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The physiological action of cystinyl peptides and guanidine derivatives

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THE PHYSIOLOGICAL ACTION OF CYSTINYL PEPTIDES AND GUANIDINE DERIVATIVES

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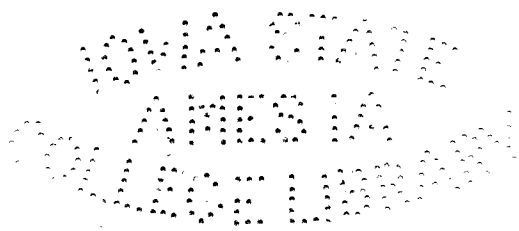

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PART I

GUANIDINE DERIVATIVES

INTRODUCTION

In his studies on the metabolic changes induced by the administration of guanidine, Watanabe (1), in 1918 noted the marked reduction in blood sugar which followed the injection of guanidine hydrochloride. Several years later Frank (2) made the suggestion that while guanidine is not identical with insulin it might play an important part in the physiological activity of insulin. Acting upon the assumption that insulin might be a guanidine compound Dubin and Corbitt (3) were led to investigate a number of guanidine derivatives and related substances. Though several of the compounds which they tested, particularly methylguanidine sulfate, showed marked hypoglucemic action, the action was delayed rather than immediate as is the case with insulin and they concluded that no relation existed between insulin and the compounds tested.

In an attempt to arrive at the chemical structure of the active group of insulin Kon and Funk (4) investigated a series of derivatives of imidazole, pyridine and cystine. A number of their compounds possessed hypoglucemic activity especially imidazolaldehyde, imidazole hydrochloride, histidine and

(1) Watanabe, J. Biol. Chem. 33, 253 (1918).
(2) Frank, Klin. Wochschr. 3, 955 (1924).
(3) Dubin and Corbitt, J. Lab. Clin. Med. 10, 1023 (1925).
(4) Kon and Funk, Chem. Zelle Gewebe 13, 89 (1926).

cysteine hydrochloride. In general they found that basic substances tended to lower the blood sugar while acidic substances had the opposite effect. From their observations they made the suggestion that the active group of insulin is in some way related to or composed of histidine and cysteine. It is interesting to note in this connection that both histidine and cystine (the oxidized form of cysteine) have subsequently been isolated from crystalline insulin (5).

The possibility that variation in chemical constitution might produce a compound having a hypoglucemic action greater than that of guanidine itself led Alles (6) to a study of the action of a series of guanidine derivatives. He found that both aminoguanidine hydrochloride and acetylguanidine hydrochloride lowered the blood sugar but the maximum effect was reached only after several hours. Alles believed the hypoglucemic action of guanidine to be a secondary effect and he thought it unlikely that insulin and guanidine were related.

Instigated by the hope that the toxic and hypoglucemic actions of guanidine might be dissociated, Frank and co-workers (7) were prompted to test the effect on blood sugar of the naturally occurring agmatine (ω-aminobutylguanidine), the decarboxylation product of the amino acid arginine. Agmatine

(5) du Vigneaud, Jensen and Wintersteiner, J. Pharmacol. 32, 367 (1928); Jensen, Wintersteiner and du Vigneaud, ibid. 32, 387 (1928).
(6) Alles, J. Pharmacol. 28, 251 (1926).
(7) Frank, Nothmann and Wagner, Klin. Wochschr. 5, 2100 (1926); Frank, Naturwisseenschaften 15, 213 (1927).

in very small doses was found to reduce the blood sugar to the convulsive level. This hypoglucemia was however preceded by an initial hyperglucemia such as had been noted with other guanidine derivatives. The compound was much more powerful and much less toxic than any which had been previously tested. From this result it appeared that the length of the side-chain on the guanidine nucleus had the effect of increasing the activity of the compound. The next member of the series, ω-aminoamylguanidine, was therefore synthesized and tested. This compound not only exerted a stronger hypoglucemic action than agmatine but also did not show the initial hyperglucemia produced by the latter.

In the preparation of ω-aminoamylguanidine and higher homologues, the formation of substances containing two guanidine groups was observed. A study of a series of these compounds culminated in the announcement by Frank (7) of synthalin, the most powerful synthetic blood sugar reducing agent known. The exact nature of synthalin was at first not disclosed, but the compound has since been identified by Frank with decamethylene diguanidine (8).

Synthalin appeared to have an action almost identical with that of insulin with the added advantage that it could be administered orally. As a result the preparation was greeted with a great deal of enthusiasm and was made the subject of a very

(8) Frank, Archiv. exptl. Path. Pharmakol 128, 33 (1928).

large number of physiological and clinical studies (9). While synthalin has been used with success in some cases it has been found to be unreliable and very often toxic.

Blatherwick, Sahyun and Hill (10) showed that the administration of synthalin resulted in injury to the liver as indicated by the inability to deaminize glycine. They suggested that the hypoglucemia may be due to the prevention of normal glycogenesis in the liver. After a study of the problem Simola (11), Debois, Defauw and Hoet (12) and Dale and coworkers (13) reached the conclusion that there is an essential difference between the action of synthalin and insulin.

A number of naturally occurring products with hypoglucemic action have been presented. Simonnet and Tanret (14) found galegine (iso-amyleneguanidine), isolated from certain plants, to possess marked hypoglucemic properties. The compound is however quite toxic. von Noorden (15) reported an extract

(9) For bibliographies on the physiological action of synthalin see: Bodo and Marks, J. Physiol. 65, 83 (1928) and Staub, Z. klin. Med. 107, 607 (1928). These works also include excellent discussions of the action of synthalin.
(10) Blatherwick, Sahyun and Hill, J. Biol. Chem. 75, 671 (1927).
(11) Simola, Z. physiol. Chem. 168, 274 (1927).
(12) Debois, Defauw and Hoet, Compt. rend. soc. biol. 97, 1420 (1927).
(13) Dale, Graham, Lawrence and Cammidge, Proc. Roy. Soc. Med. 21, 527 (1928).
(14) Simonnet and Tanret, Compt. rend. 184, 1600 (1927); Bull. soc. chim. biol. 9, 908 (1927); ibid. 10, 796 (1928).
(15) von Noorden, Klin. Wochschr. 6, 1041 (1927).

called glukhorment prepared by the fermentation of pancreas and which it was said can be used to replace insulin except in severe cases. The possibility that glukhorment contained insulin was precluded by the method of preparation. Bischoff, Blatherwick and Sahyun (16) presented evidence that the active principle of glukhorment is either synthalin or a near homologue. Hill and co-workers (17) showed creatine to possess hypoglucemic action when administered to both dogs and man but not when administered to rabbits. The compound was without toxic effects and the reduction in blood sugar was about the same whether the dose was small or large.

An insulin-like action, together with low toxicity, were claimed by Cannavo (18) for a guanidine derivative called acoin (p,p'-dimethoxy-p"-ethoxy-triphenylguanidine hydrochloride). This substance was recommended by Cannavo as a substitute for insulin, however Voigt (19) and Fuld (20) reported the compound as showing no hypoglucemic action. Cannavo (21) also studied

(16) Bischoff, Blatherwick and Sahyun, J. Biol. Chem. 77, 467 (1928).
(17) Hill, J. Biol. Chem. 78, IV (1928); Hill and Mattison, ibid. 82, 679 (1929); Peabody and Hill, ibid 82, 687 (1929).
(18) Cannavo, Arch. farmacol. sper. 44, 49 (1927) (C.A. 22, 1397 (1928)); ibid. 45, 218 (1928) (C.A. 22, 4642 (1928)).
(19) Voigt, Klin. Wochschr. 7, 1939 (1928).
(20) Fuld, Klin. Wochschr. 7, 1939 (1928).
(21) Cannavo, Boll. soc. ital. biol. sper. 3, 618 (1928) (C.A. 23, 901 (1929)); Arch. farmacol. sper. 45, 249 (1928) (C.A. 23, 2495 (1929)).

the effect of the substitution of phenyl groups upon the activity of guanidine. He showed that the introduction of phenyl groups increased the toxicity and decreased the hypoglucemic action.

Kumagai and co-workers (22) continued the search for insulin substitutes by their investigation of a series of guanidine and related pyrimidine derivatives. They found that guanidine derivatives in which both amino groups were substituted were inactive while in the case of mono-substituted guanidines increase in the length of the side chain was accompanied by increased hypoglucemic and decreased toxic action. This latter observation had previously been made by Frank (7).

Frank, Nothmann and Wagner (23) in 1928 reported a homologue of synthalin, dodecamethylene diguanidine (called synthalin B), which compound they found to be less toxic and more active than synthalin. These facts are not in agreement with the findings of Bischoff, Sahyun and Long (24).

Bischoff, Sahyun and Long (24) in a study of the effect of structure on the physiological action of guanidine compounds

(22) Kumagai, Kawai, Shikinami and Hosono, Sci. Papers. Inst. Phys. Chem. Res. 9, 271 (1928) (C.A. 23, 2425 (1929)); Kumagai, Kawai and Shikinami, Proc. Imp. Acad. (Japan) 4, 23. (1928) (C.A. 22, 4662 (1928)).
(23) Frank, Nothmann and Wagner, Klin. Wochschr. 7, 1996 (1928).
(24) Bischoff, Sahyun and Long, J. Biol. Chem. 81, 325 (1929).

compared the hypoglucemic action and toxicity of a variety of types of guanidine derivatives. They also studied the fate of the glycogen, glucose tolerance, CO_2-combining power and change in inorganic phosphorus in animals in guanidine hypoglucemia. These workers found that substitution of negative groups or aromatic nuclei was not productive of hypoglucemic action while this action was most powerful in basic derivatives with long aliphatic side chains. Derivatives of guanidine formic acid and its derivatives were non-toxic. Hypoglucemic activity, lethal dose and liver damage ran almost parallel. The toxic effect of the aromatic derivatives as shown by impaired respiration, nervous symptoms and kidney damage did not appear to be connected with the hypoglucemic activity while liver damage was definitely involved.

Of the compounds tested by Bischoff, Sahyun and Long (24) guanylpiperidine was found to more closely resemble insulin in its action than any of the others. The hypoglucemia produced by this compound occurs during the first hour after administration, as is the case with insulin, and the compound is less toxic than the other derivatives studied. These authors believed that there was a possibility that guanidine and insulin were chemically related.

Hesse and Taubmann (25) reported a study of the action of

(25) Hesse and Taubmann, Arch. exptl.Path. Pharmakol. 142, 290 (1929).

a series of biguanide derivatives on sugar metabolism. A number of their compounds produced a very marked decrease in blood sugar. The action of these biguanides, like that of guanidines, differed from the action of insulin in a number of respects. The administration of biguanides produced no glycogen synthesis. Biguanide convulsions were not abolished by the injection of adrenaline or grape sugar. Normal symptoms were not produced in pancreotomized animals.

Wantoch (26) investigated the influence of various organic and inorganic substances, principally acids, upon the blood sugar.

Braun (27) reported a considerable hypoglucemic effect without toxic action produced by p-aminophenylguanidine hydroiodide. This observation has since been corrected (28). A test of the sulfate and hydrochloride of this compound showed no activity and a further study of the hydroiodide showed inconsistent results. This inconsistency was not explained.

Ruiz, Silva and Libenson (29) studied the hypoglucemic action of a number of sulfur compounds. Several of these showed some hypoglucemic action. Assuming the possibility of the existence of a 2-thiolimidazole group in insulin these

(26) Wantoch, Arch. exptl. Path. Pharmakol. 143, 337 (1929).
(27) Braun, J. Biol. Chem. 89, 97 (1930).
(28) Parks and Braun, J. Biol. Chem. 91, 629 (1931).
(29) Ruiz, Silva and Libenson, Compt. rend. soc. biol. 104, 1029 (1930).

authors (30) investigated a series of compounds containing this group. Several of the compounds tested were found to be active.

