in a refrigerator overnight to yield 75 mg. of needles, m.p. 202–203° dec.

Anal. Calcd. for $C_{13}H_{14}N_6$: C, 61.44; H, 5.55. Found: C, 61.43; H, 5.38.

Biological Assays.—The microbiological assay procedure with *Lactobacillus arabinosus* 17–5 has been previously described in detail.[6] The general technique for the lettuce seed germination study has been reported[4,14]; however, in the present study, the pre-soaked seeds were placed on filter paper wet with water alone for germination at 30° in the dark.

(14) C. G. Skinner, F. D. Talbert and W. Shive, *Plant Physiol.*, **33**, 190 (1958).

AUSTIN, TEXAS

[CONTRIBUTION FROM THE NORTHERN UTILIZATION RESEARCH AND DEVELOPMENT DIVISION[1] AND THE VETERINARY MEDICAL RESEARCH INSTITUTE, IOWA STATE COLLEGE[2]]

Possible Toxic Factor of Trichloroethylene-extracted Soybean Oil Meal[3]

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Treatment of amino acids and peptides with trichloroethylene, under conditions known to produce the bovine aplastic anemia-causing factor in soybean oil meal and other proteins, showed that trichloroethylene reacted readily with the sulfhydryl groups of cysteine and reduced glutathione. The S-dichlorovinyl derivatives of L-cysteine and L-glutathione were synthesized and found to produce the aplastic anemia syndrome in calves typical of that produced by trichloroethylene-extracted soybean oil meal. The stability and properties of the S-dichlorovinyl derivatives are in agreement with known properties and mode of formation of the toxic principle in the meals. Enzymic hydrolysis of a toxic protein from trichloroethylene-extracted soybean oil meal resulted in the loss of a large portion of its toxicity. Chromatographic studies on the hydrolyzate indicated the presence of products resembling the synthetic compounds, and giving tests for the S-dichlorovinyl group.

Earlier studies in these laboratories[5] have shown that the bovine aplastic anemia-causing factor in trichloroethylene-extracted soybean oil meal (TESOM) is associated with the purified protein component of the meal, that it is labile to vigorous acid hydrolysis and that measurable loss in toxicity occurred on heating the alkaline (*p*H 11–12) protein dispersion at 60° for 4 hours. Independent studies[6] confirmed these findings and showed that a suspension of the meal in dilute sulfuric acid at *p*H 1.5 at 65–70° for 48 hr. retained its toxicity. Picken and Biester[7] have shown that the aplastic anemia-causing factor is formed by heating zein, casein, lactalbumin and gelatin, as well as soybean protein with trichloroethylene (TCE) at 120°, and that the presence of lysine, tryptophan and tyrosine in the protein does not appear essential for the formation of the toxic entity.

A preliminary report[8] listed the findings that prompted the synthesis of S-(dichlorovinyl)-L-cysteine which on oral administration to calves produced an aplastic anemia syndrome typical of that produced by TESOM. The present paper describes the details of these experiments.

(1) One of the Divisions of the Agricultural Research Service, U. S. Department of Agriculture, Peoria, Illinois. Article not copyrighted.

(2) A report of work done, in part, under contract with the U. S. Department of Agriculture and authorized by the Research and Marketing Act. Contract supervised by the Northern Utilization Research and Development Division.

(3) Presented before the Division of Biological Chemistry, 132nd Meeting, American Chemical Society, New York, N. Y., Sept. 8–13, 1957.

(4) Veterinary Medical Research Institute, Iowa State College, Ames, Iowa.

(5) L. L. McKinney, F. B. Weakley, R. E. Campbell, A. C. Eldridge, J. C. Cowan, J. C. Picken, Jr., and N. L. Jacobson, *J. Am. Oil Chemists' Soc.*, **34**, 461 (1957).

(6) T. A. Seto, M. O. Schultze, V. Perman, F. W. Bates and J. H. Sautter, *J. Agr. Food Chem.*, **6**, 49 (1958).

(7) J. C. Picken, Jr., and H. E. Biester, Abst. Papers, 132nd Meeting, Am. Chem. Soc., New York, N. Y., Sept. 8–13, 1957.

(8) L. L. McKinney, F. B. Weakley, A. C. Eldridge, R. E. Campbell, J. C. Cowan, J. C. Picken, Jr., and H. E. Biester, THIS JOURNAL, **79**, 3932 (1957).

Results and Discussion

Sealed Tube Experiments.—Sealed tube interaction of amino acids with TCE at 120° for 12 hr. slightly decreased the ninhydrin color of DL-asparagine, L-glutamic acid and DL-lysine hydrochloride (Table I) as determined by chromatogram assay. Interaction of cysteine with TCE in sealed tubes liberated hydrogen sulfide together with a significant amount of chloride; the ninhydrin values were inconsistent and are not included in Table I. The presence of steel wool in the cysteine–TCE reaction prevented the liberation of hydrogen sulfide and resulted in the formation of an additional ninhydrin-positive spot observed by paper chromatography.[9] This new ninhydrin-positive spot absorbed ultraviolet light and gave a color reaction with 4-(*p*-nitrobenzyl)-pyridine (4-NBP).[10]

Sealed tube experiments indicated that TCE reacted more readily with glutathione than it did with cysteine. Paper chromatograms of the glutathione–TCE reaction mixture and admixtures revealed a ninhydrin-positive spot which absorbed ultraviolet light and gave a 4-NBP test identical to that given by the compound S-(dichlorovinyl)-L-cysteine. Occasionally two spots, typical of the S-dichlorovinyl derivatives of cysteine and glutathione, appeared on paper chromatograms of glutathione–TCE reaction mixtures. Usually, only the spot associated with S-(dichlorovinyl)-L-cysteine was in evidence, indicating a breakdown of the peptide in the reaction mixture at 120° over the 20-hour period.

