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THE EFFECT OF THE pH AND SOLVENT CONCENTRATION ON THE ADSORPTION CHROMATOGRAPHIC SEPARATION OF ^{125}I-LABELLED IODOTYROSINES

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^{125}I-labelled 3-iodo- and 3,5-diiodotyrosine were separated by adsorption chromatography using Sephadex LH-20 dextran gel and ethanol-water binary eluent. The effect of the pH on the distribution coefficient vs. ethanol concentration relationship was determined and interpreted.

INTRODUCTION

Parallel to the spread of the application of ^{125}I-labelled compounds as tracers in the radioimmunoassay since the last decade, the separation of phenols from their mono- and polyiodine substituted derivatives has also become of great importance. Into iodotyrosines and iodothyronines, exhibiting phenolic hydroxyl group alike, radioiodine can be introduced via aromatic electrophilic substitution into the o and/or o' positions relative to

the hydroxyl group. On the other hand, low molecular weight compounds without phenolic hydroxyl group like steroids, prostaglandins etc. can be labelled with radioiodine in the same way in the tyrosine methyl ester side chain coupled to the molecule.

In both of the cases mentioned above the labelling procedure, e.g. the chloramine T method may result in the formation of mono- and disubstituted phenol derivatives, in some cases unidentified labelled compounds are formed and, in addition, more or less unreacted, so-called free radioiodine remains in the reaction mixture as well. In the majority of the cases, especially when the tracer exhibits a tyrosine methyl ester side chain coupled to the molecule to be labelled, only the monoiodine substituted derivative can be used as a tracer in radioimmunoassay, consequently this is to be separated from the diiodo derivative and from the starting material as well. On the other hand it is evident, that in the radioimmunoassay of the 3',5'-iodothyronines /i.e. 3',5'-diiodothyronine, 3,3',5'-triiodothyronine and thyroxine/ as well as in that of the 3,5-diiodotyrosine the tracer exhibits two iodine atoms in the o,o' positions relative to the hydroxyl group, one of them originating from the inactive starting material and the other one /i.e. radioiodine/ is introduced into the starting molecule via electrophilic substitution.

The aim of this paper is to show how the distribution coefficient of the 3-iodotyrosine and that of the 3,5-diiodotyrosine varies with the pH and the ethanol concentration of the eluent. The experimental results can be generalized to a certain extent for the separation of other radioiodine labelled tracers exhibiting phenolic hydroxyl group as well.

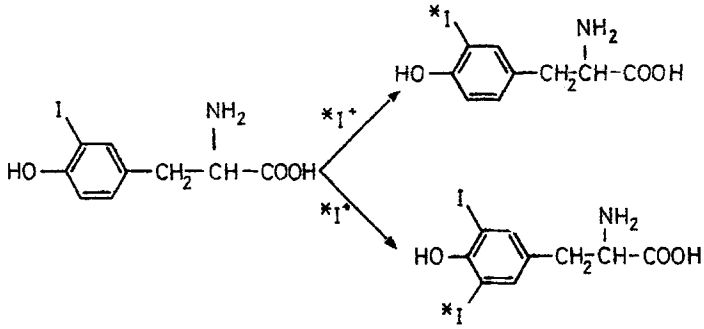

Fig. 1. ^{125}I-labelled iodotyrosines /right/ formed from 3-iodotyrosine /left/ in the course of the chloramine T labelling procedure /3-iodotyrosine is formed via aromatic electrophilic isotope exchange while 3,5-diiodotyrosine via aromatic electrophilic substitution/

EXPERIMENTAL

Reagents and labelling

Tyrosine and 3-iodotyrosine were labelled with ^{125}I using the chloramine T method[1]. In the course of the chloramine T labelling procedure from 3-iodotyrosine ^{125}I-labelled 3-iodotyrosine and 3,5-diiodotyrosine are formed, the former via electrophilic isotope exchange while the latter via electrophilic substitution /see Fig. 1./.

Chromatography

An SR/50 Pharmacia chromatographic column equipped with a thermostat jacket /I.D. 10 mm/ was filled with Sephadex LH-20 dextran gel swollen in citrate buffer /pH 4/ prior to being packed in the column. The height of the packing was 100 mm. The effluent was passed over a NaI/Tl/ scintillation crystal and the count rate was

monitored by a ratemeter and registered by an x-y plotter.

0.1-0.4 ml of the chloramine T labelling mixture was placed on the top of the column and was allowed to soak in it. After 10-20 min, i.e. when adsorption equilibrium had been attained the elution was started with ethanol-water binary eluent delivered by a peristaltic pump with a flow rate of 22-24 ml/h. The pH of the aqueous ethanol eluent was adjusted to the required value with citrate and phosphate buffer. The temperature of the column was adjusted to 25 ^{o}C by the use of an ultrathermostat connected to the jacket of the column.

