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Chemical Characterization of Isocyanate–Protein Conjugates[1]

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Chemical Characterization of Isocyanate–Protein Conjugates. TSE, C. S. T. AND PESCE, A. J. (1979). *Toxicol. Appl. Pharmacol.* **51**, 39–46. The rates of reaction of toluene diisocyanate (TDI) and hexamethylene diisocyanate (HDI) with the protein, human serum albumin, were studied. The reaction of TDI with protein was assessed by determining the total number of TDI residues bound as well as those bound in a monoureido linkage. There was a 3.5-fold greater number of residues bound in the bisureido linkage as compared to the monoureido linkage. The reaction of HDI with human serum albumin was quantified by combined assays of gas chromatography of the hexamethylenediamine formed after acid hydrolysis of the conjugate and by amino acid analyses of the protein. HDI isocyanate groups reacting with the lysyl residues and other sites on the protein molecule formed at least two reaction products, one which was easily hydrolyzable by acid and the other resistant to hydrolysis. The chemical structures proposed by these observations are similar to those of classical hapten derivatives and suggest that such derivatives may be immunogenic and/or allergenic in some workers exposed to the vapors of these reagents.

Diisocyanates are used extensively in various industries for the preparation of polyurethane foam. Two of the most important commmercial agents are toluene diisocyanate (TDI) and hexamethylene diisocyanate (HDI). These compounds are volatile and contain reactive isocyanates which may be toxic to mucous membranes especially conjunctivae and the respiratory tract (Zapp, 1957). In addition, asthma-like reactions and hypersensitivity pneumonitis may occur in workers after exposure to small amounts of various isocyanate compounds (Charles *et al.*, 1976; Peters and Wegman, 1975).

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In 1973, the National Institute of Occupational Safety and Health reported that about 5% of workers exposed to volatile isocyanate compounds developed acute or chronic respiratory symptoms and about 10% of affected workers experienced an asthmatic syndrome (NIOSH, 1973; Peters, 1975). The relatively long latent period between initial exposure and the development of signs and symptoms after exposure to low concentrations of isocyanates suggested an adaptive immune response. The latency period varied from as short as 2 weeks to as long as several years. If the concept of hypersensitivity is correct, it is presumed that isocyanates combine covalently with body proteins to form hapten–protein conjugates, the immunologic specificity of which would be primarily determined by the isocyanate haptenic moeity (Bruckner *et al.*, 1968). The current threshold limit value for TDI is 0.02 ppm, but some sensitive individuals respond to concentra-

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tions as low as 0.005 ppm (Porter *et al.*, 1975).

While numerous investigators have studied the toxicity of aromatic isocyanates such as TDI, few have examined the effects of aliphatic isocyanates, the most important example of which is HDI. Lomonova and Frolova (1968) reported that single or repeated inhalations of HDI in mice, rats, and rabbits caused inflammation of conjunctivae and respiratory mucous membranes. In other experiments (Kondrat'ev and Mustaev, 1971) eosinophilia, lymphopenia, and neutrophilia were observed in peripheral blood of sensitized animals. Although both TDI and HDI were allergenic when applied to the skin of experimental animals, HDI appeared to have a stronger sensitization potential. In these experiments, sensitization of the skin was also accompanied by a change in the amount of serum albumin. Another report (Kondrat'ev and Mustaev, 1969) noted that skin contact with HDI under industrial conditions caused allergic contact dermatitis in some workers.

The isocyanate group (NCO) of various aliphatic and aromatic molecules is responsible for the covalent attachment to proteins, which may then induce the biological effects cited above. At physiologic conditions isocyanates react with amines to form substituted ureas, or with alcohol to form urethanes. Even after isocyanate moeities are bound to protein they can hydrolyze to form an amine and carbon dioxide (Lowe, 1970). However, since the initial reaction with amine groups is so much faster than with other chemical moeities at alkaline pH, this is presumed to be the most probable derivative which produces a biological effect. As proteins contain such reactive amines and the classical work with other haptens shows that such protein derivatives can result in an immune response (Landsteiner, 1962), the formation of such derivatives may be an essential precursor for sensitization.

Since more precise chemical characterization of these hapten–protein conjugates is a mandatory prerequisite for exploring the immunologic effects of these compounds, the purpose of this investigation is to study and quantify the reaction kinetics of coupling TDI and HDI to human serum albumin.

