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Protein Thiolation and Reversible Protein–Protein Conjugation

N-SUCCINIMIDYL 3-(2-PYRIDYLDITHIO)PROPIONATE, A NEW HETEROBIFUNCTIONAL REAGENT

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A heterobifunctional reagent, *N*-succinimidyl 3-(2-pyridyldithio)propionate, was synthesized. Its *N*-hydroxysuccinimide ester group reacts with amino groups and the 2-pyridyl disulphide structure reacts with aliphatic thiols. A new thiolation procedure for proteins is based on this reagent. The procedure involves two steps. First, 2-pyridyl disulphide structures are introduced into the protein by the reaction of some of its amino groups with the *N*-hydroxysuccinimide ester side of the reagent. The protein-bound 2-pyridyl disulphide structures are then reduced with dithiothreitol. This reaction can be carried out without concomitant reduction of native disulphide bonds. The technique has been used for the introduction of thiol groups *de novo* into ribonuclease, γ-globulin, α-amylase and horseradish peroxidase. *N*-Succinimidyl 3-(2-pyridyldithio)propionate can also be used for the preparation of protein–protein conjugates. This application is based on the fact that protein–2-pyridyl disulphide derivatives (formed from the reaction of non-thiol proteins with the reagent) react with thiol-containing proteins (with native thiols or thiolated by, for example, the method described above) via thiol–disulphide exchange to form disulphide-linked protein–protein conjugates. This conjugation technique has been used for the preparation of an α-amylase–urease, a ribonuclease–albumin and a peroxidase–rabbit anti-(human transferrin) antibody conjugate. The disulphide bridges between the protein molecules can easily be split by reduction or by thiol–disulphide exchange. Thus conjugation is reversible. This has been demonstrated by scission of the ribonuclease–albumin and the α-amylase–urease conjugate into their components with dithiothreitol. *N*-Succinimidyl 3-(2-pyridyldithio)propionate has been prepared in crystalline form, in which state (if protected against humidity) it is stable on storage at room temperature (23°C).

A number of methods exist for the modification of functional groups in proteins. Many of them involve bifunctional reagents, i.e. reagents with two reactive groups that are capable of reacting with, and forming bridges between, the side chains of certain amino acids in the proteins. The cross-links or bridges may be either of the intra- or inter-molecular type.

Intramolecular cross-linking has been used to introduce additional tertiary structure into proteins, e.g. enzymes, with the hope of increasing their conformational stability (Wold, 1972; Zaborsky, 1973) and to measure inter-residue distances in proteins (Wold, 1972). Intermolecular cross-linking, on the other hand, can be used to bind proteins of the same or different kinds to each other (protein–protein conjugation) and to modify cell membranes or other macromolecular assemblies (Wold, 1972; Zaborsky, 1973). The control of intra- versus inter-molecular cross-linking, however, is very difficult to achieve with homobifunctional reagents (where the two reactive groups are identical), which type of reagent has so far been most prevalent. Some control can be achieved through appropriate choice of reaction parameters such as protein/reagent ratio, pH, ionic strength etc. With a heterobifunctional reagent, where the two reactive groups are directed toward different functional groups, one can usually conduct the coupling and the cross-linking in separate sequential steps. There have been very few reports that point this out.

In this work the synthesis and properties of a hetero directed heterobifunctional reagent *N*-succinimidyl 3-(2-pyridyldithio)propionate are described. With this reagent intermolecular cross-linkages can easily be introduced between proteins without the concomitant formation of intramolecular cross-linkages. It should therefore have great potential as a protein–protein conjugation reagent. Unlike most other conventionally used protein-

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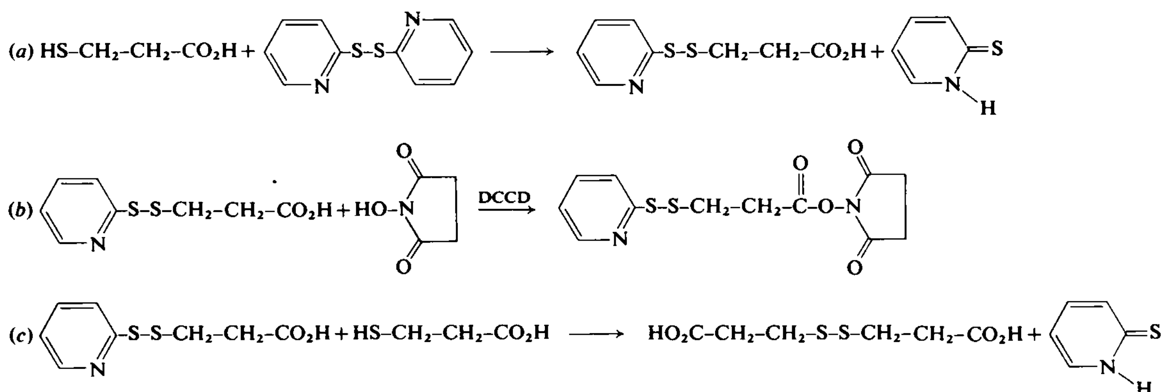
conjugation techniques, reactions involving *N*-succinimidyl 3-(2-pyridyldithio)propionate can be controlled to give conjugates of different proteins (heteroconjugates), without unwanted cross-reaction products such as the homoconjugates of each of the two proteins. The size of the conjugates can also be reasonably well controlled, e.g. bimolecular, termolecular or polymeric conjugates can be prepared. Finally, *N*-succinimidyl 3-(2-pyridyldithio)-propionate also presents a new reagent for the introduction of aliphatic thiols into amino group-containing molecules such as proteins.

Materials and Methods

2,2′-Dipyridyl disulphide was purchased from Aldrich-Europe (B-2340 Beerse, Belgium); 3-mercaptopropionic acid, *N*-hydroxysuccinimide (purum) and dicyclohexylcarbodi-imide (purum) were obtained from Fluka A.G. (Buchs SG, Switzerland). Aluminiumoxid aktiv basisch (Aktivitätsstufe 1), DC-Fertigplatten Aluminiumoxid 60F 254 (type E), Nessler's Reagent A [potassium tetra-iodomercurate(II) solution], Nessler's Reagent B (NaOH solution), soluble starch (Zulkowski), and 3,5-dinitrosalicylic acid were from E. Merck (Darmstadt, Germany). Pyridine-2-thione (2-mercaptopyridine) was purchased from E.G.A.-Chemie K.G. (Steinheim, Germany). Dithiothreitol, cytidine 2′:3′-cyclic monophosphoric acid, bovine γ-globulin (Cohn fraction II, stock no. BG-11), bovine serum albumin (crystallized and freeze-dried), α-amylase (bacterial type II-A; 4×crystallized), urease from jack beans (type III) and ribonuclease A from bovine pancreas were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Peroxidase from horseradish [Reinheitsgrad 1, suspension in $(NH_4)_2SO_4$], purchased from Boehringer G.m.b.H., Mannheim, Germany, was, before use, transferred to 0.1 M-sodium phosphate buffer/0.1 M-NaCl, pH 7.5, by gel filtration on Sephadex G-25. Sephadex G-25, G-75 and Sepharose 6B were from Pharmacia Fine Chemicals A.B. (Uppsala, Sweden). 2,2′-Dicarboxyethyl disulphide was prepared from 3-mercaptopropionic acid by oxidation with H_2O_2. Rabbit anti-(human transferrin) antibodies were obtained from Dakopatts A/S, Denmark. Other chemicals were of the highest commercial grade.

Synthesis of N-succinimidyl 3-(2-pyridyldithio)-propionate

This was performed by a two-step procedure. First 3-mercaptopropionic acid was reacted by thiol–disulphide exchange with 2,2′-dipyridyl disulphide to give 2-carboxyethyl 2-pyridyl disulphide (Scheme 1*a*). This disulphide was then converted into *N*-succinimidyl 3-(2-pyridyldithio)propionate by esterification with *N*-hydroxysuccinimide by using *NN*′-dicyclohexylcarbodi-imide (Scheme 1*b*). The product of the first reaction, 2-carboxyethyl 2-pyridyl disulphide, can undergo thiol–disulphide



Scheme 1. *Synthesis of N-succinimidyl 3-(2-pyridyldithio)propionate*
3-Mercaptopropionic acid is first reacted with 2,2′-dipyridyl disulphide (*a*). The product thus formed, 2-carboxyethyl 2-pyridyl disulphide, is then converted into *N*-succinimidyl 3-(2-pyridyldithio)propionate by esterification with *N*-hydroxysuccinimide with dicyclohexylcarbodi-imide (DCCD) as condensing agent (*b*). The main product of the first step, 2-carboxyethyl 2-pyridyl disulphide, can react with 3-mercaptopropionic acid to give the 2,2′-dicarboxyethyl disulphide (*c*). This unwanted side reaction can be almost eliminated by using an excess of 2,2′-dipyridyl disulphide in reaction (*a*) and by adding 3-mercaptopropionic acid dropwise to the 2,2′-dipyridyl disulphide solution with vigorous stirring.

exchange with 3-mercaptopropionic acid (Scheme 1*c*) to form 2,2′-dicarboxyethyl disulphide. However, this side reaction was almost completely suppressed by using a 2-fold molar excess of 2,2′-dipyridyl disulphide.

