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PROTEIN AND CELL MEMBRANE IODINATIONS WITH A SPARINGLY SOLUBLE CHLOROAMIDE, 1,3,4,6-TETRACHLORO-3a,6a-DIPHENYLGLYCOLURIL

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SUMMARY: Films of 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (conveniently "plated" in reaction vessels from methylene chloride solution) react rapidly in the solid phase with aqueous mixtures of I^- and proteins to yield iodinated proteins. Similarly applied, this reagent brings about iodination of cell membranes, although, apparently, at a somewhat lesser rate than in iodinations carried out with lactoperoxidase-glucose oxidase. The stability and sparing solubility of this chloroglycoluril in water can account for the minimal damage to proteins and living cells observed in these iodinations; further, these properties allow for elimination of the reduction step employed at the close of iodinations with soluble chloroamides such as chloramine-T.

Rapid expansion of biological membrane research and growing application of radioimmune assay have brought about a greater awareness of the importance of protein iodination under mild conditions. This communication is concerned with examination of 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (IIa) as a possible solid-phase (and thus potentially less destructive) reagent for iodination of proteins. The experiments described here include iodinations of nine proteins and two cell types with this chloroglycoluril; of especial significance are those in which IIa has been compared with chloramine-T in (a) the efficiency of iodination of rabbit IgG and in (b) the binding of iodinated mouse LPC-1 myeloma protein to rabbit antibodies specific for the idiotypic region of this protein. The results of these and related experiments will show that IIa possesses several of the attributes of an ideal reagent for protein iodination.

MATERIALS AND METHODS

Materials and their sources. Chloramine-T (Eastman Kodak); fetuin and bovine serum albumin (Sigma); chicken lysozyme and human serum albumin (Worthington); M. luteus cells and bovine γ-globulins (Miles Laboratories); keyhole lim-

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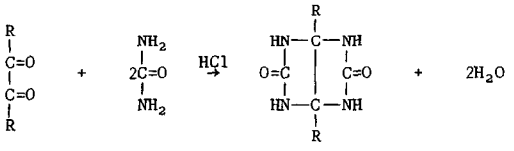
pet hemocyanin (Calbiochem); carrier-free $^{125}I^-$ in 0.1 M NaOH (Amersham/Searle); N-chlorosuccinimide (Aldrich); BALB/c mice (Charles River Breeding Laboratories); mouse plasmacytoma tumors, LPC-1, MOPC-21, and MOPC-300 were gifts from Professor Alfred Nisonoff.

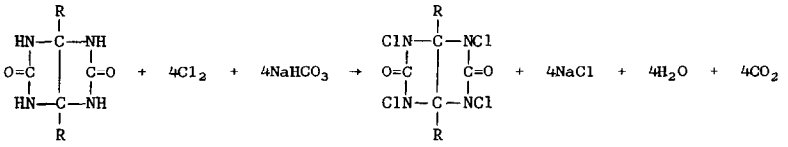
General methods. A Beckman Biogamma counter (efficiency, 65%) was used for radioactivity measurements. Protein concentrations were determined either by measuring A_{280}, which had been related to dry weight, or by the method of Lowry et al. (1). Complement activity in all sera was destroyed by heating at 56° for 10 min. Positive chlorine of the chloroamides was determined by titration of I_2, formed on reaction with excess I^-, with thiosulfate in the presence of acetic acid; samples of IIa and IIb were dissolved first in chloroform.

Preparation of IIa[1] and IIb. These substances and their immediate precursors, Ia and Ib, were prepared via the reactions indicated in Scheme 1 by slight modification[2] of procedures developed a number of years ago at the Naval Research Laboratory (2, 3).



Ia, Ib

Scheme 1



IIa, IIb

Ia, IIa, R = phenyl

Ib, IIb, R = methyl

Solubility determinations.[3] To a 5-ml aliquot of filtrate from the mixture of water and either IIa or IIb were added 0.2 g of KI and 0.2 ml of 1 M H_2SO_4; the absorbance at 466 nm, an isosbestic point for I_2 and I_3^-, was measured after dilution to 10 ml. Alternatively, 1.66 g of KI was added in order to convert virtually all I_2 to I_3^-; A_{353} then was determined after dilution. Concentrations were calculated from extinction values of Autrey and Connick (4).