METHODS

Reagents

Reagent grade TDI and HDI were donated by Mobay Chemical Corporation, Pittsburgh, Pennsylvania. 2-Naphthol-3,6-disulfonic acid disodium salt and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were purchased from Eastman Organic Chemicals, Rochester, New York. Succinic anhydride and *p*-toluidine were obtained from Aldrich Chemical Company, Milwaukee, Wisconsin. Human serum albumin (HSA, 25% U.S.P., salt poor) was acquired from Cutter Laboratories, Inc., Berkeley, California.

Preparation of Isocyanate–Protein Conjugates

All isocyanate–protein conjugates were prepared by a standard protocol. A 1% protein solution of HSA was prepared by adding 4 ml of 25% HSA to 96 ml of phosphate-buffered saline (PBS, 0.08 M sodium phosphate, 0.03 M potassium phosphate, 0.15 M sodium chloride buffered to pH 7.4). Aliquot of 10 ml of the protein solution was used as the unconjugated control (0 min). Reaction of isocyanates with HSA were carried out under a well-ventilated hood at room temperature (25 ± 1°C). Isocyanate reagents [2 ml (2.4 g) TDI; 2 (2.1 g) or 0.2 ml (0.21 g) HDI] were added to the remaining protein solution (90 ml) with constant stirring. Ten-milliliter aliquots were taken at 1, 5, 10, 15, 20, 60, and 180 min after the beginning of the reaction and an equal volume of 2 M ammonium carbonate was added to each aliquot to stop reactions. All samples were centrifuged at 3000*g* for 20 min in order to remove unreacted isocyanate and any precipitate and then were dialyzed overnight three times with 4 liters of 0.1 M ammonium carbonate.

A portion of each isocyanate–protein conjugate was further purified by precipitation with an equal volume of 20% trichloroacetic acid (TCA). The protein precipitate was then redissolved in 1.0 M sodium hydroxide and dialyzed with distilled water overnight several times. This procedure removed any small molecular weight polymers of toluene or hexylamine diisocyanate.

Aliquots (0.5 ml) of TCA-precipitated, NaOH-dissolved protein conjugates were hydrolyzed for 24 or 140 hr with 6 N hydrochloric acid at 110°C under vacuum. The hydrolyzed samples were dried under vacuum to remove hydrochloric acid and then dissolved in 0.5 ml of PBS prior to assays for isocyanate content and the amino acid residues.

Chemical Assays of Isocyanate Bound, Protein Content, Amino Acid Residues, and Free Amino Groups

A modified Gutmann assay (Modesto and Pesce, 1973) was used to measure the amount of isocyanate bound to protein by either the monoureido or bisureido linkage. Briefly, the assay was performed on 0.1 ml aliquots of samples to which were added 0.1 ml of concentrated HCl, 0.1 ml of water, and 0.4 ml of a 0.029 M solution of $NaNO_2$. After 5 min of reaction time, 1.2 ml of a 0.031 M solution of sodium 2-naphthol-3,6-disulfonate in 7.4 M NH_4OH was added. Each assay tube was mixed thoroughly, allowed to stand for 5 min, and read spectrophotometrically at 500 nm. The amount of isocyanate bound was measured from a standard calibration curve constructed from five different concentrations of *p*-toluidine.

The amount of amine bound isocyanate (hexamethylene diamine, HDA) in HDI–protein conjugates was determined by a gas chromatographic method (Sandridge, 1978) after acid hydrolysis of protein samples. Hydrolyzed samples were dissolved in 1 ml of 0.1 N aqueous NaOH and 5 μl of this solution was injected into the gas chromatographic column which was equipped with a flame inonization detector. Peak areas of the chromatograms were measured and the corresponding concentration of HDA was found by comparison with a standard calibration curve of HDA. Triplicate analyses were performed on each sample and each calibration standard. Specific conditions of gas chromatography were: column temperature, 200°C; injector temperature, 300°C; detector temperature, 320°C; carrier gas, nitrogen at column pressure of 29 psi; stainless-steel column, 0.32 cm in diameter and 2 m in length, containing 25% Apiezon L. plus 10% KOH coated on Chromosorb W-H.P. (60/80 mesh).

To determine whether isocyanate binding occurred at sites other than amino groups, some conjugates were prepared with succinylated HSA solutions which contained no free amino acid residues. These solutions were prepared by dissolving 4 ml of 25% HSA in 46 ml of half saturated sodium acetate buffer and gradual addition of 1500 mg of succinic anhydride at pH 8.0 and 4°C (Habeeb *et al.*, 1958). "Succinylated" protein solutions were dialyzed with PBS at pH 7.4 overnight and reconstituted to a final volume of 100 ml with PBS.

