# N-(4-DIMETHYLAMINO-3,5-DINITROPHENYL)MALEIMIDE: A COLOURED SULFHYDRYL REAGENT*

## ISOLATION AND INVESTIGATION OF CYSTEINE-CONTAINING PEPTIDES FROM HUMAN AND BOVINE SERUM ALBUMIN

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SUMMARY

The coloured sulfhydryl reagent, N-(4-dimethylamino-3,5-dinitrophenyl) maleimide (DDPM), has been synthesized and its reactions with cysteine and with the free SH groups of human and bovine serum albumin have been studied.

The DDPS-serum albumins, prepared from DDPM and serum albumins, were subjected to hydrolysis with pepsin. From the digests, S-DDPS-cysteine peptides were isolated by means of their yellow colour, and purified by adsorption on talc, paper ionophoresis and chromatography. The same amino acid sequence, Leu–Glu($NH_2$)–Asp–Glu–Glu($NH_2$)–Glu–CySH–Pro–Phe, has been found to occur in the peptides derived from bovine and from human serum albumin.

## INTRODUCTION

The widespread use of FDNB[3] for labeling amino groups with the yellow DNP residue and for characterizing the N-terminal amino acids in polypeptide chains indicates the value of coloured reagents in protein chemistry. It was felt that it would be desirable to find a colour label which could be applied to protein sulfhydryl groups and which could be used for the identification of cysteine residues and for the investigation of amino acid sequences containing them.

In this work, N-(4-dimethylamino-3,5-dinitrophenyl) maleimide (DDPM) has been tested as a reagent for SH groups. More than ten years ago, the colourless

Abbreviations: DDPM, N-(4-dimethylamino-3,5-dinitrophenyl) maleimide; DDPS, N-(4-dimethylamino-3,5-dinitrophenyl) succinimido-; NEM, N-ethylmaleimide; FDNB, 1-fluoro-2,4-dinitrobenzene; DNP, 2,4-dinitrophenyl; PCMB, *p*-chloromercuribenzoate; BSA, bovine serum albumin; HSA, human serum albumin. The abbreviations for the amino acid residues are those suggested by BRAND AND EDSALL[50].

* The use of DDPM as a label for SH groups in proteins was first reported at the Symposium on Sulphur in Proteins, Falmouth, Mass.,[1] May, 1958. When the work described in this paper was almost completed, BURLEY AND HAYLETT[2] published a paper on the preparation of DDPM and announced its use as a reagent for SH groups in wool.

N-ethylmaleimide[4-6] was introduced into protein and peptide biochemistry and was shown to combine specifically with SH groups[7,8]. Subsequently, other maleimide derivatives such as N,N'-(1,3-phenylene)-bis-maleimide[9] and N-(4-hydroxy-1-naphthyl) maleimide[10] have also been used with success in protein chemistry and histochemistry, respectively, on account of their specific reaction with sulfhydryl groups. In contrast to the maleimide derivatives employed so far, DDPM is a coloured compound. It contains the yellow 4-dimethylamino-3,5-dinitrophenyl grouping which, a few years ago, became known in protein chemistry as part of the amino group reagents, 4-dimethylamino-3,5-dinitrophenyl isocyanate and isothiocyanate[11,12].

In order to explore the potentialities of DDPM, its reactions with the free SH groups of bovine and human serum albumin have been studied and the sequences of amino acids which surround the labeled cysteine residues have been determined.

## MATERIAL AND METHODS

Commercial preparations of bovine serum albumin (Armour lots N 66706 and S 68108 and Sigma lot No. A 68–084) were used without further purification. Human serum albumin was kindly provided by the Academic Hospital, Groningen, in a 20% solution. Pepsin was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Subtilisin and carboxypeptidase were gifts of the late Professor K. LINDERSTRØM-LANG, Copenhagen, and of Dr. F. A. HOMMES, Groningen, respectively.

PCMB was obtained by synthesis[13] and purified by repeated precipitation from dilute sodium hydroxide solution by the addition of hydrochloric acid. NEM was purchased from Nutritional Biochemicals Corp.

For the preparation of S-DNP-cysteine from cysteine and FDNB, at pH 5.2, the method of ZAHN AND TRAUMANN[14] was used. On paper chromatograms developed with *n*-butanol/acetic acid/water (4:1:1, v/v/v) the compound had an $R_F$ value slightly less than phenylalanine.

DDPM was prepared from 4-dimethylamino-3:5-dinitroaniline which was readily obtained by following the procedure of EVANS AND REITH[11]. The last step of the synthesis, however, was carried out in a different way: N-acetyl-4-dimethylamino-3,5-dinitroaniline (10 g) was heated for 2 h on a boiling water-bath with a mixture of conc. HCl, water and 96% ethanol (75 ml each). After cooling the mixture was diluted with water (150 ml), the precipitate filtered off, washed with water and dried (6.3 g), m.p. 157–158°. On recrystallisation from dil. ethanol the red needles of 4-dimethylamino-3:5-dinitroaniline had a m.p. 157.5–158°. (AYLING *et al.*[15] reported 157°, REITH AND WALDRON[12] 155–156°, BURLEY AND HAYLETT[2] 160°.) (Found: C, 42.7; H, 4.65; N, 24.7. $C_8H_{10}N_4O_4$ requires C, 42.5; H, 4.5; N, 24.8%.)

*N-(4-Dimethylamino-3:5-dinitrophenyl) maleamic acid*

The above amine (10 g) was dissolved in anhydrous ether (65 ml) and maleic anhydride (4.85 g) added. The clear solution was left overnight in the dark, with exclusion of moisture. The orange-red crystals which had then separated, were filtered and dried (14.2 g). Recrystallized from ethanol–water, the needles had a m.p. 178–179°. (BURLEY AND HAYLETT[2] reported 176°.) (Found: N, 17.3; $C_{12}H_{12}N_4O_7$ requires N, 17.3%.)