Preparation of immunoglobulins. IgG fractions were prepared from rabbit and mouse sera by the methods of Fraker et al. (5).

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[1]This substance now is available from Pierce Chemical Co., Rockford, Ill.

[2]Details of these procedures will be furnished by the authors on request.

[3]Hydrolysis of the chloroamides with formation of hypochlorite is a possible source of error in these solubility measurements, inasmuch as they depend on oxidation of I^- to I_2; hypochlorite was not detected in vacuum distillates of the saturated solutions.

Isotonic buffers. Borate-saline buffer, pH 8.2, was prepared by mixing 5 volumes of 0.125 M sodium borate buffer, which was 0.075 M in NaCl, with 95 volumes of 0.145 M NaCl. Phosphate-saline buffers were prepared by mixing 1 volume of the 0.1 M sodium phosphate buffer with 9 volumes of 0.145 M NaCl.

Rabbit IgG iodination with IIa. A 20-µl aliquot of a methylene chloride solution[4] containing 0.4 µg of IIa (9.2×10^{-10} mole) was placed in a 10 × 75-mm test tube, and the methylene chloride was evaporated by rotating the tube in a 37° bath so that a thin film of IIa formed in the bottom.[5] To another test tube were added (all in pH 8.2 borate-saline buffer) 100 µg (6.7×10^{-10} mole) of rabbit IgG, 0.11 µg (6.6×10^{-10} mole) of KI, and 14 µCi (6.4×10^{-12} mole) of $^{125}I^-$; the final volume of this mixture was adjusted to 100 µl with borate-saline buffer. After bringing the tubes to 0-2°, reaction was initiated by transferring the IgG solution to the tube containing IIa. The reaction was allowed to proceed for 5 min at 0-2° with gentle stirring; it was terminated by decanting the mixture from the residual glycoluril. For determining the efficiency of iodination, 5-µl aliquots of the reaction mixture were mixed with 0.1 mg of bovine serum albumin, in 10 µl of borate-saline, and then with 1 ml of cold 10% trichloroacetic acid. After standing for 30 min at 0-2°, these mixtures were centrifuged. The pellets were mixed with 1 ml of fresh 10% trichloroacetic acid, and the mixtures were centrifuged again. The protein precipitates then were dissolved in 1 ml of 0.1 M NaOH, and 100-µl aliquots were counted. All determinations were carried out in triplicate.

Iodination of rabbit IgG with chloramine-T. The conditions differed from those in iodination with IIa only in substitution of chloramine-T for IIa and in termination of the reaction by reduction with bisulfite. Typically, the reaction was initiated by addition of 2 µg (7.1×10^{-9} mole) of chloramine-T in 2 µl of water solution; excess chloramine-T was reduced at the end of the reaction by addition of 2 µl of 0.1% sodium metabisulfite in water.

Preparation of anti-idiotypic antibody to LPC-1 myeloma protein. The purified LPC-1 IgG (2 mg in 1 ml of normal saline) was injected intravenously into a New Zealand White rabbit every 2 weeks for a period of 3 months. The antiserum was rendered specific for the idiotypic region of the LPC-1 IgG by absorbing it six times with normal BALB/c IgG, once with MOPC-21 IgG, and once with MOPC-300 IgG; each of these mouse IgG's had been attached to Sepharose 4B beads by cyanogen bromide activation (6). The specificity of the treated serum for the idiotypic region of LPC-1 IgG was demonstrated by the inability of 100 µg of normal BALB/c IgG's, MOPC-21 IgG, or MOPC-300 IgG to inhibit precipitation of 0.01 µg of iodinated LPC-1 IgG by anti-idiotypic antibody at the midpoint of the binding curve (net precipitation of 59% of ligand).

