2. Formulas of the (finite) recursion type are advanced which permit the calculation from their carbon content of the number of stereoisomeric and non-stereoisomeric primary, secondary and tertiary mono-substitution products of the paraffins. In using these recursion formulas to calculate the total number of such isomers of any given carbon content, the total number of isomers, both stereoisomeric and non-stereoisomeric, of every lesser carbon content must be known.

3. The total number of isomeric mono-substitution products so obtained agrees exactly through those of the decanes with the numbers required by theory as tested by actually writing the structural formulas and counting the number of stereoisomers and non-stereoisomers.

AUSTIN, TEXAS

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, YALE UNIVERSITY]

RESEARCHES ON PYRIMIDINES. CXXV. THE EFFECT OF DILUTE ACIDS AND OF LIGHT ENERGY ON THYMINE GLYCOL. SYNTHESIS OF ACETYLMETHYLDIALURIC ACID

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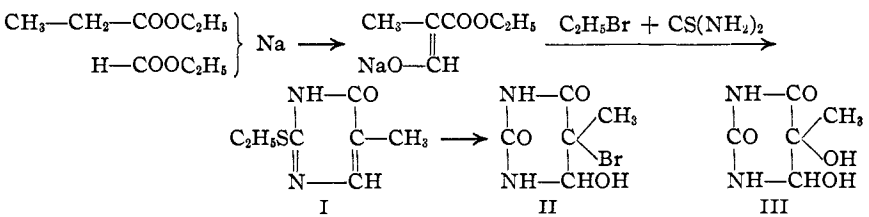
The method of preparing thymine glycol,[1] first described by Baudisch and Davidson, was modified by converting 2-ethylmercapto-5-methyl-6-oxypyrimidine I directly into bromoxyhydrothymine II by the action of bromine and then removing the bromine by the usual procedure to obtain the glycol derivative III. For the preparation of the ethyl mercapto-pyrimidine I the method of Johnson and Schmidt-Nickels[2] was used.



Method of Preparation.—Three and one-half grams of 2-ethylmercapto-5-methyl-6-oxypyrimidine I was suspended in 57 cc. of water, and with continuous stirring and gentle warming 5 cc. of bromine was gradually added. A pasty red mass was first formed which, however, on further warming went quickly into solution. The solution, colored with the excess of bromine, was filtered, concentrated *in vacuo*, and the precipitated bromoxyhydrothymine II purified by crystallization from hot water. In later preparations, instead of using silver oxide to replace the bromine in II by hydroxyl as practiced by Baudisch and Davidson, an equivalent amount of silver carbonate was used in order to avoid an alkaline reaction during the change.

[1] Baudisch and Davidson, *Ber.*, **58**, 1680 (1925).

[2] Johnson and Schmidt-Nickels, THIS JOURNAL, **52**, 4511 (1930).

In the experimental work described below, colorless thymine glycol (III), which is easily soluble in cold water, was used. In previous papers it has been shown that thymine, which is quite stable in alkaline solution and when exposed to light, becomes very sensitive toward alkali through the introduction of the two hydroxyl groups in the pyrimidine ring, and that the previously stable ring is easily broken down even in neutral solution by means of light energy (daylight or carbon arc light). In this change lactic aldehyde is formed as an intermediate decomposition product, which isomerizes into acetol. In weakly alkaline solution thymine glycol breaks down into urea, acetol and carbon dioxide. If at the same time an oxidizing agent is present (atmospheric oxygen in the presence of a catalyst), a different decomposition takes place, leading to the formation of urea, pyruvic acid and carbon dioxide. The biological importance of these characteristic decompositions is quite apparent, and it was especially desirable to learn the effect of dilute mineral acids on thymine glycol in the dark and in the light.

In order to maintain suitable biological conditions throughout our investigations, bacteria which have the power to oxidize sulfur to sulfuric acid were used as the source of acidity. Furthermore, thymine glycol in weak hydrochloric acid solution was irradiated at room temperature with a carbon arc light and the resulting changes studied. In both cases a hitherto unknown anhydride of thymine glycol was formed which is very insoluble in water. In this bacteriological research we were interested above all in a fuchsin-red compound which was formed only in very small quantities and whose chemical constitution we have not yet been able to determine. We will, however, describe carefully its characteristic reactions and especially its behavior to daylight irradiation. It is evident, from our work, that dilute sulfuric acid acts on thymine glycol to form not only the very stable anhydride of thymine glycol, but also a very labile and deeply colored intermediate compound which should be of very great biological interest.

