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Electrophoretic properties of radioiodine-labelled human serumalbumin at different iodination degrees

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SUMMARY

Progressive iodination of HSA affects its electrophoretic behaviour : however, no apparent formation of degradation products has been detected up to a substitution degree of about 24 iodine atoms per molecule of HSA.

The HSA, progressively iodinated, shows an increased mobility on agar gel, and behaves as a single and homogeneous compound by moving boundary electrophoresis. No apparent correlation exists between the electrophoretic behaviour and the biological properties of the iodoalbumins which have been previously checked in humans. On this basis, the validity of electrophoresis as a control tool for the labelled albumin may be seriously questioned.

INTRODUCTION

The effect of the progressive iodination on the metabolic behaviour of the human serumalbumin has been recently investigated and reported [1, 2].

With the aim of establishing whether the modifications of the metabolic behaviour may be correlated with electrophoretically detectable alterations of the protein, a study on the electrophoretic properties of different iodoalbumins has been carried out.

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This could cast some light on the validity of the electrophoresis as an analytical procedure to check the adequacy of radioiodine labelled human serum albumin as a metabolic tracer.

EXPERIMENTAL

An albumin lot (lot VII), especially prepared by Immuno, Wien, in the form of a dry powder, was employed. As it was previously reported [2], this lot was demonstrated to have not suffered an appreciable extent of alteration during its separation from the original plasma. It was analyzed by ultracentrifugation, revealing a single component with a ^{20}S value of 4.6.

The iodination was carried out according to an electrolytic procedure [1, 2] : 200 mg of HSA, dissolved in 10 ml of an isotonic saline solution containing 15 mg of labelled KI (about 100 μC ^{131}I), was electrolyzed at a constant current of 300 μA, for a time depending on the desired value of the iodination degree.

Moving boundary electrophoresis was carried out in a Klett-Tiselius apparatus. Before the analysis, the protein solution was submitted to a 24 hours dialysis against a Veronal buffer at pH $= 8.4$ and $\mu = 0.1$. The dialysis was prolonged until the same conductivity of the buffer was reached in the solution.

Agar-gel electrophoresis was carried out on agar plates, in a Elvi 80 cell. Albumin solutions (1-5 mg/ml) were dialyzed against a Veronal buffer at a pH $= 8.2$ and $\mu = 0.02$, before the analysis. Albumin spots were stained with Amido-Schwarz 10 B. Autoradiography, using Ferrania X films, was used to localize the radioactivity. Quantitative estimations were made by scanning of the plates, followed by integration of the recorded peak areas.

In order to evaluate mono and di-iodinated tyrosine residues, a 0.1 ml sample of each radioiodinated preparation was submitted to total hydrolysis with pancreatin in the presence of 1-metilmercaptoimidazole to prevent deiodination.

The hydrolysate was analyzed by descending paper chromatography using butanol saturated with acetic acid as eluting mixture. The spots of MIT and DIT were located by autoradiography and their relative amounts were calculated by counting the iodine activity.

RESULTS AND DISCUSSION

The patterns of the moving boundary electrophoresis, carried out on iodoalbumins at different iodination degrees (ID), are reported in Figures 1 and 2, together with the pattern of the original uniodinated albumin.

All the preparations tested showed a single and homogeneous peak, which is undistinguishable from that of the native albumin. The mobility of the albumin slightly increases with increasing iodination degree, as it is