Recently Bischoff and Long (31) published further work on guanidine structure and hypoglucemia. The article was not available at the present writing.

In addition to the work which has been mentioned a large number of animal and plant extracts have been reported as possessing hypoglucemic activity. Since these products are not of definitely known constitution they are not included in this account.

In the present work a miscellany of nitrogen and sulfur compounds have been prepared for testing as to hypoglucemic action with the hope that a substance might be obtained which had an action more closely resembling that of insulin than does the action of compounds investigated up to this time. An effort has been made not only to elaborate on types of known hypoglucemic activity but also to study types which have not previously been tested.

(30) Ruiz, Silva and Libenson, Compt. rend. soc. biol. 104, 1101 (1930); Rev. soc. Argentina biol. 6, 198 (1930) (Physiol. Abstracts 16, 46 (1931)).
(31) Bischoff and Long, J. Pharmacol. 41, 127 (1931).

EXPERIMENTAL PART

Cyanamide, NH_2CN. Cyanamide was obtained from calcium cyanamide by neutralization with sulfuric acid, followed by extraction of the concentrated solution with ether. A mixture of one kilogram of calcium cyanamide and 3 liters of water was placed in a five liter flask and mechanically stirred for one hour. The sludge was filtered and washed well with water. The cooled filtrate was made just acid to methyl red by the addition of 30% sulfuric acid and the precipitated calcium sulfate filtered and washed with water. To the filtrate was added about one cc. of acetic acid and the solution was then evaporated on the water bath under reduced pressure until the temperature (thermometer in liquid) reached 70-80°. After cooling, the remaining liquid was extracted with 1500 cc. of anhydrous ether, a few drops of acetic acid added, and the solution dried over calcium chloride. The ether was distilled, the last portion under reduced pressure, and the residue allowed to cool in a vacuum desiccator over sulfuric acid. The product solidified to a mass of crystals. The yield was 92.5 g. or 14.5% (assuming pure calcium cyanamide) of material melting at 37-40°.

Phenylguanidine, $C_6H_5NHC(:NH)NH_2$. Phenylguanidine was prepared from cyanamide and aniline hydrochloride by the method of McKee (32). A mixture of 13 g. (0.1 mole) of aniline hydrochloride,

(32) McKee, Am. Chem. J. 26, 221 (1901).

4.2 g. (0.1 mole) of cyanamide and 40 cc. of absolute alcohol was heated in a sealed tube in an oil bath at 120-130° for 2½ hours. The alcohol was evaporated on the water bath and the residue dissolved in about 75 cc. of water and treated with a concentrated solution of potassium hydroxide. An oil separated which crystallized upon cooling. After chilling well, the crystals were filtered, washed with a little water and dried in a desiccator over solid potassium hydroxide. A yield of 10 g. or 74% of material melting at 66-68° was obtained. The picrate prepared from this compound melted at 223-224°. The melting points given by McKee (32) are 66° for phenylguanidine and 221° for the picrate.

p-Bromophenyl Guanidine Nitrate, $BrC_6H_4NHC(:NH)NH_2 \cdot HNO_3$. p-Bromophenylguanidine was prepared from p-bromoaniline hydrochloride and cyanamide. A mixture of 10.2 g. (0.05 mole) of p-bromoaniline hydrochloride, 3 g. (0.07 mole) of cyanamide and 30 cc. of absolute alcohol was placed in a sealed tube and heated in an oil bath at 120-130° for 3 hours. The solution was concentrated by distillation and the residue was poured into 2-normal nitric acid. A crystalline precipitate separated which was filtered and crystallized from water. A yield of 8.5 g. or 61% of p-bromophenylguanidine nitrate melting at 185-186° was obtained.

Anal. (By Kjeldahl). Calc. for $C_7H_9BrN_4O_3$: N, 20.21. Found: N, 18.87 and 19.34.

p-Bromophenylguanidine has not been reported in the literature. The free base was obtained by dissolving the nitrate in hot water and adding concentrated potassium hydroxide. An oil separated which solidified upon cooling and was filtered and dried over solid potassium hydroxide. This compound melted at 122-124°. The free base was not analyzed.

p-Carboxylphenylguanidine, $HOOCC_6H_4NHC(:NH)NH_2$. p-Carboxylphenylguanidine was prepared from p-aminobenzoic acid hydrochloride and cyanamide. A mixture of 17.4 g. (0.1 mole) of p-aminobenzoic acid hydrochloride, 4.2 g. (0.1 mole) of cyanamide and 40 cc. of absolute alcohol was placed in a sealed tube and heated in an oil bath at 120-130° for five hours. The cooled tube contained a crystalline solid which was filtered and dissolved in water. The water solution was made alkaline and then neutralized by the careful addition of hydrochloric acid. A crystalline precipitate separated which was filtered and recrystallized from water. The compound melted at 285-286°.

p-Carboxylphenylguanidine hydrochloride was prepared by treating an absolute alcohol suspension of the free base with sufficient hydrochloric acid to effect solution. Upon the addition of ether, the hydrochloride separated as small white needles. To purify, it was dissolved in 95% alcohol and precipitated by the addition of ether. The compound melted at 273-274°.

Anal. Calc. for $C_8H_{10}ClN_3O_2$: Cl, 16.47. Found: Cl, 16.12 and 16.06.

A simplified analytical procedure was employed in the analysis of p-carboxylphenylguanidine hydrochloride and certain other guanidine hydrochlorides in the following work. The sample of about 0.2 g. was placed in a 400 cc. beaker, dissolved in a few cc. of water and 2 or 3 cc. of 10% silver nitrate added. About 100 cc. of concentrated nitric acid were then added, the beaker covered with a watch glass and the mixture allowed to digest on the steam plate until clear or only slightly yellow. The solution was finally allowed to evaporate to a volume of about 10 cc. and was then diluted to a volume of 250 cc. with distilled water and heated to boiling. The hot solution was decanted through a Gooch crucible, the precipitate washed several times with hot water by decantation and finally transferred to the crucible and washed with hot water until the washings gave no precipitate with hydrochloric acid. The crucible was dried and weighed in the customary manner.

Methylisothiourea Sulfate, $NH_2C(:NH)SCH_3 \cdot \frac{1}{2}H_2SO_4$. Methylisothiourea sulfate was prepared by the action of dimethylsulphate on thiourea according to the directions of Arndt (35). To a mixture of 76 g. (one mole) of thiourea and 50 cc. of water in

(33) Arndt, Ber. 54, 2236 (1921).

an open flask was added 63 g. (0.5 mole) of dimethylsulfate. A vigorous reaction ensued and considerable heat was evolved. When this initial reaction had subsided the mixture was heated to boiling with a small flame for about fifteen minutes. Upon cooling, the contents of the flask solidified to a compact mass of white crystals. To this was added a little water and about 200 cc. of alcohol and the crystalline product then filtered and washed with alcohol. The alcohol was distilled from the filtrate and the residue boiled until crystallization commenced. This additional product was cooled, treated with alcohol, filtered and washed as before. The yield was 122.5 g. or 88% of product melting with decomposition at 242-244°, which agrees with the melting point given in the literature (33).

Benzylguanidine Nitrate, $C_6H_5CH_2NHC(:NH)NH_2 \cdot HNO_3$. Benzylguanidine nitrate was prepared by two methods: (1) from benzylamine hydrochloride and cyanamide and (2) from benzylamine and methylisothiourea sulfate, followed in both cases by treatment with dilute nitric acid.

(1) A mixture of 14.4 g. (0.1 mole) of benzylamine hydrochloride, 5 g. (0.12 mole) of cyanamide and 40 cc. of absolute alcohol was placed in a sealed tube and heated in an oil bath at 120-130° for 3 hours. The solution was concentrated by distillation and the residue from which most of the alcohol had been removed was poured into 2-normal nitric acid. A white

crystalline precipitate separated and was filtered and crystallized from water. The yield was 13.5 g. or 63% of product melting at 149-150°.

(2) A mixture of 14 g. (0.1 mole) of methylisothiourea sulfate, 25 cc. of water and 10.7 g. (0.1 mole) of benzylamine was heated on a water bath at about 70°. A vigorous reaction took place accompanied by the evolution of methylmercaptan. After the reaction had subsided the solution was boiled for 45 minutes, cooled, filtered and the filtrate poured into 50 cc. of 2-normal nitric acid. A white crystalline precipitate separated which was filtered and dried, yielding 17 g. or 80% of material melting at 152-153°. This product was identical, as shown by a mixed melting point, with that prepared by method (1).

Anal. (By Kjeldahl). Calc. for $C_8H_{12}O_3N_4$: N, 26.41 Found: N, 24.88 and 25.06.

Methylisothiourea p-Toluenesulfonate, $NH_2C(:NH)SCH_3 \cdot CH_3-C_6H_4SO_3H$. Methylisothiourea p-toluenesulfonate was prepared by the action of methyl p-toluenesulfonate on thiourea. A mixture of 101 g. (0.54 mole) of methyl p-toluenesulfonate, 38 g. (0.5 mole) of thiourea and 50 cc. of water was cautiously heated until the reaction commenced. The reaction was very vigorous and it was necessary to cool the flask with water. When the reaction had ceased the clear solution was boiled for 15 minutes and allowed to cool. The white crystalline product which

separated was filtered and washed with a little water and the filtrate and washings concentrated by boiling until of a syrupy consistency. Upon cooling the mass solidified and was crystallized from water. The total yield was 121 g. or 92% of material melting at 141-142°.

Anal. (By Kjeldahl). Calc. for $C_9H_{14}N_2O_3S_2$: N, 10.68. Found: N, 11.00 and 10.93.

Benzylguanidine p-Toluenesulfonate, $C_6H_5CH_2NHC(:NH)NH_2 \cdot CH_3C_6H_4SO_3H$. Benzylguanidine p-toluenesulfonate was prepared by the action of benzylamine on methylisothiourea p-toluenesulfonate. The methyl mercaptan liberated in this reaction was collected as the mercury salt. Two and seven-tenths grams (0.025 mole) of benzylamine were dissolved in 25 cc. of water and to the solution were added 6.5 g. (0.025 mole) of methylisothiourea p-toluenesulfonate. The reaction flask was fitted with a reflux condenser and was then gently heated until the reaction had started. The evolved mercaptan was led into a flask containing a solution of mercuric chloride in alcohol and cooled in an ice-salt mixture. After the evolution of gas had ceased the solution was boiled for one hour. Upon cooling an oil separated which gradually crystallized. A yield of 6 g. or 75% of this product melting at about 175° was obtained. This compound was identical, as shown by a mixed melting point, with that obtained by treating a solution of benzylguanidine nitrate with p-toluenesulfonic acid.

The benzylguanidine p-toluenesulfonate separated as white plates melting at 179-181°.

Anal. (By Kjeldahl). Calc. for $C_{15}H_{19}N_3O_3S$: N, 13.05. Found: N, 12.66 and 12.84.

The mercuric methylmercaptide was filtered and washed with alcohol and ether. A yield of 4.8 g. or 66% of this material, which did not melt below 300°, was obtained.

Furfurylguanidine Sulfate. $C_4H_3OCH_2NHC(:NH)NH_2 \cdot \frac{1}{2}H_2SO_4$. Furfurylguanidine sulfate was prepared by the action of furfurylamine on methylisothiourea sulfate. A mixture of 4 g. (0.029 mole) of methylisothiourea sulfate, 3 g. (0.031 mole) of furfurylamine (34) and 20 cc. of 95% alcohol was heated on the steam plate until all of the methylisothiourea sulfate had dissolved. The alcohol was partially evaporated and the compound precipitated by the addition of ether. The furfurylguanidine sulfate was crystallized from alcohol. The yield was 1.5 g. or 38% and the compound melted at 209-210°.

Anal. Calc. for $C_6H_9N_3O \cdot \frac{1}{2}H_2SO_4$: H_2SO_4, 26.10. Found: H_2SO_4, 26.47.