Protein content of various isocyanate–protein conjugates was determined by adding 2 ml of Biuret reagent (2 g of $CuSO_4 \cdot 5H_2O$ dissolved in 100 ml of distilled water and 500 ml of 25% NaOH) to 1 ml of protein samples. Absorbance of these solutions was read at 540 nm after 30 min and the protein content determined from a standard curve constructed for known concentrations of HSA.

Amino acid assays of hydrolyzed isocyanate–protein conjugates were determined in a Durrum D-500 amino acid analyzer. Samples were reacted with ninhydrin to form high colored compounds (Spackman *et al.*, 1958). Absorbance of light at 590 and 440 nm by these compounds was recorded graphically and expressed in nanomoles of each measurable amino acid. The coefficient of variation for each of the amino acids was less than 5%.

Determination of free amino groups in protein was accomplished by the trinitrobenzene sulfonic acid (TNBS) method (Habeeb, 1966). Four percent $NaHCO_3$ was added to protein solutions or standard (HSA 10 mg/ml) at pH 9.0. Aqueous TNBS (0.1%) was added to each reactant mixture for 2 hr at 40°C. After acid hydrolysis with 1 N HCl and 10% sodium lauryl sulfate, absorbance of solutions at 344 nm was determined and substituted in a formula used to calculate the number of amino acid groups.

$$\text{Amino acid groups} = \frac{\text{absorbance} \times \text{volume of solution (ml)} \times 1000}{\text{extinction coefficient } (1.0765 \times 10^4) \times \mu\text{mol protein}}$$

RESULTS

Reaction of Toluene Diisocyanate and Human Albumin (TDI–HSA)

TDI reacts with protein by forming either mono- or bisureido protein derivatives, presumably with the ε-amino group of lysine residues within the protein molecule. The monoureido derivative forms a classical type of hapten carrier determinant while the bisureido derivative may form by either inter- or intramolecular crosslinking (Pesce *et al.*, 1976). Under the preparatory conditions above, a significant amount of the protein precipitated in the first few minutes of reaction and TDI bound protein was maximal at 20 min of reaction time. Further substitution caused more protein to precipitate from solution (Fig. 1). By quantifying the monoureido protein derivative with the Gutmann assay, the number of possible crosslinking isocyanate molecules could be estimated. The amount of bisureido protein derivative was determined by subtracting the total amount of isocyanate bound from the amount of monoureido derivative. As shown in Fig. 1, the amount of isocyanate bound increased as the time of reaction increased.

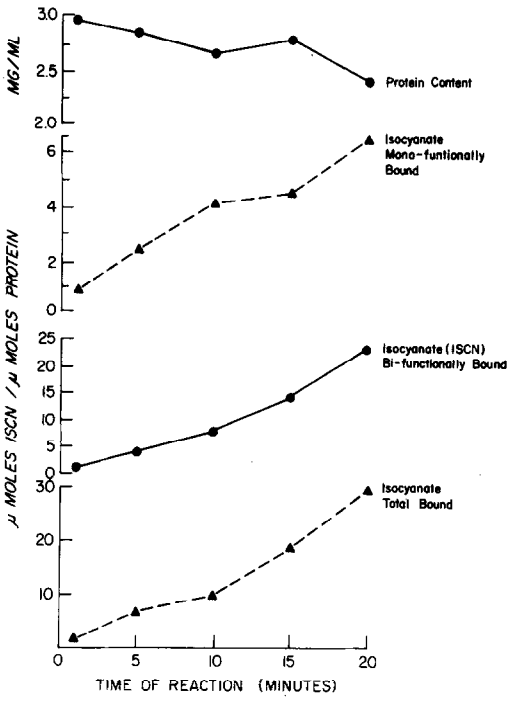

FIG. 1. Rate of reaction of toluene diisocyanate with human serum albumin (TDI–HSA). Upper Panel: protein concentration. Second Panel: μmol of isocyanate monofunctionally bound per μmol protein. Third Panel: μmol of isocyanate bifunctionally bound per μmol protein. Lower Panel: μmol of isocyanate totally bound per μmol protein.

After 20 min of reaction, a total of 29.7 μmol of isocyanate had reacted with HSA. Conjugation occurred in both bisureido (78%) and monoureido (22%) derivatized forms. The ratio of mono- to bisureido forms varied from 1 : 1 after 5 min of reaction to 1 : 3.5 after 20 min.

