Time of reaction, hr.	Yield of DNP-aspartic acid, %
0.5	86
1	91
3	93, 94
8	93
Amount of amino acid micromoles	
0.3	83, 84
1.0	93, 94
10.0	98, 97
Amount of sodium bicarbonate, mg.	
5	94
20	93, 94
Extractive procedure	
Original	85
Revised	93, 94

As previously noted, the reaction is rapid and the results are constant between 1 and 8 hr. Very probably the concentration of amino acid rather than incidental manipulative losses is responsible for the differences which appear when the amount of amino acid is altered. Thus, if one micromole is used but the concentration is reduced by using 5 ml. of water and 10 ml. of alcohol, the yield is only 85%. Further decrease in the amount of sodium bicarbonate is without effect but the revised extractive procedure apparently improves the recovery by a few per cent.

When the third procedure is used, the recoveries as shown in Table I are generally satisfactory. After this method was found to give improved results, the scope of the study was broadened to include other representative amino acids and some peptides. Of the amino acids, proline alone behaves much the same in each procedure and gives a rather low yield. Structural features may influence the result in this case as there is no reason to believe that the proline is impure. It is more difficult to assess the results from the peptides because studies of their purity have not been made.

The literature records only a few studies of the quantitativeness of the dinitrophenylation of amino acids. As already mentioned, the first procedure of the present study is essentially that of Sanger.[2] Porter and Sanger[9] studied the quantitative dinitrophenylation of valine but fail to describe experimental details. Mills[7] has obtained quantitative dinitrophenylation of all amino acids and Krol[10] has shown that glycine reacts quantitatively. In the light of the present results, Mills' description of procedure is significant. "Residual HCl (from a hydrolysate) was neutralized by the careful addition of a *slight excess* of sodium bicarbonate...." (Italics ours.) Likewise, Krol used only 30 or 40 mg. of sodium bicarbonate per 3 ml. of reaction mixture. Apparently, their quantitative results stemmed from their use of small amounts of sodium bicarbonate.

Several observations have been made which may throw some light on the reason why the reaction is more quantitative when the reagents are less concentrated. In the third procedure, the reaction mixture is homogeneous in contrast to the others. This may mean that the actual concentration of reagents is greater because the bicarbonate cannot have salted out the DNFB. Although, in both the first and third procedures, the DNFB is in excess at the end of the reaction, much less dinitrophenol is produced in the third procedure. The reduction of this competitive reaction may influence the course of the dinitrophenylation in the homogeneous reaction mixture. It may be noted that a homogeneous solution was present in that experiment in which an improved yield of DNP-aspartic acid was obtained simply by changing the amount of solvents in the original procedure from 1 to 5 ml. of water and from 2 to 10 ml. of alcohol. The change in conditions does not seem to involve the shifting of an equilibrium. Thus, when a known amount of DNP-aspartic acid was "dinitrophenylated" by both the first and the third procedures, the recovery was 94 and 95%, respectively. If an equilibrium were involved, the former value should be about 64%.

(9) R. R. Porter and F. Sanger, *Biochem. J.*, **42**, 287 (1948).
(10) S. Krol, *ibid.*, **52**, 227 (1952).

Applications

The third dinitrophenylation procedure has been used with success in the study of peptides from partial hydrolysates of gelatin.[1] The estimation of the amino acids other than the N-terminal amino acids of the peptides is generally better than 90% of the starting DNP-peptides. It is advisable, however, to use the original rather than the revised extractive procedure. When the original extractive procedure is used, dinitroaniline is removed during the extraction of the basic solution, but if the revised procedure is used, dinitroaniline is always present on the chromatograms and requires definite identification in order to distinguish it from DNP-phenylalanine. The source of the dinitroaniline is unknown and if the revised procedure is used, its presence on the chromatograms leads to unnecessary complications.

Conclusions

The investigation has shown that the quantitative dinitrophenylation of amino acids is best achieved by carrying out the reaction in a homogeneous solution of amino acid, sodium bicarbonate, DNFB, alcohol and water.

