acid (25 ml), blanketed with nitrogen at 1°, containing 164.2 mg of the labeled amide (1 mmole) was adjusted to pH 10.2 with a 10 *N* solution of sodium hydroxide. Over a period of about 10 sec, 155.2 mg of the NCA of proline (1.1 mmole) was added. The pH of the solution was maintained at 10.2 by the continued addition of aqueous alkali. Tlc of the reaction mixture on silica gel using a butanol–acetic acid–water system (10:1:3) gave radioactive spots at R_f 0.25 (prolylphenylalaninamide, 83%) and at R_f 0.41 (phenylalaninamide, 17%).

C. Reaction of an Excess of the NCA of Proline with ^{14}C-Phenylalaninamide at pH 12.5.—To a stirred, nitrogen-blanketed solution of 164.2 mg of the above ^{14}C-amide (1 mmole) in 25 ml of freshly boiled, distilled water at 1°, adjusted to and maintained at pH 12.5 with a 10 *N* solution of sodium hydroxide, were added consecutively over an 8-min period five portions of the NCA of proline consisting of 1, 2, 2, 5, and 5 moles, respectively. The reaction mixture was sampled subsequent to the addition of each portion of the NCA after no more base addition was required to keep the pH at 12.5. The aliquots were analyzed for residual ^{14}C-phenylalaninamide by tlc as described above. The results are summarized in Table V.

Reaction of Excess of the NCA of Alanine with ^{14}C-Phenylalanine.—To a solution of 165.1 mg of ^{14}C-phenylalanine (1 mmole, 100 μcuries) in 25 ml of freshly boiled water were added consecutively over about 8 min five portions of the NCA of alanine consisting of 1, 2, 2, 2, and 3 mmoles, respectively, and finally one portion of 5 mmoles of the NCA of proline. The reaction was carried out at pH 12.5 and sampled essentially as described directly above for the reactions of the NCA of proline. Radiochemical analysis of thin layer plates gave the following percentages of residual ^{14}C-phenylalanine at successive stages of the reaction: 63% (1 mmole of NCA), 8.4% (3 mmoles of NCA), 4.6% (5 mmoles of NCA), 4.2% (10 mmoles of NCA of alanine followed by 5 mmoles of proline).

Registry No.—L-Valyl-L-serine, 13588-94-8; L-leucyl-L-valine, 13588-95-9; L-alanyl-L-leucine, 3303-34-2; L-phenylalanine-L-leucine, 3303-55-7; glycyl-L-phenylalanine, 3321-03-7; L-tyrosyl-L-serine, 13588-99-3; L-alanyl-L-serine, 3303-41-1; L-tryptophenyl-L-leucine, 13123-35-8; L-prolyl-L-phenylalanine, 13589-02-1; L-isoleucyl-ε-*t*-Boc-L-lysine, 13612-78-7; L-phenylalanyl-L-arginine, 13589-03-2; L-methionyl-L-tyrosine, 13589-04-3; L-alanyl-L-phenylalanine, 3061-90-3; L-isoleucyl-L-tryptophan, 13589-06-5; L-valyl-L-histidine, 13589-07-6; N-(α-carboxyphenethylcarbamoyl)phenylalanine, 13589-08-7; α,4-dibenzyl-2,5-dioxo-1-imidazolineacetic acid, 13589-09-8; 1-(α-carboxyphenethylcarbamoyl)-proline, 13589-10-1; L-phenylalanylamide, 5241-58-7.

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Oxygen-Sensitive Reactions of Proteins and Peptides. III. Chromogenicity and Cystine-Related Structures

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Further insight into the structural requirements supporting the oxygen-sensitive color reactions of solutions of proteins or peptides containing fully combined cystine moieties in ammonia or certain other amines has been gained with studies of simpler model compounds. A generalized structure for the latter is $(S(CH_2)_nCHXCOY)_2$. To maintain the chromogenic properties, it was found that n must equal 1, though the CH_2 group could be altered to $C(CH_3)_2$, but reactivity was greatly diminished. Also, the two S atoms could not be separated by a methylene group. The group X was expanded from NHCHO to NHCOR and $NHSO_2Ar$ and was found chromogenically reactive if suitable groups were at Y. Also, X could be NHCOOR and with this substituent 1,1,3,3-tetramethylguanidine displayed not only color but also thermochromism which it failed to do when X was NHCOR. It was found that X could even be H or NH_2, but with these groups the chromogenic effect was greatly restrained. Structural variations at Y included OR, NH_2, NHR, and NR_2. The speed and intensity of color development diminished progressively in the above order; esters were the most reactive and the disubstituted amide displayed scarcely any reactivity. Finally, it was shown that Y could be H or CH_3, showing that the minimum essential structural skeleton to support the oxygen-sensitive, thermochromic reactions is $(SCH_2\underset{|}{C}HCO)_2$. Syntheses of compounds necessary for these structural studies are reported.

In paper I of this series,[1] a chromogenic thermochromic reaction of liquid anoxic ammonia was reported with proteins and peptides containing fully combined cystine groups, *i.e.*, cystine having both carboxyl and both amino groups combined in amide or peptide bonds. In paper II, it was shown[2] that similar oxygen-sensitive, thermochromic solutions resulted when such proteins or peptides were treated anoxically with primary amines containing the group CH_2NH_2. An oxygen-sensitive chromogenic reaction was observed also if 1,1,3,3-tetramethylguanidine was used as the reactive base, but these blue solutions failed to show thermochromic properties in the experiments reported. The blue solutions resulting from the reaction of keratins with anoxic ammonia have been recently investigated by the electron spin resonance technique, but no signals were observed, indicating the absence of free radicals.

In the present paper, model compounds have been studied to gain further insight regarding the minimal structural requirements in the chromogenic compounds. The fragment $SSCH_2CH(NHCOR)CONHR'$ of fully combined cystine reveals these groups: the 2-amido group NHCOR, the 1-amido group CONHR', the carbon chain, the methylene group contiguous to the disulfide group, and the disulfide group itself. All

(1) E. L. Gustus, *J. Biol. Chem.*, **239**, 115 (1964).
(2) E. L. Gustus, *ibid.*, **239**, 1114 (1964).

of these factors have been studied in the present investigation.