*DDPM (formula I)*

This was obtained by heating the maleamic acid derivative (10 g) with acetic anhydride (75 ml) and anhydrous sodium acetate (1.5 g) for 15 min on a boiling water-bath, with exclusion of moisture. The solution was then concentrated *in vacuo* at 35° to a volume of about 25 ml and diluted with water (250 ml). After standing for a few hours at room temperature, the crude product was filtered, washed with water, and dried (7.9 g). Twice recrystallized from acetone–water, the long thin orange needles had a m.p. 179–181°. (BURLEY AND HAYLETT[2] reported 181°.) (Found: C 47.4; H, 3.4; N, 18.1. $C_{12}H_{10}N_4O_6$ requires C, 47.1; H, 3.3; N, 18.3 %.) DDPM is sparingly soluble in water, but dissolves in acetone, ethanol, dimethylformamide and acetic acid, and aqueous solutions can be obtained by dilution with water. At alkaline pH values DDPM (like NEM[46]) readily hydrolyses to the corresponding maleamic acid.

*Reaction of DDPM with cysteine*

The solutions of 610 mg DDPM in 20 ml acetone and of 351 mg cysteine hydrochloride in 20 ml deoxygenated water were mixed. The mixture was adjusted to pH 5 by the addition of solid $NaHCO_3$, left at room temp. for 90 min, and evaporated to dryness *in vacuo* at a temperature not exceeding 35°. The solid material was washed with a little water and dried (0.81 g, 95 %). When triturated with warm acetone, most of the yellow substance remained undissolved. This compound, S-DDPS-cysteine (formula II), is slightly soluble in cold water. It readily dissolves in glacial acetic acid and in dimethylformamide. It may be precipitated from its solution in acetic acid with ether. For purification, some of the material was suspended in aqueous ethanol, dissolved by careful dropwise addition of 1 *N* HCl in the cold, and reprecipitated by slowly adding sodium acetate. The orange, amorphous substance which separated was washed with a little water and dried *in vacuo* over $P_2O_5$, at 78°. M.P. 169–170°. (Found: C, 40.71; H, 4.50. $C_{15}H_{17}O_8N_5S \cdot H_2O$ requires C, 40.45; H, 4.30 %.) When subjected to paper electrophoresis in 2 *N* acetic acid–0.6 *N* formic acid at pH 2 (ref. 16), it moved towards the cathode as an intense yellow band which turned purple on treatment with ninhydrin. Ionophoresis in pyridine–acetic acid buffer at pH 4.7 (ref. 24) indicated that, at this pH, the compound had no net charge. On paper chromatograms developed with the upper phase of a mixture of *n*-butanol, acetic acid and water (4:1:5, v/v/v) it showed an $R_F$ value of 0.68.

*Cleavage by silver ions of the thioether bond in S-DDPS-cysteine*

A solution of 3 mg S-DDPS-cysteine in 1.25 ml 60 % acetic acid was added to a solution of 20 mg silver sulphate in 2.5 ml warm water. The mixture was heated at 50° for 9 h and subsequently concentrated *in vacuo*. The dry residue was treated for 20 min at room temp. with a mixture of 0.75 ml 87 % formic acid and 0.075 ml 30 % hydrogen peroxide. The performic acid was then removed *in vacuo* at a temperature of 30° and the residual material twice taken up with water and brought to dryness again to remove traces of hydrogen peroxide; 10 ml of warm water (60°) and 1 drop 20 % sulphuric acid were added, and hydrogen sulphide was bubbled through the solution. The precipitated silver sulphide was centrifuged off and washed with water in the centrifuge. The clear supernatants were combined and freed of sulphuric acid by extraction with a chloroform solution of tri-*n*-octylamine[17]. As shown by paper chromatography, the aqueous solution was rich in cysteic acid.

*Rearrangement of S-DDPS-cysteine on exposure to alkali*

Even a short exposure to mild alkali (dilute sodium carbonate) has been found to transform S-DDPS-cysteine into another yellow compound which is sparingly soluble in dilute acid and does not give a colour reaction with ninhydrin. On paper ionophoresis at pH 2 it does not migrate, while at pH 4.7 it moves towards the anode with a mobility about one half that of glutamic acid. $R_F$ (in *n*-butanol–acetic acid–water, 4:1:5) 0.86. Recrystallised from acetone–petrol ether it melted at 245–247° (decomp.) (Found: C, 41.65; H, 4.05; N, 15.82. $C_{15}H_{17}O_8N_5S$ requires C 42.15; H, 4.01; N, 16.39%.) This rearrangement product has been assigned the structure of a thiazane derivative (formula III).

*Cleavage by mercuric ions of the thioether bond in the thiazane derivative III*

Treatment of III with silver ions in acetic acid at 50° followed by performic acid oxidation did not yield cysteic acid. The latter could be obtained, however, by heating III with mercuric chloride in dilute hydrochloric acid, prior to oxidation. A solution of 2.25 mg III and 1.2 mg $HgCl_2$ in 0.25 ml 0.025 *N* HCl was heated in a sealed tube at 105° for 21 h. The reaction mixture was diluted with 0.25 ml water and extracted three times with 0.5 ml ether. The orange-coloured ether layer was discarded and the nearly colourless aqueous layers oxidised with performic acid. Paper chromatography showed cysteic acid to be the ninhydrin-positive material present.