Effect of Sulfur-oxidizing Bacteria on Thymine Glycol.—The bacteria used in this research were brought to this country a few years ago by one of the authors (Baudisch) from a Mexican sulfur hot-spring in Santa Rosalia (State of Chihuahua),[3] and were investigated in Waksman's laboratory. From this spring water, or rather from the white sulfur contained therein, a new strain of sulfur-oxidizing bacteria, *Sulphomonas (thiobacillus) thiooxidans*,[4] was isolated. All of the bacteriological research described in this paper was carried out in the Laboratory of Bacteriology at Yale,[5] and

[3] O. Baudisch, "A Mexican Hot Spring," Archives of Medical Hydrology, January, 1929.

[4] See also S. Waksman and Robert L. Starkey, *J. Gen. Physiol.*, No. 3, 285 (1923).

[5] For this privilege we desire to express our appreciation of the courtesy of Professor L. Rettger.

the above-mentioned sulfur bacteria were isolated by Mr. Gibbons from sulfur water shipped recently to us from Santa Rosalia. As a culture medium the pure inorganic salt mixture used by Waksman and Joffe was prepared in the following proportions: 0.2 g. of $(NH_4)_2SO_4$, 0.2 g. of $MgSO_4 \cdot 7H_2O$, 0.25 mg. of $CaCl_2$, 5.0 g. of KH_2PO_4 dissolved in 1 liter of distilled water. The cultures were grown in small Erlenmeyer flasks which contained about 50 cc. of culture medium and about 2 g. of flowers of sulfur. The contents of the flasks were sterilized by means of a current of steam and showed a P_H of 4.6, which did not change after standing for several weeks in the incubator. If however the pure inorganic culture medium was inoculated with a loop of *Sulphomonas* (*Thiobacillus*) *thiooxidans*, then even after a few days a strong acid formation set in and the hitherto clear contents of the flasks became cloudy, which was a positive indication of an abundant growth of bacteria. If thymine glycol was introduced into the culture medium at the beginning, the growth of the sulfur bacteria proceeded normally, save that the culture medium took on a faint pink color after a few days. Upon further standing in the incubator this color became darker and darker, and after a few weeks the contents of the culture flask were colored a deep fuchsin-red. Without thymine glycol, or after the introduction of thymine, other pyrimidines, sugars, or certain amino acids into the medium, the contents of the culture flask remained colorless in spite of the intensive growth of the bacteria, and in no case except with thymine glycol were pigment formations observed.

A quantitative determination was made of the sulfuric acid formed from the sulfur present in the contents of four flasks, each of which contained 50 cc. of the culture medium and 1 g. of thymine glycol. These flasks had stood for several weeks in the incubator and the contents had become deep red in color. The filtered media of the four flasks were made up to 200 cc. (the original volume) and of this 20 cc. was used for a barium sulfate precipitation. The following results were obtained:

2.2708 g. $BaSO_4$ (from 20 cc. of medium)
−0.0140 g. $BaSO_4$ (SO_4 ions already in the medium)

2.2568 g. $BaSO_4$ (equivalent of increase in SO_4 ions).

From these figures it was calculated that in each flask about 1 g. of sulfur was oxidized to sulfuric acid; the concentration of the sulfuric acid in each flask was about 1 *N*.

In order to determine any change in the thymine glycol during growth, the contents of a flask which had stood for six weeks inthe incubator and which contained 1 g. of this pyrimidine, was filtered from the sulfur and the deep red solution decolorized by exposure to sunlight for a few hours. After evaporation of the clear, colorless solution in a vacuum desiccator, a residue consisting mainly of inorganic salts remained. After trituration with water there remained undissolved a small amount of grayish-white powder which crystallized from boiling water in glistening needles. The weight

was 0.05 g. and the substance melted at 345–350°. The results obtained by analysis indicated that we were dealing here with thymine glycol anhydride.

