BBA 75746

REACTION OF HUMAN SERUM ALBUMIN AND HUMAN ERYTHROCYTES WITH TRITIATED 2,4,6-TRINITROBENZENESULFONIC ACID AND TRITIATED PICRYL CHLORIDE

JOHN J. ARROTTI AND JAMES E. GARVIN

Department of Biochemistry, Northwestern University Medical School, Chicago, Ill. (U.S.A.)

(Received May 27th, 1971)

SUMMARY

1. Syntheses for tritiated 2,4,6-trinitrobenzenesulfonic acid (TNBS) and [^{3}H]-picryl chloride (PC) are described.

2. The time course of the reaction of [^{3}H]TNBS and [^{3}H]PC with human serum albumin was studied over a 4-h interval. At 30 min both reagents had reacted to the extent of 11.3 moles per mole of albumin. At 4 h approximately 17 moles of TNBS and approximately 33 moles of PC had reacted per mole of serum albumin.

3. The time course of the reactions of [^{3}H]TNBS and [^{3}H]PC with normal human erythrocyte suspensions in phosphate buffer were studied. Distribution of label in the medium, in the cell membrane, and in the hemolysate hemoglobin was examined as a function of time for each reagent. The dynamic labeling system which emerges from this analysis discloses a source of reagent outside the membrane, reaction of reagent with the membrane while in transit, and exhaustion of free reagent by the hemoglobin sink inside the erythrocytes. The [^{3}H]PC was found to enter the erythrocytes and label their membranes much more rapidly but the specific activity in the membranes was only about half of that achieved with the [^{3}H]TNBS.

INTRODUCTION

The use of site-specific modifying reagents, so fruitfully applied in the studies of protein structure, has recently been extended to the more complex architecture of biological membranes. In the pioneering work of MADDY[1] a non-penetrating fluorescent probe was used to monitor the amino, histidyl, and guanidyl groups accessible from the outside of the ox erythrocyte and BERG[2] has taken a similar approach using another non-penetrating reagent—^{35}S-labeled sulfanilic acid diazonium salt—in studies of the human erythrocyte. NIEHAUS AND WOLD[3] have cross-linked erythrocyte membrane proteins through their lysine residues with dimethyl adipimate before carrying out solubilization studies. MCCONNEL[4] has employed electron spin resonance to assess the environment of spin-labeled probes with a membrane. LENARD[5] has labeled sulfhydryl groups in human erythrocyte membranes with *N*-[^{14}C]ethylmaleimide. More

Abbreviations: TNBS, 2,4,6-trinitrobenzenesulfonic acid; PC, picryl chloride; DNCB, dinitrochlorobenzene; TNCB, trinitrochlorobenzene; ε-TNP-lysine, ε-trinitrophenyllysine.

recently PHILLIPS[6] has labeled tyrosine and histidine residues accessible from outside the erythrocyte membrane with ^{125}I by the elegant expedient of using the non-penetrating enzyme, lactoperoxidase, in the labeling step.

A central feature of the current membrane models has been the emphasis on a mosaic structure[7–10]. A heterogeneous array of lipids and proteins interacting differently at various loci is visualized as being primarily segregated into lipid and protein sectors extending through the thickness of the membrane. Transport as well as physicochemical studies are consistent with such a structure[11]. We undertook to examine this general model by employing a pair of reagents of roughly similar reaction specificities but differing widely in polarity. Picryl chloride and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were chosen for this comparison since they share many molecular characteristics including size and shape and both reagents form identical trinitrophenyl derivatives as reaction products. Both reagents react primarily with sulfhydryl groups and primary amines. The two reagents differ widely in polarity: at neutral pH TNBS bears a single formal charge and is highly water soluble, while PC carries no formal charge and possesses a low water solubility. Two additional advantages of these reagents are their ability to react rapidly under physiological conditions of temperature, pH, and concentration and their inability to significantly disrupt the erythrocyte membrane as determined by measurements of hemolysis. Radioactive monitoring proved to be a convenient and precise method of locating and quantitating the reaction products and therefore tritiated TNBS and PC were synthesized.

Early in this study we observed that large amounts of label were recovered from the intracellular hemoglobin as well as from the suspending medium and that the free reagent in the medium was rapidly exhausted. It soon became clear that a knowledge of the distribution of label as a function of time in the entire system—medium, membrane, and hemoglobin—was essential before an interpretation of the labeling pattern in the membrane could be undertaken. Such an analysis is the subject of this paper.

