioiodierung von Peptiden unter Verwendung von Chloramin-T und 1, 3, 4, 6-tetrachlor-3α, 6α-diphenylglycoluril (Iodogen) als Oxidans durchgeführt.

Die Iodierungsergebnisse waren von Peptid zu Peptid unterschiedlich. Bessere Ergebnisse der Iodogenmarkierung wurden bei humanem Transferrin, humanem Calcitonin, 1-84 bovinem Parathormon, Fibrinopeptid-A, F-CB3 (einem Bromcyan-Spaltprodukt des humanen Fibrinogens) und humanem Thyrotropin beobachtet. Dagegen sind die Iodogentracer von Ratten-Lutropin sowie humanem Prolactin und humanem Wachstumshormon nicht brauchbar gewesen.

Vorteile der Verwendung von Iodogen waren die längeren Markierungszeiten sowie die Verminderung der radioaktiven Abfälle (um 90%), wenn die Trennung von gebundenem und freiem Iodid mit Hilfe einer kurzen Ionenaustauschersäule erfolgte.

Eine neue „Combi-Methode" für Peptide ohne Tyrosin bzw. Histidinreste wird beschrieben, wobei N-Succinimidyl-3-(4-hydroxyphenyl)propionat zuerst bei pH 7,5 mit Iodogen markiert wurde. Anschließend wurde dieses „*Bolton-Hunter*" Reagens in einem zweiten Gefäß dem zu markierenden Peptid bei pH 8,6 hinzugefügt, wo die Iodierungsreaktion dann stattfand. In diesem Bericht werden die erreichte Iodinkorporation sowie die Qualität der hergestellten Tracer anhand von Standardkurven bzw. Elutionsprofilen mitgeteilt.

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Introduction

Iodination with chloramine-T has been carried out routinely for many years (1). The advantage of this method over its predecessors (electrolytic iodination, oxidation with iodine monochloride), was the higher yield and specific activity of the products, coupled with a rapid and simple procedure. A disadvantage is the possible alteration of the physico-chemical properties of the substance to be labelled under the strong oxidation conditions.

In order to overcome these side-effects, lactoperoxidase was substituted for chloramine-T (2–5), and although here, the reaction sequence is more complicated than with chloramine-T, the quality of the products in terms of immuno- and bio-activity is often better.

The introduction of Iodogen as oxidant (6, 7) has led to a new procedure which is both simple and effective, and which usually gives rise to iodinated tracers of high quality. When this method is used in conjunction with a short cation-exchange column for desalting the iodination mixture, up to 90% of the radioactive waste produced by chloramine-T and a conventional gel-filtration desalting step falls away.

As Iodogen is water insoluble, but is very soluble in chloroform, it can be used to plate vessel walls by pipetting a solution of Iodogen in chloroform to reaction vessels and then allowing the chloroform to evaporate off in a fume cupboard. If the resulting tubes are then stoppered and stored in a closed vessel containing silica gel at - 20 °C, they are stable for several months. Although the reaction mechanism of both chloramine-T and Iodogen is the same, the fact that the Iodogen is immobilised means that it comes into less contact with the compound to be labelled, which in turn leads to less unwanted side-effects.

Materials and Methods

Chloramine-T, sodium metabisulphite and buffer chemicals were purchased from Merck, Darmstadt, FRG. Iodogen and N-succinimidyl-3-(4-hydroxyphenyl) propionate were obtained from Pierce Eurochemie BV. Rotterdam, NL. Sephadex gels were purchased from Pharmacia, Freiburg i. Br. FRG, Ultrogel from LKB, Munich and Biogel from Bio-Rad, Munich, FRG. Sodium ^{125}iodide was obtained from Amersham-Buchler, Brunswick, FRG.

The proteins and hormones were obtained from the following sources: Human transferrin, human caeruloplasmin and human Gc-protein were purchased from Behringwerke, Marburg a.d. Lahn, FRG.

1-32 human calcitonin (hCT) and 1-34 human parathyrin (1-34 hPTH) were donated by Prof. *R. Ziegler*, Heidelberg, FRG.

1-84 bovine parathyrin (1-84 bPTH) was purchased from Inolex (Lot Nos. 1515-A001 and 1515 C001) Chicago, Illinois USA.

