CHLORAMINE-T FOR "NO-CARRIER-ADDED" LABELLING OF AROMATIC BIOMOLECULES WITH BROMINE-75,77

G. Petzold and H.H. Coenen

Institut für Chemie 1 (Nuklearchemie)
der Kernforschungsanlage Jülich GmbH, D-517 Jülich, FRG

SUMMARY

The applicability of chloramine-T (CAT) was examined for "no-carrier-added" labelling of aromatic compounds with the short-lived isotopes bromine-77 ($T_{1/2}$ = 56 h) and bromine-75 ($T_{1/2}$ = 98 min). In aqueous solution the optimum reaction conditions with respect to pH, concentration of CAT, reaction time and added bromine-carrier were determined using tyrosine as a model substrate. Highest radiochemical yields (>85%) were obtained at pH 1 within a few seconds at a CAT concentration of 10^{-3} M. At pH 7 a maximum radiochemical yield of 65% was achieved with 10^{-2} M after 2.5 minutes. Studies with some model peptides showed that only the tyrosyl residues were brominated and similar yields were obtained as in free tyrosine. Some aromatic molecules such as α-methyl-tyrosine, phenylalanine, uracil, cytosine, and phenol were radiobrominated by the CAT method with yields up to 90%. Separation and identification of the labelled products were achieved by means of radio high performance liquid chromatography.

Key Words: Amino Acids, Aromatic Bromination, Bromine-75,77, Chloramine-T, HPLC-Separations

0362-4803/81/091319-18$01.80

Received September 1, 1980
Revised October 9, 1980

INTRODUCTION

Radiohalogens are particularly important for the biochemical approach to develop radiopharmaceuticals, and the neutron deficient isotopes iodine-123 ($T_{1/2}$ = 13.3 h) and bromine-77 ($T_{1/2}$ = 56 h) find increasing interest for in-vivo applications in nuclear medicine (for a review see (1)). It has often been pointed out that bromine isotopes have some advantages over iodine isotopes (e.g. (1-3)). There are also bromine radioisotopes with high positron emission rates which are useful for positron emission tomography; among them bromine-75 with the convenient half-life of 98 minutes seems to be of particular interest, and it has recently been produced in useful quantities (4).

While many radioiodination methods exist, little is known about "carrier-free" radiobromination procedures. Classical bromination methods are often not useful because of the short half-life of the radioisotopes and the requirement of high specific activity which is generally mandatory for most in-vivo applications. Biomolecules and pharmaceuticals often contain aromatic systems forming stable bromine derivatives. Thus, simple and fast methods for aromatic bromination with high specific activities are of particular interest.

Some of the aromatic iodination procedures have already been adapted for bromination, such as the noble gas (krypton-) exposure technique (5,6) as well as the oxidation of bromide with Cl_2 (7) and $KBrO_3$ (6,8). These methods, however, are not satisfying either because of low yields and lack of specificity (exposure method), carrier problems, and the high excess of oxidation agent. We have recently reported that bromination and iodination in organic solvents can be achieved efficiently with N-chlorotetrafluorosuccinimide (NCTFS) (9) and with hypohalites such as CF_3CO_2Cl and

CF_3SO_3Cl (10). These reagents are useful for labelling with high specific activities. They can be regarded as analogues of the well-known chloramine-T (CAT) which was succesfully applied as an oxidant for the radioiodination in aqueous solution (11). A great variety of proteins has been iodinated with chloramine-T (12). While radiobromination of proteins is generally carried out via enzymes such as chloroperoxidase (3,13) and more recently by myeloperoxidase and bromoperoxidase (14,15), chloramine-T also seems to be a promising agent, and several biomolecules like tyrosine, uracil, and cytosine have recently been labelled with bromine-77 at pH 7 (3).

In this work some differences between iodination and bromination with CAT were observed and a more detailed determination of the optimum reaction parameters was necessary. Therefore we have studied the dependence of the bromination yield on pH, time and concentration. In analogy to radioiodination of proteins the possible applicability of CAT to radiobromination of these biomolecules is also of interest. Since in proteins tyrosine is predominantly labelled (16), this compound was chosen as model substrate. In addition, radiobromination of some simple peptides with CAT was studied. Although enzymatic bromination is a very mild procedure, it needs - besides other disadvantages - carefully controlled biochemical conditions. This effort is not justified for fast labelling of small molecules. Therefore, the CAT-method was also applied to radiobromination of some simple biomolecules.