*Acid hydrolysis of S-DDPS-cysteine and of the thiazane derivative III*

When S-DDPS-cysteine and the compound III were subjected to acid hydrolysis in sealed tubes (5.7 *N* HCl, 15 h, 105°) and the hydrolysates were examined by paper chromatography (*n*-butanol–acetic acid–water, 4:1:5), the same pattern of spots was obtained. In addition to a set of yellow and orange spots which did not give a colour reaction with ninhydrin and had $R_F$ values in the range between 0.80 and 0.95, 3 ninhydrin-positive spots were found, one of them ($R_F$ 0.16) being by far the strongest; this was indistinguishable from that given by synthetic 2-amino-2-carboxyethyl-mercaptosuccinic acid[46,9]. The two weaker spots had lower $R_F$ values and occurred in variable amounts. The slowest one ($R_F$ 0.07) was identified as cysteic acid. The same set of three ninhydrin-positive spots was also obtained when 2-amino-2-carboxyethyl-mercaptosuccinic acid was subjected to acid hydrolysis. In paper chromatography, using the butanol–acetic acid–water system, the 2-amino-2-carboxyethyl-mercaptosuccinic acid spot ($R_F$ 0.16) can easily be mistaken for aspartic acid ($R_F$ 0.14). With *n*-butanol–pyridine–water (1:1:1), however, the $R_F$ values are markedly different (0.09 and 0.19, resp.). On paper ionophoresis, at pH 4.8, 2-amino-2-carboxyethyl-mercaptosuccinic acid migrates towards the anode more rapidly than aspartic acid.

*Titration of SH groups in serum albumins*

The SH groups were titrated spectrophotometrically, according to BOYER[18], with PCMB in 0.1 *M* phosphate pH 6.9 or in 0.33 *M* acetate buffer pH 4.6. The PCMB solution was added with an Agla micrometer syringe, in 20-$\mu$l portions of a $5 \cdot 10^{-4}$ *M* solution, and the extinction at 250 m$\mu$ measured after each addition. Alternatively, titrations were carried out with $10^{-3}$ *M* NEM in 0.1 *M* phosphate

buffer of pH 6.7 (ref. 19–20), readings being taken at 300 mμ. The concentrations of bovine and human serum albumins in their solutions were also determined spectrophotometrically at 280 mμ, using the extinction coefficients, $[E]_{1\,cm}^{1\%}$, of 6.6 and of 5.3, respectively[21].

*Reaction of bovine serum albumin with DDPM*

To solutions of BSA ($10^{-5}$ *M*) in 0.33 *M* acetate buffer, pH 4.6, graded volumes of a 0.01 % solution of DDPM in a mixture of equal parts of 96 % ethanol and acetate buffer were added. The mixtures were left at room temp. for 1 h. Subsequently, unreacted SH groups were determined by titration with PCMB. After 1 h's contact of BSA with a 1.5-fold molar excess of DDPM, free SH groups were no longer detectable.

*Preparation of DDPS-bovine serum albumin*

15 mg DDPM were dissolved in 50 ml of ethanol. An equal volume of 0.33 *M* acetate buffer, pH 4.6, was added, and the mixture was slowly run into 100 ml of a 1 % solution of BSA in acetate buffer. After standing overnight at room temp., the clear solution was concentrated *in vacuo* at 35°. DDPS-bovine serum albumin was precipitated by the addition of 10 volumes of ethanol and, after a few hours, collected by centrifugation, washed with ethanol and acetone to remove excess DDPM, and dried.

*Enzymic hydrolysis of DDPS-bovine serum albumin and isolation of DDPS-cysteine peptides*

DDPS–BSA (1 g) was dissolved in 95 ml 0.01 *N* HCl. A solution of 10 mg pepsin in 5 ml 0.01 *N* HCl was added, the mixture adjusted to pH 2.1 and incubated at 37° for 24 h.

A preliminary fractionation of the digest was effected by passing it through a talc column. Talc (24 g) was suspended in a mixture of 2 *N* HCl and ethanol (1:1, v/v, heated on a steam-bath for 2 h and repeatedly washed with water by decantation until the washwater was no longer acidic. The talc was re-suspended in water and poured into a column 10 cm high and 1.5 cm in diameter. The digestion mixture was filtered through the talc, with gentle suction, followed by water. The yellow-coloured peptides, which had been adsorbed on the upper parts of the column, were subsequently eluted from the talc by passing through it 50 % aqueous ethanol containing 2 % acetic acid. The yellow eluate was reduced to dryness in a desiccator.

The yellow material obtained (45 mg) was further purified by subjecting aliquot parts to high-voltage (1500–2000 V) ionophoresis on paper (Schleicher and Schüll No. 2043a, 17 × 60 cm, or Whatman 3MM, 13 × 39 cm), using a formic acid–acetic acid buffer[16] of pH 2 (Fig. 1). The yields of the yellow-coloured peptides *A*, *B*, and *C* varied to some extent in different experiments. *B* usually exceeded *A*, and *C* was only a faintly coloured, minor component. When the paper strips were sprayed with ninhydrin and heated, *B* turned brown–violet while *A* did not react. Ninhydrin also revealed a number of contaminating peptide bands (not indicated in Fig. 1) which had no yellow colour and were mainly located in the region of *C*.

Peptides *A* and *B* were eluted from the paper strips with 10 % aqueous acetic acid. The eluates were taken to dryness. In most experiments the peptides were

further subjected to descending paper chromatography. When B was run in the solvent mixture, tertiary amyl alcohol–pyridine–water (7:7:6, v/v/v): for 15 h, after an equilibration period of 24 h, the main yellow-coloured and ninhydrin-reactive material had an $R_F$ value of approx. 0.50. It was neatly separated from other faintly yellow-coloured or weakly ninhydrin-reacting components.