44–68 human parathyrin (44–68 hPTH) was a generous gift from Prof. *R. D. Hesch*, Hannover, FRG.

Human prolactin (hPRL) and human growth hormone (hGH) were given by Prof. *K. von Werder*, Munich, FRG.

Fibrinopeptide-A, (FPA), Factor VIII clotting activity (FVIIIC), antibodies to Factor VIII related antigen (Anti FVIIIR) and antibodies to FVIIIC (Anti FVIIIC) were from Dr. *W. Schramm* Munich, FRG.

High molecular weight kininogen (HMWK) was from Prof. *H. Fritz*, Munich, FRG.

F-CB3, a cyanogen bromide cleavage product of human fibrinogen was generously donated by Dr. *Rotraut Gollwitzer*, Martinsried, FRG. Human choriongonadotropin was provided by Dr. *K. Mann*, Munich, FRG. Human endorphins and 1-24 corticotropin (ACTH 1-24) were bought from Serva Heidelberg, FRG.

Human thyrotropin (hTSH) was purchased from Kabi AB Stockholm, S. Rat lutropin (rLH) was supplied by Dr. *G. Emons*, Lübeck, and cortisol-3-tyrosyl methyl ester was synthesised by Dr. *G. Stalla*, Munich, FRG.

Labelling with chloramine-T and Iodogen

Chloramine-T

Dissolve 10 mg chloramine-T and 10 mg sodium metabisulphite each in 10 ml 0.05 mol/l phosphate buffer, pH 7.5 (for labelling tyrosine moieties) or pH 8.4 (for histidine moieties). Add the components to the reaction vessel in the following order: peptide to be labelled (10 µl), $Na^{125}I$ in 0.5 mol/l phosphate buffer, pH 7.5 or 8.4 (20 µl = 37.2 MBq) and chloramine-T (10 µl) and mix. After reaction (5–30 seconds) add sodium metabisulphite (50 µl), mix and transfer the reaction mixture to a Sephadex G-10 column (9 × 1 cm) and elute with 0.05 mol/l Tris-HCl buffer, pH 7.5 containing bovine serum albumin (10 mg · liter^{-1}). Count the fractions and collect the first peak. This is then either diluted for use, or rechromatographed on a second column of suitable material (e.g. Sephadex, Ultrogel or Biogel) if necessary.

Iodogen

1. Coating the vessels

Pipette 25 µl of Iodogen in chloroform (0.2 g · liter^{-1}) into polypropylene iodination vessels (Eppendorf 3811 or Sarstedt 696) and allow the chloroform to evaporate off in a fume cupboard. The vessels are then stoppered and stored in a sealed vessel containing a dessicant (silica gel) at – 20 °C until required (shelf life in excess of six months).

2. Labelling

The substance to be labelled (20 µl) is pipetted into the vessel containing the Iodogen followed by 37 MBq $Na^{125}I$ in 0.5 mol/l phosphate buffer pH 7.5 or 8.4 (20 µl). The vessel is shaken during the reaction time (15–60 seconds) and the contents then transferred to a Sephadex SP-C25 desalting column (2 × 1 cm) equilibrated to pH 3.0 with 0.01 mol/l glycine-HCl buffer. The same buffer is then used to elute off unreacted iodide, the peptide peak being eluted off using 0.5 mol/l phosphate buffer pH 7.5. The second peak is either diluted with assay buffer for use, or rechromatographed if necessary as described above for chloramine-T. The time taken up to the completion of the desalting step is 25–40 min for Iodogen and 60–75 min for chloramine-T.

Results

Effects of Iodogen concentration and time of radioiodination

Figure 1 shows the effects of Iodogen concentration and labelling time on the elution profile of 1-84 bovine parathyrin, together with the maximum binding in the fractions showing immunoreactivity. As a comparison, the labelling with chloramine-T ran identically with the Iodogen 25/90 labelling on the same column.

