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Labeling of Proteins with ^{125}I and Experimental Determination of its Specific Activity

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The purpose of the present work consists in the standardization of the labeling technique of proteins with ^{125}I and the control of the obtained products, principally their specific activities, in order to utilize them correctly in radioimmunoassays. The quantities of Chloramine-T and sodium metabisulphite were lowered, with regard to the original method, to 3·6 and 9·6 μg respectively. Under these conditions, optimal yields and radioiodinated proteins with good immunological activities were obtained.

It was found that the specific activity calculated, as usual, from the yield obtained by electrophoresis, is higher than the real value. In fact, radioiodine not bound to the protein remains in the zone corresponding to the labeled protein, when analyzed by electrophoresis. This complication did not occur when chromatographic analysis was carried out.

The knowledge of the real specific activity is important since it is necessary for the determination of the mass of protein which must be put into reaction from the absolute activity of the labeled protein. For these reasons the yields and the corresponding specific activities were determined from ascending chromatograms obtained with 70% methanol as solvent, during two hours in darkness. The radioimmunoassay displacement curves obtained with proteins labeled with the proposed method, the specific activities of which were calculated from their radiochromatographic patterns, were reproducible and gave a percentage of bound radioiodinated protein in the absence of cold protein of 50 ± 4. The non-specific combinations were always smaller than 5%.

The possibility of determining the actual specific activity by simple chromatography, shown in the present work, should be considered as one more step towards a methodological standardization of radioimmunoassay procedures.

INTRODUCTION

THE LABELING of proteins with radioiodine by means of the Chloramine-T method has been widely used for approx. 13 y.[1,2] Proteins labeled in this way are commonly utilized in radioimmunological diagnostic methods. In order to assure the success of these techniques, the following conditions should be fulfilled:

(a) The protein should maintain its immunological activity after the labeling;

(b) The specific activity must be appropriate to the requirements of each experiment;

(c) The real average specific activity of the labeled protein must be known, in order to calculate the correct amount of the labeled protein used in the radioimmunoassays, so that reproducible displacement curves can be obtained in consecutive assays.

In the present work the labeling conditions and the control procedures of the obtained

product are studied, in order to standardize and simplify the method for a wide number of protein molecules. The Chloramine-T and sodium metabisulphite concentrations were lowered with regard to the original method,[2] in order to obtain, with a good labeling yield, a protein with an appropriate immunological activity.

There are different methods for the determination of the specific activity, some of them somewhat troublesome.[3–8] A commonly used technique consists of the analysis of the labeled product by means of electrophoresis on Whatman no 1 or no 3MM paper.[9,10] However, if a labeling blank is carried out, i.e. the iodide, the chloramine-T and the sodium metabisulphite are made to react in the absence of protein, we observed a radioelectrophoretic pattern, similar to that obtained with a radioiodinated protein. Thus, the yield and therefore the specific activity calculated by this method will be greater than the real value. In a previous paper[11] we have shown that the labeling yield calculated from the radioelectrophoretic patterns may be greater than the maximum theoretically possible value.

For these reasons we have developed a simple and rapid technique, which allows the determination of the real yield and specific activity; the method consists of ascendant chromatography on Whatman no 1 paper during 2 h in darkness, using 70% methanol as solvent.[12]

In order to achieve a good reproducibility between consecutive radioimmunoassays, it is necessary to utilize always the same amount of radioiodinated protein, which is calculated from the absolute activity of the sample and its specific activity. This last parameter, determined by the proposed method, has been shown to be correct, since radioimmunoassay curves performed with successive labeled proteins exhibited a percentage of bound radioiodinated protein in the absence of cold protein, B_0, equal to 50 ± 4; the non-specific combinations accounted for less than 5%.