*Structural investigation of the DDPS-cysteine peptide* B *obtained from BSA*

*Total hydrolysis*: This was performed on peptide *B* with 5.7 *N* HCl in a sealed capillary tube for 16 h at 105° and yielded the following amino acids, identified by paper chromatography: Leu (xx), Phe (xx), Pro (xx), Glu (xxxxx), Asp (xx) and "Cys" (xx). "Cys" indicates the mixture of degradation products, arising from DDPS-cysteine on hydrolysis with HCl, which gives on paper chromatograms sprayed with ninhydrin a characteristic pattern of yellow and purple spots. The presence of aspartic acid was confirmed by paper ionophoresis at pH 4.7, and the occurrence of leucine rather than isoleucine by paper chromatography in pyridine–tertiary amyl alcohol–water (7:7:6, v/v/v)[47].

*N-terminal residue*: This was determined using the DNP-method. The peptide was reacted with FDNB in the presence of trimethylamine[22]. The DNP-derivative obtained was hydrolysed with a mixture of glacial acetic acid and conc. HCl (1:1) for 70 min at 105°. The ether-soluble yellow compounds present in the hydrolysate were characterised by paper chromatography using the phenol–isoamyl alcohol–ammonia solvent described by BISERTE AND OSTEUX[23]. The main yellow spot had an $R_F$ value of 0.32 and was identified as DNP-Leu. A minor yellow component with $R_F$ 0.22 was found to yield DNP-Leu and Glu when hydrolysed with 5.7 *N* HCl (16 h, 105°).

*The C-terminal residue*: This was identified with the help of carboxypeptidase. A solution (0.55 ml) containing 3 μg enzyme and 500 μg peptide *B* was brought to pH 8.0 by the addition of trimethylamine and incubated at 37°. At various times 0.05-ml samples of the incubation mixture were withdrawn and investigated by paper chromatography. Phenylalanine was found to be slowly released. Even after 24 h at 37° not even a trace of an amino acid other than phenylalanine could be detected.

*Cleavage by subtilisin*: A solution (0.7 ml) containing 5.6 mg *B* and 0.28 mg subtilisin was adjusted to pH 7.7 with $NaHCO_3$ and incubated at 37° for 20 h. Two drops of glacial acetic acid were added to stop the enzymic reaction and the digestion mixture was evaporated to dryness *in vacuo*. Samples of the digested peptide mixture were subjected to high-voltage paper ionophoresis (Figs. 2(a) and (b)). Four peptides which separated from each other were eluted from the paper and investigated as summarized in Table I.

On paper chromatograms of hydrolysates of the peptides $Bb_1$ and $Bb_2$ the glutamic acid spots were strikingly stronger than the spots of the other amino acids, indicating that more than one glutamic acid residue was present (Table I). This was confirmed by semi-quantitative assay. Hydrolysates of the peptides $Bb_1$ and $Bb_2$ were allowed to react with FDNB in aqueous alcohol containing $NaHCO_3$. The reaction mixtures were diluted with water and extracted with ether to remove excess FDNB. The aqueous solutions were acidified and extracted with ether again. The ether extracts which contained the DNP-amino acids were taken to dryness and dini-

TABLE I

PEPTIDES OBTAINED BY DIGESTION OF THE S-DDPS-CYSTEINE PEPTIDE *B* WITH SUBTILISIN

| *Peptide* | *Colour* | *Colour reaction with ninhydrin* | *Amino acids obtained on acid hydrolysis* | *Dinitrophenylation followed by acid hydrolysis* | |
|---|---|---|---|---|---|
| | | | | *DNP-amino acids** | *Free amino acids* |
| *Ba* | none | purple | Leu(xxx), Glu(xxx) | DNP-Leu | Glu |
| $Bb_1$ | yellow | brownish-purple | Phe(xx), Pro(xx), Glu(xxx), "Cys"(x) | DNP-Glu | Phe(xx), Pro(xx), Glu(xx), $CySO_3H$(xx) |
| $Bb_2$ | yellow | none | Phe(xx), Pro(xx), Glu(xxx), "Cys"(x) | none | Phe(xx), Pro(x), Glu(xxxx), $CySO_3H$(xx) |
| *Bc* | none | purple | Glu(xxx), Asp(xx) | DNP-Asp | Glu |

* The DNP-amino acids were characterized by paper chromatography in the solvents amyl alcohol–phenol–ammonia[23] or tert. amyl alcohol–phthalate buffer pH 6 (ref. 25) and by cleavage with ammonia[26], the resulting free amino acids likewise being identified by chromatography.

trophenol was removed from the residue by sublimation *in vacuo*[27] at 78°. The mixtures of DNP-amino acids were subjected to descending chromatography on Schleicher and Schüll paper No. 2045 bM. The solvent was *n*-butanol saturated with water. Reference samples containing various known amounts of DNP-glutamic acid, DNP-proline and DNP-phenylalanine were run side by side with the unknown mixtures. The yellow spots corresponding to the DNP-derivatives of Glu, Pro, and Phe were cut out, eluted with 5 ml of 1% $NaHCO_3$ for 15 min[28] at 55–60° and the extinctions of the eluates determined at 360 mμ (for DNP–Glu and DNP–Phe) and 385 mμ (for DNP–Pro) in a Beckman spectrophotometer. From the readings the molar ratios of Phe:Pro:Glu were calculated to be 1.12:1.12:2.00 for peptide $Bb_1$ and 1.07:0.93:2.00 for $Bb_2$. Partial acid hydrolysis (conc. HCl, 72 h, 37°) of the peptides $Bb_1$ and $Bb_2$ gave complex mixtures which were fractionated by paper ionophoresis at pH 6.5 (Fig. 5) followed by descending paper chromatography using the solvent system *n*-butanol–acetic acid–pyridine–water (4:1:1:5, v/v/v/v)[51]. The results are summarized in Table II.

