opposing views. More recently other workers[4,5] have reported the existence of certain cobaltous periodates in complete variance with the present findings.

Some revealing information regarding this question is obtained through a consideration of oxidation potentials. In Fig. 1 are shown portions of the potential–*p*H diagram for iodine and cobalt constructed from the data of Latimer[6] as described by Delahay, Pourbaix and Van Rysselberghe.[7] (Although the potential of the periodate—iodate couple is based upon purely chemical evidence, it represents a close approximation). Thus it is seen that the stability of a strongly acid solution of cobaltous and periodate ions is indicated, whereas in weakly acid solution the reduction of periodate by the cobaltous ion appears possible.

The chemical evidence indicating this reaction was the production of gelatinous, greenish-brown cobaltic hydroxide and iodate ions when solutions of divalent cobalt and periodic acid were mixed. The cobaltic hydroxide was identified on the basis of its physical properties and the knowledge of the reactants and possible products. After filtration of the cobaltic hydroxide, iodate was identified in the filtrate with silver nitrate solution by the formation of white, curdy silver iodate, difficultly soluble in nitric acid (brown silver periodate is soluble). At a *p*H of about 2 this reaction proceeded very slowly, traces of the reaction products appearing after about 15 minutes. Many hours later the reaction was still far from complete as judged by the small amount of cobaltic hydroxide formed. In more acid solution the reaction did not go at all. However at *p*H 6 it was instantaneous even in dilute solutions.

Quantitative tests were performed to firmly establish the existence of this reaction. Cobaltous acetate solution in excess reacted with a known amount of potassium metaperiodate in solution. The oxidation state of the iodine after reaction was established iodometrically. Cobaltic ion in an acid solution is an oxidizing agent and would interfere in the iodometric procedure. Removal of the gelatinous cobaltic hydroxide was not practical so the cobalt was converted to the cobaltous state. This reduction occurred spontaneously when the cobaltic hydroxide was dissolved by heating in a 0.1 *N* sulfuric acid solution. A few drops of ethylenediamine were added to assist, through its complexing action with cobalt, in the solution of the difficultly soluble cobaltic hydroxide. Controls were run with identical treatments except that the solutions were acidified before mixing to prevent the reaction under study from occurring. The results are shown in Table I.

The ratio of the average number of oxidation equivalents found after reaction to the number of oxidation equivalents taken is 0.740 compared to the theoretical value of 0.750 if the equivalents of

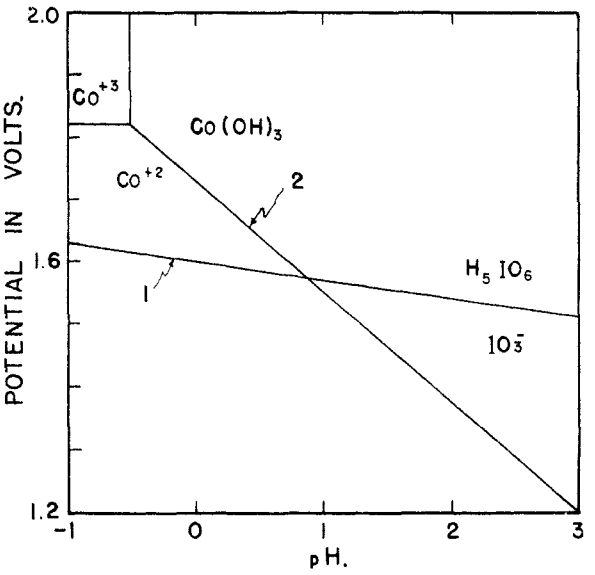

Fig. 1.—A portion of the potential–*p*H diagram for the $Co(OH)_3$–Co^{++} and the H_5IO_6–IO_3^- couples.

periodate were all reduced to iodate. The same ratio for the controls is 0.988 compared to the theoretical value of 1.000 if the periodate solution had not reacted.

TABLE I

ANALYTICAL DATA FOR THE REACTION BETWEEN Co^{++} AND IO_4^-

	Oxidation equivalents	
Taken	Found after reaction	Found in controls
0.656	0.493	0.654
	.495	.650
	.474	.635
	.479	.652
	.484	
Av.	0.485	0.648

It is believed that the above facts clearly establish the existence of an oxidation–reduction reaction between periodate and divalent cobalt. The probable reaction is indicated by the equation.

$$2Co^{++} + IO_4^- + (n+2)H_2O \longrightarrow Co_2O_3 \cdot nH_2O + IO_3^- + 4H^+$$

It is of interest to note that in agreement with the postulated formation of hydrogen ions here, a decrease in *p*H has been noted after the reaction of solutions originally at *p*H 6.