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Correlation between the Structure of Some Dinitrophenyl Peptides and their Chromatographic Behavior on Silicic Acid–Celite

BY W. A. SCHROEDER AND LEWIS R. HONNEN

RECEIVED MAY 14, 1953

During the determination of the N-terminal amino acid of lysozyme,[1] dinitrophenyl (DNP) peptides were detected in certain hydrolysates and

(1) F. C. Green and W. A. Schroeder, THIS JOURNAL, **73**, 1385 (1951).

were separated and identified in subsequent studies[2] by an extension of the methods which were devised by Green and Kay[3] for the separation and identification of DNP-amino acids. The investigation of these peptides demonstrated that the separation of unknown peptides would be greatly facilitated by a study of the chromatographic behavior of known DNP-peptides.

Consequently, although there was little intrinsic interest in any particular peptide, a variety of known DNP-peptides was chromatographed in order to give any conclusions more general validity. From the accumulated data it has been possible to deduce generalizations which permit the prediction of the chromatographic behavior of known DNP-peptides with considerable success or conversely which aid in the identification of tentatively identified DNP-peptides through the comparison of determined and predicted behaviors. This information which is the subject of the present report has been very useful in recent examinations of the peptides in partial hydrolysates of gelatin.[4]

Experimental

Sources of Peptides.—The peptides were obtained from the following sources: DL-ala-gly,[5] gly-DL-ala, gly-gly and gly-L-leu from Amino Acid Manufactures (University of California at Los Angeles); gly-L-try, gly-L-tyr, L-leu-gly, DL-leu-gly-gly and L-leu-L-tyr from Delta Chemical Works; L-ala-L-ala, tri-ala (3 L), tetra-ala (4 L), penta-ala (5 L), gly-L-ala-L-ala, gly-L-ala-gly, gly-L-lys-gly, L-lys-L-ala and L-lys-gly from Dr. Erwin Brand; tri-gly from Nutritional Biochemicals Corp.; gly-L-pro and L-hypro-gly from Dr. Emil L. Smith; lys-glu from Dr. Barbara Low; L-lys-L-val-L-phe-gly from Dr. James R. Vaughan, Jr.; di-DNP-L-lys-L-val-L-phe and di-DNP-L-lys-L-val from partial hydrolysis of di-DNP-L-lys-L-val-L-phe-gly; and D-pro-D-val from Dr. Sidney W. Fox.

Preparation of DNP-Peptides.—The method for the preparation of the DNP-peptides was similar to that of Sanger[6] for the preparation of DNP-insulin. A 5- or 10-mg. sample of peptide was placed in a 10-ml. glass-stoppered erlenmeyer flask and dissolved in one ml. of water. To this solution were added 100 mg. of J. T. Baker analyzed C.P. sodium bicarbonate and a solution of 0.1 ml. of 2,4-dinitrofluorobenzene (DNFB) (prepared by dinitration of fluorobenzene followed by distillation *in vacuo*) in 1.9 ml. of undistilled absolute alcohol (U.S. Industrial Chemicals Co.). After the reaction mixture had been shaken mechanically for 3 hr., it was transferred to a separatory funnel with 10 ml. of water and extracted with 4 × 25 ml. of ether (General Chemical Co.) to remove excess DNFB. Following acidification with one ml. of 6 *N* hydrochloric acid, the DNP-peptide was extracted with 2 × 15 and 4 × 10 ml. of ether or ethyl acetate. The nature of the DNP-peptide itself determined the choice of solvent; ethyl acetate was used if the distribution favored the aqueous phase and extraction with ether was unsatisfactory. The combined extracts were washed once with 10 ml. of water. (More recent experience has shown that it is advisable to acidify such wash water with a drop or two of 6 *N* hydrochloric acid in order to reduce re-extraction into the aqueous phase.) Finally, the solvent was evaporated from the extracts in preparation for the chromatography of the DNP-peptide. Sometimes, acidification of the reaction mixture caused precipitation of the DNP-peptide and then it was isolated by centrifugation. After washing with one ml. of water and recentrifuging, the precipitate was dissolved in acetone, transferred to a flask, and the solvent and traces of water were evaporated in preparation for chromatography.

The DNP-peptides so prepared were chromatographed without further purification and yet in only a few instances were zones of impurity observed on the chromatograms.

No attempt has been made to assess the yield of any DNP-peptide.

Chromatographic Procedures.—The chromatographic apparatus, the adsorbent, the packing and prewashing of the column and the types of developer were identical with those used by Green and Kay[3] for the separation of DNP-amino acids on silicic acid–Celite. The abbreviations used below are also those of Green and Kay.

