On the Removal of Residual Carboxylic Acid Groups from Cellulosic Membranes and Sephadex†

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ABSTRACT: A method is described for the removal of fixed negative charges from dialysis membranes and Sephadex. This is accomplished by condensing the acid groups with glycinamide with the help of a water-soluble carbodiimide derivative in a buffer solution at pH 4.75 diluted with an equal volume of dimethylformamide. The amount of glycinamide incorporated has been determined by quantitative analysis of the glycine liberated after alkaline hydrolysis The effect of complete removal of fixed charges has been demonstrated in a number of ways. With the membranes greater stability and less adsorption were noted while with Sephadex the elution volume was found to be more clearly related to diffusional volume. The effect of attaching carboxyl groups to cellophane has also been studied.

Much data from thin-film dialysis studies have accumulated which show that the rate of dialysis from calibrated cellulose membranes can be used reliably for determining diffusional size (Stokes radius) of biopolymers (Craig, 1967). Such an approach holds promise for (i) the study of molecular conformation, a phenomenon which results from the balance of intramolecular interactions of different parts of the same molecule (Craig *et al.*, 1965; Burachik *et al.*, 1970; Craig *et al.*, 1971), and (ii) the related study of intermolecular interactions such as association and binding phenomena (Guidotti and Craig, 1963; Ruttenberg *et al.*, 1966; Chen and Craig, 1971). In the meantime much has been written concerning the nature of dialysis itself and the factors which control the diffusion or retention of various solutes through semipermeable membranes (Craig, 1964; Craig and Chen, 1972). Gel filtration, the basic nature of which as yet remains vague, is undoubtedly based largely on differential diffusional parameters similar to those involved in dialysis (Ackers, 1970). The reliability of interpretations concerning molecular size from both techniques depends on comparative experiments with model compounds, where size and shape and diffusional behavior have been established by other techniques such as X-ray crystallography, free diffusion, ultracentrifugation, etc. The dialysis and gel filtration methods, however, do not give absolute measurements and must be considered as giving only difference measurements. Thus, they require extensive control experiments under the assumption that complicating parameters do not exist or at least exist only as a second-order concern. For example, adsorption and ion-exchange parameters are not completely void in the "neutral" cellulosic materials, such as the Visking dialysis membrane or Sephadex, but are presumed to be only of second-order significance. For certain solutes and purposes a particular ion-exchange medium may be much more desirable and useful than a completely neutral one. It undoubtedly complicates the results when separation on the basis of size only is desired.

The presence of negative fixed charges in the neutral dextran material is low, and the small charge effect is often ignored (Gelotte, 1960). Furthermore, the effect can be eliminated indirectly by the addition of a dilute salt solution to the system. However, this excludes the possibility of studying the solute behavior in salt-free or extremely low-salt solutions. Our recent studies with analogs of ACTH[1] and certain protamines encountered such problems (Craig *et al.*, 1971). Eaker and Porath (1967) also have observed that lysine shows an anomalous behavior in G-10 filtration in acidic solution. It therefore appears desirable to develop a simple and effective procedure for removal of residual fixed negative charges from the polymers. The nature of the negative charges in the cellulosic membrane and in Sephadex has not been well documented since the amount is low. Two kinds of negative charge groups are conceivable with such carbohydrate substances, carboxylic acid groups and sulfate groups. Since Visking membranes and Sephadex are derived from highly purified cellulose and dextran, respectively, and both are treated in a process involving alkali, unlike agarose, they probably do not contain sulfate or show the undesirable properties of such contamination as pointed out by Porath *et al.* (1971). The level of sulfate in these two substances is extremely low or not present at all. Thus, if the major negative charges are carboxylic acid groups, amidation, esterification, and catalytic reduction are possible removal procedures. However, most of the commonly used reaction conditions do not include aqueous solutions, which are required in order to maintain the native structure of the hydrophilic gel. A method for the quantitative modification of carboxylic acid groups in proteins has been reported which involves the activation of the carboxylic group by a water-soluble carbodiimide and the subsequent reaction with a nucleophile such as glycine methyl ester (Hoare and Koshland, 1967). Since this is a quantitative reaction, and can be carried out in aqueous solution under mild conditions, application of such a procedure with glycinamide to mask the carboxylic acid groups in the membrane and Sephadex seems most appropriate. Here we describe such a procedure (Figure 1) and further present data indicating the removal of charge. With such membranes dialysis rate data and effluent patterns from Sephadex chromatography can be more precisely related to diffusional size.