MATERIALS AND METHODS

Synthesis of [^3H]TNBS and [^3H]PC

[^{3}H]TNBS was synthesized using a combination of modified methods from FRANKLAND[12] and SATAKE *et al.*[13]. [^{3}H]Dinitrochlorobenzene (DNCB) was obtained from New England Nuclear at a specific activity of 87 mC/mmole. To 11.6 mg of this [^{3}H]DNCB was added 239 mg of unlabelled DNCB (Eastman), 0.82 ml of concentrated H_2SO_4 and 0.12 ml of red fuming HNO_3 (J. T. Baker). This mixture was refluxed for 16 h at 130°, and then 20 ml of cold distilled water was added to the flask. The product (PC), separated as a solid or oil which later solidified. The water was drawn off and the pale yellow product washed with 5 ml of cold water which was again drawn off. The last traces of water in the reaction flask were evacuated under vacuum. Any precipitate in the water wash was separated, dried, and added to the reaction flask.

To the dry product was added 1.5 ml of anhydrous methanol, followed by 113 mg of $NaHSO_3$. This solution was refluxed for 50 min at 80° then an additional 113 mg of $NaHSO_3$ was added and the reaction allowed to proceed for an additional 90 min. The solution was acidifed with concentrated HCl and 5 ml of methanol was added.

The mixture was evaporated to dryness under vacuum and the precipitate then redissolved in hot concentrated HCl. The precipitate that formed immediately upon cooling was a mixture of inorganic salts and was discarded. The TNBS crystallized after 2 h in the cold. The white crystals were separated, washed with heptane, and stored at 4°.

In the synthesis of PC, the first part of the TNBS synthesis was followed employing the following amounts of reagents: 4.64 mg of [^{3}H]DNCB, 195 mg of unlabeled DNCB, 1.3 ml of concentrated H_2SO_4, and 0.2 ml of red fuming HNO_3. After the reaction the solution was transferred into 25 ml of cold water. The PC which precipitated was separated, recrystallized from ethanol and stored over $CaCl_2$ at 4°.

Incubation of human serum albumin with [^{3}H]TNBS and [^{3}H]PC

Crystallized human serum albumin (Pentex) at $1.0 \cdot 10^{-6}$ M was incubated with the reagents for various lengths of time at 37°. The reactions were stopped by precipitation of the protein by addition of 0.5 ml of 50% trichloroacetic acid. The precipitates were washed twice with 5% trichloroacetic acid, placed on filter paper and washed again, dried, oxidized, and counted. All dried precipitates were oxidized to CO_2 and H_2O in a Packard Model 300 Tritium Sample Oxidizer and counted in a Beckman LS-250 liquid scintillation spectrometer. A preliminary determination of the modified amino acid residues in the human serum albumin was performed on the 4-h incubation product of each reagent as follows: The thoroughly washed precipitate was hydrolyzed at 110° in 5.7 M HCl for 12 h. The products of the hydrolysis were separated by descending chromatography on Whatman No. 3 paper using the system butanol–acetic acid–water (4:1:2, v/v/v). Picric acid (Eastman), ε-trinitrophenyllysine (ε-TNP-lysine) (Nutritional Biochemicals) and TNBS (Pierce) were also run as standards. The chromatograms were cut into segments, oxidized and counted.

Incubation of human erythrocytes with [^{3}H]TNBS and [^{3}H]PC

A flow sheet of the procedure described below is given in Fig. 1. Blood was drawn from healthy donors without regard to type, using 0.11 M EDTA as anticoagulant. After removal of the plasma and buffy coat the cells were washed 4 times with modified Krebs–Ringer phosphate (Krebs–Ringer phosphate containing 5 mM glucose, 0.7 mM Mg^{2+}, and 1.25 mM Ca^{2+} (pH 7.4)). Following the final wash, the cells were centrifuged at 800 × *g* for 30 min to obtain the red cell pack. After incubation of the cells with the appropriate reagent for a given time the incubation tubes were centrifuged at 2000 × *g* for one min and a 2.0 ml aliquot of supernatant was immediately removed and a 0.2 ml aliquot of this supernatant was obtained and saved. To the remaining 1.8 ml was added 1.0 ml of 50% trichloroacetic acid. The resulting precipitate was centrifuged and discarded while the supernatant was saved. A 1.5-ml portion of this supernatant was brought to pH 7.5, added to 1.0 ml of a human serum albumin solution (10 mg/ml) in modified Krebs–Ringer phosphate buffer and incubated at 37° for 4 h. The protein was precipitated with 1.0 ml of 50% trichloroacetic acid, centrifuged, washed twice with 5% trichloroacetic acid, filtered and dried. Oxidation and counting were carried out as described above.