Table II records the relative amounts of inorganic chloride liberated by sealed tube interaction of various amino acids and peptides with TCE at approximately 120°. No increase in chloride

(9) M. Gutcho and L. Laufer, "Paper Chromatography of Glutathione and Its Hydrolysis Products," in "Glutathione, A Symposium," Academic Press, New York, N. Y., 1954, pp. 79–87.

(10) (a) T. A. Geissman, H. Hochman and R. T. Fukuto, THIS JOURNAL, **74**, 3313 (1952); (b) J. Epstein, R. W. Rosenthal and R. J. Ess, *Anal. Chem.*, **27**, 1435 (1955).

TABLE I

NINHYDRIN RESPONSE OF SEALED TUBE EXPERIMENTS (12 HOURS, 120°)

Amino acid	Color value of amino acids[a] Sealed tube contents Standard	Soln. assay Control	Soln. assay Reaction	Chromatogram assay Control	Chromatogram assay Reaction
L-Arginine hydrochloride	105	98	97	100	102
DL-Asparagine	97	98	100	80	71
L-Aspartic acid	102	95	95	87	89
L-Glutamic acid	109	110	101	102	90
L-Histidine hydrochloride	102	104	106	77	77
DL-Lysine (free base)	89	84	86	85	85
DL-Lysine hydrochloride	111	111	104	88	80
DL-Methionine	101	102	100	98	99
L-Proline	30	30	30	21	20
DL-Serine	103	99	95	84	83
DL-Threonine	97	96	98	87	89
L-Tyrosine	96	95	94	60	59
DL-Tryptophan	61	60	61	57	58

[a] Average spectrophotometer reading (optical density of ninhydrin color reaction) × 1,000, equal to 0.1 micromole starting amino acid. Final ninhydrin reaction volume 25 ml. read at 570 mμ (proline at 440 mμ) in standard 20 × 150-mm. test-tubes.

was obtained from the TCE interactions with DL-asparagine, L-glutamic acid or DL-lysine hydrochloride, all of which previously showed slight decreases in ninhydrin color (Table I). Significant amounts of chloride were obtained only in TCE reaction mixtures containing either L-cysteine or L-glutathione.

TABLE II

CHLORIDE OBTAINED ON HEATING AMINO ACIDS OR PEPTIDES WITH TRICHLOROETHYLENE IN SEALED TUBES (20 HOURS, 120°)

Amino acid or peptide	Chloride equiv. per mole[a]
DL-Asparagine	None
L-Cysteine (free base)	0.3
L-Glutamic acid	None
L-Glutathione	1.0
DL-Lysine monohydrochloride	None
DL-Serine	None
L-Tyrosine	None
Glycyl-DL-serine	None
Glycyl-DL-threonine	None
Glycyl-DL-tryptophan	None

[a] Analyzed by the modified Volhard method that eliminates cysteine and glutathione interference in the chloride analysis.

S-(Dichlorovinyl)-L-cysteine and S-(dichlorovinyl)-L-glutathione were analyzed for chloride following the same sealed-tube treatment accorded the amino acids and peptides. Chloride was liberated from S-(dichlorovinyl)-L-cysteine on an equimolar basis whereas very little, if any, chloride was obtained from the glutathione derivative.

S-Dichlorovinyl Derivatives.—The S-dichlorovinyl derivatives of L-cysteine and L-glutathione were prepared by treating the di- and tri-sodium salts with TCE in liquid ammonia. Products, obtained in 60–70% yields, were characterized by elementary analyses, titration, ultraviolet absorption and paper chromatography. These derivatives exhibit the intense ultraviolet absorption characteristic of ethylene groups adjacent to a sulfur atom[11] as shown in Fig. 1. The molecular extinction coefficients of 3200–3400 at 258–260 mμ indicate the roles played by the sulfur and chlorine atoms in the hyperconjugation effect.

Because both compounds alkylated 4-NBP[10] to give an intense blue color typical of that obtained with mustard gases, 2–5 γ could be detected on paper chromatograms.

Attempts to prepare the hydrochloride of S-(dichlorovinyl)-L-cysteine, even with dry hydrogen chloride in ether or 1-butanol, resulted in either recovering the free base or in decomposition. Failure to form the hydrochloride might be due to the acidic properties of the β-chlorine on the vinyl group as indicated by the titration curve. S-(Dichlorovinyl)-L-cysteine slowly liberated chloride in dilute alkali at room temperature and rapidly decomposed at elevated temperatures. It was stable in dilute acid at room temperature but liberated chloride slowly at 60°. Attempts to prepare the sulfone were also unsuccessful as the compound decomposed on warming in glacial acetic acid. The basicity would be expected to be accentuated in glacial acetic acid which may encourage reaction of the amino group with the more active chlorine in the dichlorovinyl group.

Biological Response to S-Dichlorovinyl Derivatives.—The biological response of calves to the administration of graded levels of the S-dichlorovinyl derivatives of L-cysteine and L-glutathione is shown in Table III. Calves 270 and 271 were not able to tolerate the proposed dosages of 1,000 and 200 mg. per day per 100-lb. calf weight. A marked loss of weight and lack of thrift were noted almost immediately. After 5–7 days the calves were so weak they could no longer consume feed. Removal of the toxic compound from the milk for a day or two resulted in some improvement in condition and return of appetite though the loss in weight continued. Administration of the compound at reduced levels was then initiated and continued without the return of the severe effects. A clinical disease syndrome developed very rapidly and both calves were moribund on the 14th day. At necropsy examination both calves exhibited the typical severe lesions characteristic of TESOM-induced aplastic anemia of the bovine. The high dosage levels of S-(dichlorovinyl)-L-cysteine obviously produced a condensed disease syndrome.