Indirect radioimmune assay for determination of binding of anti-idiotypic antibody to LPC-1 IgG iodinated with either chloramine-T or IIa. Iodinations of the LPC-1 IgG were carried out as described above for rabbit IgG. The iodinated protein (0.01 µg in 0.2 ml of borate-saline) and 10 µl of normal rabbit serum (carrier) were added to a series of reaction tubes. Graded amounts of the purified anti-idiotypic rabbit serum in borate-saline were mixed with these

[4]Chloroform may be used as the solvent for IIa, but its use for this purpose constitutes a health hazard; further, chloroform solutions of IIa gradually decompose, in either the light or the dark, with formation of Ia, Cl_2, and phosgene. Methylene chloride solutions of IIa decompose similarly in the light but are stable for weeks when stored in the dark.

[5]It has been routine to coat a number of reaction tubes with the chloroglycoluril at one time, and to store them for future use in a desiccator over Drierite; under these storage conditions the chloroglycoluril films have undergone no apparent change over a period of several weeks.

solutions, and the mixtures were incubated for 1 hr at 37°. A slight excess of goat anti-rabbit IgG serum, which had been absorbed with normal BALB/c IgG's, was mixed with the contents of each tube. The mixtures were incubated again at 37° for 1 hr; after this they were stored at 4° overnight. Precipitated material was separated by centrifuging. The pellet was washed twice with borate-saline, centrifuging each time; it then was dissolved in 0.1 M NaOH and counted. Also counted was the solution from combination of the supernatent fractions.

Iodination of sheep erythrocytes with IIa. In typical runs 50-μg films of IIa were "plated" in 2-ml beakers. The other components of the reaction mixtures were 4×10^7 cells and 2.1×10^{-11} mole of carrier-free $^{125}I^-$ in pH 6.8 phosphate-saline buffer. The volume of these reaction mixtures was 300 μl. The iodinations were allowed to proceed for 10 min at 0-2° with gentle stirring. The mixture then was transferred to 2 ml of cold, isotonic phosphate-saline and centrifuged at 1,000 × g for 5 min. After washing the cells twice with 2 ml of cold phosphate-saline, they were resuspended in 2 ml of phosphate-saline containing 100 μg of KI and allowed to stand in this solution for 20 min in order to displace any $^{125}I^-$ present. The cells were taken up in 2.5 ml of 0.01 M, pH 7.4 Tris buffer for lysis. The crude membrane was separated from the cytosol by centrifuging at 18,000 × g for 1.5 hr.

Iodination of LPC-1 tumor cells with IIa. Conditions were similar to those for iodination of sheep erythrocytes except that no $^{125}I^-$ was present. The reaction mixture consisted of 4×10^6 cells, 50 μg of IIa, 6.6 μg (4.0×10^{-8} mole) of KI, and pH 7.4 phosphate-saline to make the volume 400 μl. The reaction time was 15 min. The reaction was terminated by transferring the mixture to 2 ml of the phosphate-saline and centrifuging at 1,000 × g. After washing twice with 2 ml of phosphate-saline, the cells were examined for uptake of trypan blue when suspended for 5 min in a 0.2% solution of this dye in isotonic saline. In a further test for viability the cells were diluted with the isotonic phosphate-saline to a concentration of 60,000 per ml, and 12 female BALB/c mice each was injected with 0.1 ml of this suspension. As a control, another group of 12 female BALB/c mice each was injected with this number (6,000) of non-iodinated LPC-1 cells. After 1 month the mice were examined for tumors.

Determination of the effect of IIa, IIb, chloramine-T, and N-chlorosuccinimide on chicken lysozyme activity. In the experiments with IIa and IIb an amount of chloroglycoluril equivalent to 10^{-5} mole of positive chlorine was "plated" in the bottom of a 10-ml beaker. After cooling to 0-2°, 1 ml of 10^{-4} M lysozyme in 0.033 M, pH 7.0 sodium phosphate buffer was added to the beaker, and the mixture was stirred gently at 0-2°. The effect of chloramine-T was determined by addition of 50 μl of freshly prepared 0.2 M chloramine-T in water to 1 ml of the buffered lysozyme solution cooled to 0-2°; that of N-chlorosuccinimide was tested in the same way except that the chloroamide concentration was 0.1 M and the volume of this solution added to the lysozyme was 100 μl. Lysozyme activities were measured by mixing 10 μl of these solutions with 3 ml of a suspension (0.13 mg per ml) of dried M. luteus cells in 0.033 M, pH 7.0 sodium phosphate buffer at 23.0°; A_{450} of this mixture then was recorded for 2 min while maintaining the temperature at 23.0°.