The labeled cells were washed three times, lysed with 15 mM Tris (pH 7.5) in a ratio of packed cells to Tris of 1/40, v/v, and centrifuged at 25000 × *g* for 30 min. This procedure was repeated three times with centrifugation for 15 min. A 0.5-ml

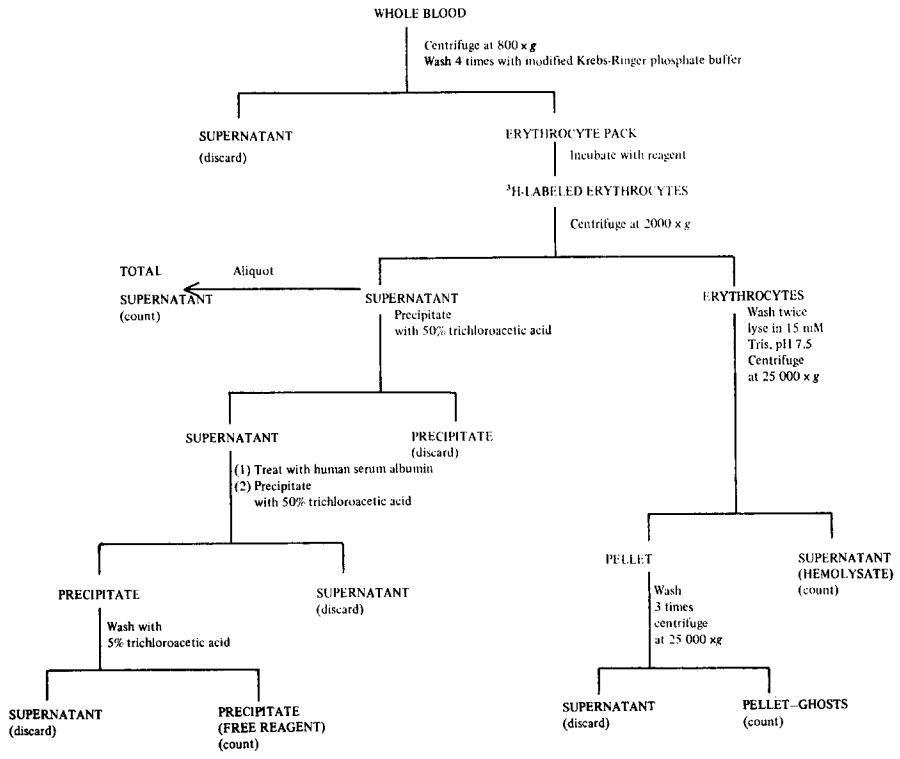

Fig. 1. Flow diagram for the preparation of radioactive fractions. (See text for details.)

portion of packed cells per tube were employed in the incubations and ghost preparations. The ghost suspension was adjusted to 1.5 ml and an aliquot of 0.5 ml was oxidized and counted as described above. Another aliquot of 0.1 ml was taken for protein determination by the method of LOWRY[14]. Hemoglobin was determined by the pyridine hemochromogen method of REMMINGTON[15] as modified by DODGE *et al.*[16]. The scintillation mixture consisted of 100 g of naphthalene (J. T. Baker), 5 g of 2,5-diphenyloxazole (New England Nuclear), 0.3 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (New England Nuclear), 730 ml of dioxane (J. T. Baker), 135 ml of toluene (Fisher) and 35 ml of methanol (Fisher).

RESULTS

Synthesis and characterization

In a typical synthesis, [^{3}H]PC was obtained in a yield of 50% with a specific activity of 2.1 mC/mmole. A melting range of 80–82° was obtained for the synthetic product which compares closely with the value of 80.6° reported by FRANKLAND[12]. A mixed melting point using a commercial preparation of PC (Aldrich) gave an identical range.

[^{3}H]TNBS was obtained in an overall yield of 64% and a specific activity of 4.2 mC/mmole. The melting range of 161–163° agreed with those obtained for two

commercial preparations (Pierce and Nutritional Biochemicals) but not with the value of 180° reported by SATAKE[13]. Additional characterization of the TNBS was carried out as described below. The derivatives, picramide and trinitrophenylglycine, were found to have melting points at 188 and 162° respectively, and R_F values of 0.78 and 0.44 respectively in the system butanol saturated with 5 % ammonia, all of which agrees with the values reported by OKUYAMA[17]. In addition, synthetic TNBS itself was found to have R_F values in two systems—butanol–acetic acid–water (4:1:2, v/v/v), R_F = 0.48; 1.5 M phosphate buffer, pH 5.4, R_F = 0.48—which agree exactly with those reported by OKUYAMA[17]. In a thin layer chromatographic system which we developed for the separation of mono-, di- and trinitrobenzenesulfonic acids as well as picryl chloride, most (96 %) of the radioactivity of the product was recovered in the TNBS spot. This system employed Silica gel G (Merck) and a solvent consisting of benzene–ethyl acetate–formic acid (40:45:15, v/v/v). The R_F values obtained were as follows: nitrobenzenesulfonic acid, 0.15; dinitrobenzenesulfonic acid, 0.19; TNBS, 0.28; and PC, 0.95. The averages of the duplicate elemental analyses of the TNBS were as follows: C, 23.4; H, 1.65; N, 13.0; O, 49.8; S, 9.4 %. $C_6H_3N_3O_9 \cdot H_2O$ requires C, 23.1; H, 1.61; N, 13.5; O, 51.5; S, 10.05 %.