The choice of a suitable solvent in which to place a DNP-peptide on the chromatographic column is determined to a large extent by the solubility of the individual DNP-peptide. In the present study, the sample solvents ranged in composition from 10 to 40 volume % of acetone in ligroin (60–70°) (abbreviated 10A-L to 40A-L) and from 5 to 10 volume % of acetic acid in benzene (5AA-B to 10AA-B). Of these, 10A-L is the poorest solvent and 10AA-B the best but for some DNP-peptides 10A-L is entirely satisfactory. More recent experience has led to the conclusion that solvents in which at least a little acetic acid is present are to be preferred over those which contain only acetone and ligroin. Most peptides are fairly strongly adsorbed and as a result 5AA-B or 10AA-B may generally be used and 2AA-10A-L certainly can. If the peptide is very insoluble, it is probably also very strongly adsorbed and solvents such as 5AA-5A-B and 10AA-10A-B which are good solvents but also strong developers are altogether satisfactory. In the majority of cases, 5AA-B or 10AA-B is very suitable. When columns about 1 cm. in diameter are used, a sample volume of 2 or 3 ml. is generally adequate. The amount of DNP-peptide which was used in each chromatogram is not known but it was sufficient to produce a nicely visible zone.

All of the chromatograms were run on 9 × 150-mm. columns of silicic acid–Celite. In general, each zone was developed with 7 *V* ml.[7] of developer and the position of the zone was measured at regular intervals throughout the development. Although each compound was chromatographed individually, only a few mixtures were studied. The chromatographic behavior was determined with the following nine developers: 2AA-10A-L, 3AA-15A-L, 4AA-20A-L, 8AA-8A-L, 8AA-4A-L, 12AA-6A-L, 10AA-B, 2AA-10A-B and 3AA-15A-B. Not all DNP-peptides have been developed with all of these developers and, likewise, duplicate chromatograms of a given DNP-peptide with a given developer have not usually been made. In those instances, in which duplicates or replicates have been made either within a short time or even after more than a year, the positions of leading and trailing edges of the zones usually did not differ by more than 5 mm.; this is a normal variation. One serious discrepancy was resolved by further chromatograms.

Results and Discussion

Representative results from several useful developers are recorded in Fig. 1. Only a portion of the available data is presented here, but the general features of the remainder will be correlated with the information of Fig. 1 in the discussion to follow. The bar graphs of Fig. 1 show the position of each zone of DNP-peptide after development with the type and quantity of developer listed.

Correlation between Structure and Chromatographic Behavior.—Even cursory examination of the data demonstrates that there is no correlation between the chromatographic behavior of dissimilar DNP-peptides on silicic acid–Celite and the length of the peptide chain: DNP-dipeptides, DNP-tripeptides, *etc.*, do not fall into distinct groups but rather there is such a wide range of adsorption affinities within each group that some DNP-dipeptides are as strongly adsorbed as DNP-tetrapeptides. Neither is the chromatographic behavior conditioned by the presence of a given

(2) W. A. Schroeder, THIS JOURNAL, **74**, 5118 (1952).

(3) F. C. Green and L. M. Kay, *Anal. Chem.*, **24**, 726 (1952).

(4) W. A. Schroeder, L. Honnen and F. C. Green, *Proc. Nat. Acad. Sci.*, **39**, 23 (1953).

(5) The abbreviations follow the suggestions of E. Brand (*Ann. N. Y. Acad Sci.*, **47**, 222 (1946)) and F. Sanger (*Adv. Prot. Chem.*, **7**, 5 (1952)).

(6) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(7) *V* ml. is the volume of solvent required to wet the column.