In contrast, the calves fed S-(dichlorovinyl)-L-cysteine at levels from 50 to 10 mg. per day per 100-lb. calf weight presented the typical clinical picture associated with the consumption of toxic TESOMs.[12] Even then the responses observed with the 50-mg. and possibly the 35-mg. level (calves 274 and 276) were more profound than normally observed with highly toxic TESOMs. The 20- and 15-mg. levels of S-(dichlorovinyl)-L-cysteine compared very favorably with levels of 190 and 127 g. per day per 100-lb. calf weight of highly toxic TESOM. Even the 10-mg. level, with deaths at

(11) C. C. Price and J. Zomlefer, THIS JOURNAL, **72**, 14 (1950).

(12) V. Perman, C. F. Rehfeld, J. H. Sautter and M. O. Schultze, *J. Agr. Food Chem.*, **4**, 959 (1956).

about 60 days, produced a more severe effect than many of the commercial TESOMs assayed,[13] indicating that the unknown toxic entity present in TESOM of low toxicity may exist in very small amounts.

Most striking is the remarkable correlation between all dosage levels of S-(dichlorovinyl)-L-cysteine and the appearance of all of the symptoms. Eliminating the 1,000- and 200-mg. levels (calves 270 and 271) from consideration because of excessive dosages, the agreements within levels and between levels are ideal. With the exception of calf 227, that appeared to be a little more resistant to the compound initially than expected, all data fit well into plots of dosage *vs.* time of appearance of symptoms. These striking correlations point specifically to the premise that S-(dichlorovinyl)-L-cysteine is acting at a specific site or sites in a direct manner. The relationship is obviously quantitative, *i.e.*, concentration of toxic entity related to effect.

S-(Dichlorovinyl)-L-glutathione was administered intravenously to conserve the supply of the compound. Earlier studies comparing the dose-effect relationships between oral and intravenous routes of administration of the cysteine derivative had shown that the intravenous route was in the order of two times as efficient as the oral route.[14]

The intravenous dosage level of S-(dichlorovinyl)-L-glutathione at 18.6 mg. per day per 100-lb. calf is molecularly equivalent to 10 mg. of the cysteine derivative, suggesting a comparative level of 20 mg. (oral) for the cysteine derivative. That the glutathione derivative was typically toxic in all respects is shown by the results presented in Table III. When compared with the 20-mg. level of the cysteine derivative it did not appear to be as toxic on a mole per mole basis. Although the thrombocytopenia did develop rapidly in comparison to the 20-mg. calf, there was a definite lag in the appearance of leucopenia and relative lymphocytosis. Clinically the terminal syndrome, temperature spike and rapid decline in condition, appeared much later than would be expected for a 20-mg. oral level. Since it was administered intravenously, there is no basis for a direct comparison, but it appeared to be in the range of $^1/_2$ to $^2/_3$ as potent as the cysteine analog.

Studies on the Enzymic Hydrolyzate of Toxic TESOM Protein.—The wet protein curd was isolated as previously described[5] and digested with pepsin, followed by crude trypsin and finally with erepsin, resulting in hydrolysis of 44% of the peptide bonds. The spray-dried hydrolyzate was fed to calf 269 and compared with feeding the unhydrolyzed protein to calf 224A as shown in Table IV. A higher dosage level was chosen for the enzymic digest because previous experience[5] had shown that toxicity might be lost during prolonged enzymic treatment. A comparison of the data in Table IV indicates that perhaps better than one-half of the toxicity was lost during the nine days of enzymic digestion. Other workers[6] have reported little, if any, loss in toxicity on digestion with pancreatin for 66 hours.

(13) J. C. Picken, Jr., and N. L. Jacobson, unpublished data.

(14) J. C. Picken, Jr., and H. E. Biester, Abst. Papers, 134th Meeting, Am. Chem. Soc., Chicago, Ill., Sept. 7–12, 1958.

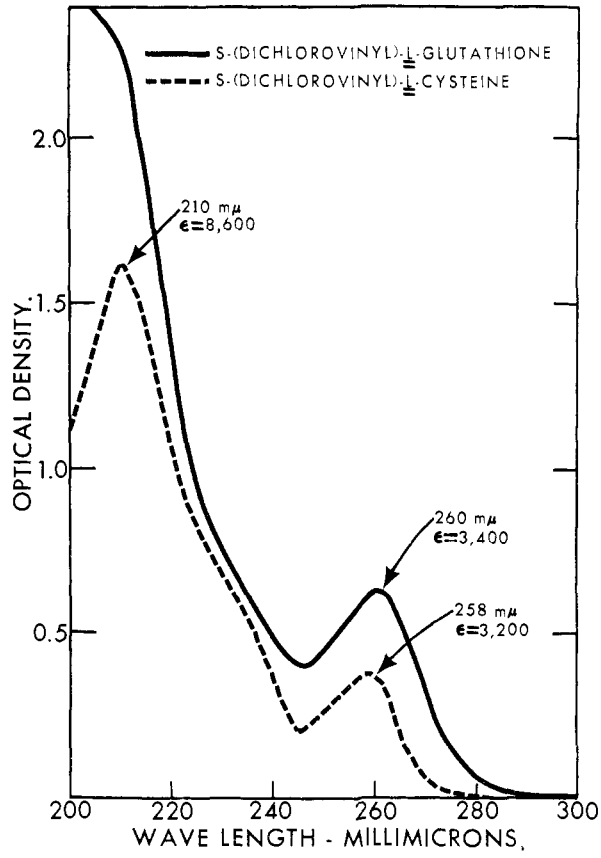

Fig. 1.—Ultraviolet spectra of S-dichlorovinyl derivatives of L-cysteine and L-glutathione.

After treatment of an aqueous solution of the enzymic digest with carbon to remove nucleic acids, the hydrochlorides were dissolved in 80% methanol and chromatographed on an alumina column. The fractions (100 ml.) were concentrated to 1–2 ml. and examined by paper chromatography. 4-NBP positive spots were found in the early fractions (3–6) with R_f values of 0.50 and 0.78; later fractions (12–16) also contained an R_f 0.50 spot. These spots were ninhydrin and iodoplatinate (sulfur) positive but negative for phosphorus. The phosphorus test was used to rule out nucleic acids and soybean inositides, which were also found to give positive tests with 4-NBP.