EXPERIMENTAL

The oligopeptides used were obtained from Serva, all other chemicals were purchased from Merck. Iodine-125 was delivered by Amersham as no carrier added NaI.

Production of bromine-77 was carried out via the $^{75}As(\alpha,2n)^{77}Br$ nuclear reaction (17) by bombarding a thick target of copper arsenic alloy with 30 MeV α-particles at the Jülich compact cyclotron CV-28 (4). After irradiation the bromine was removed from the alloy by a dry distillation technique and collected as bromide in a small amount of water (4). For the labelling procedure, generally $1.2 \cdot 10^{-6}$ mol of the substrate were dissolved in 500 µl buffer solution. About 5 to 10 µCi [^{77}Br]-bromide in 1-2 µl water were added and 10 µl of a solution containing the desired amount of CAT was added to carry out the reaction. After a given time the reaction was stopped by adding 100 µl of sodium metabisulphite solution. Exact reaction conditions are given in the figure captions.

Separation and purification of the labelled products were achieved by means of high pressure liquid chromatography. Except for the phenol system 200 µl aliquots of the reaction mixture were directly injected onto a hplc-apparatus described elsewhere (18). In the case of phenol the labelled products have been extracted before injection from water solution by $CHCl_3$. 0.5 ml fractions of the eluant were collected and measured discontinuously on an Auto-Gamma-Scintillation Spectrometer (Packard 5375). The radiochemical yield of the individual product was related to the total activity in the reaction mixture. The columns and eluants are listed in Table I. The brominated peptides had almost identical k'-values as the starting peptides. For the separation a 25 cm long (Ø 4 mm) RP-18 column and water:methanol:acetic acid (100:10:1) as eluent were used.

Table I HPLC data for the separation and purification of brominated products.

Substance	Column	Eluent	k'-values
tyrosine	Lichrosorb RP-18, 10 µm	water:methanol:acetic acid	2.2
3-bromotyrosine	(Merck), 50 cm, Ø 4 mm	100:5:1	10.0
3-iodotyrosine			14.0
α-methyltyrosine	Lichrosorb RP-18, 10 µm	water:methanol:acetic acid	4.8
3-bromo-α-methyltyrosine	(Merck), 50 cm, Ø 4 mm	100:30:1	12.1
phenylalanin	Lichrosorb RP-18, 10 µm	water:methanol:acetic acid	5.3
2-bromophenylalanin	(Merck), 25 cm, Ø 4 mm	100:30:1	14.5
3-bromophenylalanin			20.8
4-bromophenylalanin			24
histidine	Lichrosorb RP-18, 10 µm	water	1.5
bromohistidine	(Merck), 50 cm, Ø 4 mm		2.3
phenol	Lichrosorb Si-60, 10 µm	heptane:acetic acid	14.9
o-bromophenol	(Merck), 50 cm, Ø 4 mm	1000:15	1.4
m-bromophenol			16.7
p-bromophenol			19.6
uracil	Lichrosorb RP-18, 10 µm	water:acetic acid	0.9
5-bromouracil	(Merck), 25 cm, Ø 4 mm	100:1	3.0
cytosine			1.1
5-bromocytosine			5.0

RESULTS AND DISCUSSION

pH-dependence

In the iodination of proteins with CAT a strong pH-dependence around pH 7 was reported when tagging tyrosyl residues (19). We have therefore studied the influence of the pH of the reaction solution on the radiochemical yield using tyrosine as model system. Radiobromination and radioiodination were compared under idential conditions over the whole pH-range. The dependences of the [^{77}Br]-3-bromotyrosine and [^{125}I]-3-iodotyrosine yields on pH are plotted in Fig. 1.