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[1] Abbreviations used are: ACTH, adrenocorticotropic hormone; CM-, O-carboxymethylated; $GlyNH_2$, glycinamide.

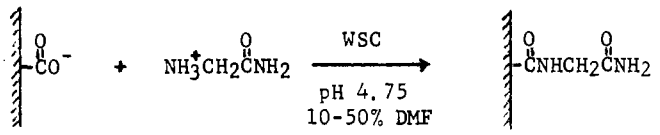

FIGURE 1: Outline of the reaction employed to block the carboxylic acid groups by peptidation with glycinamide. Two kinds of water-soluble carbodiimide (WSC) are listed in "Materials."

It seemed of interest also to study the effect of controlled addition of carboxylic acid groups into the membrane by the method described by Peterson and Sober (1961) and the subsequent change of porosity and other physical characteristics toward solutions of differing ionic strengths.

Experimental Section

Materials. The following materials were purchased from the sources indicated: Visking dialysis casings No. 18 and No. 20, Union Carbide, Chicago, Ill.; Sephadex G-10, G-25 fine, CM-Sephadex, Pharmacia Fine Chemicals, Inc.; 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate, Aldrich Chemical Co., Milwaukee, Wis.; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, Ott Chemical Co., Muskegan, Mich.; glycinamide hydrochloride, Cyclo Chemical, Los Angeles, Calif.; *N,N*-dimethylformamide, certified ACS spectranalyzed grade and chloroacetic acid, certified grade, Fisher Scientific Co., Fair Lawn, N. J.; L-tryptophan, Sigma grade, and horse heart cytochrome *c* type IV, Sigma Chemical Co., St. Louis, Mo.; [*methoxy*-^{3}H]inulin, 82 mCi/g, mol wt 5000–5500, and [1-^{14}C]glycine, 45 μCi/μmole, New England Nuclear, Boston, Mass.; cacodylic acid, C.P., Amend Drug and Chemical Co., New York, N. Y. Cupraphane sheet was obtained indirectly from the German Bemberg Co. Bacitracin A was purified by the countercurrent methods described previously (Craig *et al.*, 1969). Dimethylformamide was treated with activated molecular sieve Linde type 4A, and cacodylate buffer was filtered through Millipore (0.45 μ) before use. Ferrichrome A was a gift from Dr. T. Emery, Utah State University. We wish to thank him for the sample.

Coupling of Glycinamide to the Membranes. The cellophane membranes were attached to a glass collar and tied off at the bottom as described previously (Craig, 1967). They were washed successively with 0.5% sodium dodecyl sulfate, distilled water, 0.5% Na_2EDTA, and again with distilled water, then suspended in 60 ml of a solution containing an equal volume of dimethylformamide and 0.1 M cacodylic acid–NaOH buffer at pH 4.75 and 1 mmole of glycinamide hydrochloride. A solution containing 1 mmole of water-soluble carbodiimide in 10 ml of 0.1 M cacodylate buffer and 10 ml of DMF, with the pH adjusted to 4.75 with HCl, was added gradually with gentle stirring to the solution in which the membrane was suspended. After standing overnight at room temperature (25 ± 0.5°) or 2 hr at 65° in a water bath with gentle stirring, the membrane was removed and washed for 30 min in 0.1 M acetic acid. It was then washed successively with H_2O, a solution of 0.01 M acetic acid, and 0.1 M NaCl until no more ultraviolet-absorbing material was eluted.