Preparation of 2-*carboxyethyl* 2-*pyridyl disulphide*. 2,2′-Dipyridyl disulphide (3.75g) was dissolved in 10ml of ethanol (99.5%) and 0.4ml of glacial acetic acid added. The solution was stirred vigorously and 0.9g of 3-mercaptopropionic acid in 5ml of ethanol (99.5%) added dropwise. The reaction mixture was left at 23°C for 20h. Ethanol was then removed by evaporation (Büchi Rotavapor at 10mmHg pressure and at 40°C). In the resulting pale-yellow oil there was a small amount of precipitate. This precipitate was removed by centrifugation after the oil had been dissolved in a few millilitres of dichloromethane/ethanol (3:2, v/v). The dissolved oil was diluted with the same solvent (dichloromethane/ethanol) to 10ml and then applied to a column (2cm×24cm) containing 75ml of Al_2O_3 (equilibrated with dichloromethane/ethanol). The column was then eluted with the same solvent (dichloromethane/ethanol). T.l.c. on Al_2O_3 plates demonstrated that the main reaction product, 2-carboxyethyl 2-pyridyl disulphide, and also the by-product 2,2′-dicarboxyethyl disulphide, adsorbed strongly on the Al_2O_3 under these conditions. Thus both remained at the top of the column. 2,2′-Dipyridyl disulphide and pyridine-2-thione, however, moved with the solvent front. When all the yellow colour had been washed from the column (after about 150ml of solvent) it was considered free from 2,2′-dipyridyl disulphide and pyridine-2-thione. This was also confirmed by t.l.c. of the eluate on Al_2O_3 plates with the same system and the reference substances pyridine-2-thione and 2,2′-dipyridyl disulphide.

The main product, 2-carboxyethyl 2-pyridyl disulphide, was then eluted with dichloromethane/ethanol (3:2, v/v) containing 4ml of acetic acid/100ml. Under these conditions the by-product 2,2′-dicarboxyethyl disulphide was retarded on the column and thus separated from the main product (confirmed by t.l.c. as described above). The fractions were tested for the presence of 2-carboxyethyl 2-pyridyl disulphide by spectrophotometric determination of the 2-thiopyridone formed when 0.1ml of the eluate was shaken with a few grains of dithiothreitol in 3ml of 0.1M-$NaHCO_3$ ($\lambda_{max.}$ = 343nm, $\varepsilon = 8.08\times10^3 M^{-1}\cdot cm^{-1}$) (Grassetti & Murray, 1967; Stuchbury *et al.*, 1975). The fractions containing 2-carboxyethyl 2-pyridyl disulphide were pooled and the solvent was removed by evaporation (at 10mmHg and 30°C). The resulting viscous oil, which had the odour of acetic acid, was redissolved in a small amount of dichloromethane/ethanol. The solvent was removed by evaporation. This procedure was repeated twice. Finally the solvent was removed by exhaustive evaporation for 3h at 23°C by using an evaporator connected to an oil-pump (approx. 1mmHg) and fitted with a cold-trap. The oil obtained, which no longer had the odour of acetic acid, was dissolved in 15ml of dichloromethane. After addition of anhydrous $MgSO_4$ the solution was kept in a sealed vessel at 23°C. The 2-carboxyethyl 2-pyridyl disulphide was not isolated. However, the yield based on 3-mercaptopropionic acid was found to be 40%. This was determined by measuring the pyridine-2-thione released on reduction of a small portion of the solution.

Preparation of N-succinimidyl 3-(2-*pyridyldithio*)-*propionate*. The dichloromethane solution of 2-carboxyethyl 2-pyridyl disulphide obtained as described above was filtered to remove $MgSO_4$. To the filtrate (15ml) was added 0.43g of *N*-hydroxysuccinimide. The mixture was stirred vigorously for 20min to dissolve most of the *N*-hydroxysuccinimide. Then, 0.78g of dicyclohexylcarbodi-imide was added and the reaction was allowed to proceed at 23°C (with occasional stirring) for 3½h. The solution was dried with anhydrous $MgSO_4$, filtered, and the dichloromethane removed by evaporation (Rotavapor at approx. 10mmHg and 23°C) to yield a pale-yellow oil containing a small amount of white precipitate. The oil was dissolved in cold (4°C) dichloromethane and the solid material was (*NN*′-dicyclohexylurea) removed by filtration. The oil remaining after evaporating the solvent was dissolved in the smallest possible volume of ethanol (approx. 10ml) and the solution was cooled to −20°C. The white crystals formed were filtered off and recrystallized from ethanol. The final product was then isolated by filtration and subsequently dried over anhydrous P_2O_5 for 24h.

The product was found to be analytically pure *N*-succinimidyl 3-(2-pyridyldithio)propionate (see below). The yield from the two steps, based on 3-mercaptopropionic acid, was 30% (m.p. 78.5–80.5°C) (Found: C, 46.19; H, 3.88; N, 8.96; S, 20.02. $C_{12}H_{12}O_4N_2S_2$ requires C, 46.14; H, 3.88; N, 8.97; S, 20.53%). δ (p.p.m.) (tetramethylsilane) 2.83 (4H, s, succinimidyl H), 3.12 (4H, s, $-CH_2-CH_2-$) and 6.90–8.82 (4H, m, pyridyl H).

Introduction of 2-*pyridyl disulphide structures into proteins by N-succinimidyl* 3-(2-*pyridyldithio*)-*propionate*

The protein (0.05–2μmol) was chromatographed on a Sephadex G-25 column with 0.1M-sodium phosphate buffer containing 0.1M-NaCl, pH7.5, as eluent. (This step was required to remove low-molecular-weight material that might react competitively with the reagent.) The fraction collected

in the void volume was adjusted to a volume of 2ml with the buffer and, depending on the degree of substitution desired (i.e. mol of 2-pyridyl disulphide structures/mol of protein), 0.01–0.3ml of *N*-succinimidyl 3-(2-pyridyldithio)propionate (20mM in 99.5% ethanol) was added dropwise to the stirred protein solution. The reaction mixture was left for about 30min at 23°C. Excess of reagent was then removed by gel filtration on Sephadex G-25. The content of 2-pyridyl disulphide structures in the modified proteins was determined by adding 0.1ml of 50mM-dithiothreitol in distilled water to a properly diluted protein solution (2.0ml). This treatment effects the release of pyridine-2-thione, which has a molar absorptivity of 8.08×10^3 at 343nm (Stuchbury *et al.*, 1975). The amount of pyridine-2-thione released is equivalent to the content of 2-pyridyl disulphide groups in the protein. Since the 2-pyridyl disulphide group has its own absorbance at 280nm (Grassetti & Murray, 1967) an erroneously high protein concentration will be obtained when calculated on the basis of absorbance at 280nm. The additional absorbance can be allowed for by subtracting the A_{280} contribution of the 2-pyridyl disulphide group from the total A_{280} [as calculated by using the following expression: (concentration of 2-thiopyridone released on reduction)$\times 5100 = A_{280}$ due to 2-pyridyl sulphide group (J. Carlsson & H. Drevin, unpublished work)]. In the case of large proteins with a low degree of substitution (1–2mol of 2-pyridyl disulphide groups/mol of protein) the error, however, is not significant. The modified proteins were stored dissolved in 0.1M-sodium phosphate buffer/0.1M-NaCl, pH7.5, at 4°C.

Thiolation of proteins by N-succinimidyl 3-(2-*pyridyldithio)propionate*

2-Pyridyl disulphide groups were introduced into the protein by *N*-succinimidyl 3-(2-pyridyldithio)propionate as described above. The molar excess of reagent ([reagent]/[protein] ratio) was chosen to give the desired degree of substitution (see Table 2).