The R_f 0.50 material also was obtained by extracting the hydrolyzate with boiling 95% ethanol. Separation and concentration of this material were achieved by paper chromatography. The isolated concentrate was rechromatographed to yield well-defined spots that gave the same tests as did the S-dichlorovinyl derivatives of L-cysteine and L-glutathione. Furthermore, the concentrate inhibited the growth of a yeast in the same manner as did the synthetic derivatives.

Correlation of S-Dichlorovinyl Structure with the Toxicity of TESOM.—The data at hand indicate a strong possibility that the S-dichlorovinyl structure may be the aplastic anemia-causing factor in TESOM protein and possibly in other proteins heated with TCE.[7] However, there is yet no direct proof that S-(dichlorovinyl)-L-cysteine is the toxic entity of TESOM. Such proof must await isola-

TABLE III

BIOLOGICAL RESPONSE OF CALVES TO ADMINISTRATION OF SYNTHETIC COMPOUNDS

Biological response	S-(Dichlorovinyl)-L-cysteine[a] Calf no. 270	271	274	226	213	227	222	224	S-(Dichlorovinyl)-L-glutathione[b]
Dosage data:									
Proposed mg./100 lb./day	1000[c]	200[c]	50	35	20	15	10	10	18.61
Total mg. consumed	2940	1600	1205	1434	522	762	791	895	1226
Wt. of calf, lb., initial	115	96	103	151	122	97	91	108	127
final	87	87	109	173	166	135	150	182	171
Mg. consumed/100 lb./day	208	124	49.4	35.4	19.3	14.9	10.6	10.1	18.2[d]
	Days to develop								
Hematologic symptoms:									
Thrombocytopenia									
Initial decline	10	12	13	16	18	19	19	18	12
Below 200,000 mm.^{3}	10	12	14	18	18	24	20	22	15
150,000 mm.^{3}	10	12	15	18	20	24	20	22	18
100,000 mm.^{3}	13	12	15	19	21	25	24	25	18
50,000 mm.^{3}	Never	12	18	21	22	28	27	29	20
Leucopenia									
Below 5000	9	10	19	19	22	31	27	27	31
3000	14	12	22	24	25	40	53	60	44
2000	Never	14	22	25	Never	42	54	60	Never
Lymphocytosis									
Above 80%	13	10	19	20	21	24	31	32	25
85%	13	10	21	21	22	28	31	32	28
90%	13	10	21	24	22	28	32	32	28
95%	13	12	21	24	25	31	32	33	33
Clinical symptoms:									
Fecal blood	13	11	16	21	25	32	27	26	21
Visible hemorrhages	11	13	19	20	22	29	26	25	24
Temp. spike	13	11	21	24	25	33	56	59	44
Dead				25		44		61	
Moribund (killed)	14	14	23		27		62		49
Necropsy:									
Typical lesions of aplastic anemia of bovine	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

[a] Administered orally. [b] Administered intravenously. [c] Could not tolerate proposed dosage. [d] Administered during first 45 days.

tion from TESOM and then chemical characterization. The synthesis of S-(dichlorovinyl)-L-cysteine along with its chemical and pharmacological characterization should materially assist in experimental designs to isolate and identify the toxic factor.

That chlorinated organic solvents react readily with the sulfhydryl groups of proteins at a neutral or slightly alkaline *p*H is not a new discovery. The work of Harris and associates[15] showed that such compounds as ethylene dichloride, trimethylene dichloride and allyl chloride react readily with the sulfhydryl groups of reduced wool at 35° in either aqueous or organic media to produce thioethers.

Previous studies[16] indicated that the toxicity of TESOM did not result from the reaction of decomposition products of TCE with soybean meal. It, therefore, appeared that TCE was reacting directly with the meal to produce the toxic factor. Fractionation studies[5] showed that the toxicity was associated with the purified protein component of TESOM, indicating that the TCE was reacting with an amino acid residue in the protein. The work of Picken and Biester[7] showed that indeed TCE reacts with proteins with the aid of heat to produce an aplastic anemia-causing factor. When amino acids and peptides were heated with TCE in sealed tubes, only those containing sulfhydryl groups appeared to react. When S-(dichlorovinyl)-L-cysteine was fed to calves at levels corresponding to 75–125 p.p.m. in TESOM, toxicity symptoms were produced identical to those caused by TESOM. These levels are in agreement with the differences in chloride and sulfhydryl contents of TESOM and hexane-extracted soybean oil meal previously reported.[5]

Both the synthetic product and the toxicity factor in the meal are unstable to mild alkali and strong acid but are fairly stable to mild acid. It has been reported that immature beans more consistently produce a highly toxic meal on processing with TCE[16] while beans that have been stored a year or more produce meals of very low toxicity.[17,18] Immature beans are known[19] to be rich in sulfhy-

(15) (a) W. I. Patterson, W. B. Geiger, L. R. Mizell and M. Harris, *J. Research Natl. Bur. Standards*, **27**, 89 (1941); (b) W. B. Geiger, F. F. Kobayashi and M. Harris, *ibid.*, **29**, 381 (1942).

(16) J. C. Picken, Jr., N. L. Jacobson, R. S. Allen, H. E. Biester, P. C. Bennett, L. L. McKinney and J. C. Cowan, *J. Agr. Food Chem.*, **3**, 420 (1955).

(17) W. R. Pritchard, C. E. Rehfeld, W. E. Mattson, J. H. Sautter and M. O. Schultze, *Am. J. Vet. Research*, **17**, 430 (1956).

(18) K. Barth, "Ist mit trichloräthylen extrahiertes Sojashrot fur die Ernährung landwirtschaftlecher Natztiere Verwendbar?" Thesis, University of Munich, Feb. 27, 1957.

(19) F. B. Weakley and L. L. McKinney, *J. Am. Oil Chemists' Soc.*, **35**, 281 (1958).