Coupling of Glycinamide to Sephadex. Sephadex G-10 or G-25 was swelled in 0.1 M NaCl and washed with 0.5% Na_2-EDTA and distilled water before equilibration with 0.1 M cacodylic acid–NaOH buffer at pH 4.75. For 50 g of Sephadex, 10 mmoles of glycinamide was added to 400 ml of a pH 4.75 solution of 50% dimethylformamide in cacodylate buffer, and then a solution of the water-soluble carbodiimide, 10 mmoles in 25 ml of DMF and 25 ml of the buffer with the pH adjusted to 4.75, was added. The reaction conditions were the same as for the membrane. The reagents were removed from the treated Sephadex by washing with a solution of 0.1 N acetic acid and 0.1 M NaCl and water until the washings no longer gave a positive ninhydrin reaction.

Attachment of Carboxymethyl Groups to the Membrane. A washed wet length of the membrane was immersed in a beaker containing ice-cold 30% NaOH. After 30 min, 9 g of chloroacetic acid was added with stirring, and the mixture heated to 70° for 20 min. It was cooled, and 150 ml of 10% acetic acid was added. The salts were then washed from the membrane before incorporating it into the dialysis cell.

Static Thin-Film Dialysis Experiments. The apparatus and procedures were the same as described previously (Craig, 1967). Experiments were carried out at room temperature (25 ± 0.5°) unless otherwise specified. Radioactivities of [*methoxy*-^{3}H]inulin were measured in Bray's solution in a Tri-Carb liquid scintillation spectrometer.

Quantitative Determination of Glycinamide Attachment. Glycinamide incorporations were determined by the amino acid analysis method of Moore and Stein (1963) after hydrolysis in 1 M NaOH at 100° for 16–18 hr in an unevacuated system. Several conditions for the hydrolysis were tried: 6 N HCl at 110° *in vacuo*, 5.0 N NaOH at 110° *in vacuo*, and 2.5 M NaOH at 100°, all for 16–18 hr. In addition, hydrolyses at 100° in 1.0 M NaOH for 40 and 64 hr were investigated. All gave identical amounts of glycine. Therefore, 1.0 N NaOH at 100° for 16–18 hr in a covered plastic tube were the conditions adopted for hydrolysis. As a further check, Gly-Gly and $GlyNH_2$ yielded the quantitative amount of glycine under these conditions.

Results and Discussion

In an earlier study (Craig and Ansevin, 1963) levels of fixed charges almost below those demonstrable by potentiometric measurements had been shown in Visking cellulosic membranes. Much data obtained since then, with a wide variety of polypeptides and nucleotides, both positively and negatively charged, have supported this view. On routine calibration of a membrane, compact peptide compounds, conformationally stable toward a neutral salt (*e.g.*, NaCl) such as the antibiotics bacitracin A or gramicidin SA, were used as the testing solute. In earlier experiments these had not shown an obvious salt effect. Furthermore, if a small amount of negative charge was present, the dialysis experiments were performed either under acidic conditions (*e.g.*, 0.01 M acetic acid) or a buffer-containing salt. Occasionally we had noted that certain rolls of the cellulose casing, particularly the "seamless" cellulose tubing as compared to the "dialysis" tubing, showed a strong tendency to adsorb basic proteins such as cytochrome *c* and lysozyme in solutions of relatively low ionic strength. These membranes were simply discarded and not used for most of the previously published work. Recently in our work with newer unmodified Visking membranes it was found that bacitracin A dialyzed considerably faster in 0.01 M acetic acid than in 0.1 M NaCl. Gramicidin SA was also significantly slowed in the presence of salt. Conversely, ferrichrome A (Emery, 1967), an acidic peptide with three net carboxylic acid groups, showed an anomalously slow diffusion rate without any salt present but a faster rate with an increase of the salt concentration. These phenomena

TABLE I: Half-Escape Time of Bacitracin A in Untreated and Glycinamide-Treated Membranes.

Membrane	Treatment	t/2 (min) 0.01 M HOAc	t/2 (min) + 0.1 M NaCl
Visking No. 18	−	13.5	68
	+	50	64
	−[a]	25	70
Visking No. 20	−	5	18
	+	16	18
Cupraphane flat sheet	−	7	20
	+	12	20

[a] Glycinamide was omitted from the reaction mixture.