The protein–2-pyridyl disulphide derivative was then transferred to sodium acetate buffer, pH4.5, containing 0.1M-NaCl, by dialysis or gel filtration. Dithiothreitol dissolved in a small volume of the acetate buffer mentioned above was then added to a final concentration of 25mM. This treatment effects the reduction of the protein-bound 2-pyridyl disulphide bonds without concomitant reduction of native protein disulphide bonds. In the case of proteins lacking native disulphide bonds, e.g. α-amylase, the reduction can of course be performed at alkaline pH with a large excess of dithiothreitol or other low-molecular-weight thiol compounds. After treatment for 30–40min the thiolated protein was separated from the low-molecular-weight material by gel filtration on Sephadex G-25. The thiol content of the protein was estimated by reaction with 2,2′-dipyridyl disulphide (Grassetti & Murray, 1967; Stuchbury *et al.*, 1975).

Since the thiol group is very reactive and can take part in unwanted reactions we recommend that the modified protein be stored in the pyridyl disulphide form and be reduced just before the protein thiol is used.

Preparation of protein–protein conjugates by N-succinimidyl 3-(2-*pyridyldithio)propionate*

The technique should be applicable to all proteins containing amino groups that are reactive toward *N*-succinimidyl 3-(2-pyridyldithio)propionate. To demonstrate the method α-amylase was bonded to urease, ribonuclease to bovine mercaptalbumin and peroxidase to rabbit anti-(human transferrin) antibodies.

Preparation of ribonuclease–mercaptalbumin conjugate. Bovine serum albumin (80mg or 1.200nmol containing 490nmol of mercaptalbumin) was dissolved in 1.95ml of 0.1M-sodium phosphate buffer, pH7.5, containing 0.1M-NaCl. Ribonuclease was treated with *N*-succinimidyl 3-(2-pyridyldithio)-propionate as described above to give a derivative containing 2.1 mol of 2-pyridyl disulphide groups/mol of enzyme. This derivative (6.9mg; 500nmol) in 0.5ml of 0.1M-sodium phosphate buffer, pH7.5, containing 0.1M-NaCl was mixed with the albumin solution. The reaction was followed by measuring the pyridine-2-thione released as a result of the thiol–disulphide exchange reaction. After 40min, when the reaction was almost complete as judged from the amount of pyridine-2-thione released, the reaction mixture was gel-filtered on a Sepharose 6B column (2cm×63cm) with 0.3M-NacCl as the medium. The u.v.-absorbance and ribonuclease activity of the fractions were determined.

As a control, a mixture of native ribonuclease (6.9mg) and native albumin (80mg) in 2.5ml of 0.1M-phosphate buffer, pH7.5, containing 0.1M-NaCl was also gel-filtered on a Sepharose 6B column to determine whether any conjugate held together by non-covalent bonds was formed. To one of the fractions containing ribonuclease–albumin conjugate (1.0ml) was added solid urea and 2-mercaptoethanol to give final concentrations of 8M and 0.45M respectively. The solution was adjusted to pH8 by addition of 2M-NaOH. Under these conditions ribonuclease is completely reduced (White, 1972*a*). After 2h at 23°C the mixture was gel-filtered on a Sephadex G-75 column in 0.1M-Tris/HCl buffer, pH8.5, containing 0.3M-NaCl. The A_{280} of the fractions was determined. The fractions were left standing at about 23°C for 3h to allow the ribonuclease to

Table 1. *Values used for the calculations of the concentrations of the proteins used in the experiments*

Protein	Molecular weight		$A_{280}^{0.1\%}$	Reference
α-Amylase (bacterial)	50000		2.6	Caldwell *et al.* (1952)
Urease (jack-bean)	480000		0.589	Blakely *et al.* (1969)
Ribonuclease-A (bovine)	13700		0.73	Worthington Enzyme Manual (1972)
Albumin (bovine)	66000	($A_{278}^{0.1\%}$)	0.67	Carlsson & Svenson (1974)
γ-Globulin (bovine)	160000		1.38	Sober (1968)
Rabbit anti-(human transferrin) antibodies	160000		1.38	Sober (1968)
Peroxidase (horseradish)	40000	($A_{403}^{0.1\%}$)	2.5	Worthington Enzyme Manual (1972)

reoxidize (White, 1972*a*). Thereafter the fractions were adjusted to pH 7 and the ribonuclease activity was determined as described below.

Preparation of α-amylase–urease conjugate. Urease (1g) in 10ml of 0.3M-NaCl was gel-filtered on a Sepharose 6B column (2cm×60cm) in 0.1M-sodium phosphate buffer, pH7.5, containing 0.1M-NaCl. The fractions corresponding to the main ureolytic activity peak (160nmol of urease) were pooled and concentrated to 7ml with a collodion tube. An α-amylase–2-pyridyl disulphide derivative containing 1.3mol of 2-pyridyl disulphide groups/mol of enzyme was prepared as described above. Then 176nmol of this derivative in 2ml of 0.1M-sodium phosphate buffer, pH7.5, containing 0.1M-NaCl was mixed with the urease solution. After 30min, when the thiol–disulphide exchange reaction was almost complete as judged by the quantity of pyridine-2-thione released, the reaction mixture was gel-filtered on a column (2cm×63cm) of Sepharose 6B in 0.3M-NaCl. The A_{280} absorbance, as well as the ureolytic and α-amylase activities, of the fractions were determined. Two identical samples (1.0ml) were taken from one of the fractions containing the α-amylase–urease conjugate (see arrow in Fig. 5*a*). One of the samples was gel-filtered directly on a Sepharose 6B column (1.4cm×18cm) and the other was first treated with dithiothreitol, pH7.5 (20mM), for 30min and then gel-filtered under the same conditions. Both urease and α-amylase activities were determined in the fractions from the two gel filtrations.

Preparation of peroxidase–rabbit anti-(human transferrin) antibody conjugate. 2-Pyridyl disulphide groups were introduced into peroxidase (2.3mol/mol of enzyme) and rabbit anti-(human transferrin) antibodies (1.7mol/mol of protein) by *N*-succinimidyl 3-(2-pyridyldithio)propionate as described above.

The antibody–2-pyridyl disulphide derivative was converted into a thiol derivative by specific reduction of the 2-pyridyl disulphide groups with dithiothreitol (25mM) in sodium acetate buffer, pH4.5, for 30min. Before the conjugation the thiol-containing antibodies were transferred into sodium phosphate buffer, pH7.5, by gel filtration on Sephadex G-25. The peroxidase–2-pyridyl disulphide derivative (2.6 mg; 64nmol) in 1.2ml of 0.1M-sodium phosphate buffer, pH7.5, containing 0.1M-NaCl was mixed with the thiol-containing antibodies (11mg; 68nmol) in 0.9ml of phosphate buffer, pH7.5. After 20h reaction at 23°C the reaction mixture was gel-filtered on a Sepharose 6B column (2cm×63cm) in 0.3M-NaCl. A_{280} and A_{403}, as well as the peroxidase activity, of the fractions were determined. Protein concentrations were estimated spectrophotometrically. For calculation the data in Table 1 were used.

Enzyme activity measurements

α-Amylase activity. This was determined with 0.5% starch as substrate essentially as described by Bernfeld (1955).

Urease activity. This was determined by Nesslerization as described by Carlsson *et al.* (1974). The assay pH was 7.2. The urea concentration used was 0.13M.

Ribonuclease activity. The activity of this enzyme towards cytidine 2′:3′-cyclic monophosphoric acid (38mM) was determined titrimetrically as described by Axén *et al.* (1971). The assay pH was 7.0, which was optimal for both native and conjugated ribonuclease.

Peroxidase activity. This was determined as described in the Worthington Enzyme Manual (1972). The assay pH was 6.0 and the H_2O_2 concentration was 0.9mM. The molecular weights and extinction coefficients of the different proteins used are summarized in Table 1.

Results and Discussion

N-Succinimidyl 3-(2-pyridyldithio)propionate (Fig. 1) is a heterobifunctional reagent. It contains one *N*-hydroxysuccinimide ester moiety and one 2-pyridyl disulphide moiety. The hydroxysuccinimide ester reacts with primary amino groups (Scheme 2*c*) to give stable amide bonds, and the 2-pyridyl disulphide group reacts with aliphatic thiols to form aliphatic disulphides (Scheme 2*b*I). Both of these reactions proceed rapidly under very mild conditions in aqueous media. The *N*-hydroxysuccinimide ester moiety might also react with thiols.