TABLE IV

BIOLOGICAL RESPONSE OF CALVES FED TOXIC TESOM PROTEIN AND THE ENZYMIC DIGEST OF THE PROTEIN

Biological response	Protein Calf 224A	Digest[a] Calf 269
Dosage data:		
Total g. consumed	1702	3594
Wt. of calf, lb., initial	102	71
final	136	125
av.	119	98
G. consumed/100 lb./day	41	55
	Days to develop	
Hematologic symptoms:		
Thrombocytopenia		
Initial decline	27	No trend
Below 200,000 mm.^{3}	34	62–65
150,000 mm.^{3}	34	65
100,000 mm.^{3}	34	65
50,000 mm.^{3}	Never	Never
Leucopenia		
Below 5000	34	65
3000	Never	Never
2000	Never	Never
Lymphocytosis		
Above 80%	32	27
85%	32	27
90%	32	38
95%	34	Never
Clinical symptoms:		
Fecal blood	31	67
Visible hemorrhages	31	67
Temperature spike	33	67
Moribund (sacrificed)	35	72[b]
Necropsy, lesions:	Severe	Mild

[a] Report of enzymic digest fed is calculated to its protein content, *i.e.*, 77%. [b] Feed was stopped on 67th day.

dryl groups, and a decrease in sulfhydryl groups is noted on storage of mature beans in this study.

Experimental

Sealed Tube Experiments. (a) Ninhydrin Color.—Individual amino acids (1 mmole) were heated in sealed tubes at 120° for 12 hr. with TCE (0.1 ml.) and water (0.05 ml.). The amino acid moistened with 0.05 ml. of water served as a control. Following the heating period, the contents of each tube were diluted to 250 ml. with water (L-tyrosine to 500 ml.). Optical densities of the ninhydrin color for increasing aliquots of each solution were plotted against the respective amino acid concentrations. From each curve the average increase in optical density per unit aliquot, equal to 0.1 micromole starting amino acid, was obtained and formed the basis for comparing the effect of heat and TCE on the ninhydrin color response of the amino acid. Each amino acid reaction mixture and control was subjected to quantitative paper chromatographic analysis[20] on Whatman No. 1 paper in order that ninhydrin positive spots having the same R_f might be correlated with the color values obtained by direct assay of the solution. The results are summarized in Table I.

(b) **Chloride Liberation.**—Amino acids (1 mmole) and dipeptides (0.5 mmole) or the tripeptide L-glutathione (0.3 mmole) were moistened with 0.2 ml. (2.2 mmoles) of water-washed TCE in combustion tubes which were sealed and heated at 120° for 20 hr. Amino acids and peptides heated with toluene served as the controls. The contents of each tube were taken up in 50-ml. aqueous solution (glycyl-DL-tryptophan was dissolved by acidifying with nitric acid). L-Cysteine and L-glutathione interfered in the Volhard method of chloride analysis[21] and, therefore, were precipitated from aqueous solution with a slight excess of cupric acetate followed by removal of the chloride-free residue prior to the start of the chloride analysis. An average of the chloride liberated in the interactions of serine and dipeptides with TCE was taken as the chloride blank. S-(Dichlorovinyl)-L-cysteine liberated chloride in a molar ratio when heated with TCE or toluene under the same sealed-tube conditions. S-(Dichlorovinyl)-L-glutathione, similarly treated, liberated very little chloride. Table II records the chloride yields resulting from the sealed tube reactions.

S-(Dichlorovinyl)-L-cysteine.—Sodium and dry L-cysteine (0.10 mole) were added in portions to 250–300 ml. of dry liquid ammonia contained in a stoppered flask equipped with a magnetic stirrer (glass-covered bar) and a drying tube. The reaction flask was set in a dish so that alcohol could be applied to prevent frosting. Sodium was added first to give the characteristic blue color, which disappeared when cysteine was added to form the disodium salt. TCE (8.96 ml., 0.10 mole) was dissolved in liquid ammonia (25 ml.) and added slowly. After 30 minutes a stream of air was directed on the reaction flask to speed up evaporation of ammonia, which required about 2 hr.

The slightly colored residue was dissolved in 300 ml. of water, freed from traces of ammonia *in vacuo* and, by addition of glacial acetic acid, adjusted to *p*H 5.0 from an initial *p*H 11.9. The resulting copious precipitate was diluted with one volume of ethanol, cooled overnight in a refrigerator and isolated by filtration.

The crude precipitate was dissolved in 725 ml. of water at 70°, 1 g. of activated carbon was added, and the mixture filtered while hot. An equal volume of ethanol was added to the clear filtrate and, after overnight refrigeration, 11.16 g. of needle-like crystals was obtained. An additional 2.77 g. of crystals was obtained by evaporating the mother liquor from the crystallization for a total yield of 67%; m.p. 157–159° dec. *Anal.* Calcd. for $C_5H_7O_2NSCl_2$: C, 27.79; H, 3.27; N, 6.48; S, 14.84; Cl, 32.82. Found: C, 27.83; H, 3.29; N, 6.47; S, 14.94; Cl, 32.8. *Titration* neut. equiv. (formol), calcd. 216.03; found, 216; *p*I′, 5.4; pK'_1, 2.5; pK'_2, 8.2. Ultraviolet absorption is shown in Fig. 1. *Soly.*, g./100 ml.: H_2O, $2.5^{75°}$; $1.0^{25°}$; $0.7^{5°}$. 50% EtOH, $0.3^{5°}$. *Ascending paper chromatography:* on Whatman No. 1 paper: 70:30 1-propanol-water,[9] R_f 0.72; positive ninhydrin, 4-NBP, ultraviolet and iodoplatinate tests. Adaptation of 4-NBP as a spray reagent for paper chromatograms was achieved by using 0.01 molar alcoholic 4-NBP as the initial spray followed by drying the chromatogram, first in air and then at 110°, for approximately 10 minutes. Final spraying of the chromatogram with 0.1 *N* NaOH produced a blue spot on a white background.