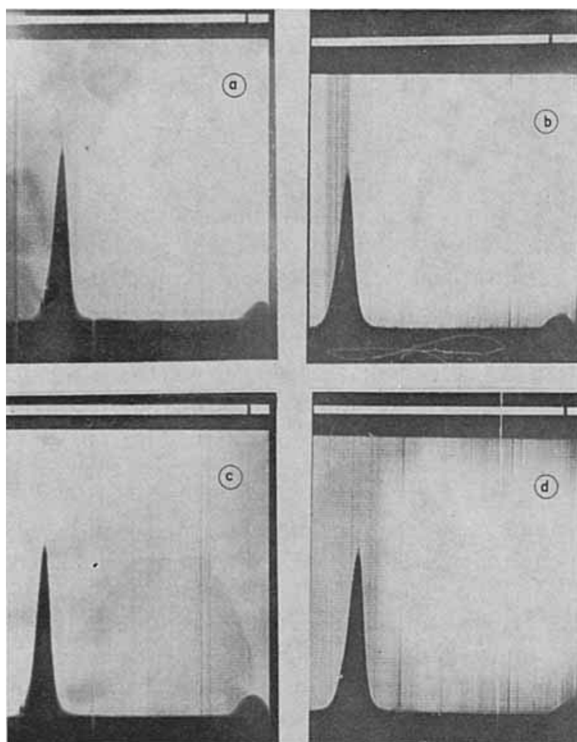

FIG. 1. Moving boundary electrophoresis of iodoalbumins at different iodination degrees : (*a*) ID = 0.5; (*b*) ID = 6; (*c*) ID = 11; (*d*) ID = 18.

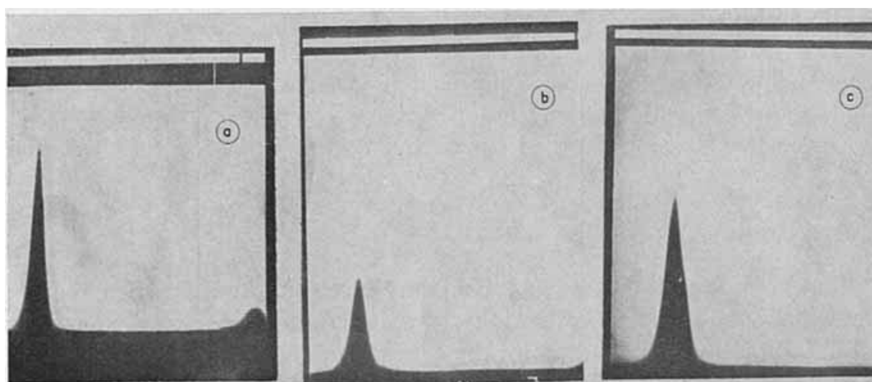

FIG. 2. Moving boundary electrophoresis of (*a*) native albumin; (*b*) iodinated albumin at ID = 24 and (*c*) mixture of the two samples.

proved by the analysis of a mixture, containing equal amounts of uniodinated and highly iodinated (ID = 24) albumin (see Fig. 2).

The significance of these findings has to be discussed in the light of the experiments on the metabolic behaviour of the iodinated albumin [3]. Using a selected human serum albumin lot and special iodination conditions, it has been found that HSA can be substituted up to an iodination degree of about 11 iodine atoms per molecule before any appreciable modification of its degradation rate in humans could be detected. Beyond such a value of iodination degree, iodoalbumins are degraded at a higher rate, but still homogeneously; rapidly degraded components appear at ID values of 18 or more. It is probable that the modifications which have affected the metabolic behaviour do not correspond to conformational changes able to modify the shape, the size and the density of charge of the molecule. An interpretation of the modification of the metabolic behaviour in the sense that the iodination involves one or more "critical sites", without any modification of the whole molecule, would seem to be favoured by these results.

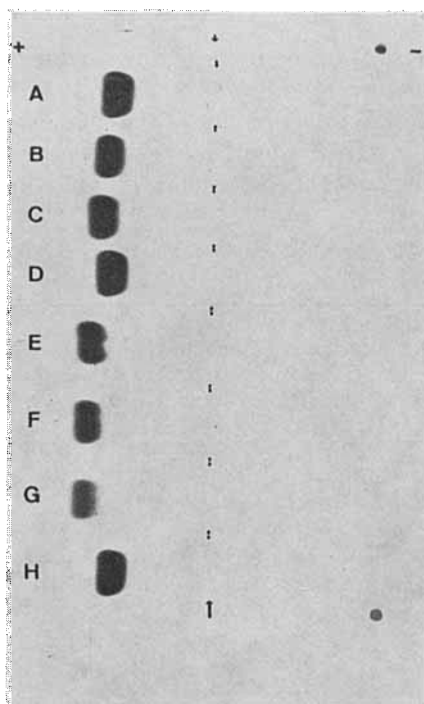

FIG. 3. Agar gel electrophoresis of iodoalbumins at different substitution degrees : (B) ID = 0.5; (C) ID = 5; (E) ID = 11; (F) ID = 17; (G) ID = 24; (A), (D), (H) : uniodinated albumin from the same lot as a reference.