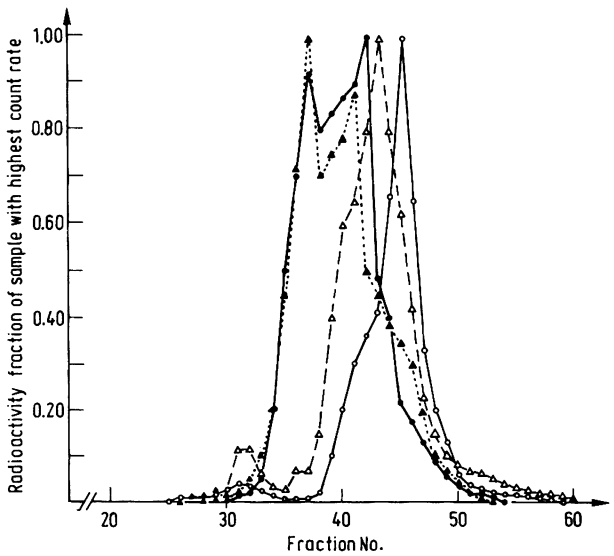

Fig. 1. The effect of the amount of Iodogen used, and of the labelling time upon the elution profile and binding characteristics of bovine parathyrin (1-84 bPTH). The column used was Ultrogel AcA-44 (130 × 2.5 cm) using 0.1 mol/l ammonium acetate buffer, pH 4.5.
o——o 25 μg Iodogen/90 s labelling-Max. binding in fractions 39–42. $B_0/T = 0.31$
△ – – △ 25 μg Iodogen/200 s labelling-Max. binding in fractions 38–41. $B_0/T = 0.28$
●——● 5 μg Iodogen/60 s labelling-Max. binding in fractions 36–39. $B_0/T = 0.32$
▲-----▲ 5 μg Iodogen/30 s labelling-Max. binding in fractions 36–39. $B_0/T = 0.34$
The void volume of the column is 118 ml which corresponds to the first 25 fractions (4.75 ml each) collected.

Tab. 1. Optimal conditions for labelling with chloramine-T and Iodogen under the conditions stated in the text.

Ligand	Amount	Chloramine-T	Iodogen
	(μg)	Labelling time (seconds)	
Human transferrin	10	5–10	30–45
1-84 Bovine parathyrin	5	10–15	30–45
*44-68 Human parathyrin	2	5–10	30–45
Human calcitonin	5	5–10	20–30
*Fibrinopeptide-A	5	15–20	30–45
Human fibrinogen fragment F-CB3	10	10–15	30–45
Human choriongonadotropin	5	10–15	30–45
Human choriongonadotropin α-subunit	5	10–15	20–30
Human thyrotropin	5	10–15	20–30
Factor VIII clotting activity (FVIIIC)	5	**	20–30
Antibodies to FVIIIC	10	15–20	30–45
Antibodies to factor VIII related antigen	10	15–20	30–45
High molecular weight kininogen	5	2– 5	10–15
α-Endorphin	2	10–15	20–30
β-Endorphin	2	10–15	20–30
Corticotropin 1-24	2	5–10	15–20
Caeruloplasmin	10	5–10	15–20
Gc Protein	5	10–15	20–30
Cortisol-3-tyrosyl-methylester	1	20–30	45–60

* Both of these peptides contained a tyrosine residue at the NH_2-terminus.
** Factor VIII clotting activity was not labelled with chloramine-T

Increasing the concentration of Iodogen and the reaction time leads to a progressive alteration of the elution profile to the detrement of the immunological activity of the tracer. The same effect is seen with increasing the reaction time using chloramine-T. The conditions for the chloramine-T labelling giving the same result as the Iodogen labelling using 25 μg for 90 seconds are shown in table 1, which also shows the optimal times of labelling, in terms of maximal incorporation with minimal damage, for several peptides under the conditions laid down in "Materials and Methods".

Quality of label produced together with iodine incorporation

Table 2 shows the maximum binding obtained with Iodogen-labelled tracers for five proteins or peptides. Figures 2 and 3 show elution profiles of tracer produced by chloramine-T for human transferrin, compared with tracer produced by the Iodogen method. Both iodinations were carried out within 30 minutes of each other using the same sodium ^{125}iodide.

The differing 50% intercepts shown in table 2 reflect the differing affinity of the peptides for the antibody after labelling.

Figure 4 shows a standard curve for parathyrin using tracer from both iodination methods.

Stability of tracers

Tracers from both methods kept under identical conditions, i.e. diluted in assay buffer to ca. 7–10 MBq/l and stored at - 20 °C were equally stable in most cases, when optimal labelling methods were used.