Furfurylguanidine sulfate and a number of other guanidine sulfates in the following work were analyzed according to a simplified procedure. The sample of about 0.2 g. was dissolved in 250 cc. of hot water, 10 cc. of concentrated hydrochloric

(34) The furfurylamine was obtained from A. P. Hewlett.

acid added, and the solution treated with an excess of boiling one-normal barium chloride, added with stirring. The precipitate of barium sulfate was allowed to settle for about one-half hour and was then filtered, washed with hot water and weighed in the customary manner.

β-Phenylethylguanidine Sulfate, $C_6H_5C_2H_4NHC(:NH)NH_2\frac{1}{2}H_2SO_4$. The preparation of β-phenylethylguanidine from β-phenylethylamine salts and cyanamide has been reported in several patents (35), however no experimental details or properties of the compound are given. The compound was prepared in this laboratory by the action of β-phenylethylamine on methylisothiourea sulfate. To a solution of 3 g. (0.025 mole) of β-phenylethylamine in 30 cc. of 95% alcohol were added 3.5 g. (0.025 mole) of methylisothiourea sulfate and the mixture heated on the steam plate until the solid had completely dissolved. A portion of the alcohol was evaporated and the remaining solution allowed to cool. A white crystalline product separated which was filtered and the filtrate diluted with absolute alcohol, whereupon an additional amount separated. The compound was recrystallized from absolute alcohol yielding 2.5 g. or 62% of β-phenylethylguanidine sulfate melting at 175.5-177°.

(35) Chemische Fabrik auf Aktien, British Pat. 279,884, Canadian Pat. 281,121 (C.A. 22, 295 (1928)); Schering-Kahlbaum, Polish Pat. 9,367 (Chem. Zentr. II, 2604 (1929).

Anal. (36). Calc. for $C_9H_{13}N_3 \cdot \frac{1}{2}H_2SO_4$: H_2SO_4, 23.11. Found: H_2SO_4, 22.07 and 21.99.

Preparation of Alcoholic Guanidine Solution. The solution of guanidine in absolute alcohol used in the preparation of a number of the following guanidine derivatives was prepared as follows. Ten grams (0.105 mole) of finely powdered guanidine hydrochloride were added to a sodium ethylate solution prepared from 2.3 g. (0.1 atom) of sodium and 50 cc. of absolute alcohol. This mixture was allowed to stand with frequent shaking for about one hour. The precipitate of sodium chloride was filtered and the clear solution used immediately.

Guanidine Benzoate, $NH_2C(:NH)NH_2 \cdot C_6H_5COOH$. Guanidine benzoate was obtained by treating guanidine with benzoic acid. Benzoic acid was added to a solution of guanidine prepared as described above. The crystals which separated were filtered and recrystallized from alcohol. The compound melted at 222-224°. A picrate prepared from this compound was identical, as shown by a mixed melting point, with that prepared from guanidine hydrochloride.

1,3-Diphenyl-3-guanidino-propanone-1, $C_6H_5C(:O)CH_2-CH(C_6H_5)NHC(:NH)NH_2$. 1,3-diphenyl-3-guanidino-propanone-1 was

(36) Analyzed by the procedure given on p.18, This Thesis.

prepared from benzalacetophenone and guanidine. A solution of 20.8 g. (0.1 mole) of benzalacetophenone in 30 cc. of absolute alcohol was treated with a solution of 0.1 mole of guanidine in 50 cc. of alcohol prepared as previously described (37). After standing overnight the solution was evaporated on the steam plate until viscous, dissolved in hot 95% alcohol and cooled. The product separated as light tan crystals and weighed 5 g. or 20% of the theoretical. The compound was purified by recrystallization from 95% alcohol and melted at 135-136°. The picrate, crystallized from alcohol, melted at 191°.

The hydrochloride was prepared by treating an ether solution of the compound with dry hydrogen chloride gas. Upon recrystallization from absolute alcohol this product melted at 202-204°.

Anal. (38). Calc. for $C_{16}H_{18}ClN_3O$: HCl, 12.01. Found: 11.14 and 11.09.

Diacetoneguanidine, $NH_2C(:NH)NHC(CH_3)_2CH_2C(:O)CH_3$. Diacetoneguanidine was prepared by the action of guanidine on mesityl oxide according to the method of Traube and Schwarz (39). Ten grams (0.1 mole) of mesityl oxide were added to a solution of 0.1 mole of guanidine in 50 cc. of absolute alcohol prepared as previously described (37) after evaporation of the

(37) See p.20, This Thesis.
(38) Analyzed by the procedure given on p.14, This Thesis.
(39) Traube and Schwarz, Ber. 32, 3168 (1899).

alcohol the solution was heated on the steam plate at 120° for 2 to 3 hours. The mass was dissolved in hot water and the solution filtered and cooled. A light brown semi-crystalline solid separated which melted at 155-158°. The yield of this material was 9 g. or 57%. According to Traube and Schwarz (39) the pure compound melted at 163°.

Acetyldiacetoneguanidine, $CH_3C(:O)NHC(:NH)NHC(CH_3)_2CH_2$-$C(:O)CH_3$. Acetyldiacetoneguanidine was prepared according to the procedure of Traube and Schwarz (39). A mixture of 3 g. (0.02 mole) of diacetoneguanidine and 3 g. (0.03 mole) of acetic anhydride was boiled under reflux for about 1½ hours. The mixture was diluted with water, neutralized with ammonium hydroxide and cooled. The product which separated was filtered and crystallized from hot water. Only one gram or 25% of this material melting at 156-157° was obtained. This melting point agrees with that previously reported (39).

Furylacrylylguanidine Hydrochloride, $C_4H_3OCH:CHCONHC(:NH)$-$NH_2 \cdot HCl$. Furylacrylylguanidine was prepared by the action of guanidine on ethyl furylacrylate according to the method of Traube (40) for the preparation of acylguanidines. Sixteen and one-half grams (0.1 mole) of ethyl furylacrylate were added to a solution of 0.1 mole of guanidine in absolute alcohol prepared

(40) Traube, Ber. 43, 3586 (1911).

as previously described (41). After standing for two weeks the solution was diluted with several volumes of ether. The solid which separated was dissolved in dilute alcohol and the solution treated with dilute hydrochloric acid. The hydrochloride of furylacryloylguanidine precipitated and was recrystallized twice from dilute alcohol. A yield of 2 g. or 10% of product, which melted with decomposition at 251°, was obtained.

Anal. (42). Calc. for $C_8H_{10}ClN_3O_2$: Cl, 16.45. Found: Cl, 16.02 and 16.02.

Cinnamylguanidine Hydrochloride, $C_6H_5CH:CHC(:O)NHC(:NH)-NH_2 \cdot HCl$. Cinnamylguanidine was prepared by the action of guanidine on ethyl cinnamate according to the method of Traube (40) for the preparation of acylguanidines. Seventeen and six-tenths grams (0.1 mole) of ethyl cinnamate were added to a solution of 0.1 mole of guanidine in 50 cc. of absolute alcohol prepared as previously described (41). After 10 days standing, the solution was concentrated to a thick syrup by evaporation under reduced pressure. Four hundred cc. of ether were added to the mixture which was well shaken and allowed to stand until the undissolved oil had solidified. This solid was filtered, dissolved in a small amount of alcohol, diluted with water to a volume of about 150 cc. and treated with an excess of dilute hydrochloric acid. A white solid precipitated which was filtered, washed with water and dried. A yield of 5 g. or 22%

(41) See p. 20, This Thesis.
(42) Analyzed by the procedure given on p. 14, This Thesis.

of crude cinnamylguanidine hydrochloride was obtained. The compound was purified by crystallization from 50% alcohol and melted at 260-261°.

Anal. (43). Calc. for $C_{10}H_{12}ClN_3O$: Cl, 15.72. Found: Cl, 15.43 and 15.48.

Formylguanidine, $NH_2C(NH)NHC(:O)H$. Formylguanidine was obtained from guanidine and ethyl formate by the method of Traube (40). Seven and four-tenths grams (0.1 mole) of ethyl formate were added to a solution of 0.1 mole of guanidine in 50 cc. of absolute alcohol prepared as previously described (44). Considerable heat was evolved following the addition of the ester and a white crystalline precipitate immediately began to separate. After standing overnight the crystals were filtered and washed with alcohol. The yield was 3.5 g. or 40% of material melting at 182°. The melting point reported by Traube (40) was 178°.

Dibenzoylguanidine, $C_6H_5CONHC(:NH)NHCOC_6H_5$. Dibenzoylguanidine was prepared by the Schotten-Baumann reaction from guanidine and benzoyl chloride according to the method of Korndorfer (45). Five grams (0.052 mole) of guanidine hydrochloride were dissolved in 50 cc. of 10% sodium hydroxide and

(43) Analyzed by the procedure given on p. 14, This Thesis.
(44) See p. 20, This Thesis.
(45) Korndorfer, Arch. Pharm. 241, 478 (1903).

the solution treated with 14 g. (0.1 mole) of benzoyl chloride added in several portions, followed by vigorous shaking. The white solid which separated was filtered, washed with water and purified by crystallization from absolute alcohol. The yield was 3 g. or 23% of product melting at 214-215°, which is in agreement with the melting point reported by Korndorfer (45).

Anhydro-diacetylguanidine, $CH_3C(NH)(N)C{:}NC(:O)CH_3$. Anhydro-diacetylguanidine was prepared from guanidine carbonate and acetic anhydride by the method of Korndorfer (45). A mixture of 5 g. (0.055 mole) of guanidine carbonate and 25 g. (0.25 mole) of acetic anhydride was heated under reflux for one hour. A violent evolution of carbon dioxide took place when the mixture was first warmed. The solid was filtered from excess acetic anhydride and recrystallized from water three times. The yield was 1.2 g. or 17% of material melting at 209°. The melting point reported by Korndorfer (45) was 210-212°.

Dicarbethoxyguanidine, $C_2H_5OC(:O)NHC(:NH)NHC(:O)OC_2H_5$. Dicarbethoxyguanidine was prepared by the action of ethyl chlorocarbonate on guanidine according to the method of Basterfield and Paynter (46). Twenty-three grams (0.15 mole) of barium hydroxide were dissolved in 200 cc. of water and to the solution were added 30 g. (0.33 mole) of guanidine carbonate.

(46) Basterfield and Paynter, J. Am. Chem. Soc. 48, 2176 (1926).

The barium carbonate was filtered and the solution concentrated under reduced pressure at 40° until water no longer distilled. Absolute alcohol was added to the residue and the solution filtered from the excess guanidine carbonate. To the solution was then added 10 g. (0.1 mole) of ethyl chlorocarbonate with shaking and cooling. After standing overnight, the white solid which had separated was filtered and washed with alcohol. A yield of 3.5 g. or 35% of this product was obtained. The compound was purified by crystallization from alcohol and was obtained as white needles, melting at 164.5-165°, which is in agreement with the melting point reported by Basterfield and Paynter (46).

β-Carboxylpropionylguanidine, $NH_2C(:NH)NHC(:O)C_2H_4COOH$. β-Carboxylpropionylguanidine was prepared from guanidine and succinic ester according to the method of Michael (47). A solution of sodium ethylate was prepared by dissolving 2.5 g. (0.11 mole) of sodium in 60 cc. of absolute alcohol and to it were added 12 g. (0.1 mole) of guanidine thiocyanate and 17.4 g. (0.1 mole) of ethyl succinate. After several days standing, a precipitate had separated which was filtered and washed with a little alcohol. This material was dissolved in hot, very dilute, hydrochloric acid. Upon cooling, a white crystalline product separated. Only one gram or 7% of this product melting at 182° was obtained. The melting point given by Michael (47)

(47) Michael, J. prakt. Chem. (2) 49, 39 (1894).

was 184-185°.