The filtrate from the crude precipitation contained 5.9 g. (0.101 mole) of sodium chloride. Paper chromatograms showed the presence of S-(dichlorovinyl)-L-cysteine along with a small amount of cysteine and cystine. No other ninhydrin positive material was present.

Paper chromatograms of the crude precipitate showed a small spot for cystine. In preparations where cystine was found in the crystalline product, it was removed by recrystallizing in the presence of a small amount of potassium cyanide but adding acetic acid to keep the *p*H at 5.0.

If care was not exercised to exclude moisture from the liquid ammonia reaction, the reaction products were highly colored, contained excess chloride, and paper chromatography revealed a spot with a low R_f value.

Attempts to prepare the hydrochloride of S-(dichlorovinyl)-L-cysteine even with dry ether or 1-butanol and HCl gas failed.

Stability of S-(Dichlorovinyl)-L-Cysteine to Alkali and Acid.—One millimole of the compound was placed in 20 ml. of either aqueous sodium hydroxide or sodium methoxide in methanol under the conditions shown in Table V. The extent of hydrolysis was determined by estimating the amount of chloride liberated or by titrating aliquots to determine the amount of alkali consumed.

One millimole of the compound was placed in 20 ml. of 4 *N* sulfuric acid for 18 hr. At 60° only 0.058 meq. of chloride was liberated compared to none at 25°.

Attempt to Prepare the Sulfone of S-(Dichlorovinyl)-L-cysteine.—Treatment of S-(dichlorovinyl)-L-cysteine with hydrogen peroxide in glacial acetic acid by the method used

(20) L. Fowden, *Biochem. J.*, **48**, 327 (1951).

(21) W. Horwitz, Editor, "Official Methods of Analysis—A.O.A.C.," 8th Ed., published by A.O.A.C., Washington, D. C., 1955, page 866.

TABLE V

STABILITY OF S-(DICHLOROVINYL)-L-CYSTEINE TO ALKALI

Alkali	Equiv. used	Hydrolysis—Time, hr.	Hydrolysis—Temp., °C.	Equiv. Cl^- liberated
H_2O	None	7 days	25	None
NaOH	3.0	4 days	25	0.39
NaOH	50	6	90	1.92
NaOMe	3.0	18	5	None
NaOMe	3.0	18	25	1.14
NaOMe	3.6	1	80	1.86
NaOMe	3.0	2	60	0.25[a]
NaOMe	3.0	4	60	1.32[a]
NaOMe	3.0	6	60	1.87[a]

[a] Values obtained by titrating the sodium methoxide used; other values obtained by estimating inorganic chloride present.

by Fuson, *et al.*,[22] and by the method of Backer, *et al.*,[23] failed to give a crystalline sulfone. Instead, as much as 1 equivalent of chloride was split out and amorphous products were obtained. Essentially the same result was obtained by treating S-(dichlorovinyl)-L-cysteine with glacial acetic acid alone.

S-(Dichlorovinyl)-L-glutathione.—Reduced L-glutathione (6.15 g., 0.02 mole) was treated with sodium in 750 ml. of liquid ammonia followed by addition of TCE (4.0 ml., 0.045 mole) in liquid ammonia (50 ml.) as described for the reaction with cysteine.[24] The ammonia-free aqueous solution of the residue (50 ml.) had a *p*H of 10.8 and was adjusted to *p*H 5.0 with acetic acid and then to *p*H 3.0 with 3 *N* HCl. Four volumes of absolute ethanol were added to the clear solution resulting in a turbid mixture. After standing in a refrigerator for 3 days, 6.82 g. of precipitate was removed. The precipitate was dissolved in 275 ml. of 50% ethanol at 70°, treated with 0.5 g. of activated carbon and filtered hot. The clear filtrate, allowed to cool to room temperature, was placed in a refrigerator overnight and yielded 4.5 g. (56%) of needle-like crystals. Evaporation of the mother liquor and crystallization yielded 1.6 g. (total 76%). Chromatograms indicated 98% purity, with the impurity being oxidized glutathione. Recrystallization from 50% ethanol, containing 0.2 g. of potassium cyanide, gave 4.5 g. of chromatographically pure crystals; m.p. 192–193° dec. *Anal.* Calcd. for $C_{12}H_{17}O_6N_3SCl_2$: C, 35.84; H, 4.23; N, 10.45; S, 7.97; Cl, 17.64. Found: C, 36.1; H, 4.23; N, 10.39; S, 8.1; Cl, 17.7. Ultraviolet absorption is shown in Fig. 1; $[\alpha]^{26}D$ +24.3° (*c* 2 054, in 1 *N* HCl). *Soly.*, g./100 ml.: H_2O, 3.1[5°]; 50% EtOH, 3.5[70°]; 0.5[5°]. *Ascending paper chromatography:* on Whatman No. 1 paper: 70:30 1-propanol–water, R_f 0.44; positive ninhydrin, 4-NBP, ultraviolet and iodoplatinate tests.

Chloride analysis of the mother liquor indicated that 0.017 mole was liberated by the reaction of glutathione with TCE. Paper chromatograms of the mother liquor showed eight ninhydrin positive spots, only one of which was 4-NBP positive (S-(dichlorovinyl)-L-glutathione); one spot corresponded to oxidized glutathione; the other six spots were small and assumed to be hydrolysis products of glutathione. Preliminary experiments showed that if care was not exercised in excluding moisture or if the trisodium salt of glutathione was allowed to stand overnight in liquid ammonia, the small spots became much larger.

Biological Response of Calves.—The development of aplastic anemia in young calves weighing about 100 lb. was followed by correlating the blood platelet decline (thrombocytopenia) with leucopenia and relative lymphocytosis as described by Perman, *et al.*,[12] and by observing the development of clinical symptoms characteristic of aplastic anemia caused by TESOM.[16] The biological response to oral administration of S-(dichlorovinyl)-L-cysteine at graded dosage levels and to the intravenous administration of S-(dichlorovinyl)-L-glutathione is shown in Table III.