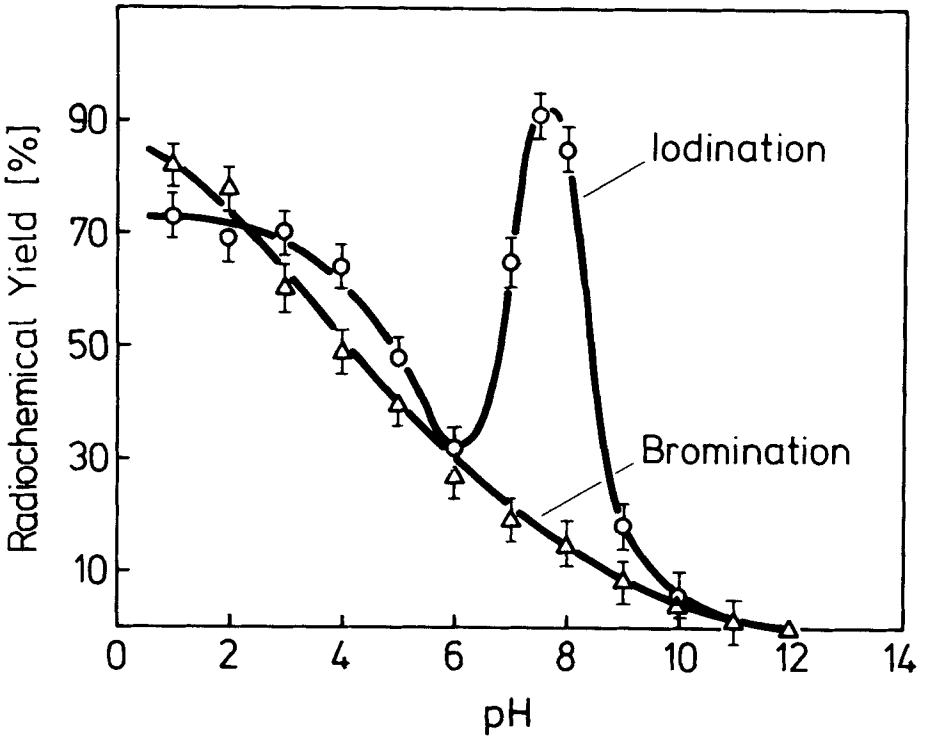


Fig. 1 Bromination and iodination of tyrosine with chloramine-T; pH-dependence of radiochemical yield.
$1.2 \cdot 10^{-6}$ mol tyrosine in 500 µl buffer solution; 22 °C; $5 \cdot 10^{-3}$ M CAT; 5 min.

The curve of the iodination product shows two regions of high radiochemical yields. The maximum of 95% at pH 7.5 is in good agreement with that found in protein iodination (19). The yield decreases to about 30% at pH 6 and then increases again to about 70% between pH 3 and pH 1. In contrast, the 85% bromination yield of [^{77}Br]-3-bromotyrosine is high at pH 1 and decreases continuously with increasing pH. Iodination is also less effective above pH 8 and both radiohalogenation reactions are completely suppressed above pH 11.

The suppression of halogenation in alkaline solution can be expected when assuming that CAT oxidizes the halides to electrophilic species which then undergo a substitution reaction at tyrosine: At higher OH^--concentration chloramine-T dissociates completely and hypochlorite anions (ClO^- and/or $^{77}BrO^-$, $^{125}IO^-$, respectively) are formed spontaneously. These species can no longer attack tyrosine. The yield maximum at pH 7.5 for the iodination was attributed to the dissociation of tyrosine forming tyrosylate anions (20). These findings, however, are different from those observed in radiobromination, which continuously decreases with increasing pH. Either the explanation for the iodination maximum is wrong, or the brominating species are principally different from the iodinating species, or, most probable, the same species are formed in both cases but the brominating ones are much less reactive. Further experiments are in progress to elucidate the reaction mechanism and the species involved. Hypochlorite (HOCl) which is often believed to be the oxidizing species, can be excluded, as could be demonstrated by analytical (21) and electrochemical experiments (22).

Time-dependence

The high yield of about 85% at pH 1 demonstrates that the chloramine-T method is an effective tool for radiobromination at least for small molecules which are stable at low pH. In view of the strong influence of the pH further studies of reaction parameters for radiobromination were carried out at pH 1 and pH 7.