γ-Bromopropylphthalimide, $C_6H_4(CO)_2NC_3H_6Br$. γ-Bromopropylphthalimide was prepared from trimethylene bromide and phthalimide according to the simplified procedure of Ing and Manske (48) for the preparation of alkyl phthalimides. A mixture of 150 g. (1.02 moles) of phthalimide, 75 g. (0.54 mole) of potassium carbonate and 500 g. (2.50 moles) of trimethylene bromide was placed in a flask fitted with a mechanical stirrer and a reflux condenser and, with stirring, was gradually heated on an oil bath. A vigorous reaction took place when the temperature of the bath had reached about 150°. The mass became very viscous and was stirred with difficulty, but upon further heating the mixture liquified. The reaction was continued at the boiling point of the mixture for a total period of about three hours. Upon steam distillation of the mixture 252 g. of trimethylene bromide were recovered. The water-oil mixture remaining after steam distillation was cooled under the tap with vigorous shaking, whereupon the oil solidified and was filtered and air dried. This crude material was extracted with 400 cc. of hot carbon disulfide to separate the insoluble diphthalimidopropane. The carbon disulfide solution was almost completely evaporated and upon cooling the bromopropylphthalimide crystallized and was filtered, washed with

(48) Ing and Manske, J. Chem. Soc. 2348 (1926).

carbon disulfide and air dried. The yield of this product was 126 g. or 46%. Crystallized from petroleum ether (B.P. 40-60°) the compound melted at 72-73°, which is in agreement with the melting point reported by Ing and Manske (48). As a by-product of this reaction 31.5 g. of crude diphthalimidopropane were obtained.

γ-Aminopropyl n-Butyl Sulfide, $NH_2C_3H_6SC_4H_9$. γ-Aminopropyl n-butyl sulfide was prepared by a modification of the method used by Schneider (49) in the preparation of γ-aminopropyl methyl sulfide. Eighteen grams (0.2 mole) of butyl mercaptan were dissolved in a sodium ethylate solution prepared from 4.6 g. (0.2 mole) of sodium and 200 cc. of absolute alcohol. To this solution were added 50 g. (0.2 mole) of powdered _-bromopropylphthalimide which dissolved with the evolution of heat accompanied by the precipitation of sodium bromide. After standing for a short time the solution was boiled under reflux for 15 minutes. Fifty cc. of water and 30 g. of sodium hydroxide were added and the mixture steam-distilled. By this treatment the alcohol was removed and the phthalimido compound was hydrolyzed to the sodium salt of the corresponding γ-phthalamidopropyl n-butyl sulfide. This solution was diluted to about 500 cc., filtered, cooled and made slightly acid with acetic acid. The oil which precipitated was separated and added to 400 cc. of

(49) Schneider, Ann. 375, 245 (1910).

hydrochloric acid prepared by diluting 250 cc. of concentrated acid. After boiling for two hours the mixture was cooled and filtered from the phthalic acid which had separated. The filtrate was concentrated to a volume of 250 cc., cooled and made alkaline by the addition of solid sodium hydroxide. The amine was extracted with ether and the ether solution dried over solid potassium hydroxide. The ether was removed on the steam bath and the residue distilled under reduced pressure. Eleven grams or 40% of γ-aminopropyl n-butyl sulfide boiling at 120°/25 mm. were obtained.

Anal. (By Carius). Calc. for $C_7H_{17}NS$: S, 21.77. Found: S, 21.41.

γ-Guanidinopropyl n-Butyl Sulfide. $NH_2C(:NH)NHC_3H_6SC_4H_9$. γ-Guanidinopropyl n-butyl sulfide was prepared by the action of γ-aminopropyl n-butyl sulfide on methylisothiourea sulfate. To a solution of 7 g. (0.05 mole) of methylisothiourea sulfate in 20 cc. of hot water were added 7.3 g. (0.05 mole) of γ-aminopropyl n-butyl sulfide. After the reaction had subsided the solution was boiled for about one-half hour and then cooled. An oil separated which crystallized to a sticky mass of white crystals upon standing in a vacuum desiccator over sulfuric acid. The yield of γ-guanidinopropyl n-butyl sulfide sulfate was 5 g. or 42%. For analysis the picrate was prepared. The picrate melted at 115-117° on crystallization from alcohol.

Anal. (50). Calc. for $C_{14}H_{22}N_6O_7S$: S, 7.65. Found: S, 7.35.

β-Phenylethyl Mercaptan. $C_6H_5C_2H_4SH$. β-Phenylethyl mercaptan has previously been prepared by the action of alkali on β-phenylethyldithiourethane (51). In this laboratory the compound was obtained by the action of alcoholic potassium sulfhydrate on β-phenylethyl bromide. Thirty-seven grams (0.2 mole) of β-phenylethyl bromide were added to 100 cc. of 3-normal alcoholic potassium sulfhydrate prepared by saturating alcoholic potassium hydroxide with hydrogen sulfide. This solution was heated on the steam bath for one hour, cooled and poured into 750 cc. of water. The oily layer was separated, dried over anhydrous sodium carbonate and distilled under reduced pressure, using a modified Claisen flask. The yield of mercaptan was 14.3 g. or 50% of product boiling at 82-83°/7 mm. According to von Braun (51) the boiling point was 105°/23 mm.

γ-Aminopropyl β-Phenylethyl Sulfide. $NH_2C_3H_6SC_2H_4C_6H_5$. γ-Aminopropyl β-phenylethyl sulfide was prepared by a method similar to that used in the preparation of γ-aminopropyl n-butyl sulfide (52). Fourteen grams (0.1 mole) of β-phenyl-

(50) Analyzed by the procedure given on p. 18, This Thesis.
(51) von Braun, Ber. 45, 1564 (1912).
(52) See p. 28, This Thesis.

ethyl mercaptan were added to a sodium ethylate solution prepared from 2.3 g. (0.1 mole) of sodium and 100 cc. of absolute alcohol. To this solution were added 25 g. (0.1 mole) of powdered γ-bromopropylphthalimide which dissolved with the evolution of heat accompanied by the precipitation of sodium bromide. The mixture was heated on the steam bath for about 15 minutes, cooled, treated with a little water and 2.5 g. of sodium hydroxide and steam distilled. This treatment removed the alcohol and at the same time hydrolyzed the phthalimido compound to the corresponding phthalamido compound . The solution was then diluted to 400 cc., cooled and just neutralized with acetic acid. After standing for a short time the water was decanted from the oil which had separated and 250 cc. of concentrated hydrochloric acid and sufficient water to make a total volume of about 400 cc. were added. The mixture was boiled under reflux for 3 hours and decanted from undissolved oil. After cooling, the phthalic acid which had separated was filtered and the filtrate made alkaline by the addition of solid sodium hydroxide. The oil which separated was extracted with ether and the solution dried first with solid potassium hydroxide and then with anhydrous sodium sulfate. The ether was removed on the steam bath and the amine distilled under reduced pressure. A yield of 6.7 g. or 34% of γ-aminopropyl β-phenylethyl sulfide boiling at 147-150°/7 mm. was obtained.

Anal. (By Carius). Calc. for $C_{11}H_{17}NS$: S, 16.41. Found: S, 17.33 and 15.27.

Sufficient material was not available for repetition of the analysis.

γ-Guanidinopropyl β-Phenylethyl Sulfide, $NH_2C(:NH)NHC_3H_6SC_2H_4C_6H_5$. γ-Guanidinopropyl β-phenylethyl sulfide was prepared by the action of the corresponding amine on methylisothiourea sulfate. A solution of 4.6 g. (0.033 mole) of methylisothiourea sulfate in 20 cc. of hot water was treated with 6 g. (0.031 mole) of γ-aminopropyl β-phenylethyl sulfide. After the vigorous reaction had subsided the solution was boiled under reflux for about 45 minutes and filtered hot. The oil which precipitated upon cooling was separated and allowed to stand in a vacuum desiccator over sulfuric acid until it had crystallized. Eight grams or 90% of crude product were obtained. The γ-guanidinopropyl β-phenylethyl sulfide sulfate was purified by crystallization from water and then from alcohol-ether. The compound softened at about 120° but showed no definite melting point.

Anal. (53). Calc. for $C_{12}H_{19}N_3S \cdot \frac{1}{2}H_2SO_4$: SO_4, 16.78. Found: SO_4, 17.17 and 17.35.

(53) Analyzed by the procedure given on p. 18, This Thesis.

Cyclohexyl Mercaptan, $C_6H_{11}SH$. Cyclohexyl mercaptan was prepared from cyclohexylmagnesiumbromide and sulfur by a modification of the method of Mailhe and Murat (54). Twelve and one-half grams (0.5 atom) of magnesium shavings, 100 cc. of dry ether and a crystal of iodine were placed in a three-necked flask fitted with a stirrer, reflux condenser and dropping funnel. A solution of 81 g. (0.5 mole) of cyclohexyl bromide in 100 cc. of dry ether was placed in the dropping funnel, a small amount added to start the reaction, and the addition continued, with stirring, at such a rate as to keep the ether refluxing gently. After all had been added the refluxing was continued for about 30 minutes. The flask was then well cooled in an ice bath and, with stirring, 16 g. (0.5 mole) of powdered sulfur added in small portions. The mixture was refluxed for one hour after the sulfur had been added and was then hydrolyzed by pouring onto a mixture of ice and 50 cc. of concentrated hydrochloric acid. Without separating the ether from the water layer the mixture was placed in a three-necked flask fitted with a reflux condenser and stirrer. Thirty-five grams of powdered zinc and 100 cc. of concentrated hydrochloric acid were added in small portions, with stirring, over a period of three hours. After standing for 36 hours the ether layer was separated and the water solution extracted once with ether. The combined

(54) Mailhe and Murat, Bull. soc. chim. (4) 7, 288 (1910).

ether solutions were extracted with 200 cc. of 10% sodium hydroxide in three portions and the alkaline solution acidified with hydrochloric acid. The mercaptan was taken up with ether and dried over anhydrous sodium sulfate. After removing the ether on the steam bath, the product was distilled at atmospheric pressure yielding 23.4 grams or 40% of cyclohexyl mercaptan boiling at 155°. Mailhe and Murat (54), using cyclohexylmagnesium-chloride, obtained a yield of 26%.

γ-Aminopropyl Cyclohexyl Sulfide, $NH_2C_3H_6SC_6H_{11}$. γ-Aminopropyl cyclohexyl sulfide was prepared by the same procedure as used in the preparation of γ-aminopropyl butyl sulfide (55). Twelve grams (0.1 mole) of cyclohexyl mercaptan were dissolved in a sodium ethylate solution prepared from 2.3 g. (0.1 mole) of sodium and 100 cc. of absolute alcohol. Twenty-five grams (0.1 mole) of γ-bromopropylphthalimide were added to the solution which was allowed to stand for several hours and was then heated on the steam plate for a short time. About 25 g. of solid sodium hydroxide and a little water were added and the solution steam distilled for a period of about 2 hours. The aqueous solution was neutralized with acetic acid and allowed to stand overnight. The water was decanted from the heavy oil which separated and a mixture of 200 cc. of concentrated hydro-

(55) See p. 28, This Thesis.

chloric acid and 50 cc. of water added. After boiling for two hours the hot solution was filtered from undissolved oil and cooled. The phthalic acid which separated was filtered and the filtrate made alkaline by the addition of solid sodium hydroxide. The amine was taken up in ether and dried first with solid sodium hydroxide and then with anhydrous sodium sulfate. After removing the ether on the steam bath the γ-aminopropyl cyclohexyl sulfide was distilled under reduced pressure. Six grams or 35% of product boiling at 135°/15 mm. were obtained. Sufficient material was not obtained for both purification for analysis and use in further syntheses, therefore the compound was not analyzed.