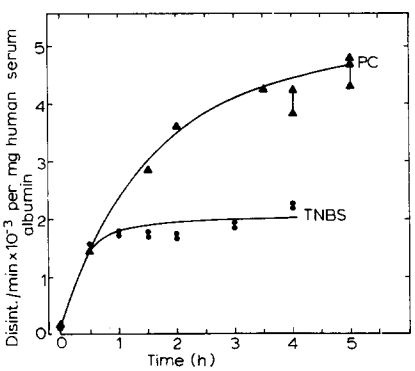

Fig. 2. Time course of the incorporation of [^{3}H]TNBS and [^{3}H]PC into human serum albumin. 1.0 ml of human serum albumin (0.2 mg/ml, $0.3 \cdot 10^{-5}$ mM) in modified Krebs–Ringer phosphate buffer were incubated with either 2.0 ml of [^{3}H]TNBS (31 μg/ml; 0.1 mM, 2.06 mC/mmole) or 2.0 ml of [^{3}H]PC (25 μg/ml, 0.1 mM; 2.06 mC/mmole) in modified Krebs–Ringer phosphate buffer. Both reactions were carried out at 37° and pH 7.4. Each point represents one experiment.

Reaction with human serum albumin

Fig. 2 shows the time course of the incorporation of each reagent when incubated separately with human serum albumin. Using the specific activity of 2.06 mC/mmole it can be calculated that at 30 min about 11.3 moles of each reagent have reacted, whereas after 4 h, 16.8 moles of TNBS and 33.2 moles of PC have reacted per mole of human serum albumin (mol. wt., 69000). Both curves display two main regions—an initial rapid phase followed by a slower but extended phase. Table I shows that pretreatment of human serum albumin with PC for 4 h reduced the moles of TNBS reacting per mole of human serum albumin from 18.4 to 3.8. The chromatographic separations of the human serum albumin hydrolysis products showed that 80 % of the applied radioactivity with both reagents appeared at the ε-TNP-lysine spot with a small amount of radioactivity at the picric acid position and almost none at either the PC

TABLE I

EFFECT OF PRETREATMENT WITH PC ON REACTIVITY OF HUMAN SERUM ALBUMIN WITH [^{3}H]TNBS

The value given for each group is the mean of 5 experiments ± S.E. All reactions were performed at 37° and pH 7.4. Group A: 1.0 ml of human serum albumin (0.2 mg/ml) in modified Krebs–Ringer phosphate buffer was incubated for 4 h with 2.0 ml of modified Krebs–Ringer phosphate buffer followed by incubation with 1.0 ml of [^{3}H]TNBS (0.5 mg/ml, 2.06 mC/mmole) for 5 h. Group B: 1.0 ml of human serum albumin (0.2 mg/ml) in modified Krebs–Ringer phosphate buffer was incubated for 4 h with 2.0 ml of unlabeled PC (50 μg/ml) followed by incubation with [^{3}H]TNBS as in Group A.

	Group A	*Group B*
Disint./min × 10^{-3}	244 ± 13	50 ± 5
Moles TNBS reacted/mole human serum albumin	18.4 ± 0.98	3.8 ± 0.38

or TNBS spots. In addition, parallel chromatograms of hydrolysates of TNBS-treated human serum albumin and PC-treated human serum albumin showed an identical distribution of radioactivity.

Reaction with intact human erythrocytes

Fig. 3 shows the amount of label found in the various fractions as a function of time during the reaction of the intact human erythrocytes with [^{3}H]TNBS. At 60 min the recovery of label in the fractions isolated averaged 69 %. The label measured in the medium (free label and total activity of supernatant) dropped rapidly as the

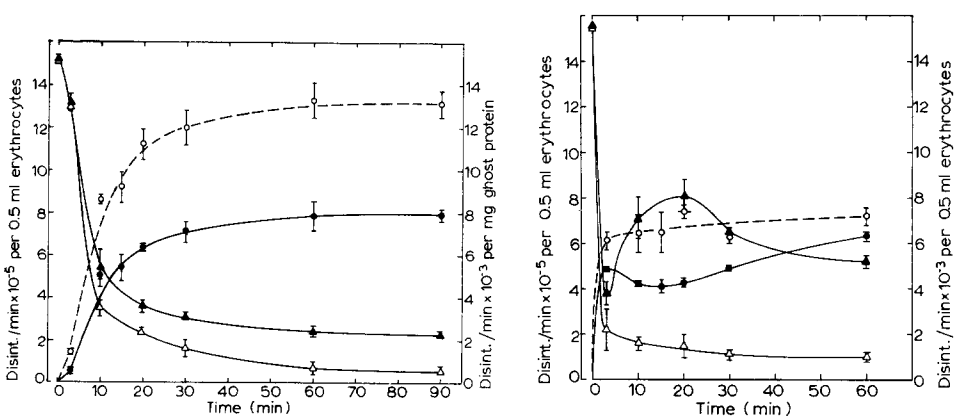

Fig. 3. Radioactivity of various fractions from erythrocyte incubation system as a function of time during treatment with [^{3}H]TNBS. 0.5 ml of packed erythrocytes was incubated with 4.0 ml of [^{3}H]TNBS (26.4 μg/ml. 0.085 mM, 2.02 mC/mmole) in modified Krebs–Ringer phosphate buffer at 37° and pH 7.4. Tubes were swirled gently during the incubation. Each tube contained 1.5 mg ghost protein. Most points represent the mean of 4 determinations ± S.E. ○—○, ghosts; ●—●, hemoglobin; ▲—▲, total supernatant; △—△, free reagent.