Amino acid analysis of unconjugated HSA and 5, 10, and 20 min TDI–HSA conjugates after 24 hr of acid hydrolysis revealed the same number of recovered lysine residues in all samples. The ease of accomplishing complete hydrolysis in both unconjugated and conjugated samples indicates that the major reaction product between TDI and HSA is an easily hydrolyzable linkage, probably of a mono- or bisureido protein derivative, although a urethane bondage cannot be ruled out from these data.

Reaction of Hexamethylene Diisocyanate and Human Serum Albumin (HDI–HSA)

The preparatory conditions of HDI–HSA conjugates were the same as TDI–HSA except that two levels of substitution were used. High (2.1 g) and low (0.21 g) concentrations of HDI per 0.9 g HSA protein were studied.

Binding of HDI to HSA determined by a chromatographic method. To determine the amount of isocyanate bound to protein at different times (0, 1, 5, 10, 15, 20, 60, and 180 min), each sample was hydrolyzed with 6 N HCl at 110°C under vacuum for both 24 and 140 hr. The kinetics of both high and low concentrations of HDI–HSA were similar in that the amount of isocyanate bound to protein as the amine (HDA) increased as the time of reaction increased (Figs. 2 and 3). However, the reaction of HDI with HSA during the first 20 min was relatively rapid followed by a slower reaction which continued throughout the 180 min of the experiment. HDA recovery from the low concentration HDI–HSA conjugate was greater when the time of hydrolysis was increased from 24 to 140 hr (Fig. 3). Even greater HDA recovery was obtained in the high concentration HDI–HSA reaction after 140 hr

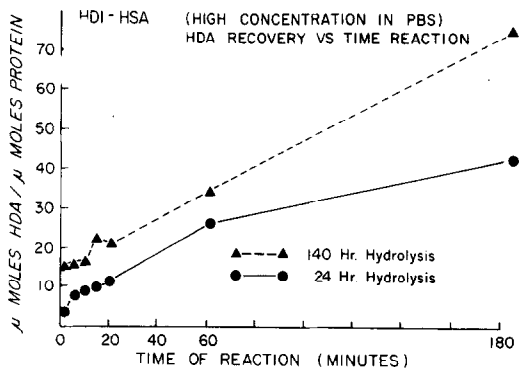

FIG. 2. Rate of reaction of hexamethylene diisocyanate (HDI 2.1 g) with 1% human serum albumin (HDI–HSA) expressed as μmol of hexamethylenediamine (HDA) bound per μmol protein. – – –, Recovery of HDA after 140 hr of hydrolysis; ——, recovery of HDA after 24 hr of hydrolysis.

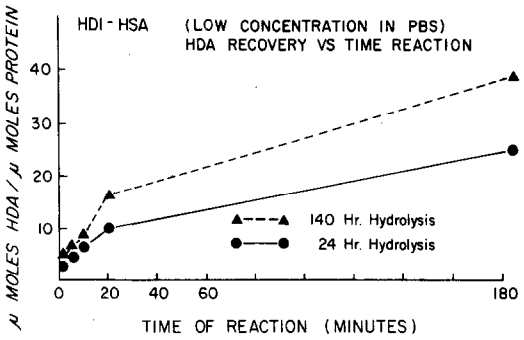

FIG. 3. Rate of reaction of hexamethylene diisocyanate (HDI 0.21 g) with 1% human serum albumin (HDI–HSA) expressed as μmol of hexamethylenediamine (HDA) bound per μmol protein. – – –, Recovery of HDA after 140 hr of hydrolysis; ——, recovery of HDA after 24 hr of hydrolysis.

hydrolysis (Fig. 2). Of the total of 75 μmol of HDA bound/μmol protein, only 42 μmol were found in the 24-hr hydrolysis sample suggesting that some products were relatively resistant to hydrolysis and therefore were nonlysyl residues.

Protein content determinations of the HDI–HSA solution showed no significant decreases, and it was concluded that protein precipitation did not occur beyond 20 min of reaction time as compared to TDI–HSA reactions.