γ-Guanidinopropyl Cyclohexyl Sulfide, $NH_2C(:NH)NHC_3H_6SC_6H_{11}$. γ-Guanidinopropyl cyclohexyl sulfide was prepared by the action of the corresponding amine on methylisothiourea sulfate. To a solution of 3 g. (0.017 mole) of γ-aminopropyl cyclohexyl sulfide in 30 cc. of 95% alcohol was added 2.3 g. (0.017 mole) of methylisothiourea sulfate. The mixture was allowed to stand on the steam plate until the methylisothiourea sulfate had completely dissolved (about 3 hours). After standing overnight several volumes of dry ether were added. An oil separated which was washed with ether, dissolved in absolute alcohol and again precipitated by the addition of ether. The γ-guanidinopropyl cyclohexyl sulfide sulfate separated as an oil which

soon crystallized and was filtered and dried in vacuum over sulfuric acid. The yield was 4 g. or 91% of material which softened about 150° but did not melt definitely. This material was analyzed without further purification.

Anal. (56). Calc. for $C_{10}H_{21}N_3S \cdot \frac{1}{2}H_2SO_4$: SO_4, 18.14. Found: SO_4, 19.35 and 19.24.

Malonylguanidine, $CH_2\begin{matrix}CONH\\CONH\end{matrix}C{:}NH$. Malonylguanidine was prepared from guanidine and malonic ester according to the method of Michael (57). Sixteen grams (0.1 mole) of malonic ester were dissolved in a sodium ethylate solution prepared from 2.3 g. (0.1 mole) of sodium and 50 cc. of absolute alcohol. To this solution was added a mixture of 12.1 g. (0.1 mole) of guanidine carbonate in a sodium ethylate solution prepared as above. The resulting mixture was heated on the steam bath for one hour. Water was added to the pasty mass which was then acidified with concentrated hydrochloric acid. After boiling for a short time the insoluble malonylguanidine was filtered and dried, yielding 8 g. or 60% of white powder which did not melt.

Thiobarbituric Acid, $CH_2\begin{matrix}CONH\\CONH\end{matrix}C{:}S$. Thiobarbituric acid

(56) Analyzed by the procedure given on p.18, This Thesis.
(57) Michael, J. prakt. Chem. (2) 49, 35 (1894).

was prepared from thiourea and malonic ester according to the method of Dox and Plaisance (58). Two and three-tenths grams (0.1 mole) of sodium were dissolved in 50 cc. of absolute alcohol and the hot solution treated with 16 g. (0.1 mole) of ethyl malonate and 7.6 g. (0.1 mole) of thoroughly dried thiourea. The mixture was heated on the steam bath for ten hours. Eighty cc. of water and 10 cc. of concentrated hydrochloric acid were added to the white pasty mass and the mixture warmed until all had dissolved. This solution was filtered and allowed to cool. The crystalline product was filtered and washed with water. The yield was 8.3 g. or 58%.

n-Butylthiobarbituric Acid, $C_4H_9CH\begin{matrix}CONH\\ \diagup\quad\diagdown\\ CONH\end{matrix}C{:}S$. n-Butylthiobarbituric acid was prepared from n-butylmalonic ester and thiourea, the method being similar to that given by Dox and Plaisance (58) for the preparation of thiobarbituric acid. Two and three-tenths grams (0.1 mole) of sodium were dissolved in 50 cc. of absolute alcohol and to the hot solution was added 21.6 g. (0.1 mole) n-butylmalonic ester and 7.6 g. (0.1 mole) of thiourea. A vigorous reaction took place with the formation of a white pasty mass. After heating for 2 hours on the steam bath the alcohol was partially evaporated and the mass treated with 50 cc. of water and 10 cc. of concentrated hydrochloric acid.

(58) Dox and Plaisance, J. Am. Chem. Soc. 38, 2159 (1916).

The white solid which separated upon standing overnight was filtered and crystallized from water. A yield of 8.5 g. or 42% of n-butylthiobarbituric acid melting at 151.5-152° was obtained.

Anal. (By Carius). Calc. for $C_8H_{12}N_2O_2S$: S, 16.00. Found: S, 16.61 and 16.79.

Iminomethyluracil, $NH{:}C\begin{matrix}NHC(CH_3)\\ NHC(:O)\end{matrix}\!\!>CH$. Iminomethyluracil was prepared from guanidine and acetoacetic ester according to the directions of Jaeger (59). A mixture of 12 g. (0.13 mole) of guanidine carbonate, 18 g. (0.15 mole) of ethyl acetoacetate and 40 cc. of absolute alcohol was heated under reflux on the steam bath for four hours. The cooled mixture was filtered and the product washed first with alcohol and then with water. The yield of this material was 15 g. or 92%. The compound was purified by crystallization from hot water and melted with decomposition at 295-296°. The melting point reported by Jaeger (59) was 270°.

N'-Allyl-N"-guanyl-thiourea, $NH_2C(:NH)NHC(:S)NHCH_2CH{:}CH_2$. N'-Allyl-N"-guanyl-thiourea was obtained by the action of allyl isothiocyanate on guanidine as described by Slotta, Tschesche and Dressler (60). A solution of sodium ethylate was prepared

(59) Jaeger, Ann. 262, 365 (1891).
(60) Slotta, Tschesche and Dressler, Ber. 63, 214 (1930).

by dissolving 2.5 g. (0.11 mole) of sodium in 65 cc. of absolute alcohol. To this solution were then added 12 g. (0.1 mole) of guanidine thiocyanate and 10 g. (0.1 mole) of allyl isothiocyanate and the mixture boiled under reflux for about one hour. The alcohol was removed under reduced pressure, 100 cc. of water added to the residue and carbon dioxide passed into the solution until the precipitate no longer formed. The solid was filtered off, washed with alcohol and then ether. Only 1.5 g. or 10% of this material melting at 78-80° were obtained. The melting point reported (60) for N'-allyl-N"-guanylthiourea carbonate was 95°.

Dipiperidyl Disulfide, $C_5H_{10}NSSC_5H_{10}N$. Dipiperidyl disulfide was prepared from piperidine and sulfur monochloride according to the method of Michaels and Luxembourg (61). A solution of 17 g. (0.2 mole) of piperidine in 100 cc. of dry ether was treated with a solution of 7 g. (0.05 mole) of sulfur monochloride in 30 cc. of ether, added slowly thru a dropping funnel. The mixture was cooled with an ice-salt bath and stirred during the addition. A precipitate of piperidine hydrochloride separated, which was filtered and the filtrate evaporated. The remaining oil was recrystallized from absolute alcohol. The yield of purified material melting at 58-59° was 2 g. or 17%. According to Michaels and Luxembourg (61) the compound melted at 64°.

(61) Michaels and Luxembourg, Ber. 28, 165 (1895).

Dipiperidyl Sulfone, $(C_5H_{10}N)_2SO_2$. Dipiperidyl sulfone was obtained in low yields by the action of sulfuryl chloride on piperidine according to the method of Tohl and Framm (62). A solution of 8.5 g. (0.1 mole) of piperidine in 100 cc. of dry ether was well cooled in an ice-salt mixture and treated with a solution of 3.5 g. (0.025 mole) of sulfuryl chloride in 40 cc. of dry ether added through a dropping funnel. The mixture was stirred during the addition and for about ten minutes after all the sulfuryl chloride solution had been added. A precipitate of piperidine hydrochloride separated which was filtered. The filtrate was evaporated and the remaining oil dissolved in alcohol. Upon diluting with water a crystalline product separated, which was recrystallized from a water-alcohol mixture. Only one gram or 17% of purified material melting at 88-89° was obtained. The melting point previously reported (62) was 93°.

Diethylaminomethyl Ethyl Sulfide, $(C_2H_5)_2NCH_2SC_2H_5$. Diethylaminomethyl ethyl sulfide was prepared from diethylamine, formalin and ethyl mercaptan by the method of McLeod and Robinson (63). Seven grams (0.1 mole) of diethylamine were gradually added to 10.6 g. (0.12 mole) of 35% formalin with cooling under the tap. To the solution were added 7.7 g. (0.12 mole) of ethyl mercaptan and the solution was then saturated with potassium carbonate. After standing for 1½ hours the non-aqueous layer

(62) Tohl and Framm, Ber. 27, 2012 (1894).
(63) McLeod and Robinson, J. Chem. Soc. 119, 1470 (1921).

was separated and dried over anhydrous potassium carbonate. The product was fractionated at atmospheric pressure using an air condenser. A yield of 9 g. or 56% of material boiling at 175-178° was obtained. The reported boiling point was 174-175° (63).

Benzimino Phenylthioether Hydrochloride, $C_6H_5C(:NH)S-C_6H_5 \cdot HCl$. Benzimino phenylthioether was prepared by the action of hydrogen chloride gas on a mixture of benzonitrile and thiophenol according to the procedure of Autenrieth and Bruning (64). A mixture of 10 g. (0.1 mole) of benzonitrile and 11 g. (0.1 mole) of thiophenol was treated with dry hydrogen chloride gas until the increase in weight amounted to 4 g. (0.11 mole). The flask was fitted with a calcium chloride tube and allowed to stand overnight. The crystalline mass was filtered, washed with ether and dried in vacuum over calcium chloride. A yield of 12 g. or 50% was obtained. The product was unstable and gradually decomposed upon standing. No melting point was recorded; according to Autenrieth and Bruning (64) the compound melted at 178°.

Diformamidine Disulfide Hydrochloride, $NH_2C(:NH)SSC(:NH)-NH_2 \cdot 2HCl$. Diformamidine disulfide hydrochloride was prepared by the action of sulfuryl chloride on thiourea according to the

(64) Autenrieth and Bruning, Ber. 36, 3464 (1903).

method of Werner (65). Seven and six-tenths grams (0.1 mole) of dry powdered thiourea and 100 cc. of absolute alcohol were cooled with ice and the mixture treated with 13.5 g. (0.1 mole) of sulfuryl chloride added slowly through a dropping funnel. The mixture was shaken constantly during the addition. After standing for a short time the white crystalline precipitate was filtered, washed with a little alcohol and dried in a desiccator. The yield was 7.5 g. or 68% of product melting at 157-159°. The melting point reported by Werner (65) was 155°.

Propionamidine Hydrochloride, $C_2H_5C(:NH)NH_2 \cdot HCl$. Propionamidine hydrochloride was prepared according to the directions of Pinner and Klein (66). A solution of 10 g. (0.2 mole) of propionitrile in 9.2 g. (0.2 mole) of absolute alcohol was treated with dry hydrogen chloride gas until the gain in weight amounted to 16 g. (0.44 mole). The flask was fitted with a calcium chloride tube and allowed to stand. After two days, crystallization had not taken place. A stream of dry air was therefore drawn through the liquid whereupon it soon crystallized. The iminoether hydrochloride thus obtained weighed 16 g. (A portion of this product was spilled).

The 16 g. (0.16 mole) of iminoether hydrochloride were finely ground, suspended in 10 cc. of absolute alcohol and

(65) Werner, J. Chem. Soc.; 101, 2171 (1912).
(66) Pinner and Klein, Ber. 11, 1484 (1878).

treated with 15 cc. of 16% alcoholic ammonia. This mixture was shaken well and allowed to stand overnight. It was then filtered to remove the ammonium chloride and the filtrate evaporated. The cooled residue solidified to a mass of white needles. A yield of 5.5 g. or 36% of propionamidine hydrochloride melting at 130-131° was obtained. Pinner and Klein (66) reported a melting point of 133°.

Physiological Results (67).

The physiological tests of the compounds herein described were made by intravenous injection into starved rabbits. In most cases three doses, large, intermediate and small, were given to three different animals. Blood sugar determinations were made just prior to the injection and at definite intervals thereafter. The results of these tests will be briefly summarized.

The following compounds had no effect on the blood sugar: p-carboxylphenylguanidine, diacetoneguanidine, furylacrylylguanidine hydrochloride, cinnamylguanidine hydrochloride, formylguanidine, dibenzoylguanidine, anhydro-diacetylguanidine, γ-guanidinopropyl butyl sulfide sulfate, n-butylthiobarbituric

(67) The compounds were tested for hypoglucemic action in the laboratories of Parke-Davis and Company.

acid, iminomethyluracil, N'-allyl-N"-guanyl-thiourea, dipiperidyl disulfide, dipiperidyl sulfone and diethylaminomethyl ethyl sulfide.