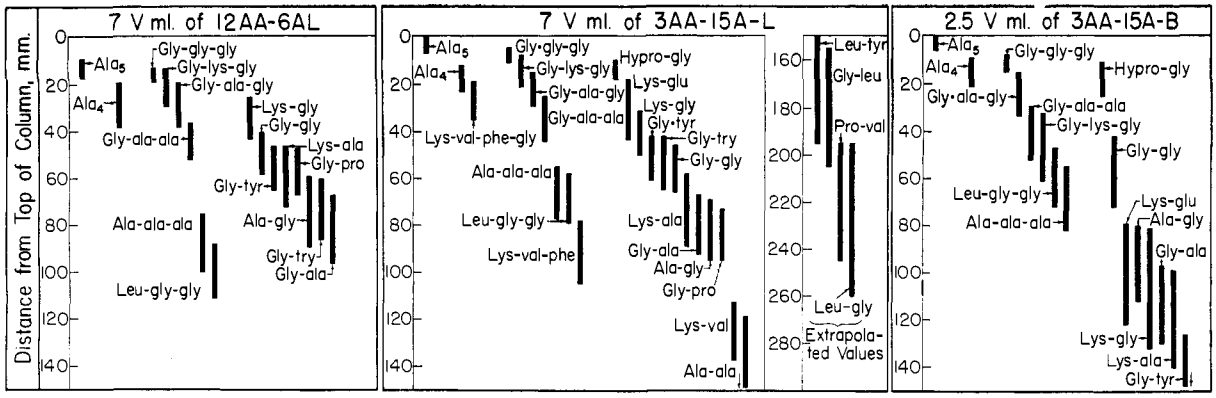

Fig. 1.—Positions of zones of DNP-peptides on columns of silicic acid–Celite after development with the type and quantity of developer listed.

amino acid in a certain position in the peptide, say, N-terminal or C-terminal: if this were so, all glycyl peptides, for example, would have similar affinities.

However, it is possible to establish a very generally applicable correlation between the relative adsorption affinities of DNP-peptides and the type of the constituent amino acids. This correlation may be stated in general form as follows: If the adsorption affinities of the DNP-derivatives of amino acids A, B, C, D, *etc.*, increases in the order listed, then the adsorption affinities of the DNP-derivatives of the peptides XA, XB, XC, XD, *etc.*, or AX, BX, CX, DX, *etc.*, (where X is any given amino acid) also increases in the order listed. Before considering this proposed correlation, attention should be called to Table I in which the relative adsorption affinities of the DNP-amino acids are listed.

TABLE I

RELATIVE ADSORPTION AFFINITIES OF DNP-AMINO ACIDS IN ACETIC ACID–ACETONE–LIGROIN DEVELOPERS

Hydroxyproline	Most strongly adsorbed
Serine	
Aspartic acid, lysine	Group I[a,b]
Threonine, tyrosine	
Glutamic acid	
Tryptophan	Group II
Glycine	
Proline	Group III
Alanine	
Methionine	
Phenylalanine	
Valine	Group IV
Leucine, isoleucine	Least strongly adsorbed

[a] The grouping is that of Green and Kay.[3] The chromatographic properties of DNP-hydroxyproline have been determined since that publication. [b] Within group I, the relative positions are subject to considerable variation or even inversion depending upon the ratio of acetic acid and acetone in the developer.

The chromatographic behavior of the DNP-amino acids with the three developers listed in Fig. 1 is as follows. Even the amino acids of group I would have been washed through the column by 2.5 V ml. of 3AA-15A-B. Only amino acids of group I would remain on the column when 7 V ml. of either 12AA-6A-L or 3AA-15A-L is used and they would be in the lower half of the chromatogram.

Let us consider first only those data which were obtained with 3AA-15A-L as the developer. In accordance with the proposed correlation, the adsorption affinities of di-DNP-lys-val, di-DNP-lys-ala, di-DNP-lys-gly and di-DNP-lys-glu increase in that order which is also the order of increase of DNP-val, DNP-ala, DNP-gly and DNP-glu. Likewise, the relative affinities of DNP-ala-ala and DNP-ala-gly; of DNP-leu-gly and di-DNP-leu-tyr; of DNP-leu-gly, DNP-ala-gly, DNP-gly-gly, di-DNP-lys-gly and DNP-hypro-gly all are in agreement. Among the six glycyl peptides the relative affinities are: DNP-gly-leu < DNP-gly-pro ~ DNP-gly-ala < DNP-gly-gly ~ DNP-gly-try ~ di-DNP-gly-tyr. Now DNP-ala and DNP-pro as well as DNP-gly, DNP-try and DNP-tyr may easily be separated,[3] and one would expect greater differences in the DNP-peptides. However, 3AA-15A-L is much too strong a developer to be used in the scheme for separating the enumerated DNP-amino acids and, in fact, it is probable that if it were tried they would group together much as do the above DNP-glycyl peptides.

If we extend the proposed correlation to the glycyl tripeptides, prediction would place di-DNP-gly-lys-gly above DNP-gly-gly-gly instead of below.