At concentrations of 10^{-2} M at pH 7 and 10^{-3} M at pH 1 the dependence on reaction time was carried out. Figure 2 shows a very fast reaction at pH 1 and a considerably slower one at pH 7. At

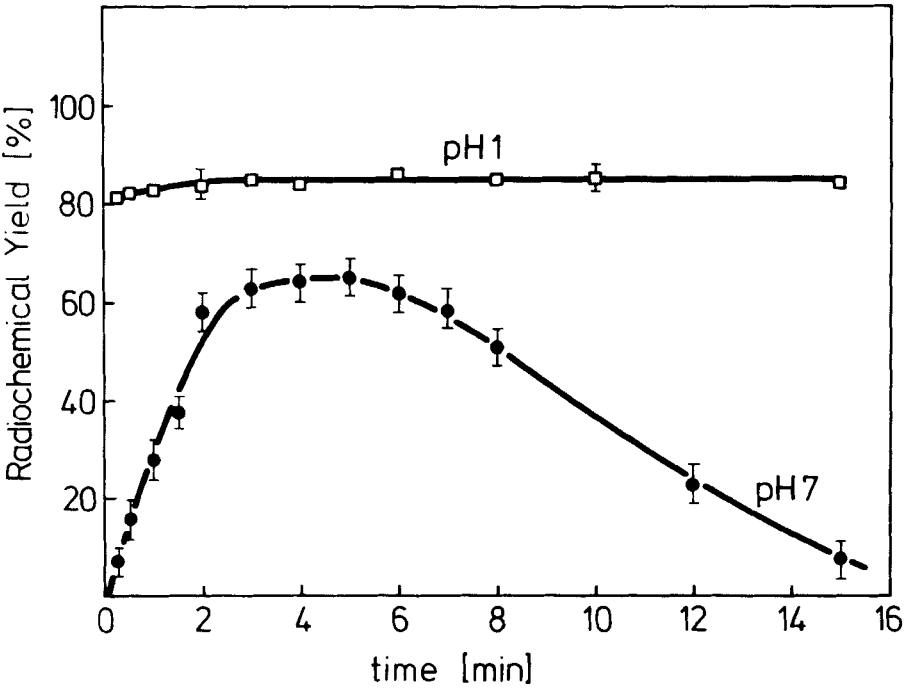


Fig. 2 Time dependence of bromination yields of tyrosine with chloramine-T at pH 1 and pH 7.
$1.2 \cdot 10^{-6}$ mol tyrosine in 500 µl buffer solution; 22 °C;
pH 1: 10^{-3} M CAT; pH 7: 10^{-2} M CAT.

pH 1 yields of >80% are obtained within a few seconds, even at the low CAT-concentration applied. Above two minutes the yield remains constant over the time period examined. A minimum of reaction time of 2.5 minutes is needed at pH 7 to reach the maximum at about 60% radiochemical yield. At longer reaction times above about 5 minutes the yield decreases again. This can be attributed to the tenfold higher chloramine-T concentration causing oxidative side reactions on tyrosine and on already formed 3-bromotyrosine.

Dependence on CAT-concentration

The influence of the CAT-concentration on the yield is demonstrated in Fig. 3. In view of the time dependence (cf. Fig. 2) 10 seconds at pH 1 and 5 minutes at pH 7 were chosen as reaction times. For pH 7 a maximum at about 10^{-2} M and a radiochemical yield of about 65% were found. The curve at pH 1 is quite different and almost unaffected by increasing the CAT-concentration. With a tenfold lower concentration of 10^{-3} M a yield of about 85% is obtained at this pH-value. The decrease of labelling yield with higher CAT-concentrations at pH 7 can be attributed to destructive side reactions with respect to the extended reaction times. It could be shown by hplc analysis that 50% of tyrosine disappeared when a fourfold excess of CAT was allowed to react for 5 minutes with the substrate. These oxidative side reactions of CAT, however, are slower than the radiobromination as can be seen from Figures 2 and 3.

The results indicate that chloramine-T can be used as radiobrominating agent even for molecules which are sensitive to strong acidic media. In our example, a [^{77}Br]-3-bromotyrosine yield of 65% can be reached within 3 minutes at pH 7 using a reagent concentration of 10^{-2} M. The best results are obtained, however,

at pH 1. Here, oxidative side reactions can be avoided using low CAT-concentrations (10^{-3} M) and a short reaction time (10-20 sec).