Binding of HDI to HSA determined by amino acid analysis. Since HDI is thought to react preferentially with lysine residues of HSA at physiologic pH, comparative amino acid analysis of unconjugated HSA (with unreacted lysine groups) and HDI–HSA (with altered lysine groups) served as another method of quantifying chemical reactivity of HDI, HDI–HSA conjugates were hydrolyzed for 24 hr *in vacuo* at 110°C and then assayed for amino acid content. Table 1 shows the recovery of amino acids from HSA before and after treatment with HDI. The only significant changes in various amino acid residues occurred in lysyl residues. Moreover, the reduction of lysyl groups determined by amino acid analysis showed that more lysyl groups were bound (i.e., inactivated) as reaction time increased. These

TABLE 1

AMINO ACID ANALYSIS DATA
HDI–HSA (LOW CONCENTRATION IN PBS)
24 hr HYDROLYSIS

Amino acid residues	Sample (min)[a]	
	0	180
Aspartic acid	57.50	57.00
Threonine	29.00	28.68
Serine	23.67	21.38
Glutamic acid	89.83	88.91
Proline	27.51	28.01
Glycine	13.92	13.70
Alanine	65.83	66.32
Valine	42.30	44.33
Methionine	6.90	6.82
Isoleucine	8.95	8.95
Leucine	66.43	66.56
Tyrosine	17.90	17.72
Phenylalanine	32.22	36.78
Histidine	15.60	16.14
Ammonia	46.14	49.09
Arginine	26.05	26.61
Lysine	47.36[b]	22.60[b]

[a] The results are given in nmol.

[b] The only marked change in amino acid residues are the lysine groups.

data also confirmed the premise that HDI reacted with ε-amino groups of lysine residues within the protein molecule.

Determination of optimal hydrolysis time. To determine the optimal time required for complete hydrolysis of the HDI–HSA conjugate, various aliquots of HDI–HSA (prepared at a reaction time of 180 min) were hydrolyzed for 24, 48, 72, 96, 120, and 144 hr and assayed for the number of lysyl groups recovered by amino acid analysis. The number of lysyl groups reached a maximum between 120 and 140 hr of hydrolysis (Fig. 5). The number of micromoles of HDA released, as determined by chromatographic assay, was maximal after 144 hr of hydrolysis (Fig. 5). Fifty-two HDA residues were recovered per mole of protein after 24 hr of hydrolysis, while only 17 lysyl residues were recovered in the same solution. Thus, 3 μmol of HDA were recovered per mole of lysine. One probable explanation is

that a number of HDI groups reacted with other sites on the protein molecule.

Binding of HDI to succinylated HSA. Possible reactivity of NCO groups with other functional sites in the protein molecule was determined by analysis of conjugates of HDI and succinylated HSA. Succinylation of HSA with succinic anhydride under mildly alkaline conditions blocked more than 98% of the amino groups in the protein when the number of amino groups of unconjugated HSA was compared with succinylated HSA by the TNBS reaction (Habeeb, 1966). HDI-Succinyl HSA conjugates were prepared by adding 2 ml (2.1 g) of HDI to the "succinylated" protein. HDA content and the reduction of lysyl groups of the succinyl conjugates were determined after acid hydrolysis. The presence of significant amount of HDA recovered after acid hydrolysis of the succinyl HDI–HSA conjugate (18 to 24 residues) provided additional evidence that NCO groups had probably reacted with nonlysyl reactive sites in the protein molecule. Furthermore, amino acid analysis of the HDI–HSA conjugate in this experiment showed essentially complete recovery of the lysyl residues after 24 hr of hydrolysis on all samples treated (0, 1, 5, 10, 15, 20, 60, 120, and 180 min), indicating that lysine groups were not inactivated or bound by HDI.

DISCUSSION

Previous experimental and clinical investigations of possible immunopathology induced by isocyanate compounds may have been seriously hampered by variability of hapten–protein conjugates prepared by different investigators. Since TDI is highly reactive and may undergo rapid autopolymerization or variable degrees of intra- and interprotein crosslinking, the degree of protein substitution with this ligand may have varied from batch to batch. These studies were therefore undertaken to determine the most optimal procedures for preparation and characterization of aromatic and aliphatic isocyanate protein conjugates. Detailed analysis of the kinetics of these chemical reactions substantiated the complexity of isocyanate–protein interactions and the mandatory requirement for proper chemical characterization of these conjugates if they are to be used as meaningful immunologic reagents.

Since preparatory conditions of all conjugates were uniform except for reaction times (5, 10 and 20 min), chemical differences between various reactant mixtures could be related to reaction time. Thus, the ratio of bis- to monoureido derivatives in TDI–HSA preparations varied from 1 : 1 after 5 min of reaction to 3.5 : 1 after 20 min of reaction. On the other hand, the ease with which hydrolysis of the bond between isocyanate groups of TDI and protein could be accomplished was independent of reaction time because the same number of lysine residues were recovered from TDI–HSA conjugates prepared at different reaction times after 24 hr of acid hydrolysis of these samples.

