TRITIUM LABELLING OF RIBONUCLEASE BY THE GAS-EXPOSURE METHOD, AS IMPROVED BY AN ELECTRIC DISCHARGE.

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SUMMARY

When lyophilized samples of bovine pancreatic ribonuclease are exposed to tritium gas, the exchange process can be markedly improved by applying high frequency electric discharges.

Labelling has been carried out for 5, 15 or 30 minutes periods. Labile radioactivity, was then removed by means of molecular sieving and repeated lyophilizations, the tritiated protein being submitted to further purification by ion-exchange chromatography.

The specific radioactivity of such purified samples is in the Curie/mmole range, and the biological activity of the enzyme remains well preserved. The distribution of tritium atoms among component aminoacids has been determined, after acid hydrolysis of the labelled protein.

I. INTRODUCTION

Tritiation of proteins by direct exposure to tritium gas has been achieved in a number of cases (1-5). However the useful range of application of this technique is seriously limited due to the rather large variety of impurities that may form during the exchange process.

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Several modifications leading to a higher efficiency have been proposed. Among these, applying the continuous high frequency electric discharge produced by a Tesla leak detector (6), successfully improved the labelling of aminoacids and other biological substances. By comparison with simple exposure to tritium gas, the latter procedure brings about a markedly higher exchange rate, while impairment of the biological activity is increased to a much lesser degree (7, 8).

The present paper describes the application of this improved technique to the tritium labelling of a well characterized protein, bovine pancreatic ribonuclease.

II. EXPERIMENTAL

a. Materials

Chromatographically purified, salt-free bovine pancreatic ribonuclease (RNase) was purchased from Koch-Light (Specific activity : 70 Kunitz units/mg (9)). Dextran gel Sephadex G-25 was obtained from Pharmacia (Uppsala, Sweden) and carboxymethyl-cellulose from Whatman. The tritium gas that was used had an isotopic purity above 98 %. All other reagents were of analytical grade.

Gradients for chromatographic separation were set up with an LKB Ultrograd 11300 unit; eluates were monitored at 280 and 206 nm, by means of an LKB Uvicord III 2089 UV-absorptiometer.

Radioactivities were measured by liquid scintillation counting in Instagel (Packard Instrument Co), using a Packard Tricarb 2021 scintillation counter.

Electric discharges were produced by a Leybold Heraeus Tesla tester, type 16415, set at approximately 60 KV and 100 µA.

b. Isotopic exchange

The lyophilized protein is spread out along the inner surface of a cylindrical Pyrex tube (20 x 100 mm) connected to a round flask, 120 mm in diameter. The electrodes are placed near the surface of the protein powder, however without making contact with it. The electric discharge runs parallel to the axis of the cylindrical tube, which is kept in a horizontal position.

Approximately 10 Curies of tritium gas were used, at a pressure

of 10 mm Hg. Samples of RNase (50 mg) were subjected to exchange at room temperature, for periods of 5, 15 or 30 minutes.

c. Purification

At the end of the exchange period, tritium gas is evacuated from the flask. The sample is then dissolved in 10 ml of water and lyophilized, this treatment being repeated three times and followed by ion-exchange chromatography.

In the latter step, the protein is applied to a column (1.6 x 40 cm) filled with carboxymethyl-cellulose equilibrated in 5 mM Tris-HCl buffer at pH 8 and 6 °C. Elution is performed by passing the same buffer at a constant rate of 45 ml/h, superimposed with a linear gradient of from 0 to 0.1 M NaCl. The protein-containing fractions are then pooled and desalted on Sephadex G-25 (2.5 x 100 cm column), in 2 mM NH_4HCO_3 at 40 °C, which further eliminates labile tritium atoms.

The lyophilized protein is finally dissolved in water, at a concentration of 2 mg/ml and pH 5.5, where it is kept at 45 ° or 50 °C during 24 h then lyophilized. This treatment is repeated three times. Constant specific radioactivity is then attained.

d. Assays

1. Enzyme activity

The modified spectrophotometric method of Kunitz was used. It is based upon the kinetics of optical density decrease at 300 nm, which is observed when RNase is acting on ribonucleic acid (9).

Assays were performed at 25 °C and pH 5.0, in 0.05 M sodium acetate buffer containing 0.05 % sodium ribonucleate. Activity is expressed in Kunitz units (K.U.) per mg, i.e. the amount of enzyme which, under the conditions of the assay, lowers in one minute the 300 nm absorbance down to its final value.