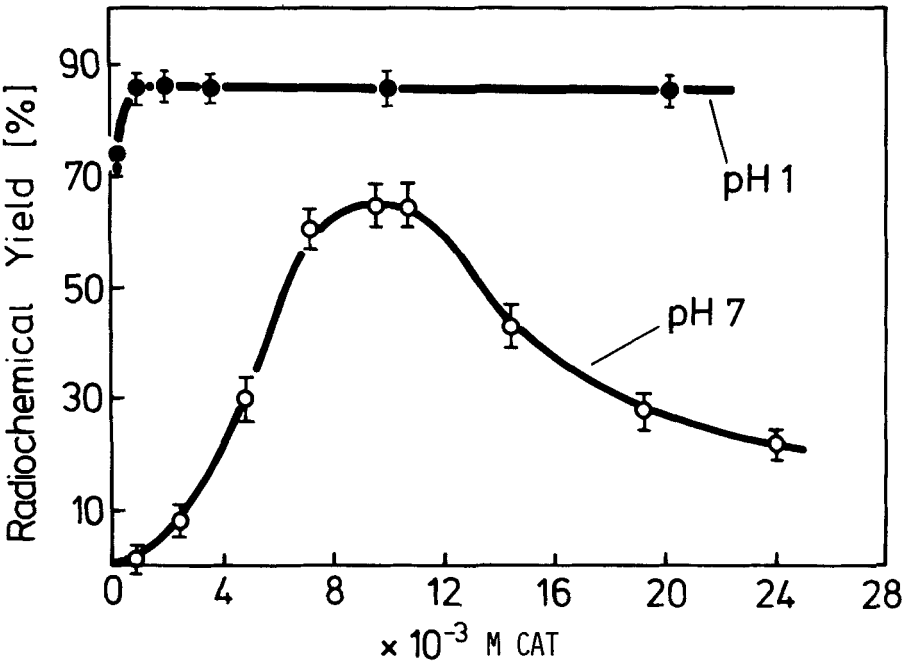


Fig. 3 Dependence of radiobromination yield of tyrosine on chloramine-T concentration at pH 1 and pH 7. $1.2 \cdot 10^{-6}$ mol tyrosine in 500 µl buffer solution; 22 °C; pH 1: 10 sec; pH 7: 5 min.

Influence of bromide-carrier

With respect to the goal of practically "carrier-free" labelling, it is interesting to study the influence of carrier addition, i.e. inactive bromide. Generally, yields increase by adding carrier, due to shifting of equilibria and/or compensating adsorption losses which occur in the 10^{-14} to 10^{-12} molar region. The dependence of radiobromination yield on the amount of added bromide was examined over some orders of magnitude. The results