The 2-Amido Group.—Compounds of structure I were used to test the chromogenic influence of the 2-amido group. Of these compounds, all showed

$(SCH_2CHXCONHCHRCOOR')_2$

	X	R	R'
Ia	$NHCOOCH_2C_6H_5$	H	C_2H_5
b	NHCHO	H	C_2H_5
c	$NHSO_2C_6H_4CH_3$-*p*	H	C_2H_5
d	H	H	C_2H_5
e	NH_2	$CH(CH_3)_2$	H
f	$NHCOOCH_2C_6H_5$	$CH(CH_3)_2$	$C(CH_3)_3$

color reactions except Ie, demonstrating that the acyl of the amido group may include sulfonyl as well as carbonyl, or it even may be omitted (Id). Although Id was much less reactive, it did develop a green color in ammonia within 1 hr at 65–70°. Colors appeared also using ethylenediamine and 1,1,3,3-tetramethylguanidine at 100° and both showed thermochromic changes. Both Ia and If also displayed the color and the thermochromism with the tetramethylguanidine. Amine Ie (N,N'-L-cystyldivaline) was negative toward ammonia after 3 days, in contrast to the positive reaction of If in 1 day, although it might become faintly colored with longer heating owing to self-acylation of the amino groups, yielding a fully combined cystine structure. However, it is clear that 2-amido groups, rather than 2-amino, greatly increase the chromogenic reactivity.

The 1-Amido Group.—The X in structure II (for fully combined cystine) has been a protonated amino group, NHR, in studies thus far, but until now the simplest examples have been omitted, namely, amides,

$(SCZ_2CH(NHY)COX)_2$

	X	Y	Z
IIa	OCH_3	$COCH_3$	H
b	OCH_3	$COOCH_2C_6H_5$	H
c	OCH_3	COC_6H_5	H
d	$OC_6H_4NO_2$-*p*	$COOCH_2C_6H_5$	H
e	$NHCH_2COOC_2H_5$	$COCH_3$	H
f	NH_2	$COOCH_2C_6H_5$	H
g	$NHCH_3$	$COOCH_2C_6H_5$	H
h	$NHCH_3$	$COCH_3$	H
i	Cl	$COOCH_2C_6H_5$	H
j	$N(CH_3)_2$	$COOCH_2C_6H_5$	H
k	$N(CH_3)_2$	$COCH_3$	H
l	$N(C_2H_5)_2$	$COCH_2Cl$	H
m	Cl	$COCH_2Cl$	H
n	$NHCH_2COOC_2H_5$	$COCH_3$	CH_3

wherein X was NH_2 and $NHCH_3$. These compounds (IIf, g, h) proved to be slowly chromogenic (18–50 hr). If X was NR_2 (IIj, k, l) a dramatic reduction in reactivity resulted. It took 14 days for III to become faintly yellow in ammonia and 6 months to become light amber, a questionably positive reaction. Incidentally, synthetic difficulties were encountered for IIj, and k, but III was obtained in crystalline form.

The methyl esters IIa, b, and c each gave rise to an intense green color in ammonia within 0.5 hr and thermochromic changes were observed. In other words, the terminal $COOCH_3$ group is considerably more reactive than the $CONH_2$ and still more than the CONHR group. The labile aryl ester IId required 18 hr to become noticeably green. It is obvious the greater speed of the lower esters is not a function of an ester–amide reaction with ammonia. If it were, IId should have been the fastest since this structure really should be classified as an acid anhydride and not an ester.

The Carbon Chain of Reactive Structures.—Three compounds, structures IIa, b, and c, were tested. Compound IIIb is Id. As mentioned earlier, Id

$(S(CH_2)_nCONHCH_2COOC_2H_5)_2$

IIIa, $n = 1$
b, $n = 2$
c, $n = 3$

gave positive response at 65–70° with ammonia, but IIIa and IIIc gave no such response with any of the bases even at elevated temperatures. In the carbon chain, therefore, n must be 2 for a possibly reactive structure.

In routine examination of starting materials, bis(thioacetic) acid and bis(4-thiobutyric) acid also gave no visible reaction with the bases at elevated temperatures, whereas bis(3-thiopropionic) acid gave frankly positive reactions with ammonia at 65° and with ethylenediamine and 1,1,3,3-tetramethylguanidine at 100°.

The Methylene Groups Contiguous to the Disulfide Group.—The next question was whether the hydrogen atoms of these methylene groups participate in the chromogenic reaction and are essential for it. Compound IIn, structured with $(SCMe_2{-}{-}{-})_2$ rather than $(SCH_2{-}{-}{-})_2$, dissolved completely in ammonia and showed no trace of color for a month. Then it gradually developed a greenish yellow color that became more intense and bluish, finally giving a bluish green color after 1 year. Thermochromic changes were observed. In ammonia at 65–70° no color appeared in 2.5 hr. With ethylenediamine and 1,1,3,3-tetramethylguanidine at 100° there was no color in 15 min. The substitution by methyl groups for the hydrogens of the cystyl methylene groups in a reactive structure IIe causes a profound reduction of the chromogenic reactivity but does not abolish it entirely in ammonia.

The Disulfide Group.—In paper I it was shown that the thiol group was unable to support the chromogenic reactions in otherwise appropriate structures. In the present study it was established that separation of the two disulfide atoms by a methylene group also caused failure of the color reactions. Methyl N,N'-dibenzoyldjenkolate, $CH_2(SCH_2C(NHCOC_6H_5)HCOOCH_3)_2$, was synthesized and was found inadequate for any color development with ethylenediamine or 1,1,3,3-tetramethylguanidine, both at 150° or with ammonia at 65–70° for 2.5 hr or at 25° for 1 year. Thus, of the sulfur functions tested, namely, SH, S, SS, and SCH_2S, only the disulfide group is capable of supporting chromogenic reactions.