A comparison has not yet been made of such peptides as DNP-AX and DNP-XA. DNP-Ala-gly and DNP-gly-ala have very similar affinities and cannot be separated completely by any developer which has been used. On the other hand, DNP-gly-leu and DNP-leu-gly separate readily if the developer is 2AA-10A-L. Thus, the arrangement of amino acid residues in the peptide influences the chromatographic behavior of the DNP-peptide but the examples are too few to permit any generalizations to be made.

Little can be said about the relative affinities of two peptides which do not have an amino acid residue in common. There seems to be general validity, however, to the idea that the chromatographic behavior of a DNP-peptide is related to the chromatographic behavior of the DNP-derivatives of the constituent amino acids of the peptide. For example, DNP-gly is more strongly adsorbed than DNP-ala so that one would anticipate the observed result that DNP-gly-gly is more strongly adsorbed than DNP-ala-ala. Consider also di-DNP-lys-val

and DNP-ala-ala. Di-DNP-lys falls in group I and is rather strongly adsorbed whereas DNP-val in group IV is rather weakly adsorbed while DNP-ala in group III is intermediate; it is not surprising then to find that di-DNP-lys-val and DNP-ala-ala have similar affinity. Such examples could be multiplied.

With only one exception it has been found that a DNP-peptide is more strongly adsorbed than the DNP-derivative of any of the constituent amino acids. DNP-Leu-gly is coincident with DNP-gly when the developer is 2AA-10A-L. Leucine in the N-terminal position may have a somewhat anomalous effect on the adsorption affinity and this may be responsible for the ready separation of DNP-leu-gly and DNP-gly-leu which has already been mentioned. Note also that DNP-leu-gly-gly is very much less strongly adsorbed than DNP-gly-gly-gly and, in fact, is less strongly adsorbed than DNP-gly-gly.

Not all of the DNP-peptides have been developed with 12AA-6A-L or 3AA-15A-B but their number is sufficient to give some idea as to whether the above generalizations apply. Broadly considered, the generalizations still apply although in certain particulars the results may seem to be discrepant. Thus, when 12AA-6A-L is used as contrasted to 3AA-15A-L, DNP-ala-ala-ala and DNP-leu-gly-gly are less strongly adsorbed than DNP-gly-ala instead of more strongly adsorbed. Likewise, the sequences of DNP-gly-gly, di-DNP-gly-try, di-DNP-lys-ala, DNP-gly-pro, DNP-ala-gly, DNP-gly-try and DNP-gly-ala differ in the two developers. Furthermore, note that when 3AA-15A-B is the developer, peptides which contain lysine or tyrosine are relatively less strongly adsorbed. These apparent inconsistencies are related to the fact that the ratio of acetic acid to acetone in the developer as well as the interchange of ligroin and benzene has such marked effects on the chromatographic behavior of DNP-amino acids as was very clearly pointed out by Green and Kay.[3] In this same way the changes in the developer must alter the behavior of the DNP-peptides. Because these developers are much too strong for most of the DNP-amino acids and, hence, their chromatographic properties have not been studied with them, it cannot be stated whether the differences between 12AA-6A-L and 3AA-15A-L are exactly what would be expected. However, the lesser affinity of lysine- and tyrosine-containing peptides in 3AA-15A-B is to be anticipated because it is known that di-DNP-lys and di-DNP-tyr are much less strongly adsorbed relative to the other DNP-amino acids of group I if benzene developers rather than ligroin developers are used.

It is of interest to compare the present results briefly with the chromatography of peptides on paper and as an example, the data of Knight[8] will be used. Inspection of Knight's data shows that a peptide on paper may either have an affinity intermediate between that of the constituent amino acids or it may have an affinity greater or less than that of the constituent amino acids even if the peptide is rather long. This behavior is in great contrast to that of the DNP-peptides. Pardee[9] has made a mathematical analysis of Knight's data and has been able with considerable success to predict the R_F value of a peptide on paper from a knowledge of the R_F values of the constituent amino acids. No such analysis has been tried in the present work in the main because most of the DNP-amino acids have not been chromatographed with the developers which are most satisfactory for the DNP-peptides. Furthermore, the strong adsorption of the DNP-peptides (equivalent to small R_F) and the weak adsorption of the DNP-amino acids (equivalent to large R_F) are the two conditions which, as Pardee points out, lead to the greatest errors in such a mathematical analysis.