However, if the reaction is carried out at pH 4–5 the reaction of a thiol with the 2-pyridyl disulphide group is usually very much faster. Amino groups and thiol groups both occur in proteins. *N*-Succinimidyl 3-(2-pyridyldithio)propionate therefore can be used to introduce 2-pyridyl disulphide moieties into non-thiol proteins and for the introduction of *N*-hydroxysuccinimide ester moieties into thiol proteins (Schemes 3*a* and 3*b*). The first-mentioned reaction, the introduction of 2-pyridyl disulphide residues, is noteworthy since two important biochemical operations can be accomplished by it. These operations are protein thiolation and protein–protein conjugation.

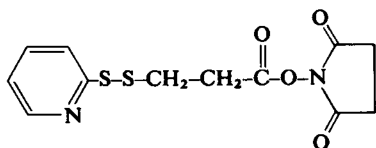
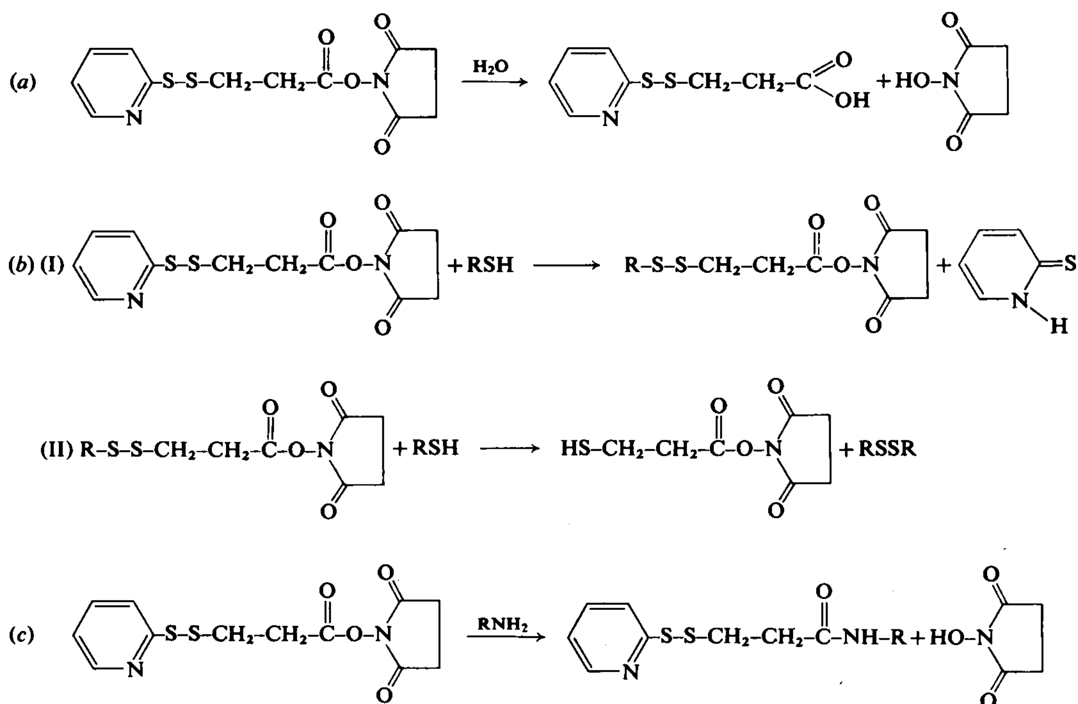


Fig. 1. *Structure of N-succinimidyl* 3-(2-*pyridyldithio*)-*propionate*

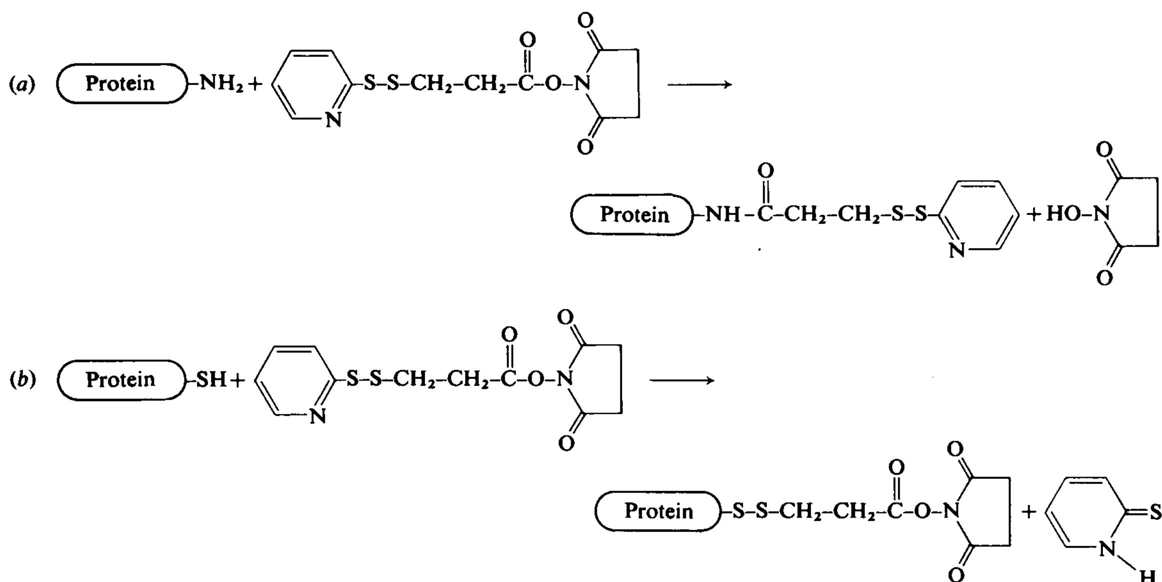
The *N*-succinimidyl 3-(2-pyridyldithio)propionate is a white powder with no tendency to deliquescence. At 23°C it dissolves in ethanol to give at least a 50mM solution and in water containing 10% ethanol to give a 5mM solution (prepared by diluting the ethanol solution 10-fold with water).

Fig. 2 shows the u.v. spectrum of *N*-succinimidyl 3-(2-pyridyldithio)propionate, which is rather similar to the one obtained with 2,2′-dipyridyl disulphide (Grassetti & Murray, 1967). It has an absorption maximum at 280nm with a molar absorptivity of $5.1\times10^3 M^{-1}\cdot cm^{-1}$. (This value was calculated from the amount of *N*-hydroxysuccinimide ester groups or 2-pyridyl disulphide moieties.) The concentration of ester structures was determined by measuring the amount of *N*-hydroxysuccinimide released by hydrolysis at pH 9 (Scheme 2*a*). The *N*-hydroxysuccinimide has an absorption maximum at 260nm with a molar absorptivity of $8.2\times10^3 M^{-1}\cdot cm^{-1}$ as determined at pH 9 in solutions



Scheme 2. *Reactions involving N-hydroxysuccinimidyl* 3-(2-*pyridyldithio*)*propionate*

(*a*) Hydrolysis leading to the release of *N*-hydroxysuccinimide. (*b*) Thiol–disulphide exchange leading to the formation of aliphatic disulphides (I). This is the case if the aliphatic thiol is of the high-molecular-weight type, e.g. a thiol protein. If the thiol is of the low-molecular-weight type and is used in large excess a further thiol–disulphide exchange reaction (II) will also occur, leading to the conversion of the reagent into its thiol form (in this case thiolysis of the ester will also occur). (*c*) Aminolysis by reaction with a primary amine leading to the release of *N*-hydroxysuccinimide and to the formation of an amide bond.



Scheme 3. *Modification of proteins by N-succinimidyl 3-(2-pyridyldithio)propionate*
(*a*) Introduction of 2-pyridyl disulphide groups into a non-thiol protein by aminolysis. (*b*) Introduction of *N*-hydroxysuccinimide ester structures into a thiol protein by thiol–disulphide exchange.