The 3-Oxoalkyl Disulfides.—The minimal structural feature for the chromogenic process as established by the above reactions is $(SCH_2\underset{|}{C}H\underset{|}{C}{=}O)_2$, the carbonyl being of amide or ester functionality. The question arises if this carbonyl might instead be part of an aldehyde or ketone group without losing its chromogenic capacity. Accordingly, 4,4'-dithiodi-2-bu-

TABLE I

MELTING POINTS AND MICROANALYSES

Compd	Mp, °C	Formula	Calcd, % C	Calcd, % H	Calcd, % N	Calcd, % S	Found, % C	Found, % H	Found, % N	Found, % S
Ia	165–166	$C_{30}H_{38}N_4O_{10}S_2$	53.09	5.64	8.25	...	53.25	5.99	8.38	...
Ib	137–138	$C_{16}H_{26}N_4O_8S_2$	41.19	5.62	12.01	...	41.03	5.84	11.81	...
Ic	184–185	$C_{28}H_{38}N_4O_{10}S_4$	46.78	5.33	7.80	...	46.62	5.42	7.72	...
Id	119–120	$C_{14}H_{24}N_2O_6S_2$	44.19	6.36	7.36	...	44.43	6.41	7.25	...
IIa	128–129	$C_{12}H_{20}N_2O_6S_2$	40.89	5.72	7.95	...	40.89	5.72	7.90	...
IIb	59–60	$C_{24}H_{28}N_2O_8S_2$	53.72	5.26	5.22	...	53.94	5.44	5.39	...
IIc	178–178.5	$C_{22}H_{24}N_2O_6S_2$	55.45	5.08	5.88	...	55.39	5.02	6.01	...
IId	138–140	$C_{34}H_{30}N_4O_{12}S_2$	54.40	4.03	...	8.54	54.24	4.05	...	8.64
IIf	199–201	$C_{22}H_{26}N_4O_6S_2$	52.16	5.17	11.06	12.66	52.31	5.20	11.09	12.61
IIg	210–211	$C_{24}H_{30}N_4O_6S_2$	53.92	5.66	10.48	11.99	53.77	5.54	10.34	11.86
IIh	280–281	$C_{12}H_{22}N_4O_4S_2$	41.13	6.33	15.99	18.30	41.42	6.38	15.80	18.32
III	127.5–128.5	$C_{18}H_{32}Cl_2N_4O_4S_2$[a]	42.94	6.41	11.13	12.74	43.22	6.61	10.96	12.50
IIIa	87–88	$C_{12}H_{20}N_2O_6S_2$	40.90	5.72	7.95	...	40.99	5.76	7.90	...
IIIc	79–80	$C_{10}H_{28}N_2O_6S_2$	47.04	6.91	6.86	15.70	46.71	6.90	7.09	15.60
IIn	263–264	$C_{22}H_{38}N_4O_8S_2$	47.98	6.96	10.17	...	47.90	6.95	10.10	...
IV	...	$C_8H_{14}O_2S_2$	46.57	6.84	...	31.08	46.63	6.61	...	30.90
V	...	$C_6H_{10}O_2S_2$	40.42	5.65	...	35.97	40.60	5.68	...	36.22

[a] *Anal.* Calcd for $C_{18}H_{32}Cl_2N_4O_4S_2$: Cl, 14.08. Found: Cl, 13.98.

tanone (IV) and 3,3′-dithiodipropionaldehyde (V) were sought for testing. Both presented experimental difficulties, especially V.

$(SCH_2CH_2COCH_3)_2$ IV $\qquad$ $(SCH_2CH_2CHO)_2$ V

Ketone IV has been mentioned[3] but with meager procedural detail and with no evidence for its homogeneity or structural validity. In attempts to duplicate the procedure no homogeneous substance could be obtained. The following different pathway proved successful: methyl vinyl ketone → 3-oxobutyl thiolacetate ($CH_3COSCH_2CH_2COCH_3$) → its semicarbazone → the mercaptan $HSCH_2CH_2C(=NNHCONH_2)$-CH_3 by transesterification with methanol → the semicarbazone of IV (by H_2O_2) → IV (by pyruvic acid). Compound IV was distillable at low pressure and gave acceptable infrared and nmr spectra.

Ammonia, ethylenediamine, and 1,1,3,3-tetramethylguanidine all gave strong color reactions and all three were reversibly thermochromic between 25 and -78° (see Experimental Section for details). All three also were oxygen sensitive and became decolorized if oxygen were admitted.

A number of synthetic pathways toward aldehyde V were devised and tested but they proved unrewarding. The successful approach to this elusive compound, but in small yields, paralleled the preparative steps for IV. From acrolein and acrolein semicarbazone was obtained 3-acetylthiopropionaldehyde semicarbazone ($CH_3COSCH_2CH_2CH=NNHCONH_2$), then the mercaptan by methanolysis and from it the semicarbazone of V, and finally V. Aldehyde V, a liquid, was distilled at low pressure and was of analytical purity. Its infrared and nmr spectra supported the assigned structure. The chromogenic reactions with ammonia, ethylenediamine, and 1,1,3,3-tetramethylguanidine (see Experimental Section) were unmistakably strong, thermochromic, and oxygen sensitive. Thus, 3,3′-dithiodipropionaldehyde becomes the simplest structure so far studied that undergoes this chromogenic reaction.

(3) R. B. Thompson, J. A. Chenicek, and T. Symons, *Ind. Eng. Chem.*, **50**, 797 (1958).

Experimental Section

Thiolacetic acid, bis(thioacetic) acid, and bis(3-thiopropionic) acid were generously supplied by Evans Chemetics, Inc., New York, N. Y. N,N′-L-Cystyldivaline, N,N′-(N,N′-bis(benzyloxycarbonyl)-L-cystyl)bis(valine *t*-butyl ester), and N,N′-(N,N′-bis(benzyloxycarbonyl)-L-cystyl)divaline were gifts from Roeske.[4] Melting points were performed in capillary tubes and are reported uncorrected. Analyses were performed by Micro-Tech Laboratories, Inc., Skokie, Ill. Crystals were dried under vacuum over P_2O_5 at appropriate temperatures. The compounds were examined for the color reactions by using anoxic liquid ammonia at 25° and at elevated temperatures, the latter being carried out in annealed glass pressure tubes instead of the bulbed tubes described in paper I. Ethylenediamine and 1,1,3,3-tetramethylguanidine were examined at 25 and at 150°, in addition to the usual reacting temperature of 100°. Thermochromic effects, when observed, were caused by chilling the colored solutions to −78° unless otherwise indicated.