TABLE II: Half-Escape Times of [*methoxy*-^{3}H]Inulin in Untreated and CM-Membranes.

Membrane	t/2 (min) 0.01 M HOAc	t/2 (min) + 0.1 M NaCl
Visking No. 20	57	50
CM-No. 20	20	26
CM-No. 20	21[a]	20[a]

[a] Dialysis experiments were carried out in 0.001 M cacodylic acid–NaOH (pH 7.0).

can be attributed to the electrostatic charge interaction between solute and membrane, a finding which stimulated the present study.

Dialysis Behavior in Glycinamide-Treated Membranes. At a pH below 7 in the absence of a neutral salt, a solute with net positive charges such as gramicidin SA and bacitracin A will partially interact with a negatively charged membrane. Such interaction accelerates the rate of dialysis in a way analogous to that observed with aromatic amino acids, where adsorption plays a role as described previously (Craig and Ansevin, 1963). On the other hand, a solute with net negative charges such as ferrichrome A will be repelled by the like charges on the membrane in the absence of salt and will show a slower dialysis rate. When the solution contains a neutral salt, the charge on the membrane will be masked and the dialysis rate will reflect more nearly the true diffusional size. With uncharged solutes the rate of dialysis is not affected by the charge on the membrane or the addition of salt. It has been observed that an unmodified membrane with a small net residual charge does not shrink in a salt solution.

As shown in Table I, particular samples of untreated Visking casing No. 18 and No. 20 show fivefold and nearly fourfold differences, respectively, in ratios of half-escape times for bacitracin A before and after addition of 0.1 M NaCl. No significant difference in half-escape times are observed in case of a neutral solute, *i.e.*, inulin (Table II). After the same membrane was allowed to react with glycinamide in order to mask the carboxylic acid groups, the large effect caused by the presence of salt is no longer observed. If a membrane was treated under the same conditions, except that glycinamide was omitted, a salt effect was still observed, suggesting that possible lactone formation does not prevail in this case, even though such reaction can occur in hydroxy acids with water-soluble carbodiimides (Taylor and Conrad, 1972). Bemberg cupraphane, widely used in hemodialyzers, has also been found to carry a low net charge which can be partially removed by the glycinamide treatment. Here the comparison was made in a flat sheet dialyzer.[2] It was observed in all cases that bacitracin A shows a slightly faster rate in the absence of salt. Although it is a rather rigid molecule, a low degree of molecular expansion by the salt effect may be possible since the thin-film dialysis method allows detection of a change in the Stokes radius on the order of 2–3% (Craig and Pulley, 1962).

[2] L. C. Craig, to be published.

With membranes demonstrated to be free of the fixed charge effect on hand it is possible to study more precisely the conformational mobility of peptides devoid of covalent cross-links. Recently from this laboratory, it has been demonstrated that highly basic polypeptides such as β^{1-24}-ACTH and salmiridine gave dialysis rates that were much too slow in a solution of low ionic strength, indicating intramolecular electrostatic repulsion which promoted a more loose or elongated conformation. Increasing the ionic strength by addition of salt shielded the charges on the molecule and promoted collapse to a more compact molecule as reflected by the relatively faster dialysis rates consistent with molecular weights of compact molecules of the same size (Craig *et al.*, 1971). The linear octapeptide [Val$_5$]angiotensin II amide behaved as if it had a rigid conformation in a solution of high ionic strength which is consistent with the presence of a hydrogen-bonded secondary structure as observed in a proton-exchange study carried out in this laboratory (Printz *et al.*, 1972).

In addition to the elimination of the charge effect on the membrane, it has been noted that the adsorptive properties of the membrane toward solutes seem to be greatly reduced. This was simply measured by the time required for the complete recovery of the solute. Without these two complications, we have investigated the basic mechanism involved in the passive transport of solute through dialysis membranes (Craig and Chen, 1972) and demonstrated that the activation energy required for the diffusion of ideal solutes through membranes follows closely that of free diffusion. With such membranes experimental evidence has been given that the factor controlling the rate in thin-film dialysis is the probability of the solute molecule finding its way into a pore at the membrane boundary on the side of the membrane containing the high concentration of the solute, and not the passage through the membrane, where resistance from the internal structure of the membrane could be expected.