Anomalous results

Figure 5 shows the labelling of factor VIII clotting activity with Iodogen. The protein peak appeared to be missing when the normal elution procedure was carried out, together with a large proportion of the radioactivity. When, however, Tween 20 (0.25%) was incorporated into the phosphate buffer, the protein was eluted, thus demonstrating its lipophilic nature.

Several peptides were not successfully labelled with Iodogen, despite several attempts. Whether this was a property of the peptides as a whole, or just of the mate-

Tab. 2. Effects of labelling with chloramine-T and Iodogen on the maximum binding (Max-B) , binding of the zero standard under assay conditions (B_0), the unspecific binding under assay conditions (UB) and the 50% intercept of the standard curves run at the same time using the two tracers.

Assay	Chloramine-T				Iodogen			
	B_0	UB	Max-B	50% intercept	B_0	UB	Max-B	50% intercept
Human transferrin	0.60	0.04	0.84	2.05 g · l^{-1}	0.85	0.03	0.99	2.01 g · l^{-1}
Human calcitonin	0.22	0.02	0.80	820 ng · l^{-1}	0.31	0.03	0.95	795 ng · l^{-1}
1-84 Bovine parathyrin	0.12	0.03	0.61	3.12 µg · l^{-1}	0.20	0.03	0.71	2.05 µg · l^{-1}
1-84 Human parathyrin	0.24	0.03	0.78	517 U · l^{-1}	0.38	0.03	0.85	526 U · l^{-1}
44-68 Human parathyrin	0.27	0.03	0.75	485 U · l^{-1}	0.38	0.03	0.82	447 U · l^{-1}

B_0 was the binding of the zero standard under assay conditions with respect to the total activity (B_0/T)
UB was the unspecific binding under assay conditions (UB/T)
Max-B was the binding of fresh tracer to an excess of antibody (B/T)
The 50% intercept of the standard curve run under assay conditions reflects the affinity of the antibody for the two tracers.
Full assay details are to be seen in l.c. (10–12).

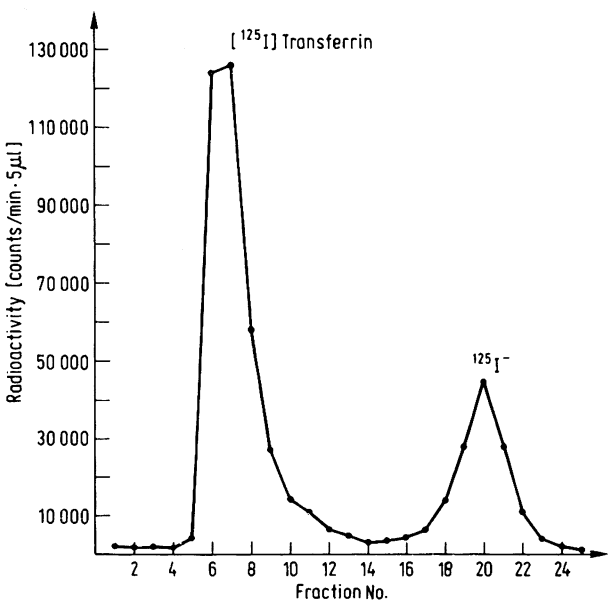

Fig. 2. Elution profile of the labelling mixture from the chloramine-T iodination of human transferrin. The first peak contains the ^{125}I-transferrin which bound to excess antibody with a B/T of 0.85. The column was Sephadex G-10 (9 × 1 cm) with 0.05 mol/l Tris-HCl pH 7.5 containing bovine serum albumin (1 g · liter^{-1}) as elution buffer. The void volume of this column was 4 ml, corresponding to the first four fractions collected.