N,N′-(N,N′-Bis(benzyloxycarbonyl)-L-cystyl)bis(glycine ethyl ester) (Ia).—To a round-bottomed, 200-ml, glass-stoppered flask containing a stirring bobbin, 0.1017 g (2 mmoles) of N,N′-bis(benzyloxycarbonyl)-L-cystine (Mann) was added. This was followed by 10 ml of purified acetonitrile and 0.405 g of dry triethylamine dissolved in 1 ml of acetonitrile. A colorless solution resulted at 25° after 15 min of stirring. It was then cooled to 0° and 1.013 g of powdered *m*-(2-ethyl-5-isoxazolylium) benzenesulfonate (Woodward's Reagent K, Pilot Chemicals, Inc.) was added. After 1 hr the solid had largely dissolved. Then 0.558 g of powdered glycine ethyl ester hydrochloride was added followed by 0.405 g of triethylamine dissolved in 1 ml of acetonitrile. Stirring was continued at 0° for 5 min and subsequently at 25°. A colorless solution resulted after 20 min which soon became cloudy and finally became an opaque suspension. After 48 hr the procedure given in paper II for use of Woodward's Reagent K was followed, yielding 0.3 g of colorless, small needle-shape crystals of mp 165–166° (lit.[5a] mp 164–165°). Reaction with ammonia, ethylenediamine, and 1,1,3,3-tetramethylguanidine yielded characteristic colored solutions. That in the tetramethylguanidine was unusual for it showed thermochromic changes, the first such with this base. It was green-blue at 100° and golden brown at −78°; warming to 25° restored the greenish color.

N,N′-(N,N′-Diformyl-L-cystyl)bis(glycine ethyl ester) (Ib) was prepared from N,N′-diformyl-L-cysteine[5b] and glycine ethyl ester hydrochloride by a procedure similar to that given above. From the same molar quantities of reactants, 0.12 g of aggregates of colorless needles or narrow blades were obtained from acetone. Melting point and analytical data for this and other compounds are given in Table I. Characteristic positive color reactions were

(4) R. Roeske, *J. Org. Chem.*, **28**, 1251 (1963).

(5) (a) A. Previero and E. Scoffone, *Gazz. Chim. Ital.*, **93**, 863 (1963); (b) W. O. Foye and M. Verdame, *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 275 (1957).

obtained with ammonia, ethylenediamine, and 1,1,3,3-tetramethylguanidine, the latter showing no thermochromic change.

N,N′-(N,N′-Ditosyl-L-cystyl)bis(glycine ethyl ester) (Ic) was prepared by an analogous method to that given for Ia, from N,N′-ditosyl-L-cysteine[6] and glycine ethyl ester hydrochloride. From the same molar quantities of reactants, 0.58 g of colorless plates were obtained from ethyl acetate. These dry crystals coalesced at 113–114° and cleared at 184–185° with bubbles (lit.[7] 183–184°). Characteristic positive color reactions were observed with each of the three bases, that from the tetramethylguanidine showing no thermochromism.

N,N′-(α,α′-Dideaminocystyl)bis(glycine ethyl ester) (Id) was prepared from purified bis(3-thiopropionic) acid (mp 154–155°) and glycine ethyl ester hydrochloride by a like procedure. The same molar quantities of reactants yielded 0.303 g of aggregates of hairlike crystals when separated from acetone.

N,N′-Diacetyl-L-cystine Methyl Ester (IIa).—The synthesis was by a method essentially similar to Cecil and McPhee's preparation of the corresponding ethyl ester,[8] substituting cystine methyl ester dihydrochloride for cystine ethyl ester dihydrochloride. It was recrystallized from ethyl acetate, mp 128–129° (lit.[9] mp 125–126°). In an examination of starting material, recrystallized L-cystine methyl ester dihydrochloride proved unreactive with ammonia after 36 hr, but heating to 70° caused the appearance of a light emerald color. In ethylenediamine the substance was unreactive at 25° or at 100°, but at 150° it gave a transient, faint green color. In sharp contrast, the solution in 1,1,3,3-tetramethylguanidine became intensely bluish green at 25° within 48 hr, which instantly darkened at 100° as the blue component increased. Thermochromic changes were observed.

N,N′-Bis(benzyloxycarbonyl)-L-cysteine Methyl Ester (IIb).—To a stirred solution containing 75 ml of water, 6.72 g of sodium bicarbonate, and 6.83 g of powdered L-cystine methyl ester dihydrochloride was added 35 ml of ether, after which 7.4 g of benzyloxycarbonyl chloride was added dropwise during 50 min. After 2 hr the oily layer was taken up in 50 ml of ethyl acetate. The aqueous layer was extracted twice with 40-ml portions of ethyl acetate. The combined ethyl acetate solutions were washed twice with saturated NaCl solution, dried ($MgSO_4$), and filtered, and the solvent was removed. The resinous residue crystallized on long standing under ether at 6°. The crystals, rinsed with ether, were dissolved in a little dry methanol and filtered; the filtrate was evaporated to small volume. On dilution with 10 volumes of ether and long standing at 6°, dendritic masses of small needle crystals were deposited. The substance was collected, washed with ether, then with pentane, and was dried under vacuum at 30°: yield, 5.1 g; mp 59–60° (lit. mp 57–61°,[10] 73–75 [11]).

N,N′-Dibenzoyl-L-cysteine methyl ester (IIc) was synthesized from diazomethane in ether and N,N′-dibenzoyl-L-cystine in methanol using the procedure of Fry:[12] mp 178–178.5°, with preliminary softening above 176° (lit. mp 176–178°,[12] 177–179°[13]).

