SC 2226

Halogenation of tyrosine during acid hydrolysis

Loss of tyrosine during acid hydrolysis of small amounts of peptides and proteins has been observed[1-3]. Thus MUNIER[2] observed losses during hydrolysis of proteins containing radioactive tyrosine. The tyrosine was converted to a substance which was identified as 3-chlorotyrosine or a similar compound. Traces of chlorine are probably present in solutions of HCl, particularly when heated in the presence of O_2. This conversion could not, however, account completely for the losses observed in other cases, for instance in the work of HIRS, STEIN AND MOORE[1].

In experiments with proteins labelled with [^{14}C]tyrosine or with [^{14}C]tyrosine hydrolysed under conditions similar to those of MUNIER[2] it was observed that some tyrosine was converted to a product (tyrosine-Y) with properties very similar to 3-chlorotyrosine. That it was not 3-chlorotyrosine was shown by mixed ionograms carried out at pH 1.85 (2% formic acid, 8% acetic acid using ionophoresis for 1 h at 4000 V under white spirit[4]). The formation of this unknown derivative and of the chlorotyrosines was found to depend markedly on the acid used to hydrolyse the material. Redistilled 6 N HCl gave 3-chlorotyrosine as the main transformation product, in amounts depending on the age of the distillate, with traces of tyrosine-Y; whereas conc. HCl (British Drug Houses "Analar" or Micro-analytical reagent) diluted to 6 N gave predominantly tyrosine-Y (Fig. 1). This unknown derivative also travelled at almost the same speed as 3-chlorotyrosine during ionophoresis at pH 8.9 (1% $(NH_4)_2CO_3$, 3500 V for 1 h) and from its ionophoretic properties it was concluded that it had amino, carboxyl and phenolic groups.

HCl (6 N, 2 l) was redistilled and the residues (80 ml) were used for hydrolysis. Increased loss of [^{14}C]tyrosine was noted and with increasing amounts of these residues relative to tyrosine the tyrosine-Y was transformed to a further product, tyrosine-YY which moved slower on ionophoresis (see Fig. 2). Chlorination of samples of [^{14}C]tyrosine-Y and tyrosine-YY eluted from ionograms was carried out by incubating with 30% H_2O_2 (5 μl) in 2 N HCl (10 μl) for 16 h at room temperature. This method has been shown[5] to give predominantly 3-chlorotyrosine with tyrosine, a finding which we confirmed in these studies. Only tyrosine-Y, like 3-chlorotyrosine, gave a new product on chlorination whereas tyrosine-YY, like 3,5-dichlorotyrosine, was unchanged. Similar results were obtained on bromination for 15 min with mixtures of constant-boiling HBr and H_2O_2. From the results of these experiments it was deduced that tyrosine-Y and tyrosine-YY were 3-bromotyrosine and 3,5-dibromotyrosine respectively. The chlorination product of tyrosine-Y was identical in ionophoretic properties with the bromination product of 3-chlorotyrosine.

To confirm these identifications the 3-bromo and 3,5-dibromotyrosines were prepared similarly to the chlorotyrosines[6,7] by adding known quantitites of bromine to a solution of tyrosine hydrochloride in acetic acid[8]. Mixed ionograms at pH 1.85 showed that a radioautograph of [^{14}C]tyrosine-Y and tyrosine-YY coincided exactly with the ninhydrin-positive spots of 3-bromotyrosine and 3,5-dibromotyrosine respectively. The rates of movement of the halogenated tyrosines during ionophoresis[4] or ion-exchange chromatography[9] are given in Table I.

Tyrosine (0.5 μmole) was hydrolysed for 16 h with 0.5, 1 or 4 ml of the 6 N HCl residues described above and the products analysed by ion-exchange chromatography. The yields of 3-bromotyrosine were approx. 9, 17 and 44% respectively of the total

material on the column. Only with the 4-ml sample was some 3,5-dibromotyrosine also formed.

These studies have shown that both chlorotyrosines and bromotyrosines are possible transformation products of tyrosine during acid hydrolysis. The formation of bromotyrosine is presumably due to traces of impurities in the HCl used. Whereas a small amount of 3-bromotyrosine was formed when bromide was added the tyrosine was completely converted to dibromotyrosine if bromide and an oxidizing agent (*e.g.* bromate) were present (Fig. 2). In the presence of large amounts of salt formed by concentration of buffer solutions of peptides many impurities may become significant relative to the microgram quantities of tyrosine and several different halogenated tyrosines as well as other products could be formed. The exact nature of the impurities responsible is not clear but probably both traces of bromide and an oxidizing agent are involved. Under appropriate conditions bromotyrosines are more probable than