Comments on Developers.—As has already been mentioned, this study was made to obtain general information and not because of interest in any peptide *per se*. On the other hand, it was of importance to learn which developers would be most likely to separate the DNP-peptides of an unknown mixture. Very much progress has been made toward this goal.

In order to compare the movements of the DNP-peptides with those of the DNP-amino acids, all DNP-peptides have been chromatographed with 8AA-4A-L which was used by Green and Kay[3] for the separation of the DNP-amino acids into groups. As a developer 8AA-4A-L is too weak because all but a few DNP-peptides were distributed throughout the region of group I. Some of the DNP-peptides have been developed with 12AA-6A-L as shown in Fig. 1; this is a stronger developer but the ratio of acetic acid to acetone is unchanged. The movement of the zones is more satisfactory with this stronger developer and many separations which are incomplete as shown in the figure could have been achieved by a greater volume of developer or perhaps by a stronger one such as 16AA-8A-L.

Some of the first chromatograms were made with DNP-gly-leu and DNP-leu-gly which behave much like the DNP-amino acids of group II. Compounds in this group develop well with 2AA-10A-L and, as mentioned above, DNP-gly-leu and DNP-leu-gly separate completely when this developer is used. Indeed, it is a rather satisfactory developer also for DNP-ala-ala and similarly adsorbed DNP-peptides. Because the ratio of acetic acid to acetone in the developer has such a profound influence on the effectiveness of the developer in producing separations, 3AA-15A-L was used as a stronger developer for the more strongly adsorbed DNP-peptides. The results in Fig. 1 again point out that a greater volume or a stronger developer would achieve many separations some better, some worse, some different than when 12AA-6A-L is used. Changing the strength of the developer by increasing the content of acetic acid and acetone while keeping their ratio constant does increase the rate of movement of the zones but it is not entirely without effect on the relative positions. However, the degree of the effect is by no means as great as that produced by changing the ratio. It has sometimes been necessary to use 4AA-20A-L or 5AA-25A-L.

(8) C. A. Knight, *J. Biol. Chem.*, **190**, 753 (1951).

(9) A. B. Pardee, *ibid.*, **190**, 757 (1951).

The movement of many DNP-peptides with 3AA-15A-B is more rapid than is generally desirable. The use of 2AA-10A-B or 1AA-5A-B accordingly is indicated. The usefulness of this type of developer lies in its ability in some instances to invert the relative positions of certain DNP-derivatives.

Applications

We have made extensive use of the above information in the study of peptides from partial hydrolysates of gelatin.[4] The chromatographic behavior of all of the peptides which have been identified has agreed very well with that which would be expected from the generalizations which have been formulated above.

In attempting to separate a mixture of unknown DNP-peptides, it has been found to be beneficial to develop first with 7 *V* ml. of 8AA-4A-L. In this way, any contaminating DNP-amino acids will largely be removed and any weakly adsorbed DNP-peptides will begin to move down the chromatogram. Further development with as much as 7 *V* ml. of 3AA-15A-L will often separate the DNP-peptides into well-defined well-separated zones but depending upon the nature of the peptides it may be necessary to change further to 4AA-20A-L or 5AA-25A-L. Each zone should then be rechromatographed with 1AA-5A-B or stronger developer of the same type. By the use of this procedure it was possible to separate 14 definite DNP-derivatives from one peptide zone which was isolated from a partial hydrolysate of gelatin by means of an initial ion exchange chromatogram of the free peptides.

Acknowledgment.—We wish to thank Miss Lois M. Kay and Dr. F. Charlotte Green for having determined the chromatographic properties of some of the DNP-peptides.

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Ethyl Trithioörthoglyoxylate