for a 2 minutes reaction period are shown in Fig. 4. Increasing

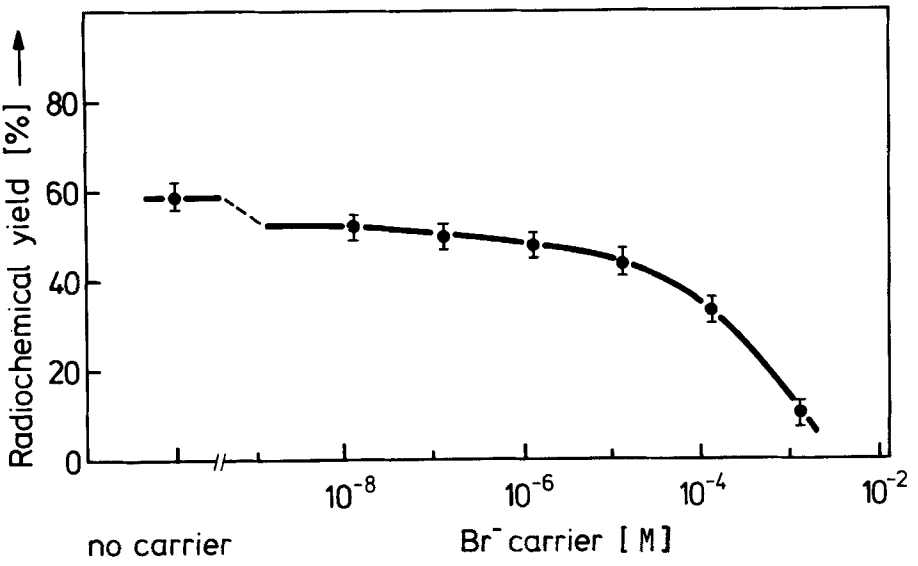


Fig. 4 Dependence of radiobromination yield of tyrosine on the amount of added bromide-carrier. $1.2 \cdot 10^{-6}$ mol tyrosine in 500 µl buffer solution; 22 °C; 10^{-2} M CAT; 2 min.

the bromide concentration to an equimolar amount of chloramin-T (10^{-2} M) leads to a decrease of the radiochemical yield. This indicates that a high chloramine-T over bromide ratio is necessary to achieve an efficient electrophile substitution. Obviously, oxidation and electrophilic substitution are fast compared to sorption processes. The findings demonstrate that the chloramine-T method is ideal for "no-carrier-added" radiohalogenation. The trend of the curve clearly indicates a high specific activity, and it can be estimated from the bromine impurities of the chemicals used in this study that at least 5000 Ci/mmol are obtained.

Bromination of oligopeptides

Although most proteins cannot be radiobrominated at pH 1, the radiochemical yields of bromotyrosine obtained at higher pH encouraged us to examine also protein labelling by chloramine-T. While it is known that there are differences in the reactions of free and protein-bound amino acids (23), we have chosen oligopeptides as model substrates for a first test. These peptides contain the most important aromatic amino acids like tyrosine, phenylalanine, histidine and tryptophane in different combination. Tyrosine and - to a smaller extent - histidine are preferentially labelled in radiohalogenation reactions (16) and are therefore examined in a different environment of the peptide chain.

The radiochemical yields of the labelled peptides at pH 1 and pH 7 are listed in Table II. It can be deduced that besides

Table II Radiobromination of oligopeptides with CAT at pH 1 and pH 7.

Peptid	Radiochemical yield pH 1	pH 7
Gly-Tyr-Gly	85 %	65 %
Ala-Tyr-Ala	78 %	64 %
Pro-His-Tyr	80 %	65 %
Gly-Phe-Gly	1 %	-
Gly-His-Gly	-	-
Glu-His-Phe-Arg-Trp-Gly	-	-

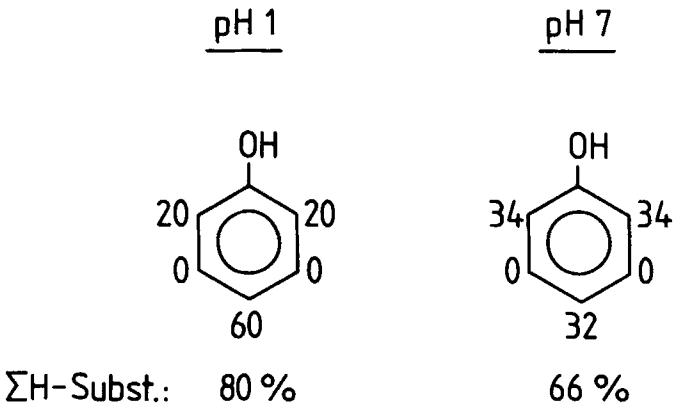
$1.2 \cdot 10^{-6}$ mol peptide in 500 µl buffer solution; 22 °C; pH 1: 10^{-3} M CAT; 2 min; pH 7: 10^{-2} M CAT; 5 min.

tyrosine no other amino acid is brominated in significant yield. In addition, the results obtained in free tyrosine are almost identical with those of the corresponding peptides. This indicates that the reaction is not influenced by the peptide-bond nor by neighbouring aliphatic (Gly and Ala) and aromatic (His) amino acids. These findings are promising in view of the application of chloramine-T for radiobromination of proteins. If the stability towards pH and oxidation of the protein to be labelled is sufficiently high, the chloramine-T method is fast, effective, and much simpler than enzymatic procedures.