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Peptides Isolated from a Partial Hydrolysate of Steer Hide Collagen

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In the work on the determination of the structure of collagen being performed in these laboratories, we have been concerned with the sequences of the amino acid residues in the polypeptide chains of

this fibrous protein. We wish to report several peptides isolated in the form of dinitrophenyl (DNP) derivatives from the neutral fraction of an acid partial hydrolysate of steer hide collagen.

Experimental

The collagen was prepared from steer hide by the method of Highberger,[1] except that repeated extraction with 10% sodium chloride solution was substituted for the treatment with trypsin. The protein contained 18.56% nitrogen on a moisture- and ash-free basis.

The partial hydrolysate was obtained by hydrolyzing the collagen for four days at 37° with concentrated hydrochloric acid using a ratio of acid to protein of 20:1. The excess acid was removed in a Craig type evaporator[2] by concentration to a thick sirup six times.

The acid digest was separated into aromatic, neutral, basic and acidic fractions by use of charcoal (treated with 20% acetic acid) and ion-exchange resins. The basic fraction was obtained by adsorption on the resin XE-64 (Rohm and Haas Co.) buffered at *p*H 6.0 with ammonium acetate and the acidic fraction was adsorbed on a column of IR-4B (Rohm and Haas Co.) treated with acetic acid and washed until the effluent remained constant at *p*H 3.5. The neutral fraction obtained in this manner contained 67.5% of the nitrogen of the hydrolysate. The nitrogen content of the neutral fraction appears to be high because of incomplete adsorption of the acidic fraction on the IR-4B column. Acidic peptides have been found in the neutral fraction, but their composition has not been completely resolved.

The dinitrophenyl derivatives of the neutral fraction were prepared by the method of Sanger.[3] During the ether extraction, it was observed that some of the yellow color remained in the acid phase. This would indicate that there are neutral peptides containing both acidic and basic amino acid residues, and the basic amino acid is the N-terminal amino acid since the basic DNP-amino acids are acid soluble. This fraction will be investigated in the future.

The DNP derivatives were separated on 10-g. columns of buffered Super-cel[4] (Johns-Manville Corp.) using ethyl acetate saturated with water as the primary mobile phase. Occasionally, the varying methyl ethyl ketone–chloroform system of Mills[5] was used and at other times, the columns were extruded and the resolved bands extracted with 2% sodium bicarbonate. In one or two instances, the columns failed to resolve a mixture of dipeptides and these were separated by paper chromatography.[6] Redistilled solvents were used in the operation of the columns and redistilled hydrochloric acid for the hydrolysis of the peptides.

In the determination of the composition and sequences of the DNP peptides, aliquots of the sub-fractions were hydrolyzed with 6 *N* hydrochloric acid at 105° for various lengths of time. Partial hydrolysates were obtained by refluxing for a period of 0.5 or 1 hour. Four hours of refluxing was used for the complete liberation of the DNP-amino acids, and 16 hours in sealed tubes for complete hydrolysis. The aqueous extracts from the four-hour hydrolysates were hydrolyzed for a further 16 hours in sealed tubes. Extractions of the hydrolysates were made with ether or ethyl acetate. The liberated DNP-amino acids were identified by their rates both on columns and by paper chromatography.[7] We have found the phenol–isoamyl alcohol–water system[8] very useful for the identification of the DNP-amino acids on paper. The amino acid residues were identified by paper chromatography.[9] The DNP-amino acids of the partial hydrolysates were identified by their rates both on columns and paper.

Results

The neutral fraction was first put through an ethyl acetate–*p*H 5.0 column and five main bands (A_1, A_2, A_3, A_4, A_5)[10] were distinguished. The last band (A_5) moved very slowly and was eluted with 1% acetic acid in ethyl acetate. The fastest band (A_1) was the largest and it was resolved into 8 sub-fractions (A_1C_1 through A_1C_8) by passage through an ethyl acetate–*p*H 7.0 column. These sub-fractions were then resolved and purified on either ethyl acetate–*p*H 7.0, methyl ethyl ketone–chloroform–*p*H 7.0 columns or by paper chromatography.