N,N′-Bis(benzyloxycarbonyl)-L-cystine *p*-nitrophenyl ester (IId) was prepared by an adaptation of the method[14] of Bodanszky and duVigneaud. The colorless needle-shape crystals were faintly yellow by transmitted light.

N,N′-Bis(benzyloxycarbonyl)-L-cystine amide (IIf) was made from the acyl chloride IIi[15] using 0.356 g of N,N′-bis(benzyloxycarbonyl)-L-cystine and 0.306 g of PCl_5 in cold, dry ether. The separated IIi was washed with cold ether and was transferred to a flask containing 25 ml of ether at −78°. A mixture containing 2 ml each of liquid ammonia and ether at −78° was added and the flask was agitated in the refrigerating bath. The acyl chloride (IIi) dissolved readily, but before complete solution occurred colorless crystals began to be deposited. After 30 min the opened flask was placed upright in a dewar containing 15 ml of liquid ammonia. After ammonia had evaporated, the ether was blown away in a stream of air. The residue was dissolved in 20 ml of hot methanol and filtered; the solution was cooled to 6°. Crystals formed, which were collected, and additional material came from the filtrate. The combined solids were extracted in a protected Soxhlet with dry acetone. The filtered extract was evaporated and the residue was repeatedly crystallized from minimal quantities of hot absolute methanol by adding an equal volume of dry acetone at 6°: yield, 0.2 g of fine needles; mp 192–193°. Schnabel[16] obtained various preparations melting at 162–172°. He reported mp 198–201° for a preparation made by ammonolysis of N,N′-bis(benzyloxycarbonyl)-L-cystine *p*-nitrophenyl ester. Repeated recrystallizations of the above IIf finally gave crystals melting at 199–201°.

N,N′-Bis(benzyloxycarbonyl)-L-cystine methylamide (IIg) was made comparably, substituting for the ammonia solution one containing 2.7 g of methylamine in 3.5 ml of dry ether. Cooling at −78° was maintained only for 10 min, then agitation was continued at −11° for 20 min, subsequently with occasional agitation at −11° for 1.5 hr. In the processing, recrystallization from hot methanol to which an equal volume of acetone was added deposited needle-shape crystals at 6°: yield, 0.25 g; mp 210–211° after first contracting at 195–196°.

N,N′-Diacetyl-L-cystine Methylamide (IIh).—A solution of 3 g of ester IIa in 65 ml of a 40% by weight aqueous methylamine was left in a glass-stoppered flask for 2 weeks at 25°, then was evaporated (under vacuum) and the oily residue crystallized under acetone. Recrystallization from methanol gave colorless prisms which were washed with chilled methanol, then ether: yield 0.4 g.

N,N′-Bis(chloroacetyl)-L-cystine Diethylamide (IIl).—To a 50-ml glass-stoppered flask were added 2 g of finely powdered N,N′-bis(chloroacetyl)-L-cystine,[17] 30 ml of dry ether at 0°, and 2.2 g of PCl_5. The mixture was vigorously agitated while chilled for 30 min. The separated acyl chloride (IIm) was quickly washed with dry ether, pressed, and put (together with the glass-fiber filter) into a large, stoppered test tube containing 20 ml of ethyl acetate at −78°. To a separate test tube, similarly chilled, were added 15 ml of ethyl acetate, 1.4 ml of triethylamine, and 1.03 ml of diethylamine. The contents of the two tubes were mixed, agitated for 30 min at −78°, then warmed to 6° for 1 hr. After filtration (Norit) to remove triethylammonium chloride, the filtrate was washed with 1% aqueous citric acid, then 5% aqueous sodium bicarbonate, and finally with saturated sodium chloride solution. After drying ($MgSO_4$), it was evaporated under reduced pressure to a viscous residue. This was dissolved in 15 ml of warm ether, then was cooled to 6°. The crystals, contaminated with amorphous material, were collected, washed with chilled ether, dried, and dissolved in warm acetone (Norit). After filtering and concentrating to 4 ml, colorless leaflets separated at 6°. They were collected, washed with cold ether, and desiccated. This sample (0.16 g) was analyzed. Additional crystals separated from the filtrate.

Bis(thioacetylglycine ethyl ester) (IIIa) was prepared by directions parallel to those for Ia, substituting purified bis(thioacetic) acid, mp 107°, for N,N′-bis(benzyloxycarbonyl)-L-cystine, and replacing solvent nitromethane by acetonitrile. Substance IIIa crystallized from a 1:2 mixture of acetone and ether as slender needles. It was recrystallized from ethyl acetate.

Bis(4-thiobutyrylglycine ethyl ester) (IIIc).—In this similar procedure, bis(4-thiobutyric) acid, mp 107–108° (prepared by peroxide oxidation of 4-mercaptobutyric acid), was used in place of bis(thioacetic) acid. Ester IIIc, needle-shape crystals from acetone, was recrystallized from ethyl acetate.

(N-Acetyl-2,2-dimethyl-DL-cysteinyl)glycine ethyl ester, $HSCMe_2CH(NHAc)CONHCH_2COOEt$, was synthesized from glycine ethyl ester hydrochloride and N-acetyl-DL-penicillamine, appropriately substituting these substances in a procedure quantitatively similar to that described above for Ia. After processing, the solid product was dissolved in a small quantity of hot acetone. On standing at 6°, needles deposited which were collected and recrystallized: yield, 0.3 g; mp 173–174°.

Anal. Calcd for $C_{11}H_{20}N_2O_4S$: C, 47.77; H, 7.29; N, 10.14. Found: C, 47.72; H, 7.11; N, 9.87.