*Investigation of the DDPS-cysteine peptide A obtained from BSA*

Peptide *A* (Fig. 1) does not give a colour reaction with ninhydrin nor has it been possible to identify a free N-terminal amino acid residue using the DNP technique. *A* appears to contain the same amino acids as *B*. Cleavage with subtilisin gave three split products closely resembling or identical with the peptides *a*, $b_2$ and *c* as derived from *B*.

*Investigation of DDPS-cysteine peptides obtained from HSA*

Human serum albumin was allowed to react with DDPM in the same way as described above for BSA except for the use of a 0.1 *M* phosphate buffer of pH 6.7 instead of an acetate buffer of pH 4.6. Peptic cleavage of the DDPS derivative of HSA, followed by the purification procedure indicated above, gave two DDPS-cysteine peptides (*B'* and *A'*) which were identical with *B* and *A*, respectively, as regards their electrophoretic mobilities, their reactions with ninhydrin, their $R_F$ values, their amino acid compositions, their N- and C-terminal residues, and the products of their cleavage by subtilisin.

## TABLE II

AMINO ACIDS AND PEPTIDES OBTAINED FROM THE S-DDPS-CYSTEINE PEPTIDES $Bb_1$ AND $Bb_2$ BY PARTIAL ACID HYDROLYSIS AND SEPARATED BY PAPER IONOPHORESIS AND PAPER CHROMATOGRAPHY

| *Ionophoresis fractions (Fig. 5)* | *Colour* | *Colour reaction with ninhydrin* | *Intensity* | *Spots obtained on paper chromatography* | $R_{Phe}$** | *Colour reaction with ninhydrin* | *Intensity* | *Amino acids obtained on total acid hydrolysis, identified by paper chromatography* | *Inferred sequence* |
|---|---|---|---|---|---|---|---|---|---|
| $Bb_1$* | brown | brown–purple | xxxx | a | 0.08 | purple | xx | "Cys" | |
| | | | | b | 0.19 | purple | xxx | "Cys" | |
| | | | | c | 0.31 | purple | xx | Pro, "Cys" | Cys–Pro |
| | | | | d | 0.42 | yellow | xx | Pro | |
| | | | | e | 0.76 | violet | trace | | |
| | | | | f | 1.00 | violet | xx | Phe | |
| | | | | g | 1.21 | yellow | xxx | Phe, Pro | Pro–Phe |
| $Bb_2$ | — | purple | xxx | a | 0.08 | violet | xx | Pro, Glu, "Cys" | Glu–Cys–Pro |
| | | | | b | 0.13 | violet | xxx | Pro, Glu(!), "Cys" | Glu–Glu–Cys–Pro |
| | | | | c | 0.18 | purple | xxxx | Glu | |
| | | | | d | 0.22 | violet | xxx | Phe, Pro, Glu, "Cys" | Glu–Cys–Pro–Phe |
| | | | | e | 0.37 | pink | x | Phe, Pro, "Cys" | Cys–Pro–Phe |
| $Bb_3$ | — | purple | xx | a | 0.05 | violet | xxx | Glu(!), "Cys" | Glu–Glu–Cys |
| | | | | b | 0.08 | purple | xx | Glu, "Cys" | Glu–Cys |
| | | | | c | 0.13 | purple | xx | Glu | Glu–Glu |
| | | | | d | 0.20 | purple | trace | | |
| | | | | | | | | | Glu–Glu–Cys–Pro–Phe |

* This fraction showed, on paper chromatography, coloured spots, which were derived from the DDPS moieties of $Bb_1$ and $Bb_2$.

** $R_{Phe}$ values of reference substances in the system *n*-butanol–pyridine–acetic acid–water (4:1:1:5, v/v/v/v): 2-amino-2-carboxyethyl-mercapto-succinic acid, 0.08; Glu–Glu, 0.13; Glu, 0.19; Pro, 0.43; Phe, 1.00.

*Preparation of DNP-bovine serum albumin*

A solution of 1 g BSA in 15 ml water was adjusted to pH 9.3 with dil. NaOH and a solution of 3.5 mg chlorodinitrobenzene in 5 ml 96 % ethanol was added. The clear mixture was left at 37° for 1.5 h and then poured into 200 ml 96 % ethanol. A yellow precipitate formed when conc. HCl was added to bring the pH to 3.5. After standing for 2 h, it was collected by centrifugation, washed and dried.

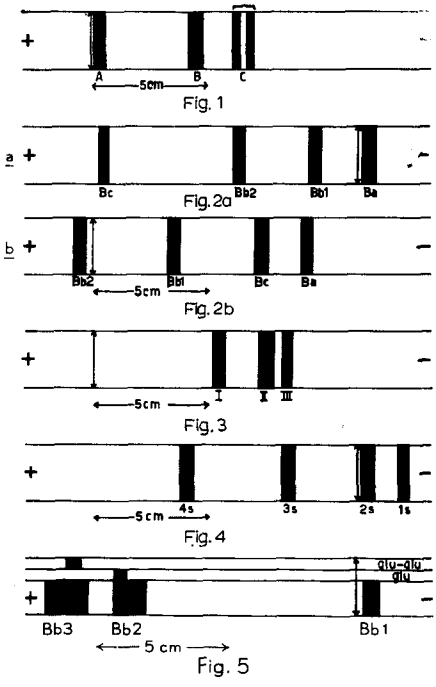



Fig. 1. Paper ionophoresis of DDPS-cysteine peptides, obtained by pepsin digestion of DDPS-bovine serum albumin. In formic acid–acetic acid, pH = 2.0 (ref. 16), 2 h, 50 V/cm, 22 mA.