EXPERIMENTAL

Instruments

A radiochromatogram Scanner Berthold II with flow-counter, automatic area integration and a bidimensional scanning accessory was utilized.*

Reagents

(a) Human Thyrotropin (HTSH), Rat Thyrotropin (RTSH), Human Luteinizing Hormone (HLH), Human Follicle-stimulating Hormone (HFSH), kindly provided by the National Pituitary Agency (University of Maryland, School of Medicine), National Institute of Arthritis, Metabolism and Digestive Diseases, U.S.A.

(b) Recrystallized Porcine Insulin, Mann Research Laboratories, U.S.A.

(c) Fab from antirabic antibody, kindly provided by Dr. E. Levin (Instituto Nacional de Farmacologia y Bromatologia, Buenos Aires, Argentina).

(d) Synthetic Human Gastrin I, from the Division of Biological Standards, National Institute for Medical Research, England.

(e) Carrier-free ^{125}I-iodide in sodium hydroxide from the New England Nuclear Corp. (NEZ 033 H).

(f) Sephadex G-25 for insulin, Sephadex G-50 for gastrin and Sephadex G-75 for HLH, HFSH, RTSH, HTSH and antirabic Fab.

(g) Buffer solution for labeling: 0·05 M phosphates at pH = 7.4.

(h) Buffer solution for the elution of Sephadex columns after the labeling of HLH, HFSH, RTSH, HTSH, antirabic Fab and Gastrin: 0·01 M phosphates, 0·15 M NaCl at pH 7·8, usually called PBS.

(i) Buffer solution for the elution of Sephadex columns after the labeling of insulin: Barbital–sodium barbital at pH = 8.6 and ionic strength equal to 0.05.

(j) Buffer solution for electrophoresis: Tris–barbital–sodium barbital, pH = 8·8, ionic strength 0·05 (Gelman Instrument Co., Michigan, U.S.A.).

(k) Solvent for chromatography: methanol–bidistilled water (70:30), freshly prepared.

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(l) One per cent Bovine Serum Albumin solution dissolved in its corresponding elution buffer (Fraction V, Sigma Chemical Co., Missouri, U.S.A.).

METHODS

(1) *Labeling*

All the proteins to be iodinated were previously fractioned into siliconed vials in amounts ranging between 1 and 5 μg of protein in a volume of 5 μl of the corresponding diluent; they were stored at −20°C.

In order to label the protein, 500–1000 μCi of radiochemically pure $^{125}I^-$, contained in 10 μl of buffer solution for labeling, were added to the vial containing the protein. Immediately afterwards 3·6 μg of Chloramine-T in 20 μl of buffer solution for labeling were added. The optimal duration of the reaction under these conditions, 30 sec, was determined in preliminary experiments. During this period the vial was shaken manually; experiments with magnetic or vortex stirrers showed an increase of the iodination yield but a decrease of the immunological activity of the labeled protein. The reaction was stopped by adding 9·6 μg of sodium metabisulphite contained in 80 μl of buffer solution for labeling, under continuous manual shaking. The chloramine-T and sodium metabisulphite solutions must be prepared immediately before use. A labeling blank, i.e. the iodination reaction in the absence of the protein, was carried out concomitantly with each experiment.

(2) *Purification of the labeled protein*

Immediately after labeling the protein, it was purified by means of gel filtration. The porosity of the utilized Sephadex was adequate for the molecular weight of each protein. The Sephadex column (50 × 0·9 cm) was always previously saturated with 6 ml of 1 % solution of Bovine Serum Albumin in the corresponding elution buffer and washed with 60 ml of the same buffer. After applying the labeled protein, it was eluted in 1 ml fractions into siliconed vials containing 50 μl of Bovine Serum Albumin solution.

(3) *Electrophoretic and chromatographic controls*

These controls were performed firstly on the $^{125}I^-$ sample, in order to check its radiochemical purity, and immediately after the labeling reactions on the labeling blank and the radioiodinated protein. The electrophoretic and chromatographic controls were carried out on Whatman no 1 paper (26 × 29 cm) using iodide and iodate carriers.