Selectivity and reactivity towards aromatic compounds

The good bromination yields observed in the tyrosine system prompted an investigation of the selectivity and reactivity of the method. Several aromatic molecules were tested at pH 1 and pH 7. Phenol was chosen as an activated "analogue" of tyrosine to study the selectivity effect. As shown in Table III the sum of [^{77}Br]-bromophenols amount to 80% at pH 1 and 66% at pH 7. No meta-product was found, thus the isomer distribution clearly indicates an electrophilic substitution reaction at both pH-values. At pH 1 a high para-selectivity of 60% is observed. This is in contrast to the isomer distribution (85% ortho, 15% para) observed in the iodination of phenol by Xe-exposed KIO_3 in strong acidic media (24). The increase of ortho-substitution and the concomitant decrease in para substitution from 40% to 68% and from 60% to 32%, respectively, when going from pH 1 to pH 7, reveal the influence of phenol dissociation which in turn causes higher ortho-activation.

Table III Isomer distribution of radiobromination of phenol with CAT at pH 1 and pH 7.



$1.2 \cdot 10^{-6}$ mol phenol in 500 µl buffer solution; 22 °C; 10^{-2} M CAT; 2 min.

The yields achieved in the aromatic compounds investigated are summarized in Table IV. In the case of activated benzene derivatives the yields are similar to those obtained for tyrosine. α-Methyltyrosine is of particular interest since it was recently reported that its [^{123}I]-3-iodo-compound is an excellent melanoma seeking radiopharmaceutical (25). Its bromine-75 analogue might be an even more useful radiopharmaceutical in conjunction with positron emission tomography. Phenylalanine is only poorly brominated. Obviously only activated aromatic compounds can be brominated by the CAT-method. In the case of histidine, the instability of its bromo derivative should be the reason, that no radiobrominated product was found (26). In the case of the nucleobases uracil and cytosine similar yields are obtained at pH 7 as previously described (3). The lower yield of 30% bromocytosine at pH 1 may be attributed to the protonation of the 4-amino group and hence deactivation of the compound. The yield of 83% bromouracil at pH 1

and pH 7 reflects the high reactivity of uracil towards electrophiles (27).

Table IV Radiobromination of some aromatic molecules with CAT.

Labelled Product	Radiochemical yield pH 1	pH 7
3-Bromotyrosine	85 %	65 %
3-Bromo-α-methyltyrosine	85 %	65 %
p-Bromophenylalanin	3 %	-
2(5)-Bromohistidine	-	-
5-Bromouracil	80 %	83 %
5-Bromocytosine	30 %	60 %
o,p-Bromophenol	80 %	66 %

$1.2 \cdot 10^{-6}$ mol substance in 500 µl buffer solution; 22 °C; pH 1: 10^{-3} M CAT; 1 min. pH 7: 10^{-2} M CAT; 5 min.

In summary, the chloramine-T method is an attractive method for no carrier added radiobromination. This is particularly true for bromination at pH 1. Even if restricted to activated molecules, advantages such as the "carrier-free" state, the speed of the reaction, the in-situ use of bromide and the high yields are obvious. The results obtained with peptides at pH 7 seem to be promising for future application to protein labelling with radiobromine.

ACKNOWLEDGEMENT

The authors are indebted to Prof. G. Stöcklin for stimulating discussions and constant support. They also want to thank Dr. G. Kloster for valuable suggestions.

REFERENCES

1. Stöcklin G. - Int.J.appl.Radiat.Isotopes 28: 131 (1977)
2. Knight L.C., Harwig S.S.L. and Welch M.J. - J.nucl.Med. 18: 282 (1977)
3. Hadi U.A.M., Malcolme-Lawes D.J. and Oldham G. - Int.J.appl. Radiat.Isotopes 30: 709 (1979)
4. Weinreich R., Alfassi Z.B., Blessing G. and Stöcklin G. - Proc. 17th Int.Ann.Meeting Soc.Nucl.Med., Innsbruck, September 1979, Schattauer Verlag, Stuttgart, New York 1979, p. 202 and:
   Weinreich R., Alfassi Z.B., Qaim S.M. and Stöcklin G. - 3rd Int.Symp.Radiopharm.Chem., St. Louis, July 1980, Abstr. No. 19.11, J.Lab.Comp.Radiopharm., in press
5. Coenen H.H., Machulla H.-J. and Stöcklin G. - 9th Int. Hot Atom Chem. Meeting, Blacksburg, 1977, Abstr. No. 31 and: Coenen H.H., Machulla H.-J. and Stöcklin G. - 10th Int. Hot Atom Chem. Meeting, Loughborough, 1978, Abstr. No. 40
6. De Jong D., Kooiman H., Lindner L., Kaspersen F.M. and Brinkmann G.A. - 2nd Int.Symp.Radiopharm.Chem. Oxford, July 1978, Abstr. No. B20, J.Lab.Comp.Radiopharm. 16: 223 (1979)
7. Lundqvist H., Malmborg P., Langström B. and Chiengmai S.N. - Int.J.appl.Radiat.Isotopes 30: 39 (1979)