(22) R. C. Fuson, C. C. Price and D. M. Burness, *J. Org. Chem.*, **11**, 475 (1946).

(23) H. J. Backer, J. Strating and J. F. A. Hazenberg, *Rec. trav. chim.*, **72**, 813 (1953).

(24) W.O. Kermack and N. A. Matheson, *Biochem. J.*, **65**, 45 (1957), recently reported the preparation of S-methylcysteine and S-methylglutathione by a similar method.

The biological response of the calf fed the spray-dried enzymic digest of toxic TESOM protein is compared with that of the starting protein in Table IV. The dosage levels reported for the enzymic digest are based on the starting protein content of the digest, *i.e.*, 77% of the actual weight used.

Enzymic Digestion of Toxic Protein.—Protein curd (14 lb., 16.0% N, dry basis) was prepared from 60 lb. of the undenatured, specially prepared toxic TESOM as previously described.[5] The curd slurry (27.7 gal., *p*H 4.5) was dissolved by adding 540 ml. of concentrated HCl to *p*H 2.35; TCE (6 ml. per gal.) was added as a preservative, followed by 47.7 g. of pepsin (1:10,000, 7 mg./g. protein) dissolved in 160 ml. of water. After digestion for 21 hr. at 52° the *p*H was adjusted to 2.58 with 250 ml. of concentrated HCl, and the digestion was continued for 48 hr. at which time the *p*H was 2.77.

The *p*H of the digest then was adjusted to 8.3 with 3.45 l. of 20% sodium hydroxide, and 196 g. of crude trypsin (4 × U.S.P., 30 mg./g. protein) slurried in 1 l. of water was added. Additional TCE (84 ml., 3 ml./gal.) was added, and the mixture was incubated 48 hr., 37°, during which time 814 ml. of 20% sodium hydroxide was required to maintain the *p*H at 8.3.

The digest was adjusted to *p*H 7.5 with 280 ml. of concentrated HCl, and 196 g. of erepsin powder (30 mg./g. protein), slurried in 1.5 l. of water, was added. An additional 56 ml. of TCE (2 ml./gal.) was added to ensure sterility. After incubation for 68 hours, 37°, with occasional stirring, the *p*H was adjusted to 7.0 with 210 ml. of concentrated HCl and stored (30.4 gal.) in a cold room (1°) from which portions were removed for spray drying over a 2.5-day period.

The percentage of peptide bonds broken as estimated after each step of the enzymic hydrolysis is shown in Table VI. The spray-dried hydrolyzate contained 13.03% nitrogen (dry basis), 5.24% of which resulted from added enzymes. Thus 1.30 lb. of hydrolyzate was equivalent to 1.00 lb. of the starting protein, *i.e.*, 77%.

TABLE VI

PEPTIDE BONDS HYDROLYZED BY ENZYMIC TREATMENT OF TOXIC PROTEIN

Step	Enzyme	Digestion time, hr.	Peptide bonds[a] hydrolyzed, accumulative %
1	Pepsin	24	11.6
2	Pepsin	48	12.5
3	Trypsin	24	31.2
4	Trypsin	48	33.5
5	Erepsin	68	43.4

[a] Percentage was calculated from Van Slyke amino nitrogen values compared with 75% of the total nitrogen found to be amino nitrogen in the completely hydrolyzed protein.

No odor of putrefaction was observed at any time during the enzymic digestion. No trace of free TCE could be found in the spray-dried hydrolyzate (Fujiwara test).

Chromatographic Studies on Enzymic Digest.—A *p*H 4.5 extract of the spray-dried enzymic hydrolyzate (25 g. in 50 ml. H_2O) was treated with activated carbon (5 g.) and adjusted to *p*H 2.5 prior to evaporating to dryness *in vacuo*. The solids (15 g.) were extracted with 45 ml. of 80% methanol and the extract was used to charge a *p*H 4.5 alumina column (4.4 × 80 cm.) packed from 80% methanol slurry. The column was developed under pressure with 4 l. of 80% methanol at 200 ml./hour. The fractions (100 ml.) were evaporated to about 1 ml. *in vacuo* and examined by ascending paper chromatography on Whatman No. 1 paper. Separate chromatograms were sprayed with 4-NBP,[10] ninhydrin[20] and with phosphorus[25] and sulfur[26] reagents.

Fractions 3–6 showed three 4-NBP positive spots at R_f 0.50, 0.78 and 0.87. Spots at R_f 0.50 and 0.78 were ninhydrin positive. Fractions 12–16 gave only the R_f 0.50 spot. All spots were negative for phosphorus but positive for sulfur. Other 4-NBP positive spots were found but gave a positive test for phosphorus and did not give a sulfur test (probably nucleic acids).

(25) D. Amelung and P. Böhm, *Z. physiol. Chem., Hoppe-Seyler's*, **298**, 199 (1954).

(26) H. M. Winegard, G. Toennies and R. J. Block, *Science*, **108**, 506 (1948).

The spray-dried enzymic hydrolyzate (50 g.) was also extracted with three successive 250-ml. portions of boiling 95% ethanol. From the combined extracts a cold alcohol-soluble fraction was isolated and evaporated to dryness. Paper chromatographic separation on Whatman No. 3 paper resulted in concentrating the 4-NBP positive material along a band at R_f 0.4–0.6. This zone was cut from the paper sheet and eluted with the propanol–water mixture. The eluate was evaporated and rechromatographed on Whatman No. 1 paper to yield five ninhydrin-positive spots, one of which (R_f 0.50) was 4-NBP, ultraviolet and sulfur positive and phosphorus negative. The concentrated eluate inhibited the growth of *Saccharomyces pastorianus*[27] in exactly the same manner as did the S-dichlorovinyl derivatives of L-cysteine and L-glutathione.