2. Aminoacid composition and radioactivity distribution

Appropriate amounts of tritiated RNase were hydrolyzed *in vacuo* at 110 °C for 24 h, using constant boiling HCl. Aminoacid analyses were performed according to the method of Spackman et al. (10), using a Beckman Unichrom II aminoacid analyzer.

The effluent from the automated analyzer was collected in 4 ml fractions, in order to determine the radioactivity of individual aminoacid peaks (except in two cases, namely serine-threonine and isoleucine-leucine, each of which has had to be pooled and be treated as a single fraction). An aliquot of each fraction was mixed with 20 ml of Instagel scintillation medium and the tritium contents was determined using a suitable internal standard.

Results are expressed in terms of normalized specific activity, or NSA, which is an indicator of the tritium distribution among individual aminoacid side chains. This parameter is derived from the complete aminoacid composition; it is defined as the ratio between the specific activity per residue of the aminoacid under consideration and the sum of the corresponding specific activities of all the aminoacids in the protein.

III. RESULTS

a. Purification of the protein and removal of labile radioactivity

Extensive exposure of proteins to ionizing radiations is known to bring about such changes as main chain disruption, polymerization, hydrophobic or hydrogen bonds breakage, and chemical modification of aminoacid side chains, all of which result in severe conformational damage. In view of these effects, tritiated ribonuclease had to be repeatedly cleaned out from possible radiation by-products.

After most of the labile radioactivity has been removed by repeated lyophilizations, the specific activity of the unfractionated protein amounts to at least some 30-40 Curies per mmole. Chromatographic separation (Fig I-b, -c, -d) reveals the presence of two major components in these preparations, the first one being a glycosylated protein; both components display exactly the same chromatographic behaviour as do their unlabelled counterparts in the original commercial sample (Fig I-a). In all three tritiated samples (5-15-30 min), the glycosylated species is observed to have a much higher specific radioactivity than does the other major one.

In addition, there appears in the elution patterns some fractions absorbing at 206 nm (Fig I-b, -c, -d), which are not present in the untreated protein (Fig I-a). These fractions, en-

dowed with high specific radioactivities, are believed to be degradation products which occur during the exchange process. They were not investigated any further.