The electrophoresis was performed perpendicularly to the paper fibers, with an electric field of 12·5 V/cm during 40 min, using the buffer solution for electrophoresis. The ascending chromatography was developed in the same direction of the paper fibers during 2 h in darkness, using 70 % methanol as solvent.

In order to study the different patterns obtained with either method, bidimensional runs were performed in the following two sequences:

1. (a) chromatography with the obtention of its monodimensional pattern.
   (b) electrophoresis on the same paper, perpendicularly to the direction of the chromatographic development. This sequence is symbolized as 1C2E.
2. (a) electrophoresis with the obtention of its monodimensional pattern.
   (b) chromatography on the same paper, perpendicularly to the direction of the electrophoretic development. This sequence is symbolized as 1E2C.

Once both runs were completed, the automatic bidimensional scanning of the paper was performed.

(4) *Determination of the labeling yield*

After step (1) of either sequence or the monodimensional electrophoresis or chromatography was completed, the yield was calculated by means of the following expression:

$$Y = \frac{I\text{–}P}{I\text{–}P + I^-} \qquad (1)$$

where:

I–P is the area integration of the curve corresponding to the protein in the radiochromatographic or radioelectrophoretic pattern.

I^- is the area integration of the curve corresponding to the iodide in the radiochromatographic or radioelectrophotetic pattern.

(5) *Determination of the specific activity* (A_{sp})

The specific activity, given in $\mu Ci/\mu g$ was calculated as follows:

$$A_{sp} = \frac{A \times Y}{M} \qquad (2)$$

where:

A is the absolute activity of $^{125}I^-$ given in μCi, utilized in the labeling.

Y is the yield defined by expression (1).

M is the mass of protein given as μg.

RESULTS

(1) *Control of* $^{125}I^-$

The radioelectrophoretic analysis of $^{125}I^-$ samples with radiochemical impurities, shows peaks other than iodide, whereas the radiochromatographic pattern of the same sample exhibits only one peak in the iodide zone. This shows that only electrophoresis is useful for the evaluation of the radiochemical purity of iodide.

(2) *Control of the labeled proteins*

Figure 1a shows the radioelectrophoretic pattern of the products obtained in a labeling of TSH. The yield is 88% and the specific activity is 208 $\mu Ci/\mu g$. Zone A corresponds to $^{125}I^-$. The two radioactive peaks of zone B are attributed to ^{125}I-HTSH.

Figure 1b shows the radiochromatographic pattern of the same iodination. The analysis shows a yield of 80% and a specific activity of 188 $\mu Ci/\mu g$. A radioactive peak corresponding to non-proteic products can be observed in zone A. The zone B exhibits only one peak attributable to the labeled protein.

The bidimensional scannings obtained from the same reaction products are also shown in the same figure.

Sequence 1E2C (Fig. 1c): Zone A corresponds to $^{125}I^-$. The two radioactive spots of the radioiodinated protein, which appears in zone B, are the same that were observed in the radioelectrophoretic pattern (Fig. 1a). The zone C is attributable to non-proteic radiochemical impurities, which have a low electrophoretic mobility but a chromatographic R_f similar to that of iodide.

Sequence 1C2E (Fig. 1d): $^{125}I^-$ is shown in zone A. The zone B exhibits a simple radioactive spot imputable to the ^{125}I-HTSH which has no electrophoretic mobility after the chromatography has been carried out. Zone C corresponds to non-proteic radiochemical impurities.

Similar results were found with all the proteins of Table 1.

TABLE 1. Percentual yields and specific activities as obtained from chromatographic and electrophoretic results