Fig. 4. Radioactivity of various fractions from erythrocyte incubation system as a function of time during treatment with [^{3}H]PC. 0.5 ml of packed erythrocytes were incubated with 4.0 ml of [^{3}H]PC (21.5 μg/ml, 0.087 mM, 2.02 mC/mmole) in modified Krebs–Ringer phosphate buffer at 37° and pH 7.4. Tubes were swirled gently during the incubation. Each tube contained 1.5 mg of ghost protein. Most points represent the mean of 4 determinations ± S.E. ○—○, ghosts; ●—●, hemoglobin; ▲—▲, total supernatant; △—△, free reagent.

reaction proceeded while there was a reciprocal rise in the activity found in the ghosts and hemoglobin. The contour of the activity curves for the hemoglobin and ghosts are similar but it should be noted that at 60 min the absolute activity in the ghosts was only about 2.5% of that of the hemoglobin fraction.

Fig. 4 displays a similar set of curves but derived from experiments involving PC. At 60 min the recovery of this label in the fractions isolated averaged 74%. The curves for the ghosts and free reagent resemble those of Fig. 3 except that changes early in the process occur much more rapidly. Thus 85% of the free PC was exhaused in 2–3 min after the zero time, whereas the comparable drop in free TNBS was not reached until 15 min. The total supernatant and hemoglobin curves also show very rapid early changes in label content but differ in contour from the homologous fractions during the TNBS reaction. The total supernatant activity is peculiar in that it falls sharply at two min, rises at 10–20 min, and then falls again gradually. The hemoglobin activity curve is likewise unusual, showing an initial rapid increase followed by a small drop and then a gradual rise. For the experiments shown in Fig. 3, the total activity found in the ghosts in each experimental tube was 1.5 times the numerator of the specific activity. Hence at 60 min the ghosts contained about 1.7% of the activity found in the hemoglobin. The incomplete recoveries of label from the experiments summarized in Figs. 3 and 4 were probably due to losses in the many manipulations of the erythrocytes and to uncounted derivatized small molecules in the hemolysates not precipitable with the hemoglobin.

In agreement with DODGE *et al.*[15] varying amounts of hemoglobin averaging about 6% were found in our ghost preparations. (See Table III). A representative value for the hemoglobin specific activity in a TNBS experiment at 60 min would be $5 \cdot 10^3$ disint./min per mg. This is clearly less than the $13 \cdot 10^3$ disint./min per mg of ghost protein given for a comparable time in the same experiment. However, the possibility that a thin shell of high specific activity hemoglobin might be adherent to the ghosts, made it necessary to determine if the amount of hemoglobin in the ghosts affected their specific activity. We proceeded in two ways: (1) by comparing ghost activity and hemoglobin contamination in duplicate tubes and, (2) by comparing ghost activity and hemoglobin contamination in two experiments differing in the number of times the ghosts were washed. The results of the first method of examination are given in Table II. The second column gives the ratios of the per cent hemo-

TABLE II

COMPARISON OF GHOST SPECIFIC ACTIVITY BETWEEN DUPLICATE TUBES IN THE SAME EXPERIMENT WITH DIFFERING AMOUNTS OF HEMOGLOBIN ASSOCIATED WITH THE GHOSTS

% Hemoglobin is defined as mg hemoglobin/mg of total ghost protein × 100 and the ghost specific activity is expressed as disint./min × 10^{-3} per mg of ghost protein. Tubes A and B were duplicates in a single experiment. Hemoglobin and ghost specific activities were measured as described in the text on aliquots of the ghost suspensions included in the data of Figs. 3 and 4. Each value below is the mean of 5 determinations ± S.E.

Reagent	$\frac{\%\ Hb\ in\ Tube\ A}{\%\ Hb\ in\ Tube\ B}$	$\frac{Spec.\ act.\ of\ ghosts\ in\ Tube\ A}{Spec.\ act.\ of\ ghosts\ in\ Tube\ B}$
TNBS	2.4 ± 0.9	0.91 ± 0.02
PC	2.4 ± 1.4	1.00 ± 0.10

globin contaminations in single tubes to the per cent hemoglobin contaminations in duplicate tubes in the same experiment. The second column of figures gives the ratios for the corresponding ghost specific activities. It is clear that tube pairs can be found in which hemoglobin contamination differs by up to 2.4-fold on the average without any change in the specific activity of the ghosts. Table III shows the results of an experiment in which ghosts labeled with TNBS were prepared with differing amounts of hemoglobin contamination by adjusting the number of washes on different aliquots. Even though the hemoglobin contamination was decreased 6.4-fold by one additional wash, there was almost no change in specific activity of the ghosts. In all of these experiments the absolute recoveries per tube of ghost radioactivity and the yields of the ghost protein remained essentially constant.