Anal. (Micro) Subs., 3.915, 4.142 mg.: CO_2, 6.17, 6.545 mg.; H_2O, 1.48, 1.58 mg. Calcd. for $C_5H_6O_3N_2$: C, 42.25; H, 4.23. Found: C, 42.98, 43.09; H, 4.23, 4.27.

When a small amount of the crystals of this anhydride was boiled with *o*-aminobenzaldehyde in alkaline solution, and the solution acidified, *o*-oxyquinaldine was extracted by ether. This experiment proves that when the anhydride of thymine glycol is warmed in alkaline solution, acetol is formed just as in the case of thymine glycol itself, and is easily identified by Baudisch's acetol test. Nothing definite regarding the constitution of this interesting substance has yet been established.

It has previously been mentioned that the deep red sulfuric acid solution of thymine glycol becomes decolorized by exposure to daylight. In order to throw further light on this characteristic photochemical change, pure thymine glycol was dissolved in *N*/5 hydrochloric acid and the solution allowed to stand for a few days in the dark at 37° in an open vessel. The hitherto clear, colorless solution gradually took on a deep red color under the influence of the acid and air. If a part of this red solution was exposed to winter daylight in a glass vessel, it was quickly decolorized, the solution assuming a faint yellow color, and did not become entirely colorless as was the case with the free bacteria experiment. It is easily shown that this decolorization is an oxidation process which is accelerated by light, as dilute hydrogen peroxide decolorizes the solution almost instantaneously. If the deep-red solution is exposed to light in an atmosphere of nitrogen or carbon dioxide, the red color remains unchanged even under intensive irradiation. The weakly yellow solution, decolorized by light from the carbon arc lamp, shows a green fluorescence in ultraviolet light. Upon standing in the incubator in contact with air, the yellow solution quickly regains its red color, although it remains unchanged in a vacuum. Atmospheric oxygen is, therefore, necessary for the formation of the red compound. It is of interest that the long wave ultraviolet rays especially, which are present in sunlight, are strongly absorbed by the red or pale yellow decomposition products of thymine glycol. As an indicator we used crystal violet leucocyanide, a colorless alcoholic solution of which becomes deep blue upon exposure to ultraviolet light. The solution was irradiated in a short glass tube with quartz windows, an iron arc light being used as the light source. If the light is passed through distilled water the leucocyanide becomes noticeably blue in one second. A freshly prepared hydrochloric acid solution of thymine glycol behaves in the same way. However, both the red solution formed on standing in acid solution and the decolorized solution act as strong light filters, and even after irradiation for several minutes the leucocyanide remains colorless. These experiments clearly show that pronounced molecular changes, concerning which we have very little knowledge, take place in the thymine glycol molecule as the result of the influence of acids.

Another indicator useful for the study of these molecular changes was found in color reactions with diazotized sulfanilic acid. Fresh 2% thymine glycol solutions couple with this reagent in alkaline solution, forming a deep red color. This reaction failed, however, if the weakly acid solutions were allowed to stand in atmospheres of nitrogen, carbon dioxide and oxygen, or in a vacuum for several weeks. Through the influence of the acid, changes had taken place in the thymine glycol molecule.

It is striking that the colorless weakly acid thymine glycol solutions remained colorless in nitrogen and carbon dioxide, and assumed only a faint pink color in a vacuum or in oxygen. In contact with the air they became in the same length of time a deep fuchsin red. Similarly the solutions which had been in carbon dioxide and nitrogen atmospheres became red in a few hours when brought in contact with the air. The fuchsin-red solutions first became colorless on making alkaline and then almost

instantaneously a deep yellowish-red due to autoxidation. If first a small amount of cysteine hydrochloride was added to the red solution and the latter then made alkaline, the decolorized solution changed to a deep red from the surface. The red compound formed by the action of acid on thymine glycol is, therefore, colorless in weakly alkaline solution and becomes deep red by autoxidation.