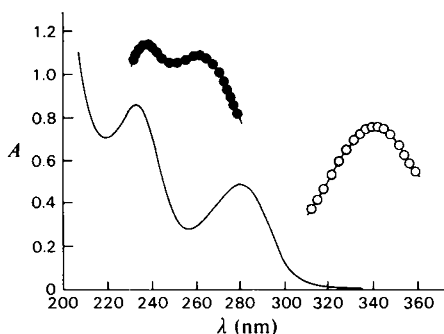


Fig. 2. *Spectra of N-succinimidyl 3-(2-pyridyldithio)-propionate*
The solid line represents the spectrum of *N*-succinimidyl 3-(2-pyridyldithio)propionate in water at pH6. The molar absorptivity at 280nm was calculated to be $5.1\times10^3 M^{-1}\cdot cm^{-1}$. ●, In 0.1M-Tris buffer, pH9 (the spectrum was recorded after 30min of incubation in the buffer). In this medium the *N*-hydroxysuccinimide ester structure is rapidly hydrolysed with the liberation of *N*-hydroxysuccinimide (molar absorptivity $8.2\times10^3 M^{-1}\cdot cm^{-1}$ at 260nm). Only part of the spectrum is shown. ○, In 0.1M-$NaHCO_3$ containing dithiothreitol (25mM) (spectrum recorded 10min after mixing the reagent with this solution). In this reducing medium, pyridine-2-thione with a molar absorptivity of $8.08\times10^3 M^{-1}\cdot cm^{-1}$ at 343nm is released. Only the spectrum around 343nm is shown.

with concentration 0.01–0.1mM, prepared from accurately weighed samples of the thoroughly dried substance. The aromatic disulphide structure also contributes to the absorption at 260nm. This contribution was determined at pH5–6, where the *N*-hydroxysuccinimide ester is rather stable, and then subtracted from the total A_{260} value obtained on hydrolysis of the ester structure. The concentration of 2-pyridyl disulphide structures was determined by treating *N*-succinimidyl 3-(2-pyridyldithio)propionate with a low-molecular-weight thiol compound (Scheme 2*b*). The pyridine-2-thione thus released has an absorption maximum at 343nm with a molar absorptivity of $8.08\times10^3 M^{-1}\cdot cm^{-1}$ (Stuchbury *et al.*, 1975) (Fig. 2).

The stability of *N*-succinimidyl 3-(2-pyridyldithio)-propionate in solid form or in water and ethanol solutions was tested by measuring the decrease of *N*-hydroxysuccinimide ester and 2-pyridyl disulphide contents as a function of time. The half-lives found for the *N*-hydroxysuccinimide ester structure in water at pH9, 7.5 and 5 at 23°C were 1min, 14min and >90min respectively. At these pH values (especially at pH5 and 7.5) the aminolysis is much faster than the hydrolysis.

The rate of decomposition of ester structures of *N*-succinimidyl 3-(2-pyridyldithio)propionate is very much lower in ethanol (99.5%) than in water. An ethanol solution of the reagent (20mM) kept at

23°C retained 80% of the initial ester content after 20 days. The reagent can thus be kept in this form at least for a few weeks when used routinely in the laboratory.

The newly prepared *N*-succinimidyl 3-(2-pyridyldithio)propionate was stored (after exhaustive drying over anhydrous P_2O_5) in a sealed glass bottle surrounded by silica gel. Kept this way at 23°C no decrease in *N*-hydroxysuccinimide ester content of the reagent was observed after 6 months.

No change in the content of 2-pyridyl disulphide structures was observed when *N*-succinimidyl 3-(2-pyridyldithio)propionate was kept in solid form or as ethanol solution for 6 months at 23°C. In water solution the disulphide structure is also more stable than the ester structure. At pH 9, 60% of the 2-pyridyl disulphide residues remained after 2 months and at pH 7.5 no decrease was observed.

The nucleophilic displacement reaction involving *N*-succinimidyl 3-(2-pyridyldithio)propionate and protein amino groups (Scheme 3*a*) in aqueous media at pH 5–9 proceeds without serious competition from side reactions such as hydrolysis. This is typical for aminolysis reactions involving *N*-hydroxysuccinimide esters and explains why they have been used as activated carboxy groups for peptide synthesis in water solutions (Andersson *et al.*, 1964) and in solid matrices for the immobilization of enzymes (Cuatrecasas & Parikh, 1972).

Table 2 summarizes some results concerning the introduction of 2-pyridyl disulphide structures into ribonuclease, α-amylase, γ-globulin, peroxidase and rabbit anti-(human transferrin) antibodies by *N*-succinimidyl 3-(2-pyridyldithio)propionate.

It is obvious that the degree of substitution (mol of 2-pyridyl disulphide structures/mol of protein)

Table 2. *Introduction of 2-pyridyl disulphide groups into proteins by N-succinimidyl 3-(2-pyridyldithio)propionate and subsequent thiolation by specific reduction*

The protein (0.05–2 μmol) was dissolved in 0.1 M-sodium phosphate buffer/0.1 M-NaCl, pH 7.5 (2 ml). An ethanol solution (0.01–0.3 ml) of *N*-succinimidyl 3-(2-pyridyldithio)propionate (20 mM) was added in portions to the stirred protein solution and the reaction proceeded for 30 min at 23°C. Excess of reagent and low-molecular-weight reaction products were removed by gel filtration on Sephadex G-25. The numbers of 2-pyridyl disulphide structures introduced were calculated from the amount of pyridine-2-thione (molar absorptivity 8.08×10^3 at $\lambda_{max.}$ 343 nm) released on treatment of the modified protein with a large excess of low-molecular-weight thiol compound (e.g. dithiothreitol). Thiolation of protein was performed by a two-step procedure. First, 2-pyridyl disulphide structures were introduced as described above. The protein-bound 2-pyridyl disulphide groups were then converted into protein-bound thiol groups by specific reduction with dithiothreitol (25 mM) at pH 4.5 for 30 min. Blank experiments with the native proteins showed that this treatment did not lead to reduction of native disulphide bonds. After removal of excess of dithiothreitol and pyridine-2-thione by gel filtration on Sephadex G-25, the number of protein-bound thiol groups was estimated by reaction with 2,2′-dipyridyl disulphide (Grassetti & Murray, 1967). Abbreviation: Py-2-S-S, 2-pyridyl disulphide group; ε, ε-amino group; α, α-amino group.

Protein	Mol of amine group/mol of protein	Amount of reagent used in aminolysis reaction ([reagent]/[protein])	Degree of substitution obtained (mol of Py-2-S-S/mol of protein)	Protein-thiol groups detected after specific reduction of protein bound Py-2-S-S structures by dithiothreitol (mol of thiol group/mol of protein)
Ribonuclease A (bovine)	10(ε), 1(α)*	1	0.8	0.75
		2.4	2.1	—
		5.5	3.0	—
		5.7	3.2	—
α-Amylase (bacterial)	18(ε), 1(α)†	1.5	1.0	0.95
		9	3.0	2.3
γ-Globulin (bovine)	50–70(ε), 4(α)‡	3	2.3	1.7
		10	3.3	—
Rabbit anti-(human transferrin) antibodies	59(ε), 3(α)‡	2	1.7	1.3
		2.5	1.7	—
		3.5	2.7	—
Peroxidase (horseradish)	4§	4.8	0.5	—
		9.6	0.7	—
		25	2.3	—

* Dayhoff (1972).
† Junge *et al.* (1959).
‡ Cohen & Porter (1964).
§ Klapper & Hackett (1965).

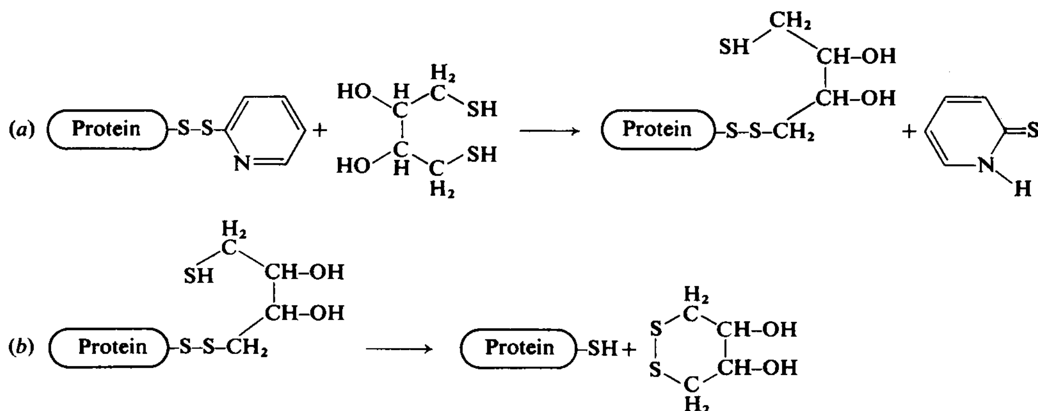
can be varied by using different amounts of reagent (see especially the ribonuclease and α-amylase experiments). Under the experimental conditions used, only a small fraction (except in the case of peroxidase) of the protein amino groups reacted with the reagent. Experiment has shown that a higher degree of substitution can be obtained by using a large excess of *N*-succinimidyl 3-(2-pyridyldithio)propionate ([reagent]/[protein] = 20–200). For example, with a 35-fold molar excess it was possible to introduce 11 2-pyridyl disulphide groups into bacterial α-amylase.