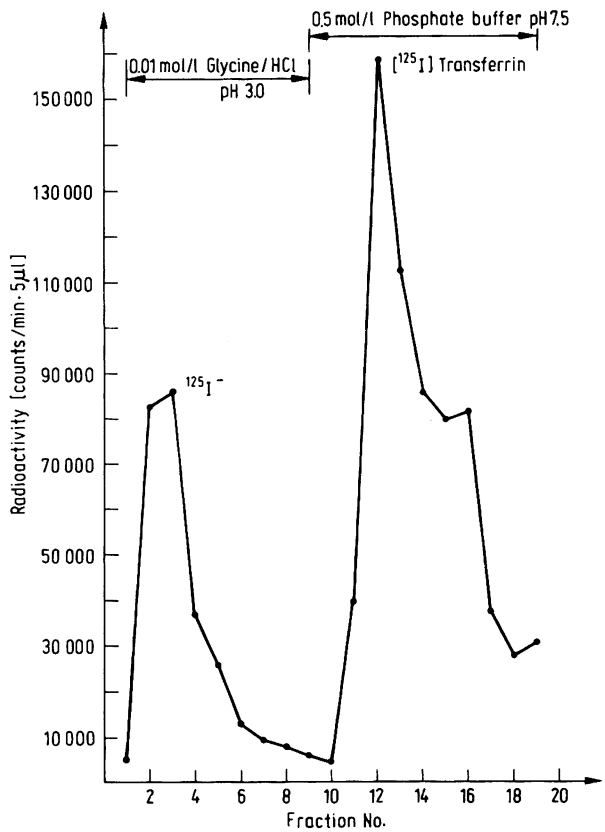

Fig. 3. Elution of human transferrin labelled with Iodogen, separated on a Sephadex SP-C25 cation exchange column (2 × 1 cm) using 0.01 mol/l glycine-HCl buffer, pH 3.0 to elute off the iodide and 0.5 mol/l phosphate buffer to elute off the labelled transferrin. The label bound to excess antibody with a B/T of 0.99. Note that in contrast to figure 2, the iodide peak is eluted from the column first.

rial which was tested, has not been investigated due to reasons of cost! Trouble was encountered with human prolactin, human somatotropin and rat lutropin, the former two producing a tracer with high incorporation but high unspecific binding whereas the latter produced an incorporation of ca. 2% despite lengthening the reaction time to 15 minutes! Other workers have also had problems (8). Where the hormones are not mentioned, labelling with chloramine-T and Iodogen gave rise to tracers of comparable quality and stability as well as similar iodine incorporation.

"Combi-labelling" with Iodogen

The immobilised Iodogen was used to facilitate the labelling of proteins and peptides with "*Bolton-Hunter*" reagent (N-succinimidyl-3-(4-hydroxyphenyl) propionate).

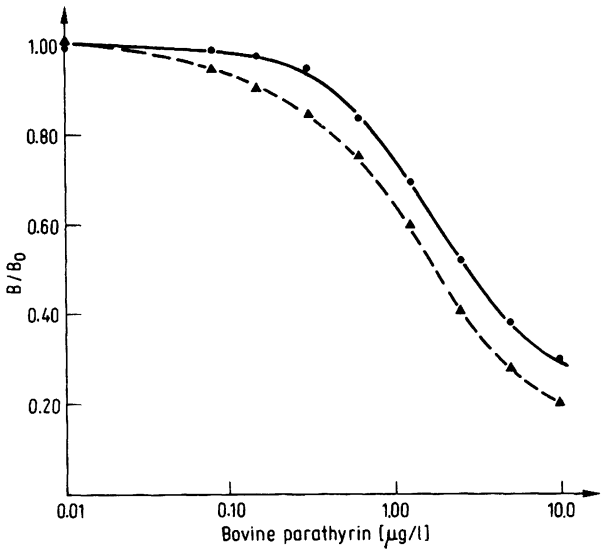

Fig. 4. Bovine parathyrin RIA standard curves run under identical conditions.
●——● Chloramine-T label, binding of zero standard in the assay. $B_0/T = 0.12$
▲– – ▲ Iodogen label, binding of zero standard in the assay. $B_0/T = 0.19$
(For full assay details see l. c. (10))