Protein	Chromatography Y_C	Chromatography $A_{sp.}$	Electrophoresis Y_B	Electrophoresis $A_{sp.}$	Ratio $Y_B:Y_C$
–	%	μCi/μg	%	μCi/μg	–
RTSH	37	75	94	193	2.6
RTSH	33	85	100	256	3.0
RTSH	59	146	95	235	1.6
HTSH	52	104	95	191	1.8
HTSH	80	188	88	212	1.1
HTSH	48	133	88	241	1.8
Insulin	38	44	86	100	2.3
Insulin	47	58	95	120	2.0
Insulin	52	55	87	82	1.7
HLH	25	88	92	284	3.7
HLH	65	98	93	140	1.4
HLH	35	74	82	172	2.3
HPSH	25	65	70	175	2.8
HPSH	52	99	84	160	1.6
HPSH	45	99	92	202	2.0
Antirabic Fab	52	122	73	171	1.4
Antirabic Fab	41	133	63	204	1.5
Antirabic Fab	58	60	79	82	1.4
Gastrin	25	126	80	403	3.2
Gastrin	61	335	91	500	1.5
Gastrin	37	177	88	415	2.4

(3) *Control of the labeling blank.*

The radioelectrophoretic pattern of the labeling blank (Fig. 2a) shows a peak of $^{125}I^-$ in zone A. In zone B (which corresponds in the Fig. 1a to the labeled protein) appears a wide radioactive zone of non-proteic impurities. The radiochromatographic pattern of the same product has a single radioactive peak (Fig. 2b).

The scannings of the bidimensional runs are also shown. Sequence 1E2C (Fig. 2c): $^{125}I^-$ appears in zone A. The zone C is attributable to non-proteic radiochemical impurities which correspond to the zone B of the radioelectrophoretic pattern (Fig. 2a). These impurities, which have a small electrophoretic mobility, when run chromatographically have a R_f approximately equal to iodide.