However, the extensively modified proteins thus formed often gave turbid solutions or sometimes even was precipitated. The reason for that might be conformational changes caused by electrostatic effects in connection with the conversion of the positively charged amino groups into neutral amide bonds. The increased hydrophobicity as a result of the introduction of several aromatic groups might also be partly responsible for the precipitation. The precipitates usually dissolved on treatment with low-molecular-weight thiol compounds.

Because the p*K* of ε-amino groups is usually higher than that of the α-amino group it should be possible to obtain some specificity in the aminolysis reaction, e.g. to modify *N*-terminals specifically by performing the reaction at more acidic pH values (5–6). This possibility has previously been examined with other *N*-hydroxysuccinimide ester derivatives (Cuatrecasas & Parikh, 1972).

The protein–2-pyridyl disulphide derivatives were found to be very stable, since no decrease in their 2-pyridyl disulphide content (as measured by the amount of pyridine-2-thione released on reduction) was observed after storage of the derivatives at pH 7.5 and 4°C for 3 months. The protein-bound 2-pyridyl disulphide structure can be converted into a protein-bound thiol compound by reduction with dithiothreitol. This reduction most probably involves two thiol–disulphide exchange reactions. The first reaction leads to the formation of a mixed disulphide of the protein and dithiothreitol and the release of pyridine-2-thione (Scheme 4*a*). The free thiol of dithiothreitol then initiates the second thiol–disulphide exchange, giving a protein-bound thiol compound and dithiothreitol in oxidized form (*trans*-4,5-dihydroxy-1,2-dithiane) (Scheme 4*b*). The overall reaction goes to completion even when only a small excess of dithiothreitol is used. This might be explained by the fact that the thiol formed in the first reaction, pyridine-2-thione, is a very poor nucleophile owing to its thiol–thione tautomerization and the oxidized dithiothreitol formed in the second reaction is a stable six-membered ring. The reactions also take place at acidic pH values at a relatively high rate. This is in contrast with thiol–disulphide exchange reactions involving aliphatic thiols and aliphatic disulphides, where virtually no reaction occurs at acidic pH values owing to the low thiolate ion concentration. The thiolate ion, rather than the thiol, is the reactive species in nucleophilic displacement reactions (Lindley, 1960; Brocklehurst & Little, 1972). The p*K* for the protonation of aliphatic thiols is 7–9. The thiolate ion concentration, and thereby the rates of the above-mentioned types of reactions, are thus very low at acidic pH.

In thiol–disulphide exchange reactions involving aliphatic thiols and 2-pyridyl disulphides the situation is different because these disulphides increase their electrophilicity at acidic pH values owing to protonization of the ring nitrogen atom (p*K* 2–3) (Brocklehurst & Little, 1972). A reasonable rate of reaction is thus obtained in spite of the low



Scheme 4. *Specific reduction of protein-bound 2-pyridyl disulphide groups by dithiothreitol*
The reduction is carried out at pH 4–5 with an excess of dithiothreitol.

concentration of thiolate ions. The protein 2-pyridyl disulphide derivative therefore can be reduced to a protein thiol derivative without concomitant reduction of native protein disulphide bonds by performing the reduction at acidic pH values. Preliminary experiments show that the protein-bound 2-pyridyl disulphide structures also can be specifically reduced at alkaline pH values by equimolar concentrations of dithiothreitol. If the protein to be thiolated lacks native disulphide bonds the protein–2-pyridyl disulphide derivative can be reduced with an excess of any low-molecular-weight thiol compound at alkaline pH values.

When ribonuclease, α-amylase and rabbit anti-(human transferrin) antibodies containing 0.8, 1.0 and 1.7mol of 2-pyridyl disulphide structures/mol of protein respectively were reduced at pH4.5 with dithiothreitol, 0.75, 0.95 and 1.3mol of thiol groups/mol of protein were obtained (Table 2). When derivatives with higher degrees of substitution were reduced usually only 50–80% of the original 2-pyridyl disulphide structures could be detected as thiol groups. The reason might be that some thiol groups become masked owing to conformational changes in the protein caused by the change in the net charge of the protein. The reaction of the protein with *N*-succinimidyl 3-(2-pyridyldithio)propionate leads to the conversion of positively charged amino groups into uncharged amide bonds. The reduction of the introduced 2-pyridyl disulphide groups then gives rise to the formation of thiol groups that at alkaline pH lose protons to become negatively charged thiolate ions. This idea is supported by the fact that the determined thiol content increased on addition of 8M-urea to the reduced proteins.

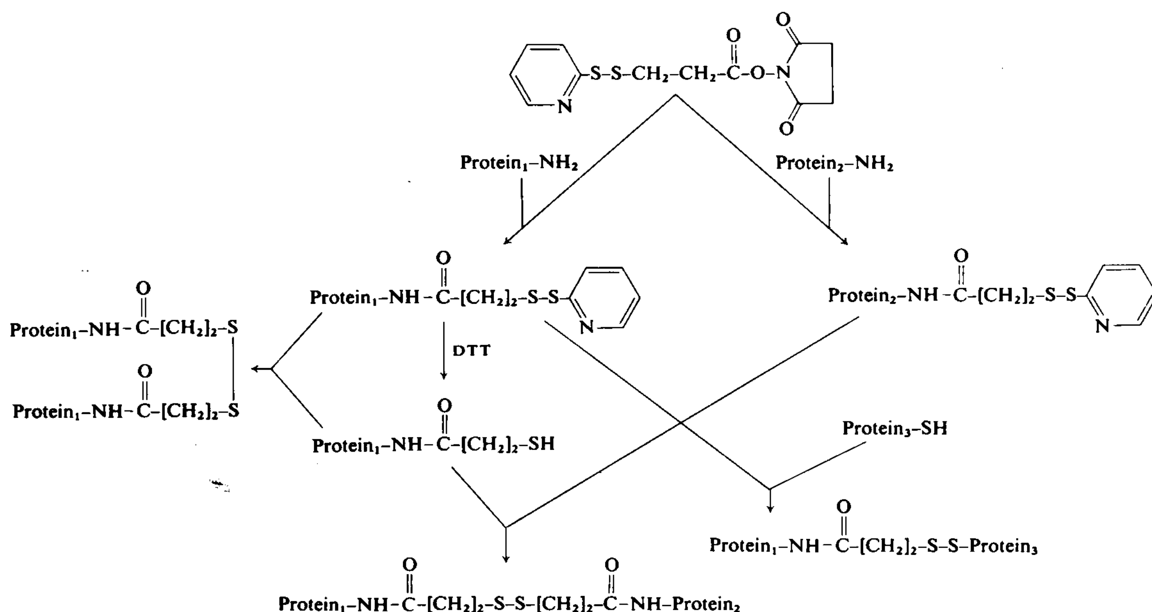
N-Succinimidyl 3-(2-pyridyldithio)propionate as a thiolating agent has several advantages over commonly used reagents such as *N*-acetylhomocysteine thiolactone (White, 1972*b*) and thiolimidates (Perham & Thomas, 1971), which also react through aminolysis.

In both of these cases a large excess has to be used to obtain a reasonable degree of thiolation (White, 1972*b*; Perham & Thomas, 1971). In the case of *N*-acetylhomocysteine thiolactone the reason is a low rate constant for the aminolysis reaction (the rate can be increased to a certain extent by using Ag^{+} cations as catalyst). The thiolimidates react more rapidly with amino groups, but alkaline hydrolysis and the reaction with its own thiol group are serious side reactions that have to be compensated for by using excess of reagent. The stability of the formed bond is also disputable (Browne & Kent, 1975).

In the course of the thiolation procedure the thiol of the thiolimidate can also take part in unwanted thiol–disulphide exchange reactions with native protein disulphide bonds. This risk is especially high since such a large excess of imidate has to be used. Furthermore, the thiol groups introduced by both *N*-acetylhomocysteine thiolactone and thiolimidates are unprotected and can easily undergo unwanted reactions during storage of the thiolated proteins. When *N*-succinimidyl 3-(2-pyridyldithio)-propionate is used, thiols blocked as 2-pyridyl disulphide groups are introduced. This structure is stable in non-reducing media (see above) and the modified proteins can therefore be stored safely in the disulphide form until use. When the disulphide groups are reduced an equivalent amount of pyridine-2-thione is released. Spectrophotometric estimation of this compound affords a simple method for the determination of the degree of substitution of the thiolated protein.