Table IV summarizes some quantitative aspects of the labeling pattern. The calculated reservoir of potentially reactive groups ($-NH_2$ and $-SH$) in the hemoglobin sink is seen to be about 60 times the number in the plasma membrane barrier,

TABLE III

COMPARISON OF GHOST SPECIFIC ACTIVITY BETWEEN TWO PREPARATIONS WITH DIFFERING AMOUNTS OF HEMOGLOBIN CONTAMINATION

% Hemoglobin is defined as mg hemoglobin/mg total ghost protein × 100 and the ghost specific activity is expressed as disint./min × 10^{-3} per mg ghost protein. Hemoglobin and ghost specific activities were measured as described in the text on aliquots of ghost suspensions included in the data of Fig. 3.

No. of washings	*% Hemoglobin**	*Specific activity of ghosts***
2	39.3 ± 2.7	11.9 ± 0.7
3	6.0 ± 0.3	13.6 ± 0.2

* Each value is the mean of 3 determinations ± S.E.
** Each value is the mean of 6 determinations ± S.E.

TABLE IV

DISTRIBUTION OF LABEL IN ERYTHROCYTES

Potentially reactive groups were calculated as all amino and sulfhydryl groups as follows: For hemoglobin the number of lysines and cysteine residues per molecule were obtained from the amino acid analyses[18]. The *N*-terminal residues were taken as four per molecule. For the ghosts, the lipid contribution was assumed to be entirely from the amino groups of phosphatidyl serines and phosphatidyl ethanolamines calculated from the values given by COOPER[19]. For the ghost protein the total amino and N-terminal groups were calculated from the amino acid analysis of total ghost protein reported by ROSENBERG AND GUIDOTTI[20] and the figure of 1.5 mg of ghost protein per 0.5 ml of erythrocytes reported in this paper; for the ghost sulfhydryls the value of VANSTEVENINCK *et al.*[21] for groups reacting with Hg^{2+} was used.

Fraction	*Potentially reactive groups (μmoles/0.5 ml erythrocytes)*	*μmoles TNBS bound/0.5 ml erythrocytes*	*μmoles PC bound/0.5 ml erythrocytes*
Hemoglobin	115	0.18 (0.16%)*	0.14 (0.12%)*
Ghosts	1.89	0.004 (0.23%)*	0.0024 (0.13%)*

* Percent of potentially reactive groups reacting in 60 min.

but that under the conditions employed no more than about 0.2% in either component did indeed combine with label. Thus, even if 75% of the calculated potentially reacting groups prove inaccessible (as for TNBS with human serum albumin) a many-fold excess of target groups would remain. In several experiments after TNBS or PC in the medium were exhausted, the cells were resuspended in fresh buffer containing labeled reagent and incubated for a second hour. In all cases, the incorporation into ghosts for two pulses was double that for one pulse, further supporting the contention that the system was far from saturation.

DISCUSSION

The identity of the synthetic TNBS appears established by direct chromatography in two systems as described by OKUYAMA[17], by characterization of two derivatives, picramide and trinitrophenylglycine[17] and finally by elemental analysis. The difference in melting points between our range of 161–163° and the 180° reported by SATAKE[13] remains unexplained, but may be due to hydration. It is interesting that the specific activities of both synthetic labeled compounds were at the theoretical maximum obtainable from [^{3}H]DNCB of 87 mC/mmole specific activity, indicating that this starting material carried no tritium on carbon six.

The data of OKUYAMA[17] and FREEDMAN[22] appear to have established that TNBS reacts exclusively with the sulfhydryl and primary amino groups of amino acids, peptides and proteins. The results of the chromatography of the acid hydrolysates of the TNBS-treated human serum albumin mentioned earlier in this paper further support this position and establish covalent bonding at the ε-amino group of lysine as the major locus for this label. It is evident from the data of Fig. 2, from which it may be calculated that 16.8 moles of TNBS are incorporated per mole of human serum albumin after 4 h, that only about 26% of the 64 moles of free amino groups[23] were readily accessible to TNBS under our conditions. This value is a maximum which would be slightly reduced by any reaction with the fractional sulfhydryl (0.6–0.7) reported for human serum albumin[24,25]. These results are parallel to the observations of GOLDFARB[26] using unlabeled TNBS and a spectrophotometric method. GOLDFARB[26] reported the slightly higher value of 19.2 moles of readily available amino groups.