As shown in Table I, we have isolated and identified six amino acids, eight dipeptides and one tripeptide as the DNP derivatives. The presence of H.Leu-Ala.OH in two different fractions may be attributed to the poor resolution of the fast moving DNP-amino acids and peptides on the ethyl acetate–*p*H 5.0 columns. Partial hydrolysis of the DNP-dipeptides resulted in the appearance of two bands. The faster band was the liberated DNP-amino acid and the second band retained the same rate as the DNP-peptide. In the instance of the tripeptide, H.Ala-Gly-Ala.OH, three bands were observed after partial hydrolysis.

TABLE I

SOME ETHER-SOLUBLE DNP-AMINO ACIDS AND DNP-PEPTIDES OF NEUTRAL FRACTION OF PARTIAL HYDROLYSATE OF STEER-HIDE COLLAGEN

Fraction	Amino acid or peptide[11]	Fraction	Amino acid or peptide[11]
$A_1C_1M_1$[a,b]	Leucine	A_1C_6	H.Ala-Gly-Ala.OH
$A_1C_1M_2$[b]	H.Leu-Ala.OH	A_1C_7-I[e]	H.Gly-Pro.OH
$A_1C_1M_3$[b]	Methionine	A_1C_7-II[e]	H.Gly-Ala.OH
$A_1C_2M_1$[b]	H.Leu-Ala.OH	$A_1C_8M_1$[d]	H.Gly-Gly.OH
$A_1C_3M_2$[c]	H.Val-Gly.OH	$A_1C_8M_2$[d]	H.Thr-Gly.OH
$A_1C_4M_1$[d]	Proline	A_2M[f]	Serine
$A_1C_4M_2$[d]	Alanine	A_2M_1[e,g]	H.Ser-Gly.OH
A_1C_5	Glycine	A_2M_2[g]	H.Hypro-Gly.OH

[a] The DNP-amino acids and DNP-peptides were separated on columns of 20 mm. diameter containing 10 g. of Super-cel and buffer.[4] A represents a column treated with 0.5 *M* phosphate buffer and developed with ethyl acetate saturated with water. C is an ethyl acetate–*p*H 7.0 (0.4 *M* phosphate buffer) column. M is the same as C, but developed with 30–75% methyl ethyl ketone–chloroform[5] saturated with water (m–e–k–chloroform). [b] Eluted with 30% m–e–k–chloroform. [c] Eluted with 45% (m–e–k–chloroform). [d] Eluted with 60% m–e–k–chloroform. [e] Paper chromatography—Whatman No. 1 paper developed with phenol—isoamyl alcohol—water[6,8] (1:1:1) at 22°. [f] Eluted with 75% m–e–k–chloroform. [g] Developed with 75% m–e–k–chloroform, and then extracted with 2% sodium bicarbonate after column extrusion.

Discussion

Schroeder, Honnen and Green[12] recently reported the following peptides from a partial hydrolysate of gelatin: -H.Thr-Gly.OH, H.Glu-Gly.-OH, H.Glu-Ala.OH, H.Gly-Glu.OH, H.Ser-Gly.OH, H.Hypro-Gly.OH, H.Ala-Gly.OH. Grassman and Riederle[13] isolated the tripeptide, H.Lys-Pro-

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Gly.OH, from a partial hydrolysate of gelatin. In an alkaline partial hydrolysate of gelatin, Heyns, Anders and Becker[14] found H.Glu-Gly.OH, H.Ala-Gly.OH, H.Gly-Asp.OH, H.Ala-Ala-Gly.OH, H.-Ala-(Gly,Glu). Of the twenty or more dipeptides and tripeptides reported present in partial hydrolysates of collagen and gelatin, only five fit the collagen sequence -P-G-R-P-G-R- suggested by Astbury[15] and Pauling and Corey[16] where P represents either proline or hydroxyproline, G is glycine and R stands for one or other of the remaining residues. The five which fit are H.Lys-Pro-Gly.OH (Grassman and Riederle), H.Gly-Asp.OH (Heyns, *et al.*), H.Gly-Glu.OH and H.Hypro-Gly.OH (Schroeder, *et al.*), and H.Gly-Ala.OH (this paper). All of the peptides, except H.Gly-Gly.OH, conform to the -G-R-P-G-R-R- sequence suggested by Bergmann and Niemann[17] for the structure of gelatin. However, this formula was based on Bergmann's value of 19.7% proline[18] in gelatin which is much higher than the currently accepted values for proline of 14.8–15.1%.[19,20] Schroeder, *et al.*,[12] have pointed out that in view of these more reliable values for proline only three-fourths of the collagen structure could have the sequence suggested by Astbury.[15] A similar consideration would apply to the Bergmann–Niemann[17] sequence. The isolation of H.-Gly-Gly.OH (present work) would indicate that other sequences in addition to those suggested must be present. At present, sufficient data have not been accumulated to justify any conclusion as to the reliability of any proposed structure of collagen.