(N,N′-Diacetyl-2,2′-dimethyl-DL-cystyl)bis(glycine ethyl ester) (IIn) was prepared by oxidation of the above thiol (0.196 g) in 95% alcohol (15 ml) and water (15 ml). Ammonia was added to a pH of 8 and 1 ml of 0.05 *M* aqueous cupric chloride solution

(6) E. W. McChesney and W. K. Swann, Jr., *J. Am. Chem. Soc.*, **59**, 116 (1937).
(7) F. Micheel and H. Haneke, *Ber.*, **95**, 1013 (1962).
(8) R. Cecil and J. R. McPhee, *Biochem. J.*, **59**, 236 (1955).
(9) E. Vinkler, J. Lazar, and F. Klyveni, *Magyar Kem. Folyoirat*, **67**, 303 (1961).
(10) K. Schlögl, J. Derkosch, and E. Wawersich, *Monatsh.*, **85**, 611 (1954).
(11) L. Zervas and I. Photaki, *J. Am. Chem. Soc.*, **84**, 3887 (1962).
(12) E. M. Fry, *J. Org. Chem.*, **15**, 438 (1950).
(13) L. Zervas, I. Photaki, and N. Ghelis, *J. Am. Chem. Soc.*, **85**, 1340 (1963).
(14) M. Bodanszky and V. duVigneaud, *ibid.*, **81**, 5688 (1959).
(15) V. duVigneaud and G. L. Miller, *Biochem. Prep.*, **2**, 76 (1952).
(16) E. Schnabel, *Ann.*, **659**, 182 (1962).
(17) J. B. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, N. Y., 1961, p 1915.

and the same of ferric chloride were added. A stream of oxygen, washed with 50% alcohol, was passed through the solution for 48 hr after which the nitroprusside test for thiols was negative. After removal of solvent, the residue was dissolved in 40 ml of hot 95% alcohol, treated with diatomaceous earth, and filtered. The colorless filtrate, on concentration in a current of air, deposited needle-shape crystals. These were washed with a little chilled alcohol and dried: yield, 0.1 g.

N,N′-Dibenzoyldjenkolic Methyl Ester, $CH_2(SCH_2CH(NHCOC_6H_5)COOCH_3)_2$.—N,N′-Dibenzoyldjenkolic acid[18] was treated in alcohol solution with diazomethane. After 1 hr, solvent and excess diazomethane were removed under reduced pressure. The residue was dissolved in a little hot acetone, filtered, and mixed with an equal volume of ether. Crystals deposited at −8° which were collected, washed with chilled 1:1 acetone and ether, and finally with cold ether. Recrystallization from hot methanol by slow evaporation of solvent at 25° gave colorless, hairlike crystals which were rinsed with cold methanol, then dried, mp 138–139°.

Anal. Calcd for $C_{23}H_{26}N_2O_6S_2$: C, 56.31; H, 5.34; N, 5.71. Found: C, 56.29; H, 5.31; N, 5.62.

3-Oxobutyl Thiolacetate, $CH_3COSCH_2CH_2COCH_3$.—Into a stirred solution of 32.4 g of thiolacetic acid in 300 ml of hexane was added dropwise during 2.5 hr a solution of 71.5 g of freshly distilled vinyl methyl ketone in 300 ml of hexane. The mixture was irradiated during the addition and for 3 hr thereafter with a 250-w spotlight. The temperature rose to 45–50°. Hexane was removed and the residue was fractionally distilled, collecting 112.4 g at 69.5–76.5° (1 mm). On redistillation, 105 g was obtained at 62–65° (0.5 mm) (lit.[19] bp 103–106° (14 mm)). This substance displayed no chromogenic reaction with the reactive bases.

Anal. Calcd for $C_6H_{10}O_2S$: C, 49.29; H, 6.89; S, 21.93. Found: C, 49.64; H, 7.03; S, 21.90.

3-Oxobutyl Thiolacetate Semicarbazone, $AcSCH_2CH_2C(CH_3)=NNHCONH_2$.—The above keto ester was treated with an excess of semicarbazide hydrochloride and sodium acetate in diluted methanol. Needle crystals that separated were washed with chilled methanol and dried, mp 143–143.5°. This compound also showed no chromogenic reaction with the bases.

Anal. Calcd for $C_7H_{13}N_3O_2S$: C, 41.36; H, 6.45; N, 20.67; S, 15.77. Found: C, 41.62; H, 6.59; N, 20.87; S, 15.68.

4,4′-Dithiodi-2-butanone Semicarbazone, $(SCH_2CH_2C(CH_3)=NNHCONH_2)_2$.—The above semicarbazone was converted to the mercaptan semicarbazone by Reid's method[20] and then, without separation, it was oxidized to the disulfide. A stirred mixture of 40.7 g of the semicarbazone and 450 ml of methanol containing 0.1 g of sodium was distilled, replacing the distillate by adding methanol until 500 ml had been collected (3 hr). To the thiol solution was added dropwise during 45 min a solution of hydrogen peroxide (11.5 ml of 32% H_2O_2 in 80 ml of methanol). Crystalline needles separated while the last third of peroxide was being added. The product, which tested negatively for thiol with nitroprusside, was filtered off and washed with methanol and with ether: yield, 32 g; mp 192° dec. It was very sparingly soluble in all usual solvents. No chromogenic reaction was observed with the appropriate bases.

Anal. Calcd for $C_{10}H_{20}N_6O_2S_2$: C, 37.48; H, 6.29; N, 26.23; S, 20.01. Found: C, 37.22; H, 6.42; N, 26.43; S, 19.91.

4,4′-Dithiodi-2-butanone (IV).—The semicarbazone groups of the above disulfide were removed by adapting Hershberg's procedure.[21] To a stirred suspension of 51.3 g of the semicarbazone, 41 g of sodium acetate, 300 ml of glacial acetic acid, and 100 ml of water was added a solution of 40 g of redistilled pyruvic acid in 40 ml of water. After 30 min the suspended material had almost completely dissolved at which point pyruvic acid semicarbazone crystals began to appear. Then 500 ml of water was added dropwise during 10 hr. After cooling to 6° these crystals were removed and rinsed with chloroform; the filtrate was diluted with 100 ml of chloroform. The chloroform layer was neutralized ($NaHCO_3$), washed with saturated sodium chloride solution, and dried ($MgSO_4$). The filtered solution was evaporated and the resulting syrup was dissolved in benzene. Solvent was again evaporated and the clear yellow liquid residue was twice fractionated *in vacuo:* bp 115–116° (0.2 mm); $n^{23}D$ 1.5212; yield, 11 g; nitroprusside test, negative.