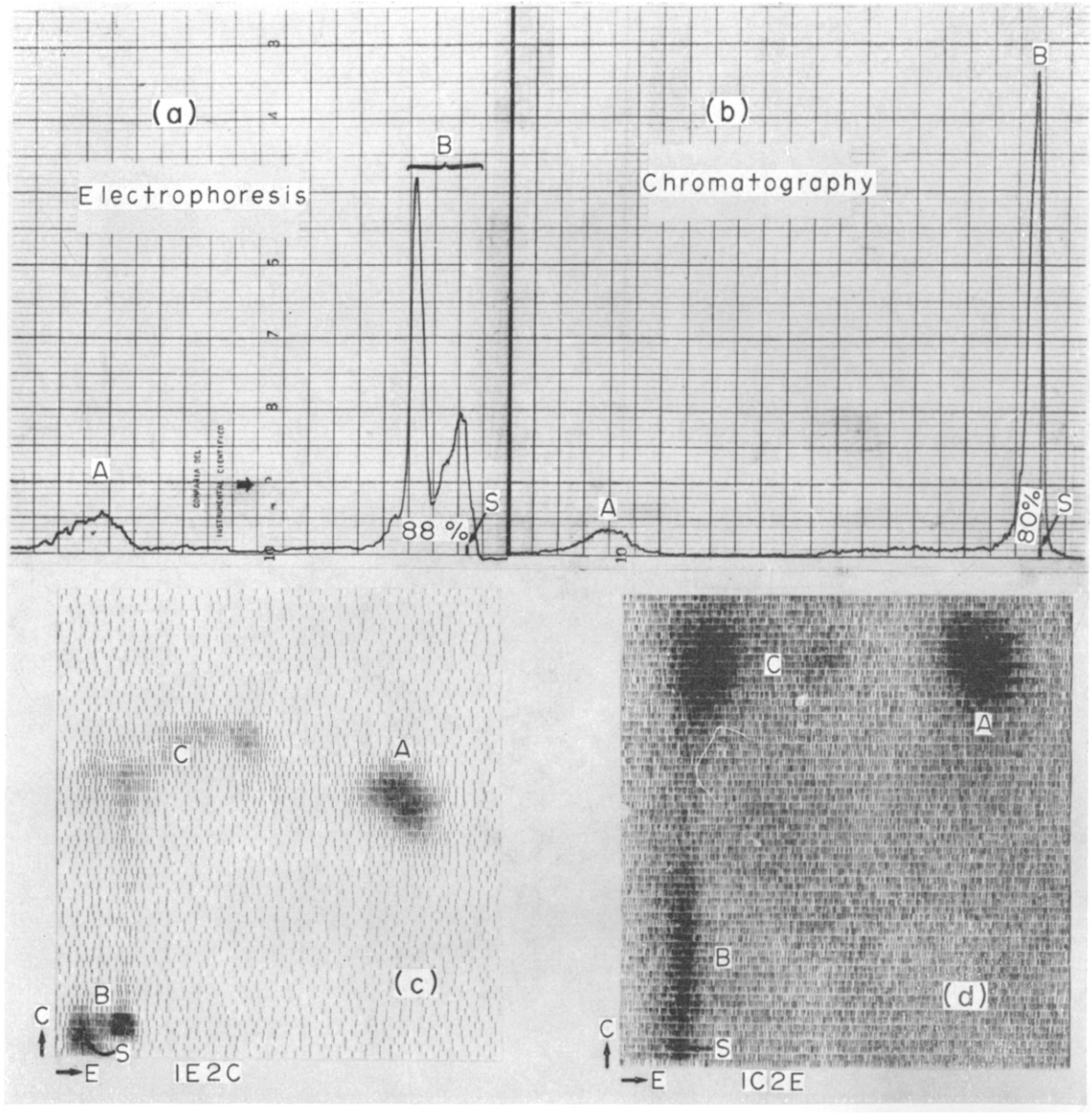

FIG. 1. Control of radioiodinated HTSH: (a) Radioelectrophoretic pattern. (b) Radiochromatographic pattern. (c) Bidimensional scanning; 1E2C sequence; (d) Bidimensional scanning; 1C2E sequence. A: zone corresponding to $^{125}I^-$. B: zone corresponding to the labeled protein. C: zone corresponding to non-protein radiochemical impurities. S: start.