8. Wong S.H., Mustaklem J.F. and Ache H.J. - Int.J.appl.Radiat. Isotopes 27: 627 (1976)

9. Coenen H.H., Machulla H.-J. and Stöcklin G. - J.Lab.Comp. Radiopharm. 16: 891 (1979)

10. Coenen H.H., El-Wetery A.S. and Stöcklin G. - 3rd Int.Symp. Radiopharm.Chem., St. Louis, July 1980, Abstr. No. 17.15, J.Lab.Comp.Radiopharm., in press

11. Hunter W.M. and Greenwood F.C. - Nature 194: 495 (1962)

12. Chervu L.R. and Murty D.R.K. - Radiolabelling of antigens, in: Radioimmunoassay, Ed. by M. Freeman & M.D. Blaufox, Grune & Stratton, New York, p. 33 (1975)

13. Knight L.C., Krohn K.A., Welch M.J., Spomer B. and Hager L.P. - in: Radiopharmaceuticals, Ed. G. Subramanian, New York, Soc.Nucl.Med., 149 (1975)

14. Mc Elvany K.D. and Welch M.J. - J.nucl.Med. 20: 663 (1979)

15. Mc Elvany K.D., Knight L.C., Welch M.J., Siuda J.F., Theiler R.F. and Hager L.P. - in: Marine Algae in Pharmaceutical Science, Ed. by H.A. Hoppe, T. Levring, Y. Tanaka; W. de Gruyter, Berlin, p. 429 (1979)

16. Krohn K.A., Knight L.C., Harwig J.F. and Welch M.J. - Biochim.Biophys.Acta 490: 497 (1977)

17. Skrabel F., Arnot R.N., Helus F., Glass H.L. and Joplin G.F. - Int.J.appl.Radiat.Isotopes 21: 183 (1970)

18. Machulla H.-J., Laufer P. and Stöcklin G. - J.Radioanal. Chem. 32: 381 (1976)

19. Ganguli P.C. and Hunter W.M. - Radioimmunoassay methods, Kirkham and Hunter (eds.), Edinburgh, Churchill Livingstone, p. 54 (1971)

20. Rudinger J. - Radioimmunoassay methods, Kirkham and Hunter (eds.), Edinburgh, Churchill Livingstone, p. 104 (1971)

21. Bishop E. and Jennings V.J. - Talanta 1: 197 (1958)

22. Koupparis M.A. and Hadjüoaunou T.P. - Anal.Chim.Acta 94: 367 (1977)

23. Means G.E. and Feeney R.E. - Chemical modification of proteins, Holden Day, San Francisco, p. 176 (1971)

24. Machulla H.-J., Shanshal A. and Stöcklin G. - Radiochimica Acta 24: 42 (1977)

25. Bockslaff H., Kloster G., Stöcklin G., Safi N. and Bornemann H. - Proc. 17th Int.Ann.Meeting Soc.Nucl.Med., Innsbruck, September 1979, Schattauer Verlag, Stuttgart, New York 1979, p. 179 and:
Bockslaff H., Kloster G., Safi N. and Blanquet P. - Radioaktive Isotope in Klinik und Forschung, Egermann, 14: 171 (1980)

26. Binkley W.W. - J.Am.Chem.Soc. 70: 2010 (1948)

27. Shik Yi Wang - J.Org.Chem. 24: 11 (1959)