A favorable increase in mechanical strength of the glycinamide-treated membrane has also been observed. The presence of like charges on the membrane, which causes the weakening of mechanical strength of the membrane, becomes more obvious when CM-membranes are examined, as will be discussed later. This added improvement of mechanical strength is particularly valuable with the thin-film countercurrent dialyzer (Craig and Chen, 1969). The countercurrent dialyzer, with the treated membrane, has been useful for stripping 2,3-diphosphoglycerate from hemoglobin, since both substances do not absorb to the treated membrane, as contrasted to untreated ones.

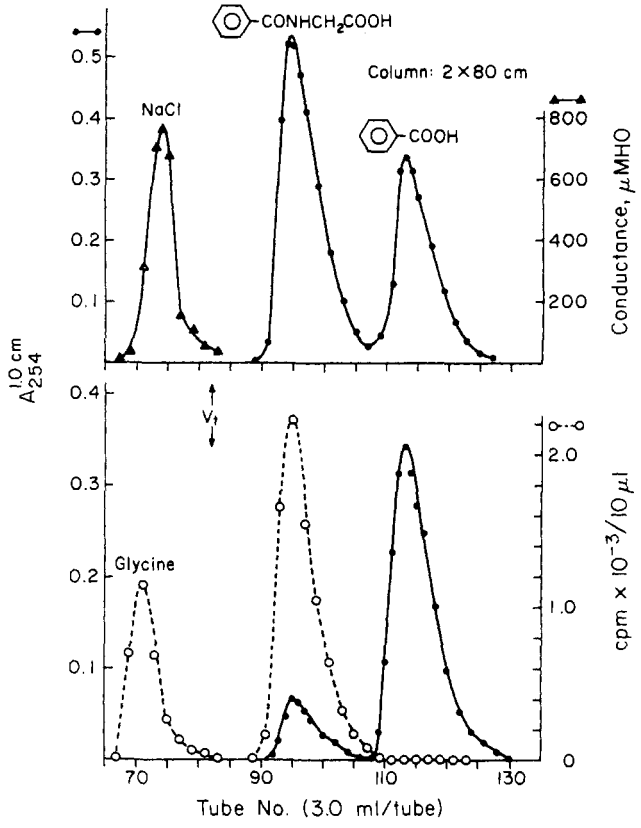

FIGURE 2: Glycinamide-treated Sephadex G-25 adsorption chromatography of hippuric acid and benzoic acid in 0.01 M acetic acid. Upper figure is the elution profiles of the calibration mixtures containing NaCl, hippuric acid, and benzoic acid. Lower figure shows the profiles of the reaction mixtures after benzoylation of glycine. Yield of hippuric acid, 0.82 μmole, 74% from glycine.

After this work was completed, Robberson and Davidson (1972) employed a similar reaction to block the carboxylic acid residues in agarose, subsequently increasing the coupling efficiency of polynucleotides onto agarose for affinity chromatography. A different method involving catalytic reduction with $LiAlH_4$ in dioxane for the preparation of "charge-free" agar was also reported by Laas (1972). Therefore, the importance of eliminating charges, both carboxylic acid groups and sulfate groups (Porath *et al.*, 1971; Laas, 1972), should be emphasized in order to make the separation of biological molecules on the solid matrix more precise and efficient.

Characteristics of the Carboxymethylated Membrane. In order to understand the characteristics of membranes with carboxylic acid groups attached, CM-membranes were prepared by the method of Peterson and Sober (1961). To determine the porosity of these membranes, it was necessary to use a neutral solute such as [*methoxy*-^{3}H] inulin. In Table II the dialysis rates of inulin both from unmodified No. 20 membrane and the CM-membrane are shown. It is obvious from Table II that the charges on the membrane, intrinsic or extrinsic, do not accelerate the dialysis rate of the neutral solute, inulin, as contrasted to charged solutes. It is of interest to observe that the CM-membrane is more porous than an untreated one. It is also of interest that although addition of 0.1 M NaCl caused a measurable contraction in the membrane, the porosity, in terms of half-escape time, did not change significantly. It should be mentioned that inulin is not strictly homogeneous in size and thus gives a curved escape pattern.