Fig. 2. Paper ionophoresis of the mixture of split products, obtained by digesting the S-DDPS-cysteine peptide *B* with subtilisin. (a) In pyridine–acetic acid, pH = 4.7 (ref. 24), 2.5 h, 25 V/cm, 36 mA. (b) In formic acid–acetic acid, pH = 2.0 (ref. 16).

Fig. 3. Paper ionophoresis of DNP-peptides, obtained by pepsin digestion of DNP-bovine serum albumin. In formic acid–acetic acid, pH = 2.0 (ref. 16), 2 h, 40 V/cm, 24 mA.

Fig. 4. Paper ionophoresis of the peptide mixture, obtained by digesting the S-DNP-cysteine peptide *I* with subtilisin. In pyridine–acetic acid, pH = 4.6 (ref. 24), 2 h, 25 V/cm, 27 mA.

Fig. 5. Paper ionophoresis of the mixture of split products, obtained by partial hydrolysis of the S-DDPS-cysteine peptides $Bb_1$ and $Bb_2$. In pyridine–acetic acid, pH = 6.5 (ref. 24), 1.5 h, 40 V/cm, 27 mA. For comparison glutamic acid and the dipeptide glutamyl-glutamic acid were simultaneously subjected to paper ionophoresis.

*An S-DNP-cysteine peptide from DNP-bovine serum albumin*

The dinitrophenylated BSA was digested with pepsin and the digest filtered through a talc column (15 cm high and 1.8 cm in diameter) under slightly reduced pressure. Material having a faintly yellow colour was adsorbed on the upper three-quarters of the talc. The column was washed with 7.5 ml water and then eluted with 2 % ammonia in 50 % aqueous ethanol. The yellow peptides slowly moved down the column, forming a sharp band. The lemon-coloured eluate was lyophilised and subjected to ionophoresis on paper (Fig. 3). Besides a considerable number of colourless peptides which only showed up after treatment with ninhydrin and are not indicated in the figure, three yellow bands were obtained. All of them gave a colour reaction with ninhydrin (I, grey–blue, weak; II, blue–green, strong; III, grey–purple, very weak). On acid hydrolysis, only I gave S-DNP-cysteine. In the other two peptides, II and III, the DNP residues were bound to groups other than SH groups. The S-DNP-cysteine peptide I was further purified by chromatography in the same manner as described above for DDPS-cysteine peptides. It had a $R_F$ value of approx. 0.50 and was clearly separated from contaminating ninhydrin-positive substances, all of which had lower $R_F$ values. It could most readily be located inspection of the chromatograms in u.v. light when it showed up as a dark spot.

On total hydrolysis with acid, peptide I gave the following amino acids: Leu (xx), Phe (xx), Pro (xx), Glu (xxxx), Asp (xx), S-DNP-cysteine (x), and cysteic acid (x). Proteolytic cleavage of the peptide with subtilisin was slow and incomplete even after prolonged incubation. The digestion mixture was subjected to paper ionophoresis (Fig. 4). The separated peptides were eluted and further examined as recorded in Table III.

In all operations involving DNP derivatives care was taken to avoid exposure to bright light.

TABLE III

PEPTIDES OBTAINED BY HYDROLYSIS OF THE S-DNP-CYSTEINE PEPTIDE *I* WITH SUBTILISIN

| *Peptide* | *Colour* | *In u.v. light* | *Colour reaction with ninhydrin* | *Amino acids obtained on acid hydrolysis* | *DNP-amino acid obtained by dinitrophenylation and hydrolysis* |
|---|---|---|---|---|---|
| *1s* | none | — | purple | Leu,Glu | DNP-Leu |
| *2s** | faint yellow | dark | grey–purple | Leu,Phe,Pro,Asp, Glu**,S-DNP-Cys | DNP-Leu |
| *3s* | faint yellow | dark | none | Phe,Pro,Glu, S-DNP-Cys | none |
| *4s* | none | — | purple | Asp,Glu | DNP-Asp |

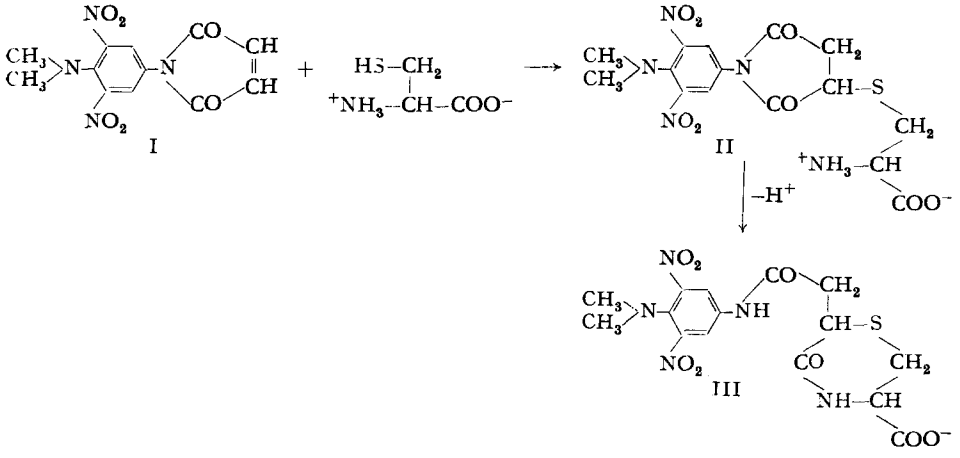
* Peptide *2s* is unchanged peptide *I*.
** The glutamic acid spot was strikingly stronger than all the other amino acid spots.