The small circular spots on the right are polyethyleneglycol, used as mobility standard. Electrophoresis conditions : 5 hours at 4 V/cm. Albumin concentration : 2 mg/ml.

Similar observations may be made when preparations of iodinated albumin at different ID were studied by agar-gel electrophoresis. In Figure 3 an experiment is reported, in which increasingly iodinated albumins, as well as native albumin from the same lot as a reference, were run on the same agar plate under highly standardized conditions.

When considering the above results, it must be kept in mind that the iodination of the tyrosyl groups is the only occurring reaction all along the albumin iodination process, at least to an ID of about 14-16; beyond these ID values, side processes take place, probably involving histidyl groups. The increased rate of migration of the HSA with increasing ID could therefore be interpreted in terms of modifications occurring in the pK of the tyrosyl residues, which have been subjected to the iodination.

The same effect occurs in the iodinated insulin and it has been considered responsible for the observed increase of the mobility of the iodoinsulin [(4)]. It has been calculated that at pH 9, about 90 % of the phenolic hydroxyl groups are dissociated in the fully iodinated insulin molecules, which then carry a higher negative charge, in comparison with the iodine free insulin molecules.

In Table I the results of the total hydrolysis carried out on the iodinated albumin preparations are reported [(1, 2)]. The relative percentage of the di-iodinated residues on the total amount of the iodinated residues (DIT X 100/ MIT + DIT) was directly evaluated by chromatographic analysis of the iodoalbumin hydrolysate. The percentage of uniodinated tyrosine residues

TABLE I. Percentages of uniodinated and di-iodinated tyrosines in iodoalbumins at different substitution degrees.

Iodination degree (ID) [a]	Percentage of uniodinated tyrosines on the total number of tyrosines [b]	Percentage of di-iodinated residues on the total number of iodinated residues [c]
1.15	91.8 ∓ 6.1	6.97 ∓ 0.7
2.00	84.4 ∓ 4.9	11.76 ∓ 1.1
3.20	76.0 ∓ 4.5	11.10 ∓ 1.1
4.19	68.4 ∓ 3.9	10.30 ∓ 1.1
5.00	65.1 ∓ 3.2	11.40 ∓ 1.2
6.37	56.4 ∓ 2.6	11.20 ∓ 1.1
7.88	42.5 ∓ 2.2	13.40 ∓ 1.4
9.72	30.5 ∓ 1.8	16.40 ∓ 1.7
11.00	22.2 ∓ 1.4	19.50 ∓ 1.8

[a] Iodine atoms per molecule.

[b] Based on the assumption that 12 tyrosines react with iodine in HSA.

[c] Calculated by the ratio of (DIT) to (MIT + DIT) residues, as evaluated by chromatography. Detailed informations on the technique employed have been reported in a previous paper (I).

still existing in each preparation, was calculated by difference, the total number of the iodinated residues being known (MIT + DIT). This was made assuming that the number of the available tyrosine residues in the undenatured HSA is 12 per molecule, as it was found by C. H. Li [5], who studied the kinetic of the HSA iodination.

As it can be seen, about 80 % of the tyrosine residues is substituted, mainly in the form of MIT, when a value of ID as high as 11 is attained. At ID = 5, when the increase in the migration rate becomes evident, about 35 % of the tyrosyl residues is substituted.