When a protein that contains a 2-pyridyl disulphide substituent reacts with a thiol compound of high molecular weight, usually only one thiol–disulphide exchange, leading to a disulphide-linked conjugate, will take place. This is most probably due to the fact that the disulphide bond formed is sterically unavailable for reaction with another high-molecular-weight thiol compound. Insoluble polymers containing 2-pyridyl disulphide structures that behave similarly to the protein–2-pyridyl disulphide derivatives in their reaction with aliphatic thiols have recently been used for the reversible immobilization of enzymes (Carlsson *et al.*, 1975) and covalent chromatography of thiol proteins and thiol peptides (Brocklehurst *et al.*, 1973; Carlsson & Svenson, 1974; Egorov *et al.*, 1975; Carlsson *et al.*, 1976).

If both of the 2-pyridyl disulphide- and thiol-group-containing components are proteins their reaction with each other will lead to the formation of a protein–protein conjugate. A general method for the conjugation of proteins can therefore be based on *N*-succinimidyl 3-(2-pyridyldithio)propionate (Scheme 5). The proteins to be conjugated ($protein_1$ and $protein_2$) are first provided with 2-pyridyl disulphide structures by reaction with *N*-succinimidyl 3-(2-pyridyldithio)propionate. One of the modified proteins ($protein_1$–S–S–2-pyridyl) is then converted into its thiol form ($protein_1$–SH) by dithiothreitol and coupled to the pyridyl disulphide-containing protein ($protein_2$–S–S–2-pyridyl) by thiol–disulphide exchange. The reaction can easily be followed quantitatively by monitoring the pyridine-2-thione released. It is clear that the pyridyl disulphide-containing proteins can also be conjugated to proteins containing natural thiol groups that are available for reaction ($protein_3$–SH). Since the two structures required for thiol–disulphide exchange are present on different protein molecules, only heteroconjugates can be formed. Theoretically, the thiol-containing protein ($protein_1$–SH or $protein_3$–SH) could oxidize to give homogeneous



Scheme 5. *Protein–protein conjugation by N-succinimidyl 3-(2-pyridyldithio)propionate*

The proteins ($protein_1$ and $protein_2$) are provided with 2-pyridyl disulphide structures by reaction with *N*-succinimidyl 3-(2-pyridyldithio)propionate. The 2-pyridyl disulphide structures in one of the modified proteins ($protein_1$–S–S–2-pyridyl) are then converted into aliphatic thiols by reaction with dithiothreitol (DTT). The thiol protein ($protein_1$–SH) thus formed is coupled to the protein 2-pyridyl disulphide derivative ($protein_2$–S–S–2-pyridyl) by thiol–disulphide exchange. Proteins containing natural thiols ($protein_3$–SH) that are available for reaction can of course be conjugated without prior modification.

aggregates. However, this does not occur because the thiol–disulphide exchange is very much faster than the oxidation. The thiol–disulphide exchange reaction can also be performed at acidic pH, where the oxidation is strongly suppressed. Homogeneous conjugates can be prepared, if desired, by allowing the thiolated form of a protein to react with the 2-pyridyl disulphide derivative of the same protein (see Scheme 5).

By providing the proteins with different numbers of 2-pyridyl disulphide and thiol groups, conjugates of various sizes can be prepared. A 'bimolecular' aggregate will thus be obtained if a protein containing 1 mol of 2-pyridyl disulphide groups/mol is reacted with a protein containing 1 mol of thiol group/mol.

It is even possible to prepare larger conjugates, such as tri- and tetra-oligomers and polymers, by increasing the number of reactive structures in the proteins. Because the proteins usually contain several amino groups that show different reactivities the reaction of proteins with *N*-succinimidyl 3-(2-pyridyldithio)propionate does not in general give a single product. For example, a protein derivative that according to the analysis contains 1 mol of 2-pyridyl disulphide groups/mol might contain a number of unsubstituted molecules as well as protein molecules with two or more groups. Owing to this the protein–protein conjugates formed by reaction of a 2-pyridyl disulphide-containing protein with a thiol-containing protein might show some heterogeneity in size. Proteins thiolated by the two-step procedure described will of course show a similar heterogeneity.

To demonstrate the possibility of using *N*-succinimidyl 3-(2-pyridyldithio)propionate for the preparation of oligomeric heteroconjugates, 2-pyridyl disulphide derivatives of ribonuclease and peroxidase were prepared. These derivatives were then reacted with mercaptalbumin and thiolated rabbit anti-(human transferrin) antibodies respectively. When the reaction mixtures were gel-filtered a large fraction of the ribonuclease and peroxidase activities was eluted at volumes corresponding to molecular weights much higher than that of the native enzymes, but coinciding with molecular weights expected for oligomeric conjugates containing 1–2 molecules of each protein (see Figs. 3 and 4). Control experiments were performed by gel filtration of a mixture

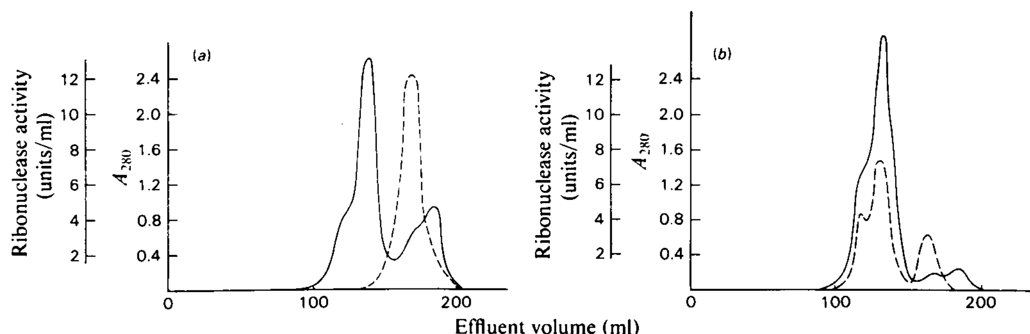


Fig. 3. *Gel-filtration experiments demonstrating the covalent conjugation of ribonuclease to albumin by N-succinimidyl 3-(2-pyridyldithio)propionate*

Chromatographic behaviour of: (*a*) a mixture of native ribonuclease (6.9mg) and albumin (80mg) in 2.5ml of 0.1M-sodium phosphate buffer, pH 7.5, containing 0.1M-NaCl on a Sepharose 6B column (2cm×63cm); the eluent was 0.3M-NaCl; (*b*) the reaction mixture after 30min reaction of a ribonuclease–2-pyridyl disulphide derivative (6.9mg containing 2.1mol of 2-pyridyl disulphide groups/mol of protein) with albumin (80mg) on the same column under the same conditions as in (*a*). The solid line represents A_{280} (protein absorbance) and the broken line represents ribonuclease activity.

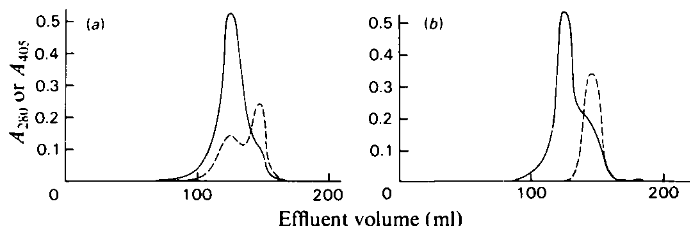


Fig. 4. *Preparation of a peroxidase–rabbit anti-(human transferrin) antibody conjugate by N-succinimidyl 3-(2-pyridyldithio)-propionate*

2-Pyridyl disulphide structures were introduced into both peroxidase (2.3mol/mol of protein) and rabbit anti-(human transferrin) antibodies (1.7 mol/mol of protein) by their reaction with *N*-succinimidyl 3-(2-pyridyldithio)propionate. The antibody 2-pyridyl disulphide derivative was converted into the corresponding antibody thiol derivative by specific reduction of the 2-pyridyl disulphide groups with dithiothreitol at pH4.5. The thiolated antibodies (11mg; 68nmol in 0.9ml) were reacted with the peroxidase–2-pyridyl disulphide derivative (2.6mg; 64nmol in 1.2ml) by thiol–disulphide exchange to produce a peroxidase–antibody conjugate. (*a*) shows the chromatogram obtained on gel filtration of the reaction mixture on Sepharose 6B. As a blank experiment (*b*), a mixture of native peroxidase and native rabbit anti-(human transferrin) antibodies (about the same quantities as used in the conjugation reaction) was gel-filtered on the same column under the same conditions. The solid line represents A_{280} (total protein absorbance) and the broken line represents A_{405} (absorbance due to peroxidase haem group).

of native albumin and native ribonuclease as well as native peroxidase and antibodies under the same conditions. The proteins then appeared at the elution volumes expected from their molecular weights. This indicated that the ribonuclease–albumin and peroxidase–antibody conjugates were not held together by non-covalent bonds. When a sample of a fraction containing ribonuclease–albumin conjugate was treated with 0.45M-2-mercaptoethanol in 8M-urea before gel filtration the ribonuclease activity (measured after reoxidation) was displaced to an elution volume corresponding to the molecular weight of the native enzyme. This experiment showed that the ribonuclease was bound to albumin by disulphide bonds and that the conjugation was reversible.