Apart from their importance in estimating the amount of autopolymerization and protein crosslinking in TDI protein complexes, the number of monoureido derivatives in these conjugates may have special immunochemical significance. Significant titers of IgE antibodies specific for the monofunctional *p*-tolyl adduct have been demonstrated recently in some TDI-sensitive workers (Karol *et al.*, 1978). If immunologic specificity of these reactions is ultimately proven to be restricted to the *p*-tolyl monofunctional derivative, it is apparent that future quantitative determination of monoureido substitution in TDI–protein conjugates will be obligatory.

Simultaneous chromatographic (HDA recovery) and amino acid analyses of two different concentrations of HDI–HSA conjugates revealed other important chemical characteristics of isocyanate–protein reactions. In these experiments comparative data could be obtained on adducts prepared under conditons of much longer reaction times

(180 min) than was possible with the TDI–HSA conjugates. Thus, it was demonstrated that recovery of both HDA and lysyl amino acid residues was relatively greater from both concentrations of HDI–HSA conjugates prepared at a reaction time of 20 min than similar adducts formed during longer reaction times (Figs. 2, 3, and 4). Since conjugates prepared under reaction times greater than 20 min were relatively resistant to prolonged acid hydrolysis, it was evident that isocyanate substitution of these adducts also occurred at sites other than the ε-amino group on the protein molecule. This was corroborated by analysis of HDI–succinyl HSA preparations in which the amino acid residues were blocked by the "succinylation" process. Recovery of HDA from various conjugates prepared at different reaction times after 24 and 144 hr of hydrolysis was similar, thereby suggesting that HDA bonds formed in these conjugates were relatively resistant to hydrolysis. In contrast, similar assays of HDI conjugated to unblocked HSA revealed increased hydrolysis and HDA recovery of samples hydrolyzed for 144 hr (Fig. 5). Taken together, these series of experiments suggest that complete characterization of isocyanate–protein conjugates will require material balance studies to account for ligand substitution at protein sites (e.g., hydrosyl or carboxyl) other than lysyl residues.

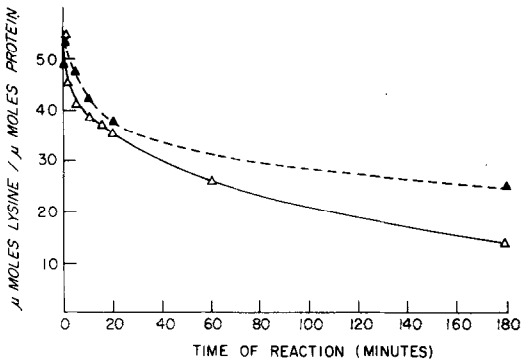

FIG. 4. Recovery of lysine residues from HDI–HSA conjugates prepared by varying incubation times. Samples were hydrolized for 24 hr. Data are expressed as μmol lysine per μmol protein. ▲, Low concentration of HDI, 2.1 g in 1% HSA; △, high concentration of HDI, 0.21 g in 1% HSA.

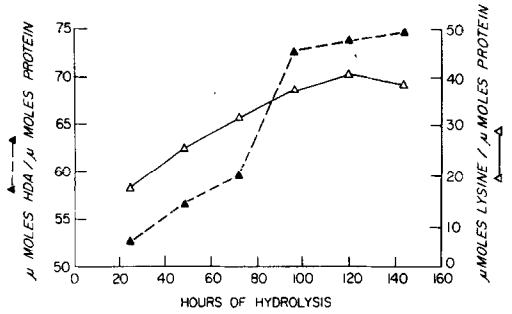

FIG. 5. Recovery of lysine residues and HDA from HDI–HSA conjugates as a function of hydrolysis time. The conjugate prepared by incubation for 180 min with a high concentration of HDI (2.1 g in 1% HSA) was hydrolized for increasing periods of time. Left ordinate (▲) is the recovery of HDA. Right ordinate (△) is the recovery of lysine residues.

In summary, it is concluded that reaction time is the most important determinant of protein conjugation with both aromatic and aliphatic isocyanates. Although ligand binding to lysyl residues is an important chemical reaction of these hapten–protein conjugates, we also found evidence to support isocyanate substitution at nonlysyl sites. Despite these problems of complete chemical characterization, we believe that a standard protocol of preparing and analyzing isocyanate protein conjugates is required to obtain stable and immunologic reagents suitable for experimental and clinical investigations of isocyanate hypersensitivity reactions.

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