The mono- and diiodotyrosine were identified by thin layer chromatography using silica plates /Merck 5553 DC-Alufolien Kieselgel 60/ and 75:15 mixture of phenol-water. The distribution coefficients of the mono- and diiodotyrosine were calculated according to Eq. /1/

$$k = \frac{V_e - V_o}{W} = \frac{V_e - 7.1}{2} \qquad /1/$$

where V_e, V_o and W stands for the elution volume, the dead volume and the weight of the packing, respectively.

RESULTS

The distribution coefficients of the mono- and diiodotyrosine as a function of the ethanol concentration of the eluent are shown in Fig. 2. The same results are portrayed on a log-log scale in Fig. 3. Figs 2. and 3. show that the distribution coefficient decreases monotonously with an increasing ethanol concentration.

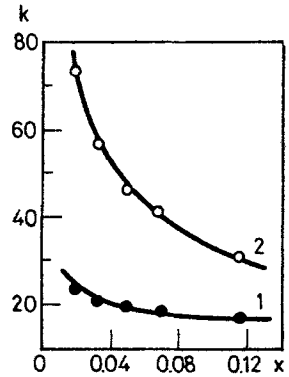

Fig. 2. The distribution coefficient of the 3-iodo- and 3,5-diiodotyrosine as a function of the ethanol concentration expressed in mole fraction at pH 4 /Curve 1: 3-iodotyrosine, Curve 2: 3,5-diiodotyrosine/

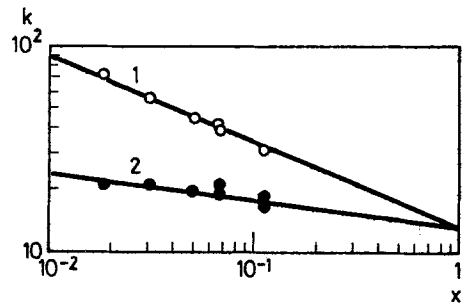

Fig. 3. The distribution coefficient as a function of the ethanol concentration plotted on a log-log scale

The inspection of the log k vs. log x plots shown in Fig. 3. indicates that log k is linearly dependent on log x. The k vs. x relationship shown in Fig. 3. obeys Eq. /2/:

$$\log k = \log k_o - n \log x \qquad /2/$$

where k stands for the distribution coefficient, x for the ethanol concentration expressed in mole fraction,

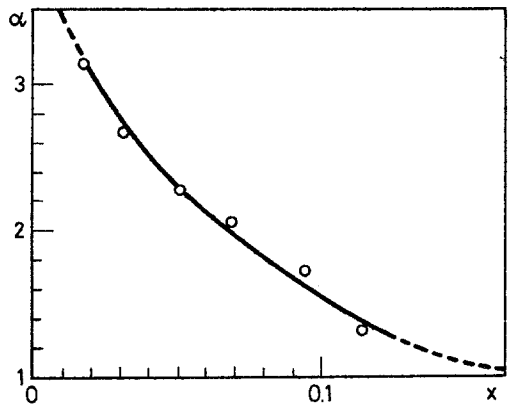

Fig. 4. The selectivity of the separation of the 3-iodotyrosine and 3,5-diiodotyrosine as a function of the ethanol concentration of the eluent, pH = 4

while n and k_o are constants, the latter corresponds to the distribution coefficient obtained by extrapolation to x = 1, i.e. to the pure ethanol eluent. Substituting n and k_o for the actual values into Eq. /2/, the following equations can be obtained:

$$\log k = 1.1 - 0.13 \log x \quad \text{/for 3-monoiodotyrosine/} \qquad /3/$$

$$\log k = 1.1 - 0.42 \log x \quad \text{/for 3,5-diiodotyrosine/} \qquad /4/$$

Being the straight lines representing the log k vs. log x relationship for the mono- and diiodo-tyrosines divergent, it follows, that the selectivity of the separation of these two iodotyrosines increases with a decreasing ethanol concentration. The selectivity of separation defined as the ratio of the distribution coefficients as a function of the ethanol concentration is shown in Fig. 4. From this figure the conclusion can be drawn that by choosing the proper ethanol concentration the selectivity can be adjusted to any realistic value.

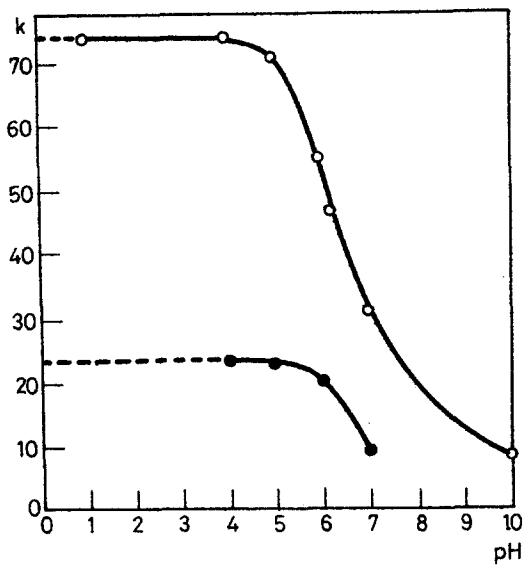

Fig. 5. The distribution coefficient of the 3-iodotyrosine and 3,5-diiodotyrosine as a function of the pH of the eluent

The effect of the pH on the distribution coefficient is demonstrated in Fig. 5. according to which a sudden decrease takes place near the pK_{OH} of the mono- and diiodotyrosines.