The infrared spectrum showed absorption bands for a methyl ketone at 5.83 and 7.31 μ. The nmr data (in CCl_4) showed two resonances in the ratio of 4:3 at 2.81 and 2.19 ppm downfield from tetramethylsilane due to methylenic and methyl hydrogens, respectively. In benzene solution the methyl resonance appeared as a singlet at 1.69 ppm while the methylenic hydrogens appeared as a 12-line AA′BB′ pattern symmetrically disposed about the center at 2.56 ppm.

Substance IV in ammonia (0.03 g in 0.25 g) at 25° rapidly gave a clear solution, then turned yellow, cherry red, and eventually brownish red after 1 hr. Twelve hours later the color was deep green. At −78° the color became light orange. Green reappeared at 25°. On placing the chilled open tube in an atmosphere of oxygen[1] above liquid ammonia the green color gradually disappeared at 25° and became light orange.

One drop of IV in diluted methanol containing an excess of semicarbazide hydrochloride and sodium acetate rapidly deposited crystals of mp 192° dec, mmp 192° dec. The analysis (C, 37.67; H, 6.49; N, 26.52) agreed with the above starting material, further establishing its identity.

A solution of IV in ethylenediamine (0.04 g in 0.22 g) paralleled the colors reported in ammonia except color development at 25° was faster than in ammonia, an unusual observation with this base. On opening the tube to air at 25° the green color gradually disappeared and became colorless. A solution of IV in 1,1,3,3-tetramethylguanidine (0.04 g in 0.3 g) paralleled the colors in ammonia except this solution at 25° became a deep olive green after 12 hr. At −78° the color became brown and the green color reappeared at 25°. After opening the tube to air the green color disappeared within 24 hr.

3-Acetylthiopropionaldehyde Semicarbazone, $AcSCH_2CH_2CH=NNHCONH_2$.—Directions paralleled those described above for 3-oxobutyl thiolacetate except that methanol was the solvent. To it was added a few drops of an aqueous solution of benzyltrimethylammonium hydroxide as catalyst. The thiolacetic acid (in C_6H_6) was added with stirring to a methanolic suspension of acrolein semicarbazone,[22] mp 174–175°, keeping the temperature below 30° while irradiating the solution with a tungsten lamp, subsequently continuing stirring and irradiation for 1 hr. The clear solution deposited crystals at 6° which were washed with benzene and ether, mp 100.5–101°, which, after cooling, remelted at 105.5–106°. Recrystallization from methanol gave needle-form crystals which melted at 109° with preliminary softening.

Anal. Calcd for $C_6H_{11}O_2N_3S$: C, 38.08; H, 5.96; N, 22.20; S, 16.94. Found: C, 37.85; H, 5.86; N, 22.09; S, 17.26.

Semicarbazone of V, $(SCH_2CH_2CH=NNHCONH_2)_2$.—The above compound was transformed to the mercaptan by methanolysis and the latter was oxidized, without isolation, using hydrogen peroxide following the procedure described above for 4,4′-dithiodi-2-butanone. From 94.6 g there was obtained 91.2 g of a very sparingly soluble microcrystalline powder, mp 185–186° dec. Some of it was recrystallized from hot water and dried under vacuum at 100°, mp 189° dec. No chromogenic reaction was observed with the anoxic bases.

Anal. Calcd for $C_8H_{16}N_6O_2S_2$: C, 32.86; H, 5.52; N, 28.74; S, 21.93. Found: C, 33.12; H, 5.47; N, 28.60; S, 22.18.

3,3-Dithiodipropionaldehyde (V).—A stirred mixture (25°, N_2 atmosphere) of 8.77 g of the semicarbazone of V, 50 ml of glacial acetic acid, 10 ml of redistilled pyruvic acid, and 10 ml of water became clear in 12 min. The flask was chilled, rotated during solidification of the solution, and then connected to an evaporator at reduced pressure to remove acetic and pyruvic acids and water, using a bath at 0° while the receiver was held at −78°. To the residue was added 150 ml each of water and chloroform and the suspended solid was filtered off. Powdered sodium bicarbonate was added to the filtrate with stirring until the latter was faintly alkaline. The organic layer was separated, washed successively with water, saturated sodium chloride, then dried ($MgSO_4$), and filtered; the chloroform was removed at reduced pressure. The clear liquid was dissolved in 15 volumes of ether, except for a pasty residue. The decanted ether solution of V was concentrated at reduced pressure, leaving 1.2 ml of a clear yellowish liquid. It was purified by bulb to bulb distillation in a microstill at 0.02 mm, yielding 0.25 ml which displayed the expected infrared spectrum: λ_{max}^{film} 3.44 (CCH_2C), 3.53 (SCH_2C), 3.65, 5.81 (CHO), 7.50, 7.84, 8.57, 9.48, and 11.77 μ. The nmr spectrum

(18) A. G. vanVeen and A. J. Hijman, *Rec. Trav. Chim.*, **54**, 493 (1935).

(19) J. Druey and H. U. Daeniker, U. S. Patent 3,098,078 (July 16, 1963); *Chem. Abstr.*, **61**, 4225 (1964).

(20) E. E. Reid, "Organic Chemistry of Bivalent Sulfur," Chemical Publishing Co., Inc., Vol. 2, New York, N. Y., 1958, p 30.

(21) E. B. Hershberg, *J. Org. Chem.*, **13**, 542 (1958).

(22) K. von Auwers and P. Heimke, *Ann.*, **458**, 202 (1927).

of the substance (in $CHCl_3$) displayed two resonances in a ratio of 1:4 at 9.09 and 3.00 ppm downfield from tetramethylsilane due to the aldehydic and methylenic hydrogens, respectively. In benzene solution the methylenic hydrogens appeared as an eight-line AA'BB' pattern. An analytical sample was prepared by an additional short-path distillation at 0.03 mm: bath 80–95°; $n^{23}D$ 1.5452. This material (12 mg) was reconverted to its semicarbazone in the usual way, mp and mmp 189° dec.