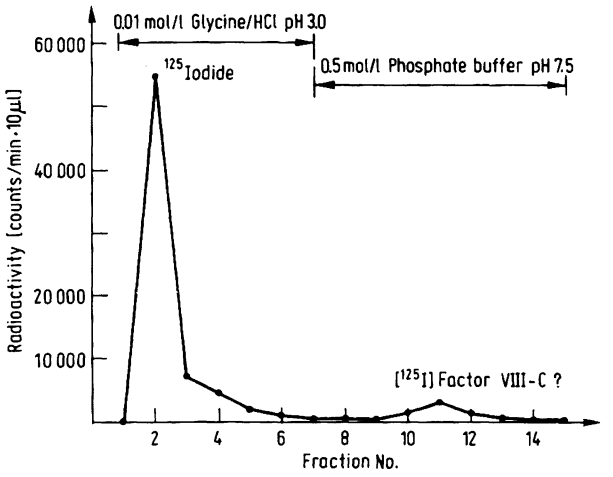

Fig. 5. Elution of factor VIII clotting activity (FVIIIC) after Iodogen labelling. The total activity was 204600 counts/10 s, the remaining activity on the vessel after labelling 20000 counts/10 s. From the elution profile both the labelled protein peak and ca. 100000 counts/10 s are missing. This activity can be washed from the column with 0.5 mol/l phosphate buffer, pH 8 containing 2.5 g/l Tween 20, and demonstrates the lipophilic nature of factor VIII clotting activity.

Here time and money could be saved by first labelling the "*Bolton-Hunter*" reagent with Iodogen at pH 7.4 with subsequent transfer of tube contents to a second vessel containing the peptide to be labelled at pH 8.6–9.0 (9). As the Iodogen is bound to the tube wall, it is not transferred with the "*Bolton-Hunter*" reagent, thus preventing transfer of unwanted oxidation agents to the second vessel.

Figures 6 and 7 show the labelling of 1-34 human parathyrin with chloramine-T at pH 8.4 (labelling of histidine residues), and using the "combi-labelling" method. An excess "*Bolton-Hunter*" reagent must be added to compensate for loss due to hydrolysis during iodination.

Discussion

Although the mechanism of action of Iodogen and chloramine-T is similar, namely by the action of chlorinium ions which oxidise iodide to atomic iodine and to iodinium ions, the immobilisation of Iodogen on the vessel wall reduces the potential hasard of structural changes during iodination (fig. 8). The longer incubation time of the reagent with Iodogen reduces the hectic which can occur during labelling with chloramine-T. In several cases, the quality of tracer produced by Iodogen is better than that from chloramine-T, although there are cases where the Iodogen method produced a tracer which could not be used in a radioimmunoassay, but where tracer produced by reacting the material with chloramine-T and iodide was excellent in the same assay.

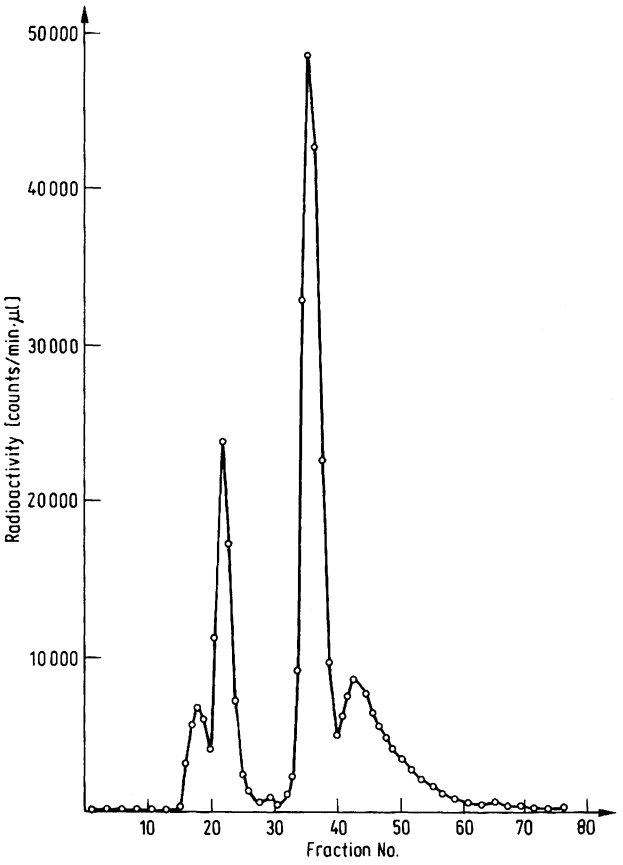

Fig. 6. Elution of human parathyrin 1-34 peptide on Ultrogel AcA 54 after labelling with chloramine-T at pH 8.4. The large peak contains the immunoreactive peptide ($B_0/T = 0.59$, $UB/T = 0.06$) and the two preceding peaks aggregated material with high unspecific binding (UB). The column dimensions were the same as described in figure 1 as was the elution buffer.