Colorimetric Correlation of Sulfhydryl Activity in Old and New Soybeans.—The dye, 2,6-dichlorophenol-indophenol, was used to estimate the free sulfhydryl group activity in old and new beans in accordance with the principle of Basford and Huennekens.[28] In a typical study 5% aqueous slurries of Clark variety soybeans (1956 and 1957 crops, *i.e.*, approximately 14 months and 2 months after harvesting) were prepared under nitrogen in a high-speed homogenizer and subjected to colorimetric assay with the dye. Thiol activity of a 5-ml. aliquot from each homogenate was abolished by 10-minute interaction, preferably in a glass-stoppered reaction flask, with 2.5 ml. of 0.005 molar *p*-chloromercuribenzoic acid (*p*-CMB) in a slight excess of alkali. Two milliliters of aqueous dye solution (0.004 molar) was added to each flask. After 6 minutes 5 ml. of *p*H 4 acetate buffer and 10 ml. of xylene were added in rapid succession. After vigorous shaking, the sample was centrifuged to separate the layers. The xylene layer was isolated, dried with anhydrous Na_2SO_4 and read in a colorimeter at 500 mμ against a blank provided by substituting water for dye in the assay. A measure of the normal dye decolorizing activity of the homogenate was obtained by substituting water for *p*-CMB in the assay. Thus, devoid of preliminary treatment with the thiol blocking reagent, *p*-CMB, soybean homogenates from both the 1956 and 1957 crops produced identical colorimetric changes with the dye. Where *p*-CMB treatment of the homogenate preceded reaction with the dye, a 79% decrease in bleaching action was observed with the homogenate of the 1957 soybeans in contrast with only 60% inhibition from the 1956 soybeans.

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(27) L. L. McKinney, A. C. Eldridge and J. C. Cowan, THIS JOURNAL, **81**, in press (1959).

(28) R. E. Basford and F. M. Huennekens, *ibid.*, **77**, 3873 (1955).

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Studies on *myo*-Inositol Phosphates of Natural Origin

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myo-Inositol monophosphates previously obtained by enzymic and basic hydrolysis of phytin have been prepared as pure crystalline cyclohexylamine salts. These two compounds have been shown to be identical with each other and with the *myo*-inositol 2-phosphate first prepared by Iselin. A *myo*-inositol monophosphate has been obtained as a crystalline salt from a base hydrolysis of soybean phosphoinositide. It is optically active and is shown to be *myo*-inositol 1-phosphate. This suggests that the ester linkage in the phosphoinositide is to position 1 of the *myo*-inositol ring, a structural feature that has long been in question. Chromatographic analysis of base hydrolysates of phosphoinositides from beef heart, liver and wheat germ indicates that the same linkage is present in the lipid from these different sources. *myo*-Inositol 1- and 2-phosphate have been shown to be readily interconverted by acid-catalyzed phosphate group migration, while the 5-phosphate does not undergo such migration. The action of dicyclohexylcarbodiimide (DCC) on *myo*-inositol 1- and 2-phosphate produces the same cyclic 1,2-phosphate. Base hydrolysis of this cyclic phosphate yields mostly the 1-phosphate, which explains why the major inositol phosphate component in the base hydrolysis of the phosphoinositide is the 1-phosphate. Treatment of the 5-phosphate with DCC leads to unstable cyclic phosphate intermediates, with the eventual formation of the 1,2-phosphate.

myo-Inositol monophosphates have been obtained by alkaline,[1] acid[2] and enzymic[3] hydrolysis of *myo*-inositol hexaphosphate, by acid and alkaline hydrolysis of phospholipids[4] and by synthesis.[5,6] Only in the case of one synthetic compound[5] has the structure been defined (as *myo*-inositol 2-phosphate),[7] although it has been claimed[8] that the product obtained by enzyme hydrolysis of phytic acid[3] is identical with the Iselin phosphate, and that made by alkaline hydrolysis of phytic acid is different.

We have now prepared *myo*-inositol monophosphates by several of the above mentioned procedures and have attempted to interrelate them structurally. All compounds were obtained as crystalline cyclohexylamine salts. The monophosphates prepared by the methods of Iselin,[5] Carter[3] and Desjobert[1] were found to be identical by infrared analysis, rate of periodate oxidation, chromatographic properties, melting point and microscopic appearance of the cyclohexylamine salts. Thus, accepting the Iselin compound as being *myo*-inositol 2-phosphate (from mode of synthesis), it follows that the Carter and Desjobert compounds have the same structure.[9]

The *myo*-inositol phosphate prepared from soybean phosphoinositide by alkaline hydrolysis gives a cyclohexylamine salt that differs from the 2-phos-

(1) A. Desjobert, *Bull. soc. chim. biol.*, **36**, 1293 (1954).

(2) R. J. Anderson, *J. Biol. Chem.*, **12**, 447 (1912); **18**, 441 (1914).

(3) M. H. McCormick and H. E. Carter, *Biochem. Preps.*, **2**, 65 (1952); S. Posternak and T. Posternak, *Helv. Chim. Acta*, **12**, 1165 (1929).

(4) D. W. Woolley, *J. Biol. Chem.*, **147**, 581 (1943); J. N. Hawthorne and E. Chargaff, *ibid.*, **206**, 27 (1954); J. M. McKibbin, *ibid.*, **220**, 537 (1956).

(5) B. Iselin, THIS JOURNAL, **71**, 3822 (1949).

(6) K. Horiuchi, *J. Biochem. (Japan)*, **14**, 163 (1931–1932).

(7) The *myo*-inositol ring was numbered as in I when Iselin named his substance the 5-phosphate. The presently accepted way for numbering the inositol ring, shown in II, makes Iselin's compound the 2-phosphate.

I

II

(8) P. Fleury, A. Desjobert and J. Lecocq, *Bull. soc. chim. biol.*, **36**, 1301 (1954).

(9) Drs. D. M. Brown and G. E. Hall of the University Chemical Laboratory, Cambridge, have informed us that they have come to the same conclusion.