RESULTS AND DISCUSSION

Figure 1 presents a comparison of rabbit IgG iodinations with IIa and with chloramine-T. These data show that it is indeed possible to carry out protein iodinations with a virtually water-insoluble chloroamide. These results reveal also that the reagent of choice, IIa, is more efficient in the iodination of

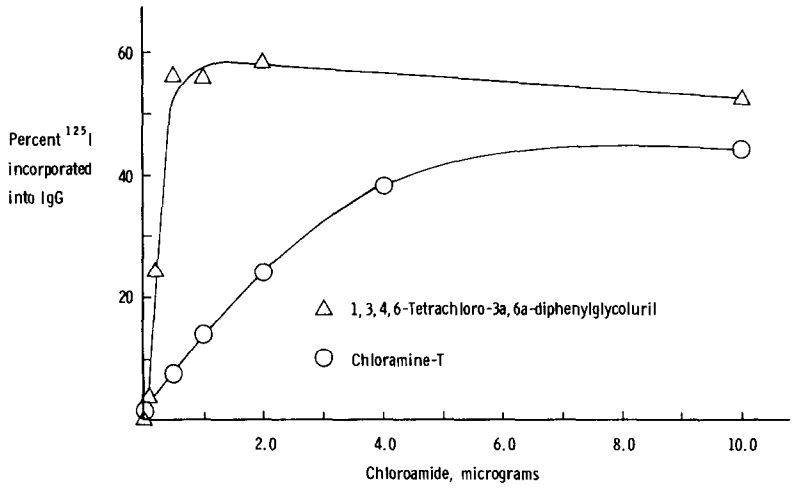

Figure 1. Iodination of rabbit IgG with IIa and with chloramine-T. These iodinations were carried out as described in Methods, except that the amount of chloroamide was varied.

this protein than is chloramine-T: for maximum iodination with IIa the molar ratio of positive chlorine to protein was 5.5; with chloramine-T as the iodinating reagent this ratio was about 37. This difference between IIa and chloramine-T in iodination efficiency may derive from a difference in the rate of reaction of I^- with the chloroamides. A lower rate with chloramine-T can be explained by the necessity for interaction of I^- in these reactions with the neutral (conjugate acid) form of chloramine-T which, because the pK_A for chloramine-T is about 4.6, is in very low concentration around pH 7.

Shown in Table 1 is the effect of carrier I^- in iodinations of rabbit IgG with IIa, an as yet incompletely understood phenomenon that has been observed prior to the present investigation in iodinations of erythrocytes with lactoperoxidase-glucose oxidase (7). Data for iodination of several proteins with IIa appear in Table 2.

Figure 2 shows the difference in binding between mouse LPC-1 IgG, iodinated with either IIa or chloramine-T, and rabbit antibodies specific for the idiotypic region of the mouse protein. This difference in binding indicates

TABLE 1

Effect of Carrier I^- on the Incorporation of ^{125}I into Rabbit IgG[a]

Molar ratio of $^{127}I^-$ to $^{125}I^-$	^{125}I incorporated, %
0	27
50	50
100	55
300	41
600	27

[a]The procedure was that described in Methods, except that the concentration of $^{127}I^-$ was varied.