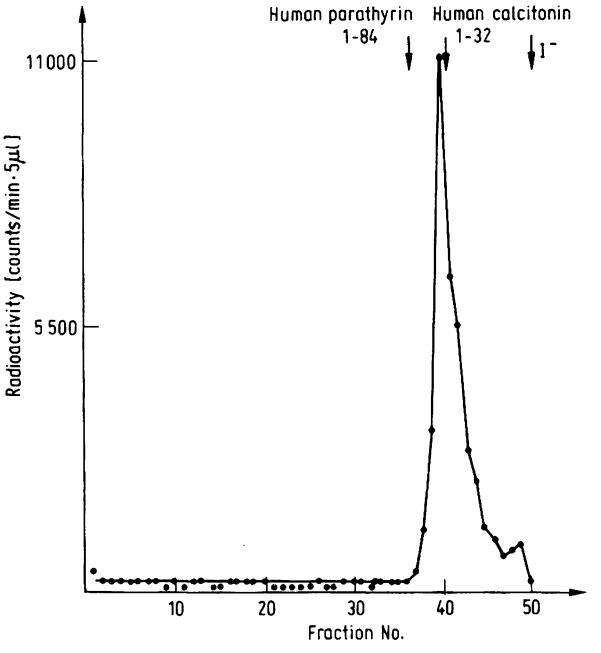

Fig. 7. Elution of human parathyrin 1-34 peptide on Ultrogel AcA 44 after "combi-labelling" with Iodogen and *Bolton-Hunter* reagent. The large peak contained all the immunoreactive peptide, which showed a relatively high unspecific binding (B_0/T = 0.62, UB/T = 0.13). Marker proteins, 1-84 bovine parathyrin (1-84 bPTH), and 1-32 human calcitonin (1-32 hCT) indicate that the labelled 1-34 human parathyrin elutes at the expected point. The void volume of the column was 158 ml, and the dimensions and elution buffer the same as in figure 1.

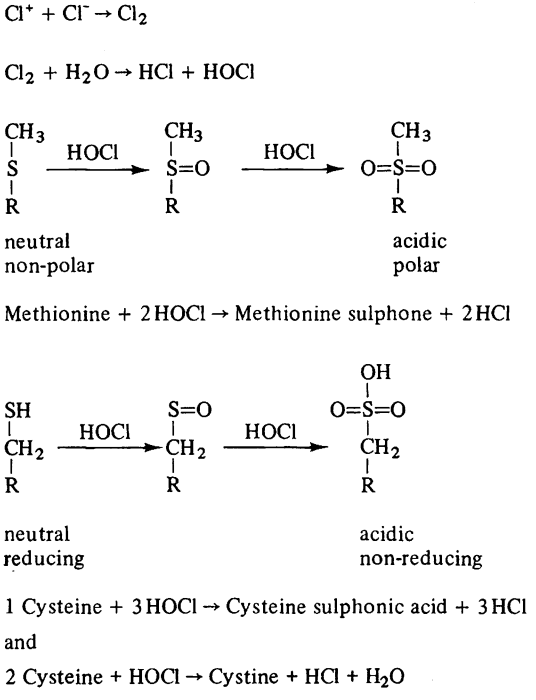

Fig. 8. Possible unwanted side effects which may lead to structural changes in peptides during labelling with chloramine-T and Iodogen.

For proteins and peptides which are resistant to oxidation, Iodogen labelling may offer a more comfortable method of iodination, coupled with a drastic reduction in the volume of radioactive waste when the reaction is coupled with the desalting step using the short cation-exchange column here described.

There are cases where, due to a lack of tyrosine or histidine residues, or where a non-oxidative iodination is desired to prevent groups such as the -SH of cysteine and the $-S-CH_3$ groups of methionine being destroyed. Here the free NH_2-groups (ϵ-NH_2 of lysine) must be iodinated, and the "combi-method" may prove to be useful in such cases.

To conclude, an alternative to chloramine-T and lactoperoxidase is presented by the introduction of Iodogen, although, like many innovations, it has its advantages and disadvantages which can only be tested by trial and error.

The full details of the assay conditions for parathyrin (10), human calcitonin (11) and human transferrin (12) have been published in full elsewhere.

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