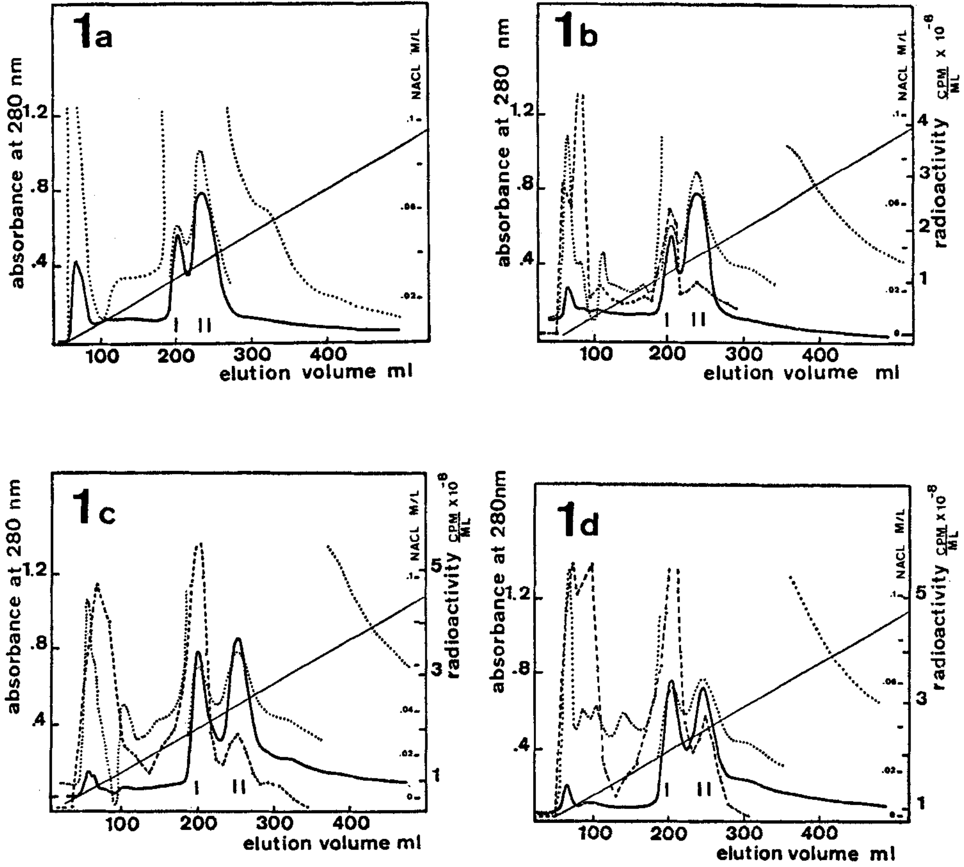

Figure I

Analytical chromatography of ribonuclease
(carboxymethyl-cellulose CM-11; for conditions, see methods)

(a) commercial RNase; (b) tritiated RNase, 5 min exchange;
(c) same 15 min; (d) same 30 min.

———————— : absorbance at 280 nm
.... : absorbance at 206 nm
----------- : radioactivity in cpm per ml of eluate

In order to eliminate buffer salts as well as residual labile radioactivity, each of the two major components was then submitted to gel chromatography on Sephadex G-25 at 40 °C (Fig 2) (11). The corresponding specific radioactivities are summarized in table I. The total recovery of protein, as given by the sum of the two purified components, is in the range 50 to 75 %.

Since some exchangeable tritium atoms still might remain buried within the compact globular structure of the native protein, samples have been routinely incubated for 24 hours, in water at pH 5.5, near the transition temperature of the protein. Repeating this treatment leads to constant specific radioactivity (Table I).

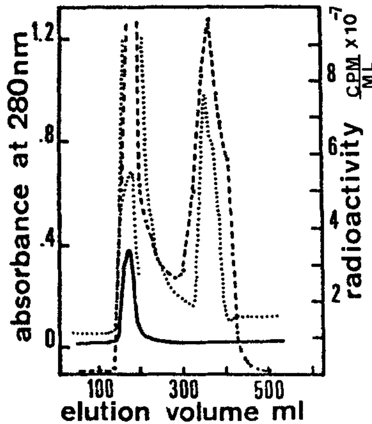

Figure 2

Typical elution pattern of tritiated ribonuclease
(First major component; Sephadex G-25 at 40 °C; symbols as in Fig 1)

b. Enzyme activity

The enzymic activity of each sample was measured after complete removal of labile radioactivity by means of chromatography and repeated lyophilizations. Table II summarizes these results, which are expressed in Kunitz units and also with reference to the original activity in the commercial enzyme. It should be remembered that incubating an enzyme under conditions close to its transition temperature has a denaturing effect which depends upon such factors as incubation time and specific radioactivity of the sample. Clearly, the latter accounts for the significant loss

of activity in the glycosylated component.

Problems arising from self-radiolysis and from storage conditions of the tritiated protein still remain to be investigated.

Exchange duration	5 min	15 min		30 min	
Component	II	I	II	I	II
Specific radioactivity in Curies per mmole, after treatment as follows :					
G-25, 40 °C [a]	2.61	15.32	4.46	11.8	3.47
(recovery) [d]	44 %	29 %	46 %	24 %	30 %
water, 45 °C [b]	2.78	14.35	2.78	7.7	-
water, 45 °C [b]	2.56	10.33	2.58	5.5	-
water, 50 °C [c]	2.55	10.33	2.55	6.3	-

Table I

Specific radioactivity of tritiated ribonuclease after chromatographic purification.

I and II refer respectively to the glycosylated enzyme and to RNase D (16). The samples were treated as follows : (a) molecular sieving on dextran gel Sephadex G-25 at 40 °C; (b) incubation for 24 h in water at pH 5.5 and 45 °C; (c) same as (b), but at 50 °C. Amount of protein, (d), is expressed as percent of total starting material.

Exchange duration	0	5 min	15 min		30 min	
Component	I + II	II	I	II	I	II
Activity :						
Kunitz U.	72	66	23	41.3	27	49
Relative	100 %	92 %	32 %	57 %	38 %	68 %

Table II

Enzymic activity of tritiated ribonuclease after complete elimination of labile radioactivity.

I and II refer respectively to the glycosylated enzyme and to RNase D (16); activity is expressed in Kunitz units per mg, and relative to that of the commercial sample.

c. Specific activity of aminoacids in tritiated ribonuclease

It seemed worthwhile investigating the distribution of radioactivity among aminoacids, to evidence a possible relationship between their specific degree of labelling and their hydrophobic of hydrophilic character.