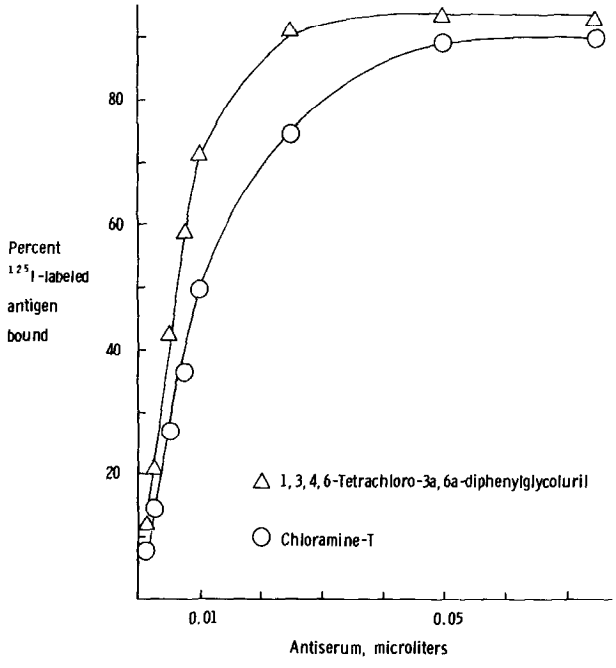

Figure 2. Binding of mouse LPC-1 IgG, iodinated with either IIa or chloramine-T, to rabbit antibodies specific for the idiotypic region of the LPC-1 protein.

TABLE 2

Iodination[a] of Various Proteins with IIa

Protein	^{125}I incorporated, %
Bovine IgG	53
Mouse LPC-1 IgG	53
Mouse MOPC-21 IgG	53
Keyhole limpet hemocyanin	32
Fetuin	27
Chicken lysozyme	57
Bovine serum albumin	16
Human serum albumin	12

[a]The procedure was that described in Methods for rabbit IgG.

that iodination with IIa alters the LPC-1 protein's structure in the idiotypic region to a lesser extent than does iodination with chloramine-T. The dramatic differences in diminution of chicken lysozyme enzymatic activity that were observed on exposure of this protein to chloroamides of differing solubility at relatively high protein concentration and high ratio of positive chlorine to protein, shown in Table 3, provide further evidence for the destructiveness of soluble chloroamides to proteins.

The following results were obtained in iodinations of living cells with IIa: In iodinations of sheep erythrocytes, typical incorporation of ^{125}I by the ghosts was 0.43%; uptake by the cytosol protein was 0.04%. Addition of carrier I^- did not augment ^{125}I incorporation under the conditions of these experiments. The efficiency of these iodinations was less than that reported by Hubbard and Cohn (8) for human erythrocyte iodination with lactoperoxidase-glucose oxidase, although it was of the same order of magnitude for the same reaction time; the ratio of ghost to cytosol iodination was nearly the same as

TABLE 3

Effect of Various Chloroamides on the Enzymatic Activity of Chicken Lysozyme

	Solubility, moles per l		Activity remaining, %	
	Saturation	After 5 min		
Chloroamide	at 25°	at 0-2°	After 15 min	After 30 min
IIa	8×10^{-6}	6×10^{-7}	96	92
IIb	7.6×10^{-5}	4.3×10^{-6}	56	12
Chloramine-T	Soluble		[a]	
N-Chlorosuccinimide	Soluble		[a]	

[a]The enzyme precipitated in 1 min; no activity remained.

that Hubbard and Cohn observed when Cl^- was present in their reaction mixtures. No change in the morphology of the sheep erythrocytes was observed as having occurred during these reactions, as determined by examination under the light microscope. Similarly, the LPC-1 tumor cells appeared to remain viable to a large degree when carried through the iodination procedure with IIa and $^{127}I^-$. Thus in this instance the proportion of viable iodinated cells, as measured by uptake of trypan blue, was 97%; from the results of the experiments involving tumor development in BALB/c mice the viability of the iodinated LPC-1 cells was greater than 80%—at the end of 1 month 9 of the 12 animals injected with iodinated LPC-1 cells displayed large tumors and were near death, whereas the number of animals in the control group having large tumors was 10. The results of these latter experiments constitute further evidence for the mildness of IIa as a reagent for protein and living cell iodinations.

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