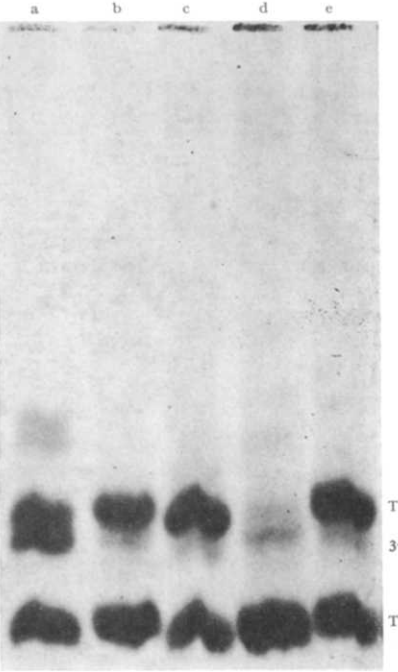

Fig. 1. Radioautograph of ionogram of [^{14}C]tyrosine after heating 16 h in a sealed tube at 105° with 0.1 ml 6 N HCl (a) freshly prepared from technical conc. HCl, (b) as in (a) but aged 3 weeks in a stoppered tube, (c) residues from distillation of "Analar" HCl, (d) redistilled "Analar" HCl, (e) microanalytical reagent HCl diluted to 6 N.

TABLE I

RATES OF MOVEMENT OF HALOGENATED TYROSINES

	Distance (cm) moved during Ionophoresis		*Peak position (ml at maximum) on 15-cm ion-exchange column**
	pH 1.85, 4000 V, 1 h	*pH 8.9, 3500 V, 1 h*	
Tyrosine	22.5	12.3	17.5
3-Chlorotyrosine	19.3	21.3	26.0
3,5-Dichlorotyrosine	16.5	23.2	33.3**
3-Bromotyrosine	18.0	20.7	31.5**
3,5-Dibromotyrosine	14.8	21.7	47.5
3-Iodotyrosine	17.0	19.6	42.7
3,5-Diidotyrosine	13.0	20.1	87.5
3-Chloro-5-bromotyrosine	15.7		39.5

* Beckmann-Spinco instrument operated according to SPACKMAN, STEIN AND MOORE[4] using citrate buffer (pH 5.28). Lysine emerged at 52 ml, ammonia at 77.5 ml.

** 3-Bromotyrosine is not resolved from 3,5-dichlorotyrosine.

Fig. 2. Radioautograph of ionogram of [^{14}C]tyrosine heated with 0.1 ml 6 N HCl (a) redistilled, (b) redistilled containing 5 μl 0.1 M KBr, (c) redistilled containing 5 μl 0.1 M KBr and 5 μl 0.01 M $KBrO_3$, (d) as in (c) but with 10 μl 0.1 M thioglycollic acid first added, (e) as in (c) but with 10 μl 0.1 M phenol first added.

chlorotyrosines. Under strong oxidative conditions both may be formed together with chlorobromotyrosine and further oxidation products, which migrate relatively slowly on ionophoresis at pH 1.85.

This loss of tyrosine could be avoided in our experiments by the addition of small amounts of compounds that are readily oxidized, *e.g.* thioglycollic acid, or halogenated, *e.g.* phenol or hydrazine (Fig. 2). These additives may prove generally useful in preserving tyrosine during acid hydrolysis.

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Loss of the 4-β hydrogen in the conversion of [4-β-^{3}H] cholesterol to cortisol by the guinea-pig adrenal gland homogenate

The conversion of pregnenolone to progesterone, considered one of the principal reactions in the biosynthesis of cortisol from cholesterol, has been shown to occur in perfused beef-adrenal glands[1] and in adrenal-gland slices[2]. It has also been effected by two bacterial enzymes acting consecutively: an NAD-linked 3β-ol-Δ^5-steroid dehydrogenase which oxidizes the 3β-hydroxy to a 3-keto group[3], and a 3-ketosteroid Δ^5-Δ^4 isomerase (steroid Δ-isomerase, EC 5.3.3.1) which shifts the double bond from the 5–6 to the 4–5 position, a process that involves an intramolecular hydrogen transfer from C-4 to C-6 (see ref. 4).

With the aid of cholesterol labeled with tritium almost exclusively in the β-position of C-4, by the procedure described by IRELAND *et al.*[5], we have studied certain aspects of the mechanism of Δ^5-3-ketosteroid isomerization in the intact guinea pig. Animals that had been fed a mixture of [4-^{14}C]cholesterol- and [4-β-^{3}H]-cholesterol for 10 days excreted [4-^{14}C]cortisol and 6β-[4-^{14}C]hydroxycortisol containing negligible amounts of ^{3}H. Since guinea-pig urine is alkaline, and the ethyl acetate used to extract the steroids was washed with 0.1 N NaOH, the possibility