The following compounds increased the blood sugar: 1,3-diphenyl-3-guanidino-propanone-1 and hydrochloride, β-carboxylpropionylguanidine, γ-guanidino-propyl cyclohexyl sulfide sulfate, malonylguanidine, thiobarbituric acid, benzimino phenylthioether hydrochloride, diformamidine disulfide hydrochloride and propionamidine hydrochloride.

Phenylguanidine in large doses lowered the blood sugar but was toxic; in small doses it was ineffective. p-Bromophenylguanidine nitrate showed some hypoglucemic action but was toxic. Benzylguanidine nitrate in large doses lowered the blood sugar considerably but was toxic; in small doses it was ineffective. Furfurylguanidine sulfate was active to some extent even in small doses without toxic effect; the activity, however, did not approach that of synthalin or insulin. β-Phenylethylguanidine sulfate was slightly active but toxic. Guanidine benzoate in a very large dose produced a marked hypoglucemic action but was very toxic; in small doses it was ineffective. Acetyldiacetoneguanidine reduced the blood sugar slightly. Dicarbethoxyguanidine was inconsistent in its action on the blood sugar; it produced a marked hyperglucemia in one case and a hypoglucemia in another. γ-Guanidinopropyl β-phenylethyl sulfide sulfate showed a slight hypoglucemic action but was toxic.

SUMMARY AND CONCLUSIONS

A series of guanidine derivatives and related compounds were prepared in an effort to find a substance that would more satisfactorily serve as a substitute for insulin than does synthalin.

The effect of these compounds on the blood sugar of rabbits was determined.

Compounds which had no effect on the blood sugar were: p-carboxylphenylguanidine, diacetoneguanidine, furylacrylylguanidine hydrochloride, cinnamylguanidine hydrochloride, formylguanidine, dibenzoylguanidine, anhydro-diacetylguanidine, γ-guanidinopropyl butyl sulfide sulfate, n-butylthiobarbituric acid, iminomethyluracil, N'-allyl-N"-guanyl-thiourea, dipiperidyl disulfide, dipiperidyl sulfone and diethylaminomethyl ethyl sulfide.

Compounds which raised the blood sugar were: 1,3-diphenyl-3-guanidino-propanone-1 and hydrochloride, β-carboxylpropionylguanidine, γ-guanidino-propyl cyclohexyl sulfide sulfate, malonylguanidine, thiobarbituric acid, benzimino phenylthioether hydrochloride, diformamidine disulfide hydrochloride and propionamidine hydrochloride.

Compounds which lowered the blood sugar were: phenylguanidine, p-bromophenylguanidine nitrate, benzylguanidine nitrate, furfurylguanidine sulfate, β-phenylethylguanidine sulfate,

guanidine benzoate, acetyldiacetoneguanidine, dicarbethoxy-guanidine, and γ-guanidinopropyl β-phenylethyl sulfide sulfate.

While certain of the compounds tested showed hypoglucemic action, this action in no case approached that of synthalin or insulin. The action was also, in most cases, accompanied by toxic effects which rendered impossible the use of the compound as an insulin substitute.

PART II

CYSTINYL PEPTIDES

INTRODUCTION

Insulin, the hormone which controls carbohydrate metabolism in the animal body, was first extracted from the pancreas by Banting and Best (1) in 1922. Three years later Abel and co-workers (2) succeeded in obtaining the hormone in the form of minute crystals which they believed was the pure substance. The announcement of the preparation of crystalline insulin was followed by a series of studies by these workers on the physical, chemical and physiological properties of the pure hormone (3) - (13).

Purified insulin as first prepared by Abel and co-workers (3) (4) was a dimorphous, optically active, crystalline compound

(1) Banting and Best, J. Lab. Clin. Med. 7, 251 (1922).
(2) Abel and Geiling, J. Pharmacol. 25, 423 (1925); Abel, Geiling, Allee and Raymond, Science, 62, 169 (1925).
(3) Abel, Proc. Nat. Acad. Sci. 12, 132 (1926).
(4) Abel, Geiling, Rouiller, Bell and Wintersteiner, J. Pharmacol. 31, 65 (1927).
(5) du Vigneaud, Jensen and Wintersteiner, J. Pharmacol. 32, 367 (1928).
(6) Jensen, Wintersteiner and du Vigneaud, J. Pharmacol. 32, 387 (1928).
(7) Wintersteiner, du Vigneaud and Jensen, J. Pharmacol. 32, 397 (1928).
(8) du Vigneaud, Geiling and Eddy, J. Pharmacol. 33, 497 (1928).
(9) Jensen and Geiling, J. Pharmacol. 33, 511 (1928).
(10) Jensen, Wintersteiner and Geiling, J. Pharmacol. 36, 115 (1929).
(11) Jensen and De Lawder, J. Biol. Chem. 87, 701 (1930).
(12) Geiling and De Lawder, J. Pharmacol. 39, 369 (1930).
(13) Jensen and De Lawder, Z. physiol. Chem. 190, 262 (1930).

melting at 233° and possessing a physiological activity of 40 international units per milligram. This value for the activity has since been shown to be high. Jensen, Wintersteiner and Geiling (10) and Harington and co-workers (14) placed the activity of crystalline insulin at approximately 24 international units per milligram.

Abel and co-workers (4) considered the possibility that the activity of their product was due to the adsorption of some very powerful substance, but they believed this unlikely since upon recrystallization the product contained almost all of the potency. Dingemanse (15), however, claimed to have succeeded in preparing a product of extremely high activity (140 to 180 units) by adsorption on charcoal followed by extraction with phenol. du Vigneaud, Geiling and Eddy (8) were unable to obtain a product, by adsorption on charcoal, which was more active than the original. Jensen and De Lawder using the same charcoal and insulin and the same procedure as Dingemanse were unable to substantiate her claim.

In an attempt to gain an insight into the active group of insulin, considerable work has been done in the study of the chemical behavior of insulin. Dingemanse (16) showed that the

(14) Harington, Scott, Culhane, Marks and Trevan, Biochem. J. 23, 384 (1929).
(15) Dingemanse, Arch. exptl. Path. Pharmakol. 128, 44 (1928).
(16) Dingemanse, Biochem. Z. 163, 422 (1925).

activity of insulin was destroyed by acetylation, benzoylation and long heating with hydrochloric acid. Scott (17) inactivated insulin by benzoylation and by treatment with carbon disulfide, nascent hydrogen, sulfur dioxide, sodium persulfide, nitrous acid and formaldehyde. In no case was he able to restore the activity by acid hydrolysis or reduction.

Blatherwick and co-workers (18) produced inactivation by treatment with alkaline diazocompounds, phenylhydrazine, ultraviolet light, heat, trypsin, iodine and nitrous acid. They found that insulin which had been inactivated by hydrogen sulfide could not be restored by treatment with air or oxygen and that benzoylation by the Schotten-Baumann method produced complete inactivation. They also stated that Scott had informed them by private communication of his success in restoring the activity of benzoylated insulin by treatment with dilute hydrochloric acid. He was, however, unable to repeat this work.

In a study on the acetylation of crystalline insulin Jensen and Geiling (9) prepared an acetyl insulin with an activity of 8 units per milligram. This material was deacetylated to yield a product with an activity of 25 units per milligram. These authors suggested that the acetylation may take place on a hydroxyl, amino or imino group.

(17) Scott, J. Biol. Chem. 65, 601 (1925).
(18) Blatherwick, Bischoff, Maxwell, Berger and Sahyun, J. Biol. Chem. 72, 57 (1927).

Bischoff and Sahyun (19) found that treatment of insulin with cold concentrated sulfuric acid or sulfuric acid solutions of formalin, sodium cyanide, methylsulfate or sodium cyanide produced a partially inactivated product. In some cases this product gave no biuret test.

Freudenberg, Dirscherl and Eyer (20) by deacetylation of acetylated insulin obtained a product which was more stable toward dilute alkali than insulin. These workers also found that insulin was inactivated by formaldehyde; the activity was partially restored by treatment with dilute hydrochloric acid. They also noted a variation in the optical activity of insulin upon standing with sodium hydroxide, ammonia, and zinc and hydrochloric acid. They suggested that a relation existed between physiological activity and optical activity.

Carr and co-workers (21) found that insulin was inactivated by treatment with mixtures of strong acids and primary or secondary alcohols. The product obtained by treatment with ethyl alcohol and hydrochloric acid was completely reactivated by treatment with dilute sodium hydroxide.

Jensen and De Lawder (13) found that the inactivation of insulin by means of alkali was accompanied by the splitting out of ammonia. They believed there was a relation between loss of

(19) Bischoff and Sahyun, J. Biol. Chem. 81, 167 (1929).
(20) Freudenberg, Dirscherl and Eyer, Naturwissenschaften 17, 603 (1929).
(21) Carr, Culhane, Fuller and Underhill, Biochem. J. 23, 1010 (1929).

activity and loss of ammonia. These workers prepared a benzal derivative of insulin by treatment with benzaldehyde and dilute alkali. This product was inactive and the activity could not be restored. Jensen and De Lawder were unable to restore the activity to benzoylated insulin by treatment with either acid or alkali. They found the activity of acetylated insulin was higher when the acetylation was carried out at low temperatures. When deacetylated with sodium hydroxide only part of the activity was regenerated and the product was not the same as insulin.

Freudenberg, Dirscherl and Eyer (22) made a study of a number of reactions of insulin. Inactivation by dilute alkali was accompanied by splitting out of ammonia and a marked change in optical activity. These authors also believed there was a relation between activity and loss of ammonia. Methylation with diazomethane produced an inactive product. Reduction with zinc and hydrochloric acid produced inactivation accompanied by a fall in optical rotation. The activity was not restored by oxidation. Insulin, inactivated by formaldehyde, was partially reactivated by treatment with dilute hydrochloric acid. Acetylation with pyridine and acetic anhydride produced an inactive product which was partially restored by dilute sodium hydroxide. It was suggested that the regenerated product was a partially

(22) Freudenberg, Dirscherl and Eyer, Z. physiol. Chem. 187, 89 (1930).

deacetylated insulin of less activity than free insulin. The hydrochloride of insulin prepared by treatment with dilute hydrochloric acid contained most of the activity of the original. Freudenberg, Dirscherl and Eyer concluded from their study that the active group of insulin contains an acid-stable, alkali-sensitive amino group which is in the neighborhood of at least one asymmetric carbon atom and which can be easily hydrolyzed.

The chemical property of insulin to which much attention has been directed is the extreme lability of the sulfur in the insulin molecule. Abel and co-workers (2) first noted that upon boiling insulin with dilute sodium carbonate the sulfur linkage was so altered that upon treatment with dilute acid hydrogen sulfide was evolved. These authors stated that this labile or "sodium carbonate" sulfur appeared to be proportional to the activity of the insulin and they believed that it was involved in the physiological action of insulin. Brand and Sandberg (23) showed that while the sulfur of insulin was much more labile than that of cystine it still might be due to the presence of cystine in insulin. They found the lability of sulfur in dialanylcystine and dialanylcystine dianhydride was much greater than in cystine itself. The extreme sensitivity of cystine derivatives to dilute alkali has also been noted in the present work.

(23) Brand and Sandberg, J. Biol. Chem. 70, 381 (1926).

Blatherwick and co-workers (18) believed that sulfur was present in insulin in other forms than cystine. These workers found that the labile sulfur could be destroyed to a large extent without affecting the activity of insulin and that the activity could be destroyed without affecting the labile sulfur. In regard to the latter fact it had previously been pointed out by Brand and Sandberg (23) that other factors besides the removal of sulfur might play a part in the alkaline destruction of the activity of insulin. du Vigneaud (24) stated that labile sulfur is a general property of proteins and that there was no evidence that the disulfide linkage was responsible for the activity of insulin. He suggested that the activity might be due to guanidine or imidazole groups. He also pointed out the similarity between the action of insulin and cystine derivatives with respect to the splitting out of sulfur.