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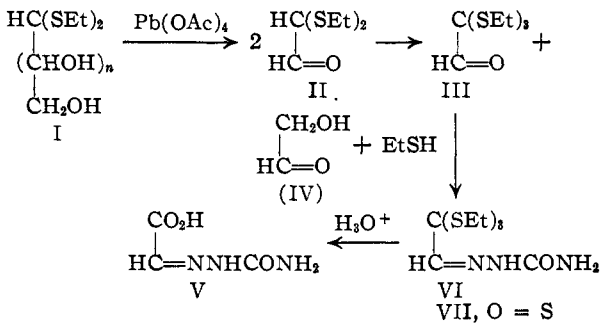
Fischer and Baer[1] synthesized the mono-(diethyl acetal) of glyoxal by subjecting DL-glyceraldehyde diethyl acetal to lead tetraacetate oxidation. The compound was a liquid that polymerized on standing. Schmidt and Wernicke[2] prepared glyoxal mono-(dibenzyl thioacetal) as a crystalline solid by the oxidative scission of 4,5-isopropylidene-D-fucose dibenzyl thioacetal with lead tetraacetate. In attempts to obtain the diethyl analog of this substance by the corresponding scission of aldose (D-galactose, L-arabinose and glyceraldehyde) diethyl thioacetals, the only product isolated (as the semicarbazone VI or thiosemicarbazone VII) was ethyl trithioörthoglyoxylate. Its structure was proved by the partial acid hydrolysis of its semicarbazone to the known glyoxylic acid semicarbazone (V). It is probable that the ethyl trithioörthoglyoxylate (III) arose as a bimolecular disproportionation product of the intermediate glyoxal mono-(diethyl thioacetal) (II). Fischer and Baer[1] demonstrated that two moles of glyoxal mono-(diethyl acetal), on treatment with alkali, underwent such a disproportionation to produce one mole of acid; the presence of glyoxylic acid and of glycolaldehyde were demonstrated in the acidified reaction mixture by these workers. The appearance of the thioörthoester in the glyoxylic acid moiety obtained by us is unexpected.



Experimental

Oxidative Scission of Aldose Thioacetals.—An amount of 2.86 g. (0.01 mole) of D-galactose diethyl thioacetal (mercaptal)[3,4] was dissolved in 500 ml. of dry dioxane in an apparatus protected from moisture. Lead tetraacetate (17.6 g., 0.04 mole) was added portionwise under stirring while maintaining the reaction mixture at 10–20°. After the addition, the cooling bath was removed and stirring was maintained for 2 hr. Lead diacetate was separated by filtration and the solvent was removed, under reduced pressure, from the filtrate. The reaction product was recovered by distillation under reduced pressure, yield 1.1 g., b.p. 100° (1 mm.).

From the product there were isolated, in low yield, the crystalline semicarbazone and thiosemicarbazone described below. These were also obtained: from the reaction mixture before distillation; on similar oxidation in benzene solution or in abs. chloroform–acetic acid solution; and on oxidation of the diethyl thioacetals (mercaptals) of L-arabinose and of glyceraldehyde.[5]

Ethyl Trithioörthoglyoxylate Thiosemicarbazone (VII).—Following Wolfrom and Tanghe,[6] the above distilled oil was dissolved in 100% ethanol, and thiosemicarbazide was dissolved in a 30% solution of acetic acid in ethanol. The two solutions were heated just short of boiling and then mixed. After cooling to room temperature, water was added to incipient turbidity and crystallization was effected at ice-box temperature; m.p. 140.5–141°.

Anal. Calcd. for $C_9H_{19}N_3S_4$: C, 36.33; H, 6.44; N, 14.13; S, 43.10. Found: C, 36.47; H, 6.37; N, 14.14; S, 43.26.

Ethyl Trithioörthoglyoxylate Semicarbazone (VI).—Semicarbazide hydrochloride (1 g.) was dissolved in 10 ml. of *N* potassium hydroxide and then more solid semicarbazide hydrochloride was added until the solution was just acid. The above distilled oxidation product (1 g.), dissolved in 10 ml. of 95% ethanol, was warmed to 90° and the semicarbazide solution was added to it. More water was added to effect complete solution at that temperature and a crystalline product separated on slow cooling. Pure material was obtained on recrystallization from 95% ethanol; m.p. 195°.

Anal. Calcd. for $C_9H_{19}ON_3S_4$: C, 38.41; H, 6.81; N, 14.94; S, 34.18. Found: C, 38.63; H, 6.84; N, 14.92; S, 33.89.

(1) H. O. L. Fischer and E. Baer, *Helv. Chim. Acta*, **18**, 514 (1935).
(2) O. T. Schmidt and E. Wernicke, *Ann.*, **556**, 179 (1944).
(3) E. Fischer, *Ber.*, **27**, 673 (1894).
(4) M. L. Wolfrom, THIS JOURNAL, **52**, 2466 (1930).
(5) H. W. Arnold and W. L. Evans, *ibid.*, **58**, 1950 (1936).
(6) M. L. Wolfrom and L. J. Tanghe, *ibid.*, **59**, 1601 (1937).