## RESULTS AND DISCUSSION

The preparation of DDPM (formula I) does not present any difficulty. Its reaction with cysteine, however, has been found to be not quite clear-cut. In slightly acidic solutions a yellow-coloured neutral compound is formed which gives a purple colour reaction with ninhydrin and has properties consistent with the expected structure of a S-[N-(4-dimethylamino-3:5-dinitrophenyl)-succinimido]-cysteine (formula II). At an alkaline pH, however, and even in neutral solutions II readily rearranges into an acidic compound which does not react with ninhydrin and to which is assigned formula III. The rearrangement is presumed to be initiated by the removal of a proton from the $NH_3^+$ group of DDPS-cysteine (II) and to proceed via an intramolecular acylation of the $NH_2$, the succinimide ring being opened and a thiazane ring formed*.

DDPS-cysteine and the thiazane derivative arising from it in alkaline solution were subjected to the action of silver sulphate in acetic acid followed by oxidation with performic acid. This procedure had previously been used to break the thioether bonds in cytochrome *c*[29] and in hemopeptides derived from it[30]. With DDPS-cysteine this treatment was found to give a good yield of cysteic acid, whereas with its rearrangement product none was produced. Cysteic acid could be obtained from the thiazane derivative, however, by reacting it with mercuric ions, under conditions (0.025 *N* HCl, 105°, 21 h) where cleavage of the thioether linkage was accompanied by hydrolytic opening of the CO–NH bond, followed by performic acid oxidation.

* During the preparation of this manuscript, Professor J. S. FRUTON kindly informed us that experiments in his laboratory (D. G. SMYTH, A. NAGAMATSU AND J. S. FRUTON, unpublished) have shown that the cysteine adduct of N-ethyl maleimide, upon exposure to alkali, is transformed into a thiazane derivative corresponding to III.




These findings are in good agreement with the formulae II and III, respectively, assigned to the two compounds.

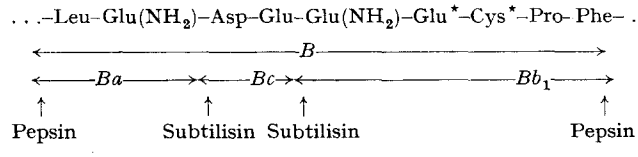
Bovine and human serum albumin were used to examine the reaction of DDPM with SH groups of cysteine residues in proteins. It is well known that in the absence of denaturing agents approximately two-thirds of one SH group per molecule of bovine or human serum albumin will react with SH reagents such as silver in Tris or in ammonia solution[31,31A], PCMB[18], or NEM[19]. With the serum albumin samples employed in this investigation, titrations with PCMB gave values of 0.72 SH/mole BSA (at pH 4.6), 0.65 SH/mole BSA (at pH 6.9), and 0.60 SH/mole HSA (at pH 6.9). When HSA was allowed to react with NEM, 0.63 SH/mole were found to have combined within 45 min. When DDPM was added to solutions of HSA or BSA and the mixtures were allowed to stand for 1 h at room temperature, 1.5 moles of the maleimide reagent per one mole of albumin were found adequate for a quantitative reaction; subsequent titration of the serum albumins with PCMB gave evidence for the complete absence of residual easily accessible SH groups. The necessity for using a SH reagent in excess had been reported before for NEM[20].

When the S-DDPS derivative of BSA was subjected to hydrolysis with pepsin and the digestion mixture worked up, two S-DDPS-cysteine peptides, *A* and *B*, were mainly obtained (Fig. 1). The relative amounts of *A* and *B* were different in different experiments, and there are indications that *A* may arise from *B* in the course of the isolation procedures. Both peptides had the same amino acid composition (Leu, Phe, Pro, Glu, Asp, Cys) and, on enzymic hydrolysis, gave identical split products, which indicated that they had the same sequence of amino acids. *B* gave a purple colour with ninhydrin and readily reacted with FDNB. Acid hydrolysis of DNP-*B* gave DNP-Leu-Glu. *A*, on the other hand, did not react with ninhydrin nor could a DNP derivative be obtained from it. It obviously lacks a free $\alpha$-$NH_2$ group. An unexpected reaction presumably involving the DDPS group appears to have masked the amino-terminal leucine residue. Since the ready re-arrangement of S-DDPS-cysteine (II) to the thiazane derivative (III) indicates that the DDPS group has acylating properties, it may be presumed that the DDPS moiety in the peptide *B* is able to acylate the free N-terminal $\alpha$-amino group. It appears likely that this acylation occurs intramolecularly rather than intermolecularly, a large-

sized ring being formed. Surprisingly, treatment of *A* with subtilisin produced the same fragments as treatment of *B*, indicating that the enzyme is capable of severing the acid amide bond in which the amino group of the terminal leucine residue appears to be involved. Carboxypeptidase was found to release phenylalanine from both *A* and *B*. Thus, in both peptides phenylalanine is in the C-terminal position.