Jensen and Geiling (9) found that the sulfur in acetylated insulin was more labile than in insulin itself. Freudenberg, Dirscherl and Eyer (20) found that while the sulfur content of insulin increased with increase in purity, the splitting of labile sulfur had no relation to the decline in activity. They

(24) du Vigneaud, J. Biol. Chem. 75, 393 (1927).

also detected the presence of a thiomethoxy group in insulin.

Karr, Belk and Petty (25) believed that insulin contained sulfur in a form other than cystine. Neither these workers nor Mathis (26) found that insulin contained sulfur in the form of ethereal sulfate.

Peek (27) found no relation between labile sulfur and activity while Jensen and De Lawder (13) stated that part of the sulfur content was concerned in the physiological activity of insulin.

Freudenberg, Dirscherl and Eyer (22) upon further study of the thiomethoxy content of insulin found that this group was due to the presence of methionine in the protein of impure insulin. They found no thiomethoxy group in crystalline insulin or its hydrochloride. These workers also found that the sulfur content of insulin was practically unchanged after inactivation with dilute sodium hydroxide and they believed that no relation existed between the splitting out of sulfur and inactivation or change in optical activity.

du Vigneaud, Jensen and Wintersteiner (5) (6) succeeded in the isolation of tyrosine, cystine, arginine, histidine and leucine from crystalline insulin. They also stated that the

(25) Karr, Belk and Petty, J. Pharmacol. 36, 611 (1929).
(26) Mathis, Biochem. Z. 213, 72 (1929).
(27) Peek, Arch. neerland. physiol. 14, 294 (1929) (C.A. 24, 4899 (1930)).

presence of lysine was indicated. Previous to this Sandberg and Brand (28) determined the arginine content of insulin and Abel and co-workers (4) obtained negative tests for cysteine and tryptophane.

Sandberg and Brand (28) found that urea was split out upon boiling insulin with dilute sodium carbonate. They suggested that the formation of urea by the hydrolysis of arginine may be concerned in the inactivation of insulin. These authors also stated that insulin probably contains cystine, possibly linked by peptide linkage to arginine, histidine or tyrosine. Jensen and De Lawder (15) found that the arginine content did not seem to be reduced in the inactivation of insulin with alkali.

Basing their calculation upon ammonia split out Freudenberg, Dirscherl and Eyer (22) placed the molecular weight of insulin at about 8,000. More recently Sjogren and Svedberg (29) by means of the ultracentrafugal method arrived at a value of 35,100 for the molecular weight of insulin. This value and other constants determined by them showed insulin to be a well-defined protein of the complexity of egg albumin.

From a study of the work which has been done on insulin

(28) Sandberg and Brand, Proc. Soc. Exptl. Biol. Med. 24, 373 (1926-27).
(29) Sjogren and Svedberg, J. Am. Chem. Soc. 53, 2657 (1931).

it would appear that the insulin molecule is a protein made up from the various amino acids mentioned above and containing free amino and free carboxyl groups. Whether the activity is due to a specific group or to the special arrangement of a number of groups can not be said. The latter, however, would appear to be the case since tests of the effect of various amino acids on the blood sugar in no instance showed an effect approaching that of insulin.

Tests of the following amino acids showed them to be ineffective in reducing the blood sugar: histidine hydrochloride (30), cystine hydrochloride (31), alanine (32), sodium aspartate (32), creatine (33) and arginine (33). While having no effect on the blood sugar of rabbits, creatine was shown to reduce that of dogs and man to some extent (34) (35) (36). Cysteine hydrochloride and histidine also showed some hypoglucemic action (31). Little work has been done on the effect of polypeptides on the blood sugar, however the following were found to have no effect: glutathione (31), reduced glutathione (31), dialanyl-

(30) Dubin and Corbitt, J. Lab. Clin. Med. 10, 1023 (1925).
(31) Kon and Funk, Chem. Zelle Gewebe 13, 39 (1926).
(32) Wantoch, Arch. exptl. Path. Pharmakol. 143, 337 (1929).
(33) Bischoff, Sahyun and Long, J. Biol. Chem. 81, 325 (1929).
(34) Hill, J. Biol. Chem. 78, IV (1928).
(35) Hill and Mattison, J. Biol. Chem. 82, 679 (1929).
(36) Peabody and Hill, J. Biol. Chem. 82, 687 (1929).

cystine (23) and dialanylcystine dianhydride (23).

In the present work polypeptides of cystine with other amino acids found in insulin have been prepared in the hope of finding some grouping which would show insulin-like action. Cystine was included in all of these compounds since it was thought possible that the very reactive disulfide linkage might in some way be concerned as a catalyst or oxygen carrier in the metabolic oxidation of sugar.

EXPERIMENTAL PART

Dicarbethoxytyrosine, $C_2H_5OCO_2C_6H_4CH_2CH(NHCO_2C_2H_5)COOH$. Dicarbethoxytyrosine was prepared by the action of ethyl chlorocarbonate on tyrosine according to the method of Havestadt and Fricke (37). A solution of 9 g. (0.05 mole) of tyrosine in 100 cc. of normal sodium hydroxide was well cooled in an ice-salt freezing mixture. To the cold solution were then added 11.5 g. (0.11 mole) of ethyl chlorocarbonate in small portions with intermittent shaking and cooling. It was found necessary to add an additional amount of normal sodium hydroxide in order to keep the solution basic. When the odor of the acid chloride had disappeared the solution was acidified with 5-normal hydrochloric acid. A white solid precipitated which was extracted with ether and the ether solution dried over anhydrous sodium sulfate. Upon evaporation of the ether a syrupy residue remained which solidified upon cooling. This was broken up and well washed with dry petroleum ether (B.P. 30-60°). A yield of 15 g. or 90% of product melting at about 75-80° was obtained. According to Havestadt and Fricke (37) the pure compound melts at 96-97°.

(37) Havestadt and Fricke, Ber. 57, 2048 (1924).

Dicarbethoxytyrosyl Chloride, $C_2H_5OCO_2C_6H_4CH_2CH(NHCO_2C_2H_5)$-$COCl$. Dicarbethoxytyrosyl chloride was prepared by the action of phosphorus pentachloride on dicarbethoxytyrosine according to the method of Havestadt and Fricke (37). A mixture of 3.25 g. (0.01 mole) of dicarbethoxytyrosine and 25 cc. of freshly distilled acetyl chloride was well cooled in an ice-salt freezing mixture. Three grams (0.014 mole) of powdered phosphorus pentachloride were then added and the mixture shaken until all had dissolved. The solution was evaporated under reduced pressure until the residue solidified. This was broken up and washed in the distilling flask with five portions of dry petroleum ether (B.P. 30-60°). The material was then dissolved in about 40 cc. of dry chloroform and used immediately.

Cystine Diethylester Dihydrochloride, $(SCH_2CH(NH_2)$-$CO_2C_2H_5 \cdot HCl)_2$. Cystine diethylester dihydrochloride was prepared by the action of dry hydrogen chloride gas on an absolute alcoholic suspension of cystine, according to the method of Friedmann (38). A suspension of 6 g. (0.025 mole) of cystine in 500 cc. of boiling absolute alcohol was treated with a rapid stream of dry hydrogen chloride gas until all of the cystine had dissolved. The hot solution was filtered and allowed to

(38) Friedmann, Beitr. chem. Physiol. Path. 3, 17 (1902).

cool. The crystalline product was filtered and washed with alcohol and then ether. The filtrate was evaporated to about one-half the volume and was again treated with dry hydrogen chloride gas for about one hour. Several volumes of ether were added and the additional product allowed to crystallize. It was filtered and washed as before. A total yield of 9 g. or 90% was obtained.

Cystine Diethylester, $(SCH_2CH(NH_2)COOC_2H_5)_2$. Free cystine diethylester was obtained in a manner similar to that used by Fischer and Suzuki (39) in the preparation of free cystine dimethylester. A mixture of 4 g. (0.011 mole) of cystine diethylester dihydrochloride and 15 cc. of absolute alcohol was well cooled in an ice-salt freezing mixture. To the mixture was then added a cold sodium ethylate solution prepared by dissolving 0.46 g. (0.02 atom) of sodium in 20 cc. of absolute alcohol. An excess of sodium ethylate, as evidenced by a yellow color, was avoided. After standing for about 30 minutes, the mixture was treated with several volumes of dry ether and shaken well to coagulate the precipitate of sodium chloride. The latter was then filtered and the clear filtrate evaporated under reduced pressure without heating. The clear, yellow, oily product was dissolved in 30 cc. of dry chloroform and used immediately.

(39) Fischer and Suzuki, Z. physiol. Chem. 45, 405 (1905).

Bis-(dicarbethoxytyrosyl)-cystine Diethylester, $(C_2H_5OCO_2-C_6H_4CH_2CH(NHCO_2C_2H_5)C(:O)NHCH(CO_2C_2H_5)CH_2S)_2$. Bis-(dicarbethoxytyrosyl)-cystine diethylester was prepared by the action of dicarbethoxytyrosyl chloride on cystine diethylester. The chloroform solutions of dicarbethoxytyrosyl chloride and cystine diethylester prepared as described above were cooled in an ice-salt freezing mixture. The solution of acid chloride was then, in small portions with shaking, added to that of the ester. After all had been added the cystine diethylester dihydrochloride which had separated was filtered (1.8 g. of this material were recovered). The filtrate was evaporated under reduced pressure to a volume of about 20 cc. and was then diluted with dry petroleum ether (B.P. 30-60°). A white flocculent precipitate separated which was filtered, washed with petroleum ether and dried. A yield of 4 g. or 90% was obtained.

The compound was purified by dissolving in hot ethyl acetate. Upon cooling, a white almost crystalline solid separated which melted at 177-178°.

Anal. (By Carius). Calc. for $C_{40}H_{54}N_4O_{16}S_2$: S, 7.03. Found: S, 7.16 and 6.98.

Attempts to hydrolyze this ester, using dilute sodium hydroxide, ammonium hydroxide, alcoholic sodium hydroxide and alcoholic ammonium hydroxide were unsuccessful. Where any action at all was observed the compound appeared to be decom-

posed, as evidenced by the liberation of hydrogen sulfide upon acidification of the alkaline hydrolysis solution.

Dicarbethoxycystine, $(SCH_2CH(NHCO_2C_2H_5)COOH)_2$. By the action of ethyl chlorocarbonate on an alkaline solution of cystine, Gortner and Hoffman (40) obtained a solid product melting at about 63° and which analysis indicated to be tetracarbethoxycystine. From 2.0 g. (0.0083 mole) of cystine and 2.2 g. (0.0204 mole) of ethyl chlorocarbonate these authors obtained a yield of 3.55 g. of pure product. Assuming this to be the tetracarbethoxy derivative, 3.55 g. (0.0067 mole) would require at least 2.89 g. (0.0268 mole) of ethyl chlorocarbonate which is 0.69 g. (0.0064 mole) more than that actually used in the reaction.

A procedure similar to that of Gortner and Hoffman (40) yielded a viscous syrup. Analysis of derivatives of this material in every case showed them to be dicarbethoxy compounds rather than tetracarbethoxy. A solution of 12 g. (0.05 mole) of cystine in 300 cc. of normal sodium hydroxide was cooled in an ice-salt freezing mixture. To the solution were then added 12 g. (0.11 mole) of ethyl chlorocarbonate in small portions with shaking. About twenty minutes were required for the complete addition. It was necessary to add an additional amount

(40) Gortner and Hoffman, J. Biol. Chem. 72, 437 (1927).

of sodium hydroxide to keep the solution alkaline. After shaking intermittently for 30-40 minutes the solution was acidified with 5-normal hydrochloric acid and extracted with ether. The ether solution was dried with anhydrous sodium sulfate and was then evaporated, the last portion under reduced pressure. A residue of clear amber colored, viscous syrup remained. The yield of this material was 18.5 g. or 97%.