One gram of thymine glycol was dissolved in 100 cc. of $N/5$ hydrochloric acid and the colorless solution placed in the incubator for four weeks, after which time a deep red coloration developed. The flask was then lightly stoppered and kept for two months in a dark room. At the end of this time beautiful ruby-red and also some brown crystals had separated from the remaining 25 cc. of the deep red solution. The aqueous solution was poured off, the crystals thoroughly washed with absolute alcohol, and the ruby red crystals separated from the brown. The melting point of the red crystals was almost the same as that of the thymine glycol used, and the micro-analysis showed the same composition. The brown crystals were soluble in absolute alcohol with the formation of a faint yellow color. When the solution was evaporated to dryness in a vacuum desiccator, a bright yellow residue which could not be crystallized remained. A micro-analysis gave values which agreed well with those of the anhydride of thymine glycol.

In order to determine further the influence of dilute mineral acids on thymine glycol, air was passed through a solution of one gram of thymine glycol in 200 cc. of $N/5$ hydrochloric acid in a quartz cylinder, and the solution irradiated with a strong arc light for several hours under efficient cooling. The solution remained entirely clear and colorless, revealing however an aldehyde-like odor. It was evaporated to dryness in a vacuum desiccator at room temperature. The solid residue was colored a strong pink and was noticeably crystalline. While the original thymine glycol was easily soluble in water, a part of the pink residue remained undissolved. This was filtered off and washed with water, yielding a white powder which crystallized from water in glistening snow-white crystals melting at 340–345°. The weight was 0.25 g.

Anal. (Micro) Subs., 4.377 mg.: 0.758 cc. of N_2 (26°, 746 mm.). Subs., 4.620 mg. ash-free (0.015 mg. ash), 4.081 mg. (0.012 mg. ash): H_2O 1.77, 1.54 mg.; CO_2, 7.19, 6.35 mg. Calcd. for $C_5H_6O_3N_2$: C, 42.25; H, 4.23; N, 19.72. Found: C, 42.44, 42.43; H, 4.29, 4.23; N, 19.41.

Although the new compound is also very insoluble in cold water, it dissolves quickly in alkali and is reprecipitated on acidification. If the alkaline solution is warmed, acetol is split off, which can be identified by Baudisch's acetol reaction.

The filtrate from the crystals above, which had become pink on standing, was again irradiated with cooling. It decolorized rapidly, with the development of an unmistakable aldehyde-like odor. After evaporation in a vacuum desiccator, glistening white crystals, identical with the above-mentioned substance, remained behind and melted at 340–345°.

The Action of Phosphoric Acid on Thymine Glycol.—One gram of thymine glycol was dissolved in 50 cc. of commercial phosphoric acid and the clear, colorless solution lightly stoppered and placed in the incubator. Gradually color changes set in and at the end of three months the solution became dark brown and cloudy. It was then diluted with one liter of water, filtered, and the white, insoluble part recrystallized from boiling water, yielding snow-white glistening crystals with a melting point of 345–350°.

Anal. (Micro) Subs., 3.915, 4.142 mg.: H_2O, 1.48, 1.58 mg.; CO_2, 6.17, 6.545 mg. Calcd. for $C_5H_6O_3N_2$: C, 42.25; H, 4.23. Found: C, 42.9, 42.09; H, 4.23, 4.27.

As can be seen from these results, the anhydride of thymine glycol is also formed in phosphoric acid solution, without, however, the formation of the fuchsin-red com-

pound which served as a colorimetric indicator of the acid formation in the sulfur bacterial culture. As has been previously mentioned, the color does not appear in carbon dioxide or nitrogen atmospheres; it is probable that the color is due to the formation of compounds similar in constitution to quinhydrone, which are formed, however, only in the dark and under a certain partial pressure of oxygen. In daylight the fuchsin-red compound quickly takes up more oxygen and is decolorized.

The research below was also carried out in order to clear up further the chemical reactions occurring in an acid thymine glycol solution. The assumption was made from the beginning that at the same time an oxidation process was being dealt with, that the secondary alcohol groups of the thymine glycol molecule were involved first with formation of methyldialuric acid, which has thus far not yet been described in the literature. The preparation and some knowledge of the chemical behavior of this pyrimidine was, therefore, very desirable. We tried to prepare this compound by the direct oxidation of thymine glycol and also by synthesis. Thus far no method which we have applied has given us the desired compound.