On investigating V, it was found to dissolve promptly in anoxic ammonia (4.3 mg in 146 mg) and the clear solution at 25° displayed a greenish tint in 3 min and became deep emerald green within 15 min. At −78° the color changed to light orange and the deep green color returned at 25°. An appreciable quantity of a colorless microcrystalline material appeared in a few minutes. This suspension was solidified by liquid nitrogen, the tube was opened, and transferred to chilled liquid ammonia in a large tube flushed with oxygen, and then the outer tube was sealed. On warming to 25° the yellow color in the small tube changed to a greenish color momentarily, then disappeared entirely in less than 1 hr while the ammonia deposited additional opaque colorless material on the walls of the inner tube.

The solution of V in anoxic ethylenediamine (5 mg in 137 mg) at 25° displayed a yellow color which changed to emerald green after 16 hr. Chilling the solution to −78° formed a canary yellow solid and returning to 25° restored the green color. On opening the tube in air at 25° the green color disappeared within 12 hr, leaving a light yellow solid.

Similarly, 5.8 mg of V in 180 mg of 1,1,3,3-tetramethylguanidine displayed first yellow then emerald green in 3 min at 25°. Chilling to −78° caused it to become faintly greenish yellow while warming restored the intense emerald green color. On opening the tube to the air the green color disappeared within 12 hr but retained a light cherry red color.

Registry No.—Ia, 2790-85-4; Ib, 13961-87-0; Ic, 13961-90-5; Id, 6513-21-9; IIa, 6367-15-3; IIb, 3693-95-6; IIc, 5673-91-6; IId, 13961-95-0; IIf, 13961-96-1; IIg, 13976-38-0; IIh, 13028-62-1; III, 13976-40-4; IIn, 13976-41-5; IIIa, 13976-42-6; IIIc, 13976-43-7; IV, 13976-44-8; V, 13976-45-9; semicarbazone of V, 13976-46-0; $HSCMe_2CH(NHAc)CONHCH_2CO_2Et$, 13976-47-1; $CH_2(SCH_2CH(NHCOC_6H_5)CO_2CH_3)_2$, 13976-48-2; $AcSCH_2CH_2CH{=}NNHCONH_2$, 13976-49-3; $CH_3COSCH_2CH_2COCH_3$, 13976-50-6; $AcSCH_2CH_2C(CH_3){=}NNHCONH_2$, 13976-51-7; $(SCH_2CH_2C(CH_3){=}NNHCONH_2)_2$, 13976-52-8.

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Isolation of the 11-Carbon Acyclic Isoprenoid Acid from Petroleum. Mass Spectroscopy of Its *p*-Phthalimidophenacyl Ester[1]

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Gas chromatographic examination of appropriate distilled fractions of methyl naphthenates from a California petroleum failed to reveal the presence of any of the C_{10} isoprenoid acid, 3,7-dimethyloctanoic acid. The ester of the C_{11} isoprenoid acid, methyl 4,8-dimethylnonanoate, was isolated but only to the extent of 0.04% of the naphthenate mixture. Final purification by crystallization of the *p*-phthalimidophenacyl ester was necessary and this ester proved satisfactory for structural determination by mass spectrometery. Features of the fragmentation pattern of this type of ester are described. For comparison with the isolated product, 4,8-dimethylnonanoic acid was synthesized from citronellol.

Recent investigations in this laboratory of the component acids in a California petroleum have resulted in the identification of only one nonisoprenoid structure—geometric isomers of 3-ethyl-4-methylcyclopentylacetic acid.[2] In addition to one cyclic isoprenoid acid, 2,2,6-trimethylcyclohexylacetic acid,[3] the C_{14}, C_{15}, C_{19}, and C_{20} acyclic isoprenoid acids have been isolated.[4] The C_{14} and C_{19} acyclic acids could arise by degradative oxidation of the C_{15} and C_{20} acids;[5] however, such oxidation from the chain end could not yield the C_{12}, C_{13}, C_{17}, or C_{18} acids by a simple direct route. These four acids must be present in extremely low concentration, if at all, in the California petroleum under investigation, for our efforts have not yet resulted in their isolation. In contrast the C_{11} and C_{16} acyclic isoprenoid acids could arise by oxidation from the chain end. Examination of fractions in which the C_{10} and C_{11} isoprenoid acids should be located is the subject of the present report. A search for the C_{16} isoprenoid acid, which resulted in isolation of a C_{12} bicyclic acid, will be reported subsequently.

The stereoisomers of 3-ethyl-4-methylcyclopentylacetic acid were isolated[2] from a fraction of methyl naphthenates boiling in the range 97–107° (11.5 mm). A prominent band in the glpc of this ester fraction (refer to Figure 1 of the cited publication, 33-min band) was at the retention time expected for the ester of the 11-carbon acyclic isoprenoid acid; however, rechromatography of the material collected in this band showed the presence of a particularly formidable mixture. Examination of higher boiling fractions suggested a less difficult separation, and the desired component was eventually isolated, as described in the Experimental Section, with somewhat less difficulty from the fraction which had bp 107–118° (11.5 mm). The second fraction from glpc on neopentyl glycol succinate (NPGS) was rechromatographed on silicone. The second and third fractions (IV-2-B and IV-2-C) from this second chromatography were examined in some detail.

(1) This investigation was supported by a grant from the Petroleum Research Fund, administered by the American Chemical Society.

(2) J. Cason and A. I. A. Khodair, *J. Org. Chem.*, **31**, 3618 (1966).

(3) J. Cason and K.-L. Liauw, *ibid.*, **30**, 1763 (1965).

(4) J. Cason and D. W. Graham, *Tetrahedron*, **21**, 471 (1965).

(5) In principle, the C_{15} and C_{20} acyclic isoprenoid acids could arise by terminal oxidation of the hydrocarbons farnesane and phytane; however, it is also possible that the hydrocarbons arise by reduction of the analogous acids. The latter route has been proposed as the origin of the normal hydrocarbons [J. E. Cooper and E. E. Bray, *Geochim. Cosmochim. Acta*, **27**, 1113 (1963)].