IODINATION OF A MIXTURE OF SOLUBLE PROTEINS BY THE [^{125}I]-LACTOPEROXIDASE TECHNIQUE

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Summary A mixture of 4 purified proteins at equal concentrations was radiolabelled with [^{125}I] and lactoperoxidase and analysed by SDS-polyacrylamide gel electrophoresis. Bovine serum albumin took up 9 times as much label as ovalbumin or lysozyme and 3.3 times as much as α-chymotrypsinogen A. These results suggest that when applying the [^{125}I]- lactoperoxidase technique to labelling unknown mixtures of proteins, such as may exist on the surfaces of cells, caution should be exercised in interpreting the degree of labelling of particular proteins in terms only of surface abundance or accessibility.

Since the introduction (1,2,3) of the lactoperoxidase (LPO)[1] technique for radioiodinating proteins, the method has been applied to labelling surface proteins of erythrocytes (2 - 6), lymphocytes (7,8), tissue culture cells (6, 9 - 11), platelets (12), bacteria (13,14) and viruses (15 - 18). The method has also been used for labelling erythrocyte ghosts (19 - 22) and bacterial protoplasts (23,24). Individual purified proteins vary in the extent to which they become labelled under the same conditions (1,25). However the labelling of a mixture of several purified proteins of known equal concentrations has not been reported. Such a study would have a bearing on interpreting the labelling patterns of the complex mixtures of proteins found on cell surfaces, and which currently are explained mainly in terms of surface abundance and accessibility. This report records the distribution of radioactivity among the components of a mixture of equal concentrations of BSA, Ea, Ch, and Lz labelled by the [^{125}I]-LPO method, with glucose and GO as a source of H_2O_2.

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1 The abbreviations used are : LPO, lactoperoxidase; BSA, bovine serum albumin; Ch, α-chymotrypsinogen A; Ea, ovalbumin; GO, glucose oxidase; Lz, lysozyme; and SDS, sodium dodecyl sulphate.

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METHODS

Proteins and enzymes were all obtained from Sigma. LPO and GO were assayed for enzyme activities as described in (3). Protein concentrations were estimated as in (26) using BSA as standard. Radioiodination of the protein mixture was based on the method of Hubbard and Cohn (3). The complete reaction mixture (1 ml) contained (in 0.1M Na phosphate buffer, pH 7.4) a mixture of 1 mg each (as determined colorimetrically) of BSA, Ea, Ch and Lz, added to 10 milliunits LPO, 10 milliunits GO, 10 μCi carrier-free Na $[^{125}I]$ (Amersham) and 5 μM glucose, in that order. Control mixtures lacking one or more of the reagents were prepared in parallel. Each mixture was incubated for 1 h at 37°, whereupon it was transferred to a sac of Visking tubing and dialysed at 4° over a magnetic stirrer against 5 l of distilled water containing 10^{-5}M $Na_2S_2O_3$. After 1 h the diffusate was replaced by 5 l of distilled water. This was changed twice more before dialysis was considered complete.

After dialysis, the mixture of radioiodinated proteins was adjusted to 2 mg protein per ml and prepared for electrophoresis by adding 0.5 ml to 0.5 ml 0.125 M tris-HCl buffer pH 6.8 containing 4% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 20% (v/v) glycerol and 0.002% (w/v) bromophenol blue. The mixture was heated at 100° for 5 min. Disc gel electrophoresis with a discontinuous SDS buffer system was based on the method of Laemmli (27) as modified by Ames et al (28). Separating and stacking gels contained 11% and 5% (w/v) acrylamide respectively. Both the gels and the electrode compartments contained 0.1% (w/v) SDS. The volume of sample applied to the gels was 40 μl and electrophoresis was performed at 1 mA per gel. Staining and destaining was done by the method of Weber and Osborn (29). Gels were scanned with a Joyce-Loebel Densitometer fitted with a 280 nm interference filter. They were then cut into 1 mm slices with a Mickle gel slicer. $[^{125}I]$ was counted on a Nuclear Enterprises NE 8312 automatic beta gamma spectrometer optimized for $[^{125}I]$ and with a counting efficiency of 50%. Gel slices were swollen in the scintillation vials by adding 1 ml 1N NaOH.

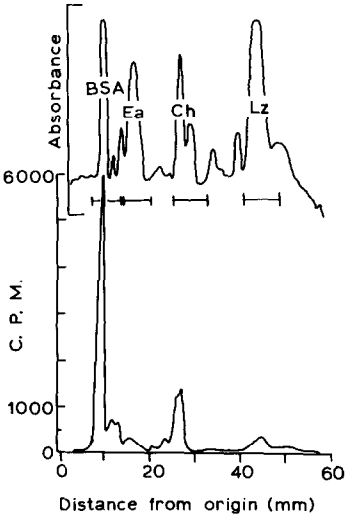
RESULTS

Table 1 records the extent of $[^{125}I]$ -incorporation by the protein mixture in a typical experiment, expressed as a percentage of the isotope added and as c.p.m./μg protein. The complete reaction mixture gave 16% incorporation, a figure which was closely duplicated in two repeat experiments under the same conditions. In a separate experiment, BSA alone at 4 mg per reaction mixture incorporated 46% of the $[^{125}I]$. Omission of LPO from the mixture caused a drastic reduction in labelling which was only slightly reduced still further if GO and glucose were also omitted. However, a system containing LPO, but lacking GO and glucose gave 10% isotope incorporation, i.e. about 60% of the level attained by the complete reaction mixture.