Cleavage of *B* with subtilisin gave three fragments, *Ba*, *Bb* (appearing in two forms, $Bb_1$ and $Bb_2$), and *Bc* (Fig. 2 and Table I). *Ba* is a dipeptide containing leucine and glutamic acid. Leucine is N-terminal indicating that the dipeptide is derived from the N-terminal portion of *B*. On ionophoresis at a pH of 4.7 (Fig. 2(a)) *Ba* has been found not to migrate. Thus, it is Leu-Glu($NH_2$) rather than Leu-Glu. $Bb_1$ and $Bb_2$ are both yellow-coloured and they contain the same amino acid residues: 2 Glu, 1 Cys (in the form of S-DDPS-cysteine), 1 Pro, and 1 Phe. The occurrence of phenylalanine shows that $Bb_1$ and $Bb_2$ are derived from the C-terminus of the polypeptide chain of *B*. In partial acid hydrolysates of $Bb_1$ and $Bb_2$, the dipeptide Pro-Phe could be identified. Thus, the C-terminal phenylalanine residue is preceded by proline. (This finding is consistent with the slow release of phenylalanine from *B* under the action of carboxypeptidase. Proline residues adjacent to C-terminal phenylalanine have previously been reported to retard the release of the latter by carboxypeptidase while C-terminal proline residues themselves are known to be entirely resistant to the action of this enzyme[32].) The entire pentapeptide sequence in the peptides $Bb_1$ and $Bb_2$ can be easily deduced from the results of partial acid hydrolysis summarised in Table II. $Bb_1$ and $Bb_2$ show the following differences: $Bb_1$ gives a purple colour reaction with ninhydrin while $Bb_2$ does not react; using the DNP technique, one of the two glutamic acid residues of $Bb_1$ has been proved to be N-terminal, while $Bb_2$ does not react with FDNB; furthermore, paper ionophoresis reveals that $Bb_2$ is more acidic than $Bb_1$. These findings can be accounted for by assuming that the N-terminal residue in $Bb_1$ is glutamine rather than glutamic acid, and in $Bb_2$ is pyroglutamic (pyrrolidone carboxylic) acid. Glutamine residues which occupy a N-terminal position in peptides are known to undergo readily a cyclisation to pyroglutamic residues[33,34]. In this transformation the free $\alpha$-amino group is lost which is responsible for the reactions with ninhydrin and FDNB and which, at the pH used in ionophoresis, bears a positive charge. *Bc* is an acidic dipeptide composed of aspartic and glutamic acid residues. It must be derived from the middle part of the polypeptide chains of *A* and *B*. Aspartic acid is N-terminal. Carboxypeptidase has been found to split *Bc* into free aspartic acid and glutamic acid. Therefore, the free acids rather than their amides, glutamine and asparagine, appear to build up the dipeptide. The cleavage of dipeptides, using relatively high concentrations of carboxypeptidase, has been reported previously[52,53].

From the results obtained the following amino acid sequence can be deduced:




Cys* signifies the cysteine residue which has been labeled by reaction with DDPM. It has not been established whether the amino acid residue symbolized by Glu* is

a glutamyl or a glutaminyl residue. The arrows below the formula point to the peptide bonds which have been found susceptible to cleavage by the proteolytic enzymes, pepsin and subtilisin. The split linkages conform to the known specificities of the enzymes used: subtilisin has previously been reported to attack, among others, peptide bonds involving the carboxyl groups of glutamine[35,36] and glutamic acid[37] residues, whereas pepsin is known to split bonds adjacent to aromatic amino acid residues[38-40].

In order to confirm these results, experiments were carried out to label the SH group of bovine serum albumin with a reagent other than DDPM. For this purpose, 1-chloro-2:4-dinitrobenzene was chosen. It was allowed to react with the albumin in a molar ratio of approximately 1.2:1. When the dinitrophenylated protein was digested with pepsin and the digest worked up, a S-DNP-cysteine peptide, *I*, was obtained (Fig. 3). The yield of this peptide was low and its colour very faint, and it was accompanied by two DNP-peptides which carried the DNP group on functional groups other than SH. This shows that the specificity of chlorodinitrobenzene for SH groups, even under relatively favourable conditions, is inferior to that of DDPM. (The same is true for the use of FDNB as a SH reagent. ZUBER, TRAUMANN AND ZAHN[48] who studied the reaction of FDNB with wool, at a pH of 5.2, were unable to effect specific and complete dinitrophenylation. Treatment of serum albumin with FDNB at pH 5.5 has been reported by ŠORM, KÖRBL AND MATOUSEK[49] to yield a yellow product which contained three dinitrophenyl residues per mole of albumin as estimated spectrophotometrically; this compares well with our finding of three DNP peptides in the peptic digest of serum albumin treated with chlorodinitrobenzene.) The structural investigation of the S-DNP-peptide *I* has given results analogous to those obtained with the DDPS-cysteine peptide *B* and has thus been valuable in corroborating the conclusions drawn above (Table III, Fig. 4). Attention may be drawn to the fact that in the subtilisin digest of peptide *I* a S-DNP-cysteine peptide, 3*s*, quite analogous to the S-DDPS-cysteine peptide, $Bb_2$, was found. 3*s* was negatively charged at a pH of 4.7, gave no colour reaction with ninhydrin and appeared not to have a free α-amino group. It may be assumed that the lack of an α-amino group in both 3*s* and $Bb_2$ is similarly due to the cyclisation of a N-terminal glutamine residue resulting in a pyroglutamic acid residue.

When the S-DDPS derivative of human serum albumin was degraded with pepsin in the same manner as the derivative of bovine serum albumin, exactly the same DDPS-cysteine peptides were obtained. The finding that the amino-acid sequences in the S-DDPS-cysteine peptides *B* (from BSA) and B′ (from HSA) were identical was not expected. The two serum albumins are known to be similar but they have been reported to differ with respect to amino acid composition[41,42], N-terminal[43] and C-terminal amino acid sequences[44], u.v. light absorption[21], and the composition of peptides in partial hydrolysates[45].

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