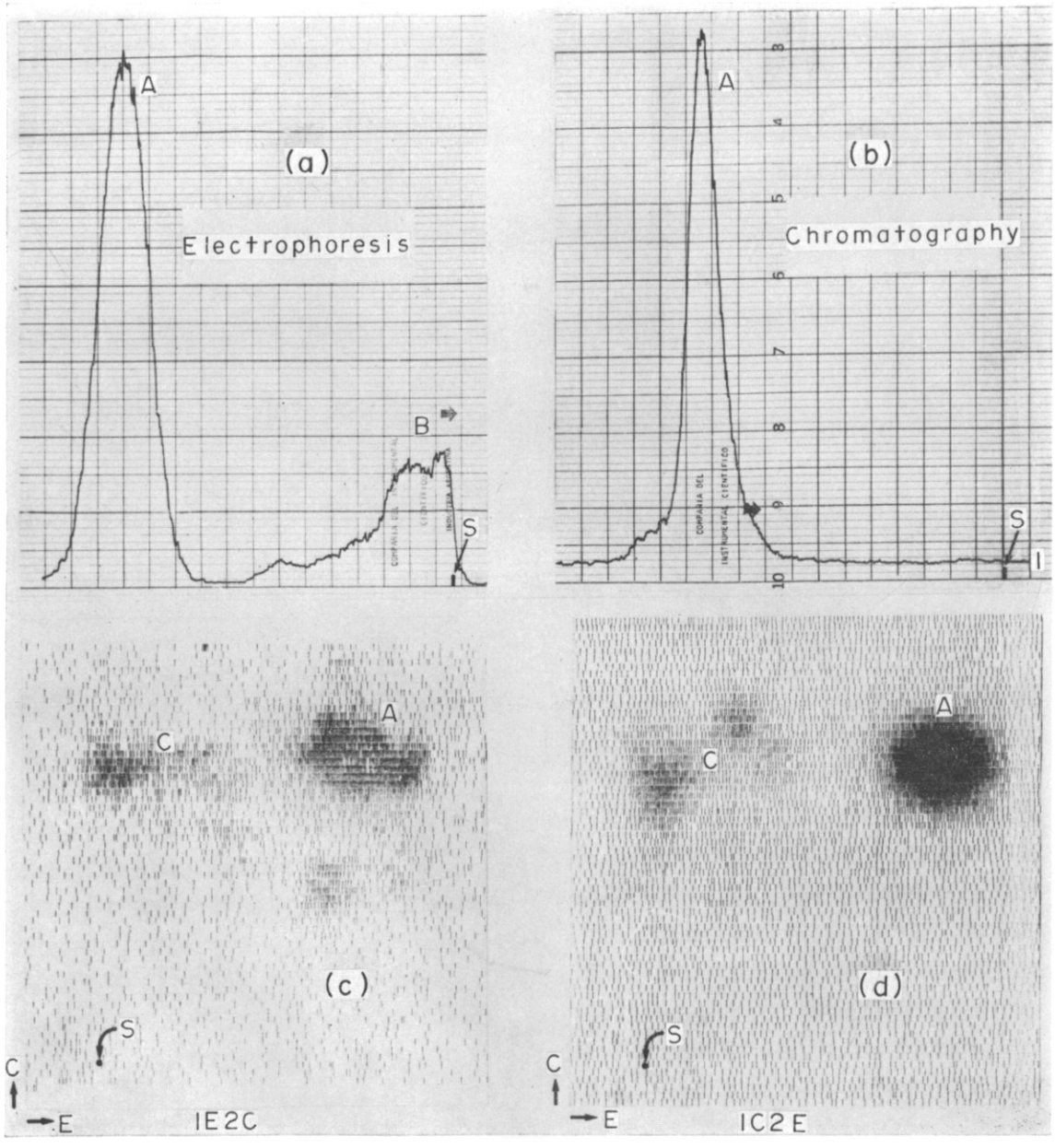

FIG. 2. Control of the labeling blank. (a) Radioelectrophoretic pattern. (b) Radiochromatographic pattern. (c) Bidimensional scanning; 1E2C sequence. (d) Bidimensional scanning; 1C2E sequence. A: zone corresponding to $^{125}I^-$. B and C: Non-protein radiochemical impurities. S: Start.

Sequence 1C2E (Fig. 2d): Zone A is $^{125}I^-$. Zone C are non-proteic radiochemical impurities. The single peak which appears in the radiochromatographic pattern (Fig. 2b) corresponds to the sum of zones A and C. This peak is separated into its components during the electrophoresis.

(4) *Yield and specific activity results*

The labeling yields obtained from electrophoresis and chromatography, Y_E and Y_C respectively, and their corresponding specific activities, are shown in Table 1 for the different proteins labeled with the proposed method. The ratio between both yield values are also indicated.

DISCUSSION

The purpose of the present work consists in an attempt to standardize the methodology of the Chloramine-T labeling technique and the evaluation of the specific activity of the radioiodinated proteins, in order to use them correctly in radioimmunoassays.

Firstly it should be mentioned that the $^{125}I^-$ must have less than 5 per cent of radiochemical impurities, if a labeled protein with good immunological activity, is to be obtained. It could be demonstrated that the control of the $^{125}I^-$ samples should be carried out by electrophoresis since chromatography does not detect the presence of some radiochemical impurities. We observed also that the labeling yield decreases as the $^{125}I^-$ ages.

On the other hand we obtained better results if the Chloramine-T and sodium metabisulphite quantities were lowered. The optimal yield and best immunological activities were obtained with 3·6 μg of Chloramine-T and 9·6 μg of sodium metabisulphite, which represents 28 and 25 times less, respectively, than the quantities given by GREENWOOD *et al.*[2] Under the conditions mentioned in the present work we labeled HTSH, RTSH, HLH, HFSH, Insulin, antirabic Fab and synthetic human Gastrin I. Radioiodinated proteins with appropriate specific activity, that allowed the obtention of sensitive and reproducible radioimmumoassay curves with a non-specific combination smaller than 5% were obtained in all cases.