The slight difference in the migration rate between the albumin at ID = 0.5 and the native one may hardly be explained on this basis : it would seem that in the first step of the process, when the iodine is consumed in side oxidative effects on the sulphydryl groups, something may intervene to modify the electrophoretic properties. The small differences observed do not allow, however, a deeper discussion of the phenomenon.

Some interesting observations can be drawn from the autoradiographies of the agar plates, as reported in the Figure 4. A marked trailing effect can be observed in the preparation at ID = 24. This agrees with the results of the experiments on the metabolic properties, from which it has been established that the preparation at ID = 24 was degraded as a highly inhomogeneous product, whilst the preparation at ID = 18 showed an abnormally high, but relatively homogeneous, trend.

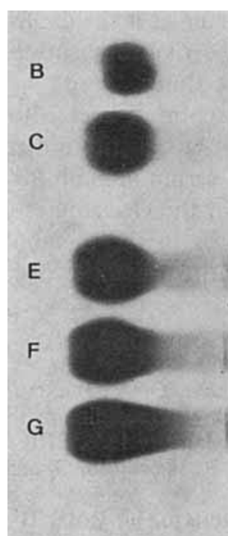

FIG. 4. Autoradiography of the agar plate reported in Fig. 3, after electrophoresis of progressively iodinated albumins : (B) ID = 0.5; (C) ID = 5; (E) ID = 11; (F) ID = 17; (G) ID = 24.

All the samples were only very slightly ^{131}I labelled.

From the practical point of view, some observations concerning the validity of the electrophoretic control on the iodinated proteins can be drawn. The moving-boundary electrophoresis has been employed by several authors in order to check previously the good quality of iodinated albumin preparations. On the contrary, our experiments show that preparations of IHSA, which revealed a different metabolic behaviour, were substantially undistinguishable as far as electrophoretic properties are concerned. As already pointed out, phenomena other than those which may affect the electrophoretic properties are responsible for the differences in the metabolic behaviour.

Conversely, many authors have concluded that the iodinated serum-albumin is definitely different from the native one, just on the basis of experiments of comparative electrophoresis carried out on various supporting materials. That this criterium is not acceptable, is proved by the fact that in the experiment reported in Figure 3 the two serum albumin samples, B and C, which definitely differ from each other, as far as the migrability on agar gel is concerned are both treated in the same way by the living organism [3]; both show the same value of the metabolic degradation rate, which is in close agreement with those commonly accepted for the serum albumin, according to the works of Cohen *et al.* [5] and Freeman [6].

Conclusion

One of the most troublesome problems of the producers of labelled serum albumin for metabolic studies is the control of the final product characteristics : the electrophoresis on various supporting materials is one of the most employed analytical procedures.

From the results above reported it can however be concluded that the electrophoretic analysis does not constitute a reliable test to check the adequacy of radioiodine labelled serum albumin as a metabolic tracer. Actually, the progressive modification of the electrophoretic properties does not necessarily correspond to differences in the metabolic behaviour, such as to be put in evidence at the light of the present knowledge.

BIBLIOGRAPHY

1. Rosa, U., Pennisi, F., Scassellati, G. A., Bianchi, R., Federighi, G., Donato, L., Rossi, C. A. and Ambrosino, C. — Proceeding of the conference on Labelled proteins in trances studies — Pisa, Italy, January 1966. EUR 2950. d, f, e — EURATOM 1966.
2. Rosa, U., Pennisi, F., Bianchi, R. Federighi, G. and Donato, L. — *Biochim. Biophys. Acta*, **133** : 486 (1967).
3. Bianchi, R., Federighi, G., Donato, L., Rosa, U., Pennisi, F. and Scassellati, G. A. — *J. Labelled Compounds*, **1** : 314 (1965).
4. Li, C. H. — *J. Am. Chem. Soc.*, **67** : 1065 (1965).
5. Cohen, S., Halloway, R. C., Matthews, C., and Mc Farlane, A. S. — *Biochem. J.*, **62** : 135 (1956).
6. Freeman, T. — *J. Labelled Compounds*, **1** : 312 (1965).