TABLE III: Elution Behavior Independent of 0.1 M NaCl in Glycinamide-Treated Sephadex G-25 Gel Chromatography.

Conditions[a]		V_e/V_t[b]		
$GlyNH_2$ Treatment	0.1 M NaCl	Cytochrome *c*	Bacitracin A	Tryptophan
−	−	∞	1.08	1.39
−	+	0.39	0.57	1.61
+	−	0.39	0.68	1.60
+	+	~0.39	0.66	1.71

[a] Solvent, 0.01 M acetic acid; Sephadex G-25, 50 g in a column of 2 × 80 cm; flow rate, 36 ml/hr. [b] V_t is the total effective volume as determined from the peak position of tritiated water. V_e is the elution volume of solutes.

The CM-membrane swells and becomes more elastic in the absence of salt, but reverts back to normal in the salt solution. The length and diameter of the wet casing was found to increase by 12 and 28%, respectively, on going from the salt to the salt-free solution. It is conceivable that the presence of charge on the membrane induces more hydration or hydrophilicity, a property well known in ion-exchange resins. The repulsion of like charge and increasing hydration could explain why a charged membrane swells more and is more elastic on carboxymethylation. Unfortunately, the CM-membrane does not have the wet strength of the unmodified membrane.

It was found that the thin-film escape patterns of positively charged solutes such as lysozyme and bacitracin A in the CM-membrane were not of a linear type and also gave a relatively slow dialysis rate. Moreover the rate was slowed by a lower salt concentration. This is a phenomenon which should be expected from the behavior of ion exchangers, yet seems to contradict the preceding statement that interaction between solute and membrane accelerates the rate of dialysis. This discrepancy can best be resolved by considering the solutes of interest (*e.g.*, bacitracin and lysozyme) as replacers in an ion-exchange process. When the charge on the membrane is low, a small amount of solute is first attracted to the pore by the opposite charge on the membrane and immediately is pushed on to the low concentration side of the membrane by the predominant amount of solutes in the high concentration side. On the other hand, if more solutes are tightly absorbed to the membrane by much higher charge density the amount of free solute becomes so low that the rate of dialysis is controlled by the rate of dissociation from the charged membrane. The dissociation process in this case should be slow and salt dependent.

Chromatographic Behavior of Glycinamide-Treated Sephadex G-25. The presence of carboxylic acid groups in dextran gel has long been recognized. A recent detailed study on the elution patterns of amino acids conducted by Eaker and Porath (1967) revealed that both acidic and basic amino acids, while opposite in effect, showed variable distribution coefficients which depended on pH and ionic strength. Cytochrome *c*, a highly basic hemoprotein, tends to adsorb to the gel in salt-free solution. As shown in Table III, horse heart cytochrome *c* was absorbed completely by the untreated G-25 in 0.01 M acetic acid, pH 3.3. Under the same conditions, bacitracin A, which has one net positive charge, was slightly, adsorbed ($V_e/V_t \approx 1.08$). In the presence of 0.1 M NaCl,

however, both cytochrome *c* and bacitracin A were eluted at positions expected from their molecular size. After glycinamide treatment to remove residual carboxylic acid groups in the Sephadex, both cytochrome *c* and bacitracin A were eluted at the expected positions, either in the presence or in the absence of 0.1 M NaCl. The recovery of cytochrome *c* and bacitracin A from treated G-25 was quantitative. When water was used as the eluent instead of 0.01 M acetic acid the position of the peaks for these two substances did not change. However, only 70% of the cytochrome *c* was recovered in the eluent. The remaining 30% was found mainly on the top part of the column bed, where the fine gel particles were concentrated.