The interpretation of the time course of the incorporation of [^{3}H]PC shown in Fig. 2 in terms of accessible amino groups requires some preliminary comments regarding specificity. This is so because although PC reacts readily with free primary amino groups it has also been shown to react with tyrosine and histidine residues by OKUYAMA[17], and human serum albumin (mol. wt. = 69000) has been found to contain 15.7 moles of histidine residues and 18.0 moles of tyrosine residues per mole[23]. The prospect of interference from these two classes of residues does not appear to be very great however, for in studies of the reaction of PC with the free amino acids OKUYAMA[17] also found that the formation of *O*-TNP-tyrosine and *im.*-*N*-TNP-histidine required much more rigorous conditions than the formation of products such as ε-TNP-lysine which result from reaction with primary amino groups. In addition, IKENAKA[27] has reported that the reaction of the related reagent dinitrochlorobenzene (DNCB), to produce *O*-DNP-tyrosine and *im.*-*N*-DNP-histidine is greatly reduced below pH 8, reaching essentially no reaction at all with Taka-amylase A at pH 7. Finally, we have found that 80% of the radioactivity from PC-treated human serum albumin acid

hydrolysates was in the chromatographic location for ε-TNP-lysine with a distribution identical to that obtained with the TNBS hydrolysates. Therefore barring special properties of histidyl and tyrosyl residues in serum albumin[28], it appears likely that the bulk of the PC label was associated with free amino groups of human serum albumin.

With these qualifications in mind, Fig. 2 can be interpreted to mean that PC reacted with about twice as many free amino groups as TNBS during a 4-h incubation. A partial answer to the question of whether the two reagents combine with the same or different amino groups is given by the data in Table I. There it is shown that PC pre-empted all but four of the groups available to TNBS. It is an attractive hypothesis to suppose that in addition to reacting with most of the amino groups in relatively exposed positions, the PC because of its apolar character would have had easier access to many of the amino groups "buried" in the apolar regions of the human serum albumin molecule. Possibly these would have been in the hydrophobic regions described by FOSTER[29]. The alternative hypothesis, that unlike TNBS, the PC caused conformational changes which exposed additional amino groups, cannot be excluded by the data available.

Fig. 3 shows that the concentration of free [^{3}H]TNBS in the medium dropped rapidly during the early minutes of incubation with the erythrocytes and that simultaneously the label appeared in combination with the hemoglobin and ghosts, although some remained in the medium in a form other than free starting reagent. As the concentration of free [^{3}H]TNBS dropped the reaction rate slowed and by 60 min had essentially ceased. A schematic diagram of such a system involving a penetrating reagent is shown in Fig. 5. It consists essentially of three compartments: (1) a source, (2) a barrier and, (3) a sink. In the case of the incubation of erythrocytes in phosphate

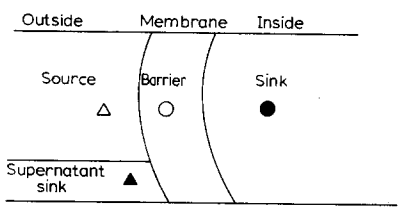

Fig. 5. Schematic diagram of the compartments of the erythrocyte incubation system. The areas are coded to match Figs. 3 and 4.

buffer containing TNBS, the source is the incubation medium, the barrier is the plasma membrane of the erythrocyte, and the sink is the hemoglobin and other molecules inside the erythrocyte whose sulfhydryl and amino groups react with the entering TNBS. Labeling of a membrane by a penetrating radioactive derivatizing reagent can occur at the outer surface adjacent to the source, at the inner surface adjacent to the sink compartment, or anywhere on the pathway of transit taken by the reagent through the membrane. The degree of labeling will depend upon the effective concentration maintained by the reagent in each zone of the membrane, the accessibility of the target groups, and the intrinsic rate of the reaction. For any individual reagent the relative effect of these factors may be difficult to determine. Consideration of this general model, as well as the data presented in this paper, emphasize that the concentration of a penetrating reagent in the medium may fall quite rapidly during in-

cubation. The rate of fall will depend upon the concentration of the reagent added, the volume of the extracellular compartment, the permeability of the membrane, the number of reactive groups on the membrane and their rate of reaction, and the mass and reaction rate of the sink components. Recently KRUPKA[30] has presented evidence that during incubation of erythrocytes with dinitrofluorobenzene, the concentration of the reagent may fall sufficiently to affect important kinetic conclusions regarding the rate of inhibition of glucose transport by this reagent.

The reciprocal contours of the curves for the incorporation of [^{3}H]PC in the hemoglobin and total supernatant fractions in Fig. 4 suggest that these two components may have been exchanging label. The finding that dinitrofluorobenzene, TNBS and related compounds[31] are hydrolyzed by human erythrocyte carbonic anhydrases makes it possible to propose the following mechanism: Upon entry into the erythrocyte the PC is hydrolyzed to picric acid which quickly diffuses out and is measured at intermediate times in the total supernatant. A slow reaction of picric acid with hemoglobin would then reverse the flow, explaining the return to higher activities in the hemoglobin at 60 min. The failure to find a similar effect with TNBS may be due to a slower rate of penetration, slower hydrolysis, or both.