The amount of pyridine-2-thione released as a result of the thiol–disulphide exchange reactions corresponded quite well to the amount of thiol (mercaptalbumin, thiolated anti-transferrin) added to the reaction mixture. Since approximately equimolar concentrations of reactants were used, this indicates that the reactions proceeded to completion. However, analysis of the fractions from the gel-filtration experiments showed that only 70% of the ribonuclease and 45% of the peroxidase activity

Table 3. *Preparation of protein–protein conjugates by N-succinimidyl 3-(2-pyridyldithio)propionate*

α-Amylase–urease, ribonuclease–albumin and peroxidase–anti-transferrin conjugates were prepared by introducing 2-pyridyl disulphide groups into α-amylase, ribonuclease and peroxidase (see Table 2) and then reacting the enzyme–2-pyridyl disulphide derivatives with urease, albumin and thiolated anti-transferrin by thiol–disulphide exchange. Some data for the resulting conjugates are given in the Table. Anti-transferrin contains no native thiol groups and was therefore thiolated (to 1.3 mol of thiol/mol of protein) before reaction with the peroxidase–2-pyridyl disulphide derivative. The thiolation was performed by reaction with *N*-succinimidyl 3-(2-pyridyldithio)propionate followed by specific reduction with dithiothreitol. The specific activity of the urease was 250 units/mg. Abbreviations used: SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; T–D, thiol–disulphide; Py-2–S–S, 2-pyridyl disulphide group.

	Specific activity			Total enzyme activity		Distribution of enzyme activity after gel filtration of T–D-exchange reaction mixture on Sepharose 6B (%)	
Enzyme	Native enzyme (units/mg)	Enzyme–Py-2–S–S derivative prepared by reaction with SPDP (units/mg)	Mol of Py-2–S–S/mol of enzyme	Enzyme–Py-2–S–S derivative added to the thiol protein	Reaction mixture after finished T–D exchange	Conjugated enzyme	Unconjugated enzyme
α-Amylase (conjugated with urease)	165*	152	1.3	1450	1300	49	51
Ribonuclease (conjugated with albumin)	67†	36	2.1	245	228	73	27
Peroxidase (conjugated with anti-transferrin)	2710‡	2410	2.3	6170	2740	45	55

* One unit of α-amylase activity liberates an amount of reducing groups from starch (0.5%) equivalent to 1 mg of maltose in 1 min at pH 7 at 23°C.

† One unit of ribonuclease activity is the amount of enzyme that catalyses the hydrolysis of 1 μmol of cytidine 2′:3′-cyclic monophosphoric acid (38 mM) at pH 7.0 at 23°C in 1 min.

‡ One unit of peroxidase activity is the amount of enzyme that catalyses the decomposition of 1 μmol of H_2O_2 (1 mM) per min at pH 6 at 23°C.

present in the final reaction mixtures was conjugated (Figs. 3 and 4, Table 3). This discrepancy might be due to the heterogeneity of the product described above and obtained as a result of modification of the proteins with *N*-succinimidyl 3-(2-pyridyldithio)-propionate. The values might also be only apparently low owing to diffusion control of the conjugated enzymes. Diffusion control is known to occur as a result of immobilization of enzymes (especially enzymes with high turnover numbers) to polymers (Sundaram *et al.*, 1970; Axén *et al.*, 1970; Carlsson *et al.*, 1972).

An α-amylase–urease conjugate was also prepared by reaction of an α-amylase–2-pyridyl disulphide derivative with urease. Urease is composed of 6 (or 8) subunits, each containing at least one thiol group that reacts with 2-pyridyl disulphide groups. The reaction therefore led to a heterogeneous product containing α-amylase–urease conjugates of different sizes. The reaction mixture was gel-filtered (Fig. 5) and two identical samples were taken from a fraction containing oligomeric conjugate. One sample was gel-filtered again and the other was reduced with dithiothreitol and subsequently gel-filtered. In the first case α-amylase and urease activities were eluted together at an elution volume corresponding to about 500000. After reduction of the conjugate the α-amylase activity separated from the urease activity and appeared at an elution volume corresponding to the position of native α-amylase. These experiments, which agree well with the ribonuclease–albumin experiments discussed above, show that the urease conjugate was held together by disulphide bonds.

Upon reduction of the α-amylase–urease conjugate the urease activity decreased, whereas the α-amylase activity increased (Fig. 5).

The decreased urease activity is hard to explain since it is known that native urease (which lacks native disulphide bonds) is quite stable or even activated in reducing conditions (Reithel, 1971).

The most likely explanation for the increase in α-amylase activity on reduction of the conjugate is that the conjugated α-amylase is less active towards starch because of steric hindrance. Similar results have previously been presented for α-amylase immobilized to dextran (Marshall, 1976) and agarose (Carlsson *et al.*, 1975).

The ribonuclease–albumin and α-amylase–urease conjugates were prepared to demonstrate the conjugation technique. However, protein conjugation by means of *N*-succinimidyl 3-(2-pyridyldithio)propionate should have several useful applications. One is the preparation of enzyme-labelled antibodies and antigens to be used in enzyme immunoassays. Particularly important is the possibility of preparing 'low-molecular-weight' conjugates while avoiding cross-reactions leading to unlabelled aggregates. The peroxidase–rabbit anti-(human transferrin) antibody conjugate discussed is an example of such a conjugate. This conjugate showed both enzymic and immunochemical activity.

The technique described should also allow the synthesis of aggregates of sequentially operating enzymes. Kinetic studies on such aggregates in solution and immobilized to solid supports by previously introduced techniques for random immobilization (Mosbach & Mattiasson, 1970; Mattiasson & Mosbach, 1971) might improve the understanding of enzyme systems *in vivo*.

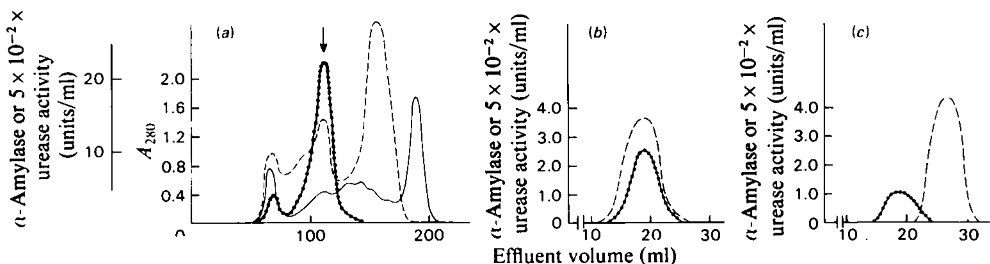


Fig. 5. *Preparation of an α-amylase–urease conjugate by N-succinimidyl 3-(2-pyridyldithio)propionate*
2-Pyridyl disulphide structures were introduced into α-amylase (1.3 mol/mol of protein) by reaction with *N*-succinimidyl 3-(2-pyridyldithio)propionate at pH 7.5. The α-amylase derivative obtained (176 nmol in 2 ml) was reacted with urease (160 nmol in 7 ml) [purified by gel filtration of commercial Sigma (type III) urease on Sepharose 6B] at pH 7.5 for 30 min at 23°C. (*a*) The reaction mixture was then gel-filtered on Sepharose 6B. The solid line represents A_{280} and the broken line represents α-amylase activity of the different fractions. Symbol: ●, urease activity. Two identical samples were taken from one of the fractions containing α-amylase–urease conjugate (arrow in *a*). One of the samples was gel-filtered directly on a Sepharose 6B column (*b*) and the other after treatment with dithiothreitol (20 mM) for 30 min (*c*). Both urease (●) and α-amylase activities (broken line) were determined in the fractions from the two gel filtrations.

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