The densitometer scan and radioactivity profile of the mixture after

TABLE I. $[^{125}I]$-incorporation by a mixture of BSA, Ea, Ch and Lz when reacted with the complete labelling mixture of LPO, GO, glucose and Na $[^{125}I]$, and with reagents omitted.

Reagent omitted	Radioactivity (c.p.m./μg protein)	$[^{125}I]$-incorporation (%)
None	470	16
LPO	3	0.1
GO + glucose	284	10
LPO, GO + glucose	2	0.07



Distribution of stained polypeptide bands (densitometer trace, above) and of radioactivity (below) in a SDS-polyacrylamide gel electrophoretogram of a mixture of equal amounts of BSA, Ea, Ch and Lz labelled by the $[^{125}I]$-LPO technique. The four horizontal brackets indicate the points where a gel similar to the one illustrated was sliced so as to obtain broad bands corresponding to each of the proteins in the mixture. Counts on these bands are in Table II.

SDS-polyacrylamide gel electrophoresis are shown in the Figure. The densitometer trace shows that the 4 proteins gave 4 main peaks of about the same height, together with several minor peaks. The latter, in separate

experiments were shown to originate from the BSA and Ch. The lower part of the Figure, where radioactivity is plotted, shows one main peak and three minor peaks. It is clear that BSA became labelled to a relatively much greater extent than the other proteins and also that Ch took up significantly more label than Ea or Lz.

To obtain a quantitative estimate of the amount of label in each protein, a gel similar to the above was cut (as indicated on the Figure) so that each main peak of staining was contained in a single thick slice. The counts from these slices (Table II) show that if the relative amount of label on BSA is taken as 100, then that on Ch is 30 and Ea and Lz both have 11.

When labelling was done with LPO and $[^{125}I]$ without GO and glucose, the distribution of label among the peaks was in the same ratios as those found with the complete system.

DISCUSSION

Although for maximum labelling it was necessary to use LPO together with a source of H_2O_2 (GO + glucose), about 10% of the label was incorporated with $[^{125}I]$ and LPO alone. A similar phenomenon has also been reported in the labelling of mycoplasma membrane proteins (14) where it was suggested that the bacteria themselves might have generated H_2O_2. Marchalonis (1) found 3% $[^{125}I]$ incorporation in human γG when H_2O_2 was omitted, but Hubbard

TABLE II. Radioactivities of individual protein peaks cut from a SDS-polyacrylamide gel as shown in the Figure.

Peak	c.p.m.	c.p.m. relative to BSA = 100
BSA	12,290	100
Ea	1,360	11
Ch	3,632	30
Lz	1,350	11

and Cohn (3) obtained essentially no labelling of washed erythrocytes when either GO or glucose was left out. Insufficient iodinatable substrate may give indirect halogenation via iodine (30) but this should not have been a factor in our experiments.

The percentage incorporation of $[^{125}I]$ by the complete reaction mixture (16 - 18%) is somewhat lower than might have been expected from previous reports in which certain purified proteins were labelled. However Marchalonis (1) found uptakes ranging from 2.5% for human α_2-macroglobulin to 97.3% for human γG and David (25) reported a similar diversity in the labelling of other proteins. In the present investigation, when BSA alone at 4 mg per reaction mixture was subjected to the same labelling procedure as used for the protein mixture, the uptake of $[^{125}I]$ (from 50 μCi) was 46%.

Labelling the 4 proteins as a mixture has shown by direct experiment that proteins may vary substantially in the extent to which they take up $[^{125}I]$ under "competitive" conditions. According to Phillips and Morrison (2), the $[^{125}I]$ is inserted mainly into tyrosine residues, with histidine taking up smaller amounts. The proteins studied here have the following mole percentages of tyrosine and histidine respectively: BSA, 3.36, 3.00 ; Ea, 2.33, 1.8 ; Ch, 1.62, 0.81 ; and Lz, 2.33, 0.78. These values do not correspond closely to the relative ease of labelling, which suggests that the neighbourhood of the tyrosine and histidine residues in the native proteins may exert a strong modifying influence. Fritz (18) in a study of AMV virus proteins concluded that differences in degree of labelling of two proteins of the virus was not due to differences in surface exposure but to one of the proteins being refractory to labelling.

Several papers (3,4,5,9,12,13,24) in which intact cells or membrane preparations were labelled by the $[^{125}I]$-lactoperoxidase technique contain the implicit assumption that the relative radioactivity of the protein peaks in electrophoretograms is an index of the surface abundance or accessibility of the particular protein species. The experiments reported here suggest

that such conclusions should be reached with caution, for in this model system of 4 proteins at equal concentration, BSA behaves as a "major protein" in its response to labelling while Ea and Lz might be characterized as "minor components".

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