Examination of the ghost specific activity curves for [^{3}H]PC in Fig. 4 suggests that the derivatization reaction was complete within 3 min. The more rapid entry of the relatively apolar and uncharged PC is consistent with the general rule that passage through the erythrocyte membrane is favored for low polarity compounds. The theory of dual passage for non-electrolytes[11] suggests that for simple diffusion, the erythrocyte membrane behaves as a mosaic having polar and apolar tesserae, and that the lipid soluble compounds pass through the apolar tesserae. Whether PC and TNBS take the same or different paths in transit through the erythrocyte membrane cannot be decided from the data presented here. However, it seems reasonable to suppose that, by analogy with their behavior towards human serum albumin, the PC would have more ready access to apolar regions in the membrane while the polar regions would be more accessible to TNBS.

The conceptual approach emphasized in this paper involving total system analysis would appear to have general application in the interpretation of derivatization experiments on whole cells and cell fractions employing penetrating reagents.

ACKNOWLEDGEMENTS

This work was submitted by John J. Arrotti in partial fulfillment of the requirements for the degree of Doctor of Philosophy of Northwestern University and was supported in part from the Edris Fund.

REFERENCES

1 H. A. MADDY, *Biochim. Biophys. Acta*, 88 (1964) 390.
2 H. BERG, *Biochim. Biophys. Acta*, 183 (1969) 65.
3 W. NIEHAUS AND F. WOLD, *Biochim. Biophys. Acta*, 196 (1970) 170.
4 W. L. HUBBELL AND H. M. MCCONNELL, *Proc. Natl. Acad. Sci. U.S.*, 64 (1969) 20.
5 J. LENARD, *Biochemistry*, 9 (1970) 1129.
6 D. R. PHILLIPS AND M. MORRISON, *Biochemistry*, 10 (1971) 1766.
7 D. GREEN, D. ALLMANN, E. BACHMAN, H. BAUM, K. KOPACZYK, E. KORMAN, S. LIPTON, D. MACLENNAN, D. MCCONNELL, J. PERDUE, J. RIESKE AND A. TZAGOLOFF, *Arch. Biochem. Biophys.*, 119 (1967) 312.

8 J. LENARD AND S. J. SINGER, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 1823.
9 D. F. H. WALLACH AND A. GORDON, *Fed. Proc.*, 27 (1968) 1263.
10 E. KORN, *Science*, 153 (1966) 1491.
11 R. WHITTAM, *Transport and Diffusion in Red Blood Cells*, Arnold, London, 1964, p. 156.
12 P. FRANKAND AND F. GARNER, *J. Soc. Chem. Ind. London*, 39 (1920) 257T.
13 K. SATAKE AND T. OKUYAMA, *Kagaku-no-Ryoiki, Electrospectrophotometry in Biological Chemistry*, Vol. II, Nankodo, Tokyo, 1958, p. 63.
14 O. LOWRY, N. ROSEBROUGH, A. FARR AND R. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
15 C. RIMINGTON, *Br. Med. J.*, 1 (1942) 177.
16 J. DODGE, C. MITCHELL AND D. HANAHAN, *Arch. Biochem Biophys.*, 100 (1963) 119.
17 T. OKUYAMA AND K. SATAKE, *J. Biochem. Tokyo*, 47 (1960) 454.
18 M. O. DAYHOFF, *Atlas of Protein Sequence and Structure*, Vol. 4, National Biomedical Research Foundation, Silver Spring, Md., 1969, pp. D-39, D-49.
19 R. A. COOPER, *Seminars Hematol.*, 7 (1970) 296.
20 S. A. ROSENBERG AND G. GUIDOTTI, *J. Biol. Chem.*, 243 (1968) 1985.
21 J. VANSTEVENINCK, R. I. WEED AND A. ROTHSTEIN, *J. Gen. Physiol.*, 48 (1965) 617.
22 R. B. FREEDMAN AND G. K. RADDA, *Biochem. J.*, 108 (1968) 383.
23 G. MARKUS, D. K. MCCLINTOCK AND B. A. CASTELLANI, *J. Biol. Chem.*, 242 (1967) 4395.
24 W. L. HUGHES, *J. Am. Chem. Soc.*, 69 (1946) 1836.
25 T. P. KING, *J. Biol. Chem.*, 236 (1961) PC5.
26 A. GOLDFARB, *Biochemistry*, 5 (1966) 2574.
27 T. IKENAKA, *J. Biochem. Tokyo*, 46 (1959) 177.
28 L. A. COHEN, *Annu. Rev. Biochem.*, 37 (1968) 697.
29 J. F. FOSTER, *The Plasma Proteins*, Academic Press, New York, 1960, p 221.
30 R. M. KRUPKA, *Biochemistry*, 10 (1971) 1148.
31 P. HENKART, G. GUIDOTTI AND J. T. EDSALL, *J. Biol. Chem.*, 243 (1968) 2447.