The Oxidation of Thymine Glycol

Oxidation with Bromine Water.—Three hundred and twenty milligrams of thymine glycol ($^1/_{500}$ mole) was dissolved in 10 cc. of water and warmed for a short time with 320 mg. of bromine. Neither immediately nor upon standing for fifteen days did the bromine react, as the unchanged color of the solution showed. After evaporation *in vacuo* over sulfuric acid and potassium hydroxide the unchanged thymine glycol was recovered.

Oxidation with Chromosulfuric Acid.—Eight hundred milligrams of thymine glycol ($^1/_{200}$ mole) was dissolved in 40 cc. of water, 2 cc. of concd. sulfuric acid added and 5 cc. of 6.6% solution of chromic oxide ($^1/_{200}$ mole of active oxygen). After gentle boiling for about two hours the original red color had changed to green and the mixture gave no test with potassium iodide–starch paper. Barium hydroxide in the calculated amount was added to the gently boiling mixture so that the solution gave a neutral reaction and was free from barium and sulfate ions. After filtering, the filtrate was evaporated to dryness in a vacuum and the residue taken up with a little hot water and filtered. Upon cooling, 153 mg. of a crystalline compound separated out. This was crystallized from 35 parts of water, and separated in the form of interlocking plates. The compound was neutral to litmus, gave no acetol or pyruvic acid test, and did not melt upon heating to 260°, although it sintered at about 175–180°. By means of a green precipitate which it gave with copper ion and by analysis, this substance was recognized as cyanuric acid.

Anal. Subs., 101.2 mg.: 27.5 cc. of N_2 (22°, 764 mm.). Calcd. for $(CHON)_3$: N, 32.56. Found: N, 32.50.

The formation of the cyanuric acid can be explained by the hydrolysis of thymine glycol by the acid, forming acetol, urea and carbon dioxide. The urea was then oxidized by the chromic acid. In the reaction mixture we were able to identify acetol by means of the Baudisch reaction.

Oxidation with Red Mercuric Oxide.—Eight hundred milligrams of thymine glycol ($^1/_{200}$ mole) was dissolved in 500 cc. of water and the solution heated to boiling for a time with a suspension of freshly-prepared mercuric oxide until the oxide dissolved. The solution was then filtered and cooled, when an amorphous, mercury-containing product precipitated, the solution having first become cloudy white. It was impossible to crystallize this substance. For every mole of thymine glycol, one mole of mercury oxide was used, no more going into solution. An analysis of the compound indicates that it has the empirical formula $C_5H_6O_4N_2Hg$.

Anal. (Micro) Subs., 118.7 mg.: 8.35 cc. of N_2 (22°, 762 mm.). Calcd. for $C_5H_6O_4N_2Hg$: N, 7.83. Found: N, 8.16.

Synthesis of Acetylmethyldialuric acid.—(1) An attempt was made to condense chloromethylmalonic acid with urea by means of phosphorus oxychloride according to Grimaux's method. At the end of the experiment a crystalline product separated out which was identified as biuret by means of its crystal structure, reaction, and melting point.

Anal. Calcd. for $C_2H_5O_2N_3$: N, 40.78. Found: N, 41.02, 41.1.

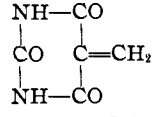
(2) We then attempted to condense ethyl chloromethylmalonate with urea by means of sodium ethylate following the method of Michael: 10.4 g. of chloromethylmalonic ester was mixed with a solution of 1.15 g. of sodium in 25 cc. of absolute alcohol, and the resulting mixture added to a solution of 3 g. of urea in 15 cc. of absolute alcohol. This mixture after heating for seven hours under a reflux condenser gave no condensation product that could be isolated.