One of the methods used to determine the specific activity consists in its calculation from the yield obtained in electrophoretic runs. However, the radioelectrophoretic pattern of the product obtained in the labeling blank, where a single peak corresponding to $^{125}I/$ (Fig. 2a) should be observed, shows actually a radioactive zone in the place where one would expect to find the labeled protein (zone B). Thus, in the electrophoresis of the radioiodinated proteins (Fig. 1a), the B zone corresponds to the sum of the radioactivities attributable to the labeled protein and that appearing in the same zone when the labeling blank is analyzed. These results demonstrate that the yield, and therefore the specific activity, calculated by this method, is higher than the real values. It should be mentioned that these findings seem to support the hypothesis that those fractions classically considered as "damaged" radioiodinated protein in the electrophoretic pattern, correspond to some extent to the zone B obtained in the radioelectrophoretic record of the labeling blank. The same conclusion may be derived from the analysis of the bidimensional scannings. The electrophoresis performed on cellogel and Whatman no 3MM paper gave the same results.

These problems induced us to perform the analysis of the labeled proteins and the labeling blank by means of several chromatographic methods. We found that the quickest and simplest technique consists in the ascending chromatography on Whatman no 1 paper, using 70% methanol as solvent.[12] The radiochromatographic pattern of the labeling blank exhibits a single peak in the iodide zone. No radioactivity appears at or near the start of the chromatogram (Fig. 1b). Figure 1b shows that the radiochromatographic pattern of the labeled proteins exhibits a radioactive peak corresponding to the labeled protein (zone B) and another neat peak in the $^{125}I^-$zone (zone A).

From the analysis of the bidimensional scannings of the labeling of different proteins and the labeling blank (Figs. 2c and d), it may be deduced that the single peak of non-proteic radioactivity which appears in the chromatographic pattern is decomposed into different components having dissimilar electro-phoretic mobilities. On account of the fore-going

discussion and even though the difference between the radioelectrophoretic and radiochromatographic patterns of the labeling blank has no clear explanation, it is evident that the radioactive products found in zone B of the radioelectrophoresis run as iodide in the radiochromatography; it is also apparent that the products remaining at or near the start of the chromatograms where the labeled proteins are analyzed, are actually the radioiodinated proteins.

In the electrophoresis of the labelings of HTSH and other proteins, two protein fractions can be observed, whereas in the chromatographic patterns there is a single peak corresponding to the labeled protein. This difference is more apparent in the bidimensional scans, where the 1E2C sequence shows two protein spots but the 1C2E sequence exhibits only one. These results should be attributed to the precipitation of the proteins by the methanol used as chromatographic solvent.

It is known that the displacement curves of successive radioimmunoassays are reproducible only if the same amount of labeled protein, calculated from its specific activity, is made to react. If the Y_E/Y_C ratio, which is equal to the specific activity ratio, would be always constant, the quantity of radioiodinated protein used in successive assays would be equal, independently from the method used to calculate the specific activity, provided the same method is used for all the assays. However, since the Y_E/Y_C ratio varies from one radioiodination to another (see Table 1), there will be substantial differences if the specific activities are calculated by either method. It could be demonstrated with all the proteins used in radioimmunoassays, labeled according to the proposed method, that the percentage of bound radioiodinated protein in the absence of cold proteins, B_0, was equal to 50 ± 4, provided the amounts of the labeled proteins used for the radioimmunoassay and the titration of the antiserum were determined from the specific activities obtained from the radiochromatographic patterns. If the specific activities are calculated from electrophoretic results, substantial dissimilarities in the different B_0 values appear. Therefore, we may conclude that the specific activities calculated by the chromatographic method give real values, which allow a better standardization of the radioimmunoassays. It is clear that these specific activities, as well as those obtained from electrophoresis, are really average values, as noted in the introduction of the present paper. It is also evident that for the routine analysis of the radioiodination, it is sufficient to perform a monodimensional ascendant chromatography, which is a simple, rapid and accurate method for the determination of the specific activity of radioiodinated proteins.

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