Attempts to crystallize this material from hot iso-butyl alcohol or by dissolving in ether and precipitating by diluting with petroleum ether were unsuccessful. The crude material was not analyzed.

Dicarbethoxycystinyl Chloride, $(SCH_2CH(NHCO_2C_2H_5)COCl)_2$. Dicarbethoxycystinyl chloride was prepared by the action of phosphorus pentachloride on dicarbethoxycystine. The 18.5 g. (0.048 mole) of dicarbethoxycystine prepared as described above was transferred to a 500 cc. glass-stoppered flask by means of a little warm chloroform. One hundred cubic centimeters of freshly distilled acetyl chloride were added and the mixture well cooled in an ice-salt freezing mixture. The mixture was then treated with 30 g. (0.14 mole) of powdered phosphorus pentachloride added in small portions with shaking. After all had been added the flask was placed in an ice bath and allowed

to stand for several hours. A fine white precipitate gradually formed. This was filtered and washed with acetyl chloride containing a little acetic acid and then with dry petroleum ether (B.P 30-60°) and dried in vacuum over phosphorus pentaoxide. A yield of 16 g. or 76%, based on cystine originally used, was obtained. The compound melted with decomposition at 115-116°. For analysis the dicarbethoxycystinyl chloride was crystallized from hot chloroform.

Anal. Calc. for $C_{12}H_{18}Cl_2N_2O_6S_2$: S, 15.28. Found: S, 15.44.

A method of alkaline fusion was used in the analysis of dicarbethoxycystinyl chloride and certain other cystine derivatives in the following work (41). The sample of about 0.2 g. was mixed with about 4 g. of powdered potassium hydroxide in a nickel crucible. The mixture was gradually heated on the hot plate until the frothing subsided. The temperature was then raised to the fusion point and the mass treated with about one gram portions of potassium nitrate added periodically until the contents of the crucible had become a clear liquid. About 10-12 g. of potassium nitrate were added in all. Upon cooling, the contents of the crucible solidifed to a white crystalline mass. This was dissolved in about 300 cc. of water and acidified with

(41) This is a modification of Liebig's method of sulfur analysis as given in Treadwell and Hall, "Analytical Chemistry," John Wiley and Sons, Inc., New York, 1924, Vol. II, p. 326.

concentrated hydrochloric acid, 10 cc. in excess being used. The solution was boiled until the carbon dioxide and nitrogen oxides were completely driven out. The sulfate was precipitated by the addition of hot barium chloride solution and after standing for 30 minutes was filtered and weighed in the customary manner.

Tyrosine Ethylester, $HOC_6H_4CH_2CHNH_2CO_2C_2H_5$. Tyrosine ethylester was prepared by the action of hydrogen chloride gas on a mixture of tyrosine and absolute alcohol according to the method of Fischer (42). Five grams (0.028 mole) of tyrosine were suspended in 35 cc. of absolute alcohol and the mixture saturated with dry hydrogen chloride gas. Fifty cubic centimeters of absolute alcohol were then added and the solution boiled under reflux for 6-7 hours. The alcohol was evaporated under reduced pressure until the residue solidified. This was dissolved in about 100 cc. of water and treated with an excess of potassium carbonate. The free ester was extracted with 99% ethyl acetate and the solution dried over anhydrous sodium sulfate. The ethyl acetate solution was concentrated on the steam bath to a volume of about 50 cc. Upon diluting with dry petroleum ether (B.P. 30-60°) the tyrosine ethylester separated

(42) Fischer, Ber. 34, 451 (1901).

as a crystalline precipitate which was filtered and dried. A yield of 4.5 g. or 66% of material melting at 103-105° was obtained. Fischer (42) reported a yield of 85% and a melting point of 108-109° (corrected).

Dicarbethoxycystinyltyrosine Diethylester. $(SCH_2CH(NHCO_2C_2H_5)CONHCH(CO_2C_2H_5)CH_2C_6H_4OH)_2$. Dicarbethoxycystinyltyrosine diethylester was prepared by the action of dicarbethoxycystinyl chloride on tyrosine ethylester. A solution of 4.2 g. (0.02 mole) of tyrosine in 75 cc. of dry chloroform was cooled in an ice-salt freezing mixture. To the solution were then added 1.8 g. (0.0043 mole) of dicarbethoxycystinyl chloride. The mixture was well shaken and allowed to stand at room temperature for about two hours. The precipitate which had separated was filtered, washed with chloroform and dried. This material weighed 5 g. The dried solid was powdered and well mixed in a mortar with about 50 cc. of water. After standing overnight the insoluble material was filtered and dried. A yield of 3 g. or 91% was obtained. This material was purified by dissolving in hot ethyl acetate. The compound slowly separated as a white, almost crystalline solid melting at 179-182°.

The above water filtrate was made alkaline with potassium carbonate and extracted with ethyl acetate. Upon evaporation of the ethyl acetate 1.2 g. of tyrosine ethylester were recovered.

Anal. (By Carius). Calc. for $C_{34}H_{46}N_4O_{12}S_2$: S, 8.35. Found: S, 8.37 and 8.60.

Dicarbethoxycystinyltyrosine. $(SCH_2CH(NHCO_2C_2H_5)C(:O)-NHCH(COOH)CH_2C_6H_4OH)_2$. Dicarbethoxycystinyltyrosine was prepared by two methods: (1) the hydrolysis of dicarbethoxycystinyltyrosine diethylester and (2) the action of dicarbethoxycystinyl chloride on an alkaline solution of tyrosine.

(1) One and six-tenths grams (0.002 mole) of dicarbethoxycystinyltyrosine diethylester were dissolved in 12 cc. of normal sodium hydroxide. The solution was filtered rapidly and immediately neutralized with 12 cc. of normal hydrochloric acid. A white solid separated which was filtered and washed with water. This product weighed 1.4 g. or 98% of the theoretical. The compound was purified by dissolving in hot water, upon cooling a gelatinous precipitate formed. This material melted at 200-202°.

(2) A solution of 3.6 g. (0.02 mole) of tyrosine in 40 cc. of normal sodium hydroxide was diluted with 20 cc. of water and cooled in an ice-salt freezing mixture. To the solution were then added 4.2 g. (0.01 mole) of dicarbethoxycystinyl chloride in four portions. Each portion was preceded by an additional 5 cc. of normal sodium hydroxide. The mixture was vigorously shaken after each addition. After all had been added the solution was allowed to stand until almost clear. It was then filtered and acidified with dilute hydrochloric acid. The mixture was

extracted with ether to remove any dicarbethoxycystine and the water was then decanted from the gummy mass which had separated. The remaining material was extracted with hot water. Upon cooling an oil separated which gradually solidified. Only 2 g. or 28% of product melting at 193-195° were obtained. A mixed melting point with the compound prepared by method (1) showed them to be the same.

Anal. (By Carius). Calc. for $C_{30}H_{38}N_4O_{12}S_2$: S, 9.01. Found: S, 9.42 and 9.49.

Histidine Methylester Dihydrochloride, $\begin{matrix} CHNH \\ | \\ N-CH \end{matrix}\!\!>\!CCH_2CH(NH_2)-CO_2CH_3 \cdot 2HCl$. Histidine methylester dihydrochloride was prepared by the action of hydrogen chloride gas on a suspension of histidine dihydrochloride in absolute methyl alcohol according to the method of Fischer and Cone (43). Five grams (0.028 mole) of histidine dihydrochloride were suspended in 75 cc. of absolute methyl alcohol. The boiling solution was treated with dry hydrogen chloride gas until saturated. Upon cooling in an ice-salt freezing mixture the product crystallized and was filtered. By concentration of the filtrate under reduced pressure an additional amount was obtained. The yield was 4.8 g. or 90% of product melting at 200-202°.

(43) Fischer and Cone, Ann. 363, 108 (1908).

Dicarbethoxycystinylhistidine Dimethylester. ($SCH_2CH(NHCO_2C_2H_5)C(:O)NHCH(CO_2CH_3)CH_2C$  $)_2$. Dicarbethoxycystinylhistidine dimethylester was prepared by the action of dicarbethoxycystinyl chloride on histidine methylester. Four grams (0.017 mole) of histidine methylester dihydrochloride were dissolved in 40 cc. of hot absolute methyl alcohol. The solution was rapidly cooled and before crystallization had started was treated with a sodium methylate solution prepared from 0.75 g. (0.033 atom) of sodium and 20 cc. of methyl alcohol. The mixture was diluted with 40 cc. of dry ether, allowed to stand about 20 minutes and then filtered. The clear filtrate was evaporated under reduced pressure, treated with 5 cc. of dry chloroform and again evaporated. The free ester was then dissolved in 60 cc. of dry chloroform and treated with 1.6 g. (0.004 mole) of dicarbethoxycystinyl chloride. The mixture was well mixed by means of a spatula and allowed to stand overnight. The solid which had separated was filtered and dried. This material weighed 4 g. It was ground in a mortar with water and the suspension allowed to stand several hours. The water-insoluble material was filtered and dried, yielding 0.93 g. or 33%. To purify, the compound was dissolved in 30-40 cc. of absolute methyl alcohol and the hot solution diluted with 75 cc. of 99% ethyl acetate. Upon cooling the product separated as a gelatinous mass which was filtered and

dried in vacuum. The compound melted at 167-168°.

Anal. (44). Calc. for $C_{26}H_{38}N_8O_{10}S_2$: S, 9.36. Found: S, 8.93 and 8.59.

Dicarbethoxycystinylarginine, $(SCH_2CH(NHCO_2C_2H_5)C(:O)-NHCH(CO_2H)CH_2CH_2CH_2NHC(:NH)NH_2)_2$. Dicarbethoxycystinylarginine was prepared by the action of dicarbethoxycystinyl chloride on an alkaline solution of arginine. A solution of 2.1 g. (0.01 mole) of arginine monohydrochloride in 20 cc. of normal sodium hydroxide was cooled in an ice-salt freezing mixture. To the solution were then added 4.2 g. (0.01 mole) of dicarbethoxycystinyl chloride in four portions. Each portion was preceded by 5 cc. of normal sodium hydroxide. The mixture was well shaken and cooled after each addition. After standing for about one hour, undissolved material was filtered and the filtrate was acidified with normal hydrochloric acid. The mixture was extracted with ether to remove dicarbethoxycystine. The water was then decanted from the gummy mass which had separated and the solution evaporated under reduced pressure.

The residue from the evaporation and the original gummy mass which was obtained upon acidification were extracted with boiling alcohol. Upon diluting the cooled and filtered solution

(44) Analyzed by the procedure given on p. 65, This Thesis.

with ether a white non-crystalline product was obtained. This was redissolved in absolute alcohol and precipitated as before. A yield of 1.5 g. or 43% was obtained. The compound decomposed at 115-120° but did not melt definitely.

Anal. (45). Calc. for $C_{24}H_{44}N_{10}O_{10}S_2$: S, 9.22. Found: S, 9.42 and 9.25.

Physiological Results (46)

The physiological tests of the compounds described were conducted according to the method previously given (47).

These tests as well as others to be made with these compounds have not yet been completed.

(45) Analyzed by the procedure given on p. 65, This Thesis.
(46) The compounds were tested for hypoglucemic action in the laboratories of Parke-Davis and Company.
(47) See p. 43, This Thesis.

SUMMARY AND CONCLUSIONS

A number of peptides consisting of cystine in combination with other amino acids found in insulin were prepared in the hope of finding a grouping which would show the hypoglucemic action of insulin.

The compounds prepared were: dicarbethoxytyrosylcystine diethylester, dicarbethoxycystinyltyrosine ethylester, dicarbethoxycystinyltyrosine, dicarbethoxycystinylhistidine methylester and dicarbethoxycystinylarginine.

The physiological tests of these compounds have not been completed.