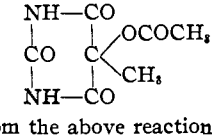
(3) Finally the following method was used. Methylbarbituric acid was prepared and then converted into bromomethylbarbituric acid. After shaking this bromo compound for about twenty hours with silver carbonate, the precipitate was centrifuged off and washed many times with water. After evaporating the solution so made, no residue worth mentioning was obtained. It was then attempted to wash out the centrifugalized residue with hot water, which could not be done however because of the difficulty in filtering. Then the entire precipitate was suspended in water and saturated with hydrogen sulfide. The solution was filtered, evaporated *in vacuo* and the residue redissolved in hot water. On cooling a compound crystallized out in needles which was recrystallized from hot water. It contained sulfur, became yellow at 200–210° and melted with decomposition at about 240–245°. On treatment with alkali no acetol could be identified by the Baudisch test.

Anal. (Micro) Subs., 4.401, 4.950 mg.: H_2O, 1.08, 1.23 mg.; CO_2, 5.625, 6.290 mg. Subs., 4.592 mg.: 0.636 cc. of N_2 (26°, 743 mm.). Calcd. for $C_5H_6O_2N_3S$: C, 34.48; H, 3.45; N, 16.08. Calcd. for $C_5H_5O_2N_3S$: C, 34.68; H, 2.89; N, 16.18. Found: C, 34.79, 34.66; H, 2.75, 2.78; N, 15.45.

The results of analysis point to the formation here of a sulfur compound with bimolecular structure and thus far we have come to no definite conclusions regarding its structure.

(4) The following method finally led to the desired result. The bromomethylbarbituric acid was dissolved in 50% acetic acid and a 20% excess of finely pulverized silver acetate added. The mixture was then kept for six hours at 80° with continuous stirring. The silver chloride was filtered off and the excess of silver acetate removed by the careful addition of hydrochloric acid. The solution was then evaporated in a vacuum and the residue taken up in hot water. Upon cooling a crystalline product separated out, which was purified by recrystallization from hot water. It separated as long needles which sintered upon rapid heating to 230°, and decomposed gradually while melting. The properties of this compound and nitrogen determinations indicated that we were not dealing here with a pure substance. The substance reacted as a mixture of the methylene and acetylpyrimidines represented by the formulas





In order to obtain a definite compound from the above reaction product, we then proceeded as follows.

(a) **Acetylation.**—Five hundred milligrams of the above described product was heated for five hours under a reflux condenser with 50 cc. of acetic anhydride which had previously been purified by vacuum distillation. The solvent was removed by vacuum distillation, and the dried residue again taken up in a little acetic anhydride. From the hot filtered solution there separated on cooling 230 mg. of a product which was crystallized in prisms arranged as twins. This melted sharply at 244–245°, solidified again upon cooling, and then melted again about half a degree higher than before.

Anal. (Micro) Subs., 107.6, 108.8 mg.: 13.1 cc. of N_2 (28°, 763 mm.); 13.0 cc. of N_2 (22°, 759 mm.). Calcd. for $C_7H_8O_5N_2$: N, 14.00. Found: N, 13.86, 13.81.

(b) **Saponification.**—Five hundred milligrams of the above described acetyl compound was boiled for five hours under a reflux condenser with 50 cc. of 2 *N* hydrochloric acid, the solvent then distilled off *in vacuo*, the residue taken up in 5 cc. of hot water, and the small amount of the crystalline product which separated on cooling filtered. It was evidently impossible after evaporation in a vacuum desiccator to obtain a crystalline compound. Further work is now in progress, and it is our intention to perfect a practical method of synthesizing this interesting pyrimidine.

Summary

1. It has been shown previously that thymine glycol is easily broken down in alkaline solution giving urea, acetol and carbon dioxide.

2. In acid solutions the pyrimidine ring of thymine glycol is more stable than in the presence of alkali.

3. Irradiation in acid solution leads to the formation of an anhydride of thymine glycol whose exact constitution is unknown.

4. When thymine glycol is introduced into a culture solution of *Sulphomonas thiooxidans* the latter takes on during incubation a deep red coloration and from the medium the anhydride thymine glycol anhydride can be isolated.

5. Thymine glycol alone in hydrochloric acid solution undergoes the same coloration when allowed to stand in the dark.

6. The red coloration formed in all cases is destroyed by the action of daylight.

7. A method is described for the preparation of acetylmethyldialuric acid.

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