When the separation is aiming at the production of ^{125}I-labelled 3,5-diiodo-tyrosine of high radioactive concentration and high radiochemical purity it is recommended to elute free radioiodine and ^{125}I-labelled 3-iodo-tyrosine with 10% aqueous ethanol /pH = 4/ which is followed by the elution performed with 30% aqueous ethanol /pH = 4/.

When tyrosine is labelled with ^{125}I only the formation of the 3-iodotyrosine is to be taken into account provided the starting material, i.e. tyrosine is in excess as compared to ^{125}I. Thus in this case it is advisable

to elute free radioiodine with an aqueous buffer /pH = 4/ which should be followed by the elution of the labelled 3-iodo-tyrosine with 30% aqueous ethanol.

DISCUSSION

The adsorption of phenol derivatives on dextran gels has been investigated by several authors, mainly aiming at explaining which parts of the gel structure interact and how the phenol gets bound to the gel. As for the former question Determenn and Walter have shown that the adsorption takes place on the hydroxy-ether groups which cross-link the dextran chains[2]. Later Brook and Housley suggested that in the phenol derivatives it is the hydroxyl group which forms hydrogen bond with the gel resulting in a stronger adsorption of phenols as compared to the adsorption of aromatic compounds without phenolic hydroxyl group[3]. At pH = 4, i.e. where the hydroxyl group is unionized, phenols are more strongly bound to the gel than in alkaline solution where phenols exist as anions. Similar drop in the distribution coefficient due to the ionization of the carboxyl or amine group can also be observed in case of aromatic acids and bases[4]. The k vs. pH plot of the p-aminobenzoic acid, e.g. shows a maximum at pH 3.5-5.5, at lower pH the protonation of the amine group while at higher pH the dissociation of the carboxylic group results in the decrease of the distribution coefficient[4].

Halogen substituents like iodine atoms increase the adsorption affinity towards the dextran gel, the increase is proportional to the number of halogen

atoms per molecule[5,6]. Accordingly the elution order of the thyroidal iodo amino acids corresponds to the increasing number of iodine atoms per molecule[5-7].

The experimental results presented in this paper indicate, in agreement with the exceptions, that the distribution coefficient of the 3,5-diiodotyrosine is considerably higher than that of the 3-iodotyrosine. Besides, near the pK_{OH} values the distribution coefficient falls drastically.

Surprisingly the ionization of the phenolic hydroxyl group does not hinder the formation of the hydrogen bond between the hydroxy-ether group of the dextran gel and the hydroxyl group only cancels the interaction, thus far not satisfactorily clarified between the dextran gel and the iodine substituent/s/ as well. On the other hand, the ionization of the carboxylic or protonation of the amine group of the alanine side chain of the tyrosine does not influence the distribution coefficient at all. This is clearly demonstrated in Fig. 5. according to which no decrease of the distribution coefficient can be observed below pH 5, albeit the dissociation of the carboxylic group starts /as it is shown in Fig. 6./ at pH 1 and is completed at pH 4. Neither does the protonation of the amine group. taking place between pH 5-9, result in the decrease of the distribution coefficient. Quite to the contrary parallel to the protonation of the amine group a monotonous increase of the k values can be observed when lowering the pH from 9 to 5 which can be attributed to the decrease of the degree of ionization of the phenolic hydroxyl group. From the experimental data presented above the conclusion can be drawn that the adsorption of the iodotyrosines on Sephadex LH-20

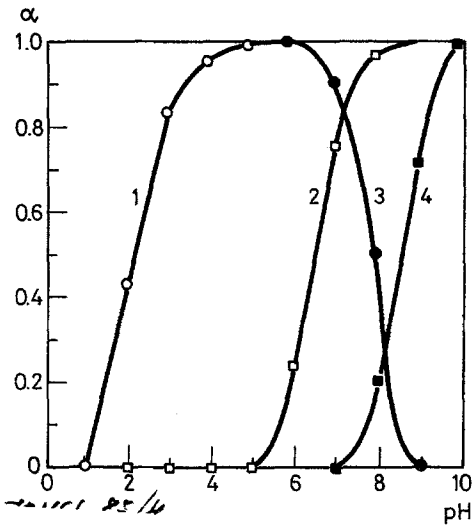

Fig. 6. The degree of ionization of the phenolic hydroxyl, carboxyl and amine group of the iodotyrosines. Curve 1: carboxyl group; Curve 2: amine group; Curve 3: phenolic hydroxyl group of the 3-iodotyrosine; Curve 4: phenolic hydroxyl group of the 3,5-diodotyrosine. /The α vs. pH relationship was calculated on the basis of the pK values reported by Ortner et al.[8]

dextral gel is governed by the phenolic hydroxyl group and the iodine substituent/s/ only and is not influenced by the alanine side chain.

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