From the elution position of tryptophan, the affinity of tryptophan toward glycinamide-treated gel seems to increase slightly over that of the untreated one. The addition of 0.1 M NaCl obviously enhanced adsorption as one would expect from a hydrophobic-type interaction. Using the same column, a complete separation of hippuric acid from the glycine and benzoic acid that contaminate the crude synthetic product was achieved as shown in Figure 2. This procedure was conveniently used in the preparation of a microquantity of salt-free [^{14}C]hippuric acid. With untreated gel the two right-hand bands overlapped.

Glycinamide Incorporation into Dialysis Membranes and Sephadex. The amount of carboxylic acid groups in membranes and Sephadex has been considered to be low. In the case of Sephadex, 20–30 μmoles/g of dry gel was found by titration (Gelotte, 1960). For membranes, it is known that the acetylated membranes carry very little, if any, fixed charge. However, the amount of carboxylic acid groups in unmodified membranes was not known. Since the coupling reaction is considered quantitative with respect to the number of carboxylic acid groups in completely unfolded proteins (Hoare and Koshland, 1967), it is reasonable to expect that it would also react quantitatively with carboxylic acid groups in membranes and Sephadex. As shown in Table IV, glycinamide incorporation into Sephadex is only 0.3 μmole/g, which is less than 0.02 that reported from titration. This discrepancy cannot arise because the conditions of the coupling reaction are not optimal since the same reaction using CM-Sephadex was carried out under two conditions which differed 50-fold in the amount of reactants, yet gave the same glycinamide incorporation, which is in fact close to the theoretical amount (4.2 mmoles/g) given by the manufacturer. It could be that the recent sample of Sephadex carries less charge or that the majority of intrinsic fixed negative charges reside at dense regions of the matrix where a steric factor could prohibit the coupling reaction. On the other hand, carboxymethyl groups are exposed more on the surface of the matrix and thereby are accessible to the reagents. Nevertheless the treated materials become essentially uninfluenced by the presence of salt, indicating that at least the functionally important groups have been masked. Thus, the values cited in Table IV can only be considered to have covered the "effective" carboxylic acid groups. It should be mentioned that the intrinsic content of glycine in dialysis membranes is appreciable and not the same from roll to roll. Data shown in Table IV were obtained from a single roll which gave 0.35 μmole/g. On the other hand, Sephadex G-25 contained 0.08 μmole/g.

Since the coupling reaction could be controlled by some steric factor existing in the matrix, it is also interesting to investigate the amount of accessible groups at an elevated temperature and also after swelling in 64% $ZnCl_2$ solution (Craig, 1967), assuming that these factors would cause a more open structure. From Table IV Sephadex G-25 shows very little difference, if any at all, when the reaction was carried out at 65°. The incorporation of glycinamide into untreated and $ZnCl_2$ swollen membranes increased moderately when the temperature was elevated from 25 to 65°. However, $ZnCl_2$ swelling did not increase the accessibility as deduced from the coupling reaction.

TABLE IV: Amount of Glycinamide Incorporation into Dialysis Membranes and Sephadex.

Sample	Temperature of Reaction (°C)	$GlyNH_2$ (μmoles/g)[a]
Sephadex G-10	25	0.35
Sephadex G-25	25	0.30
	65	0.31
CM-Sephadex	25	3144
No. 20 membrane	25	0.48
	65	0.76
No. 20 membrane-$ZnCl_2$ swollen[b]	25	0.36
	65	0.76

[a] Intrinsic glycine contents were substracted. See text. [b] Prepared according to the procedure described by Craig (1967).