No correction was made for loss of radioactivity resulting from back-exchange during acid hydrolysis, but it has been shown that this process is significant for only a few aminoacids under the conditions of hydrolysis used here (12). The values for aspartic acid, tyrosine and glutamic acid reported in table III are therefore probably underestimates. Values for isoleucine-leucine, glycine, methionine and histidine appear to be somewhat higher than average.

Taking the above remark into consideration, the present distribution of radioactivity among aminoacids may be considered as rather homogeneous in comparison with that obtained by catalytic labelling of the enzyme in solution (13) or by the free-radical interceptor technique (14). There is thus no evidence towards a correlation between the ease in labelling by the present method and the polar or non-polar character of individual aminoacids.

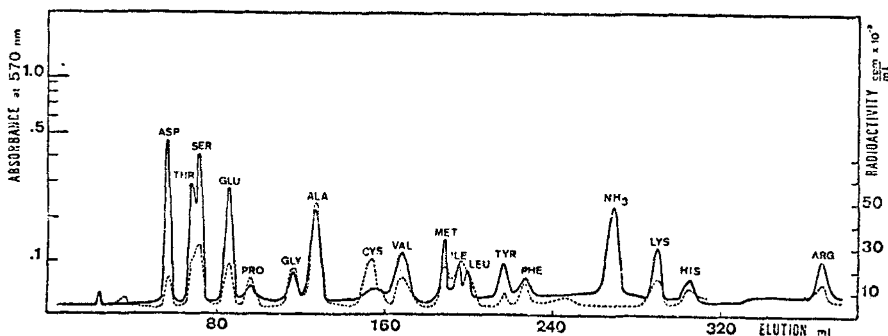

Figure 3

Chromatography of aminoacids from tritiated ribonuclease

———————— : absorbance at 570 nm (ninhydrin reaction)

------------ : radioactivity in cpm per ml of eluate

Component / Amino-acid	Specific activity $(\frac{dpm \cdot 10^{-6}}{micromole})$		N.S.A.[c]	
	I	II	I	II
Aspartic acid	25,2	4,3	2,9	2,5
Thr + ser[a]	29,1	6,5	3,4	3,7
Glutamic acid	31,9	7,0	3,7	4,0
Proline	56,9	10,0	6,6	5,7
Glycine	101,6	19,0	11,9	10,9
Alanine	68,3	12,5	8,0	7,2
Valine	39,6	7,8	4,6	4,5
Methionine	92,7	18,1	10,8	10,4
Ile + leu[b]	109,5	25,8	12,8	14,8
Tyrosine	43,2	14,1	5,0	8,0
Phenylalanine	64,8	14,6	7,6	8,4
Lysine	41,2	7,4	4,8	4,3
Histidine	97,8	18,1	11,4	10,4
Arginine	55,2	9,4	6,4	5,4

TABLE III

Specific activity of aminoacids, from hydrolysates of tritiated ribonuclease.

I and II refer respectively to glycosylated and unglycosylated components. (a) threonine • serine; (b) isoleucine + leucine; (c) Normalized Specific Activity, calculated as described in the text. The reproductibility in measuring aminoacid activities is better than 20 %.

When comparing components I and II of the enzyme, the distribution of radioactivity among aminoacids is practically undistinguishable except for tyrosine. The reason for this difference is not clear.

IV. CONCLUSION

The present results, although preliminary, indicate that isotope exchange with tritium gas, activated by applying high fre-

quency electric discharges under well defined conditions, may be used successfully for labelling biological molecules such as ribonuclease.

The above method yields a fast incorporation of tritium into the enzyme, the final specific radioactivities of purified samples reaching the Curie per mmole level. The biological activity of the enzyme remains however reasonably well preserved provided the exchange time does not exceed some 5 minutes.

Inspection of the crystallographic model of ribonuclease makes it apparent that a majority of polar aminoacids are located on the outer surface of the macromolecule while most hydrophobic side-chains are found inside (15). Our observation of a rather uniform distribution of radioactivity, with a slightly higher level for some hydrophobic residues, suggests that aminoacids located near the surface of the protein molecule are by no means preferentially labelled. Complete lack of influence of molecular conformation on radioactivity localization would have to be tested by means of a comparative study, the same labelling procedure being applied to both native and denatured enzyme.

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