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Reactions of 2,4,6-Trinitrobenzenesulfonate Ion with Amines and Hydroxide Ion†

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ABSTRACT: The reactivity of 2,4,6-trinitrobenzenesulfonate ion (TNBS) with hydroxide ion and a series of primary amines has been examined as a function of pH, nucleophile concentration, ionic strength, and temperature. Its reaction with hydroxide ion is first order in both hydroxide ion and TNBS with a second-order rate constant of $6.33 \pm 0.16 \times 10^{-2}$ M^{-1} sec^{-1} at 25° (μ = 1.0), and appears to follow the usual path for nucleophilic aromatic substitution involving the rate-limiting formation of a tetrasubstituted ring carbon intermediate. The reaction of TNBS with primary amines having pK_a values higher than ~8.7 proceeds by a similar mechanism. Reactivity increases as a function of amine basicity according to the relationship log k (M^{-1} sec^{-1}) = 0.23· pK_a − 1.18. Similar amines with lower pK_a values follow a complex rate law apparently due to partitioning of the tetrasubstituted intermediate between breakdown to products and reversion to reactants. The reaction of TNBS with amines is favored as compared to its reaction with hydroxide ion by low ionic strength, low temperature, and pH values at least 0.5 unit below the amine pK_a value. Complexation of *N*-alkylpicramides by sulfite ion involves its addition to ring carbon number three. The second-order rate constant for complexation of sulfite with *N*-trinitrophenyl-β-alanine is 5.4×10^3 M^{-1} sec^{-1} at pH 8.0 (μ = 0.50) and 25.4°. Reactions of TNBS with proteins are discussed based on these results.

2,4,6-Trinitrobenzenesulfonate (TNBS)[1] was shown by Okuyama and Satake (1960) and Satake *et al.* (1960) to react with amino groups of amino acids, peptides, and proteins. Little or no reaction was observed with several other nucleophilic groups. TNBS has since become an important protein- and peptide-modifying reagent in large part due to this reaction selectivity. Hydrolysis of TNBS, for example, is relatively slow and does not usually interfere greatly with spectrophotometric determination of its reaction with amines (Habeeb, 1966; Freedman and Radda, 1968; Fields, 1971; Means and Feeney, 1971). In this study we have quantitatively evaluated parameters which influence the reactivity of TNBS with amines and with hydroxide ion and also attempted to characterize and assess the importance of complexes formed by *N*-alkylpicramides and sulfite ion, the two products of the TNBS reaction with amines.

Experimental Section

Materials. TNBS (picrylsulfonic acid, sodium salt dihydrate) was purchased from Aldrich Chemical Co. and used without additional purification. Neopentylamine was a product of Alfred Bader Chemicals. Other amines were reagent grade or better, obtained as free amines or as hydrochloride salts. TNP-amines were prepared by the reaction of TNBS with the corresponding amine or amine hydrochloride in water containing a slight excess of sodium carbonate. After the reaction was complete (several hours at room temperature for the less reactive amines) the deep red-orange solutions were acidified with dilute HCl and the resulting precipitate was filtered and washed with cold water. Recrystallization was accomplished in ethanol or ethanol–water. TNP derivatives of *tert*-butylamine, *N*,*N*-dimethylamine, and piperidine were recrystallized from cyclohexane in order to separate them from unknown high melting contaminants. Products were examined by nuclear magnetic resonance (nmr) and their melting points compared to previously published values. Nmr peak positions, intensities, and splittings were consistent with the expected structures.

Methods. Reactions of TNBS with hydroxide ion were monitored spectrophotometrically with a Cary 14 spectrophotometer from the increase in absorbance at 355 nm due to the formation of picrate ion. Pseudo-first-order conditions with hydroxide ion in large excess were maintained in all cases. The formation of product appeared completely first order. Reactions of TNBS with amines were followed similarly at the absorption maximum of the respective TNP–amine sulfite complexes at approximately 410 nm for most amines. Under pseudo-first-order conditions in the presence of excess amine the formation of product appeared first order except during the initial 0.05–0.1 absorbance change wherein the equilibrium between the picramide and sulfite complex was established. Addition of 10^{-4} M sulfite ion rendered the appearance of product first order throughout but was not used routinely as this early part of the reaction was observable only with very slow reactions. Rate constants of the amine reactions determined in phosphate buffer (μ = 0.5, pH 12.6) were identical with those determined in 10^{-3}–10^{-2}

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[1] Abbreviations used are: TNBS, 2,4,6-trinitrobenzenesulfonate ion; TNP, 2,4,6-trinitrophenyl; μ = ionic strength.