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Pyridazinemonocarboxylic Acids and Derivatives

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In connection with recent studies of nitrogen heterocyclic amides[1] and hydrazides, new syntheses of pyridazine-3-carboxylic acid and pyridazine-4-carboxylic acid were developed. Gabriel and Colman[2] first made pyridazine-3-carboxylic acid by the permanganate oxidation of 3-*p*-hydroxyphenylpyridazine.

In the present work two alternate routes to the 3-acid were explored. Permanganate oxidation of 3-hydroxymethylpyridazine and reductive dehalogenation of 6-chloropyridazine-3-carboxylic acid were found to give identical yields of the desired acid. The choice of the route depends, therefore, upon the availability of intermediates. 3-Hydroxymethylpyridazine can be prepared in two steps and 33% over-all yield from furfuryl acetate according to Clauson–Kass,[3] and we have confirmed this. Our yield of 6-chloropyridazine-3-carboxylic acid from levulinic acid in five steps was 40%. Pyridazine-3-carboxamide and pyridazine-3-carbohydrazide were prepared from the acid *via* the ethyl ester.

Partial decarboxylation of pyridazine-4,5-dicarboxylic acid[4] gave 4-carboxypyridazine. This new acid was converted to the ethyl ester, amide and hydrazide.

The pK_a values of the isomeric 3- and 4-carboxypyridazines are 3.0 and 2.8, respectively. Data on the basicities and reduction potentials of the corresponding amides have been previously reported.[1]

The hydrazides were tested for antituberculous activity in a standardized mouse assay by Dr. M. Solotorovsky of the Merck Institute for Therapeutic Research and found to be inactive.

Experimental

6-Chloropyridazine-3-carboxylic Acid.—The route to this acid involves the following steps, all previously described: preparation of 6-hydroxy-3-methyldihydropyridazine,[5] dehydrogenation to 6-hydroxy-3-methylpyridazine,[6] conversion to 6-chloro-3-methylpyridazine[7,8] and oxidation to 6-chloropyridazine-3-carboxylic acid.[9] The yields obtained for the reactions were 94, 76, 80 and 60%, respectively.

Two details deserve special mention. In synthesis of 6-chloro-3-methylpyridazine, the temperature of the reaction mixture containing phosphorus oxychloride is critical and should not exceed 100°. In workup of the 6-chloropyridazine-3-carboxylic acid preparation, the product must be extracted immediately after pouring the reaction mixture onto ice. Failure to observe these precautions results in very poor yields.

Pyridazine-3-carboxylic Acid. A. By Dehalogenation of 6-Chloropyridazine-3-carboxylic Acid.—Thirty grams of 6-chloropyridazine-3-carboxylic acid and 25 g. of Raney nickel were added to a cooled solution of 15.9 g. of sodium hydroxide in 330 ml. of water. The mixture was shaken at once with hydrogen at 40 p.s.i. Reduction was completed in two hours. The catalyst was removed by filtration and washed with water, then the combined filtrate and washings were concentrated to 80 ml. The concentrate was cooled to 35° and acidified to *p*H 2.5 with hydrochloric acid. After two hours standing at 0°, the product was removed by filtration and washed with a little water. When recrystallized from 525 ml. of boiling water, 19.0 g. (81%) of off-white acid, m.p. 195° (dec.), was obtained. A second recrystallization gave 13.4 g. of colorless product, m.p. 201° (dec.), and a second crop, 5.0 g., m.p. 194° (dec.). The reported melting point of pyridazine-3-carboxylic acid is 200–201°. The lower melting material obtained was satisfactory for ester preparation.

Anal. Calcd. for $C_5H_4N_2O_2$: N, 22.57. Found: N, 22.36.

B. By Oxidation of 3-Hydroxymethylpyridazine.—A solution of 22.4 g. of 3-hydroxymethylpyridazine (m.p. 60°) in 1 l. of water was added with stirring to a solution of 48 g. of potassium permanganate in 2 l. of water at 75° over a period of 10 minutes. After an additional 5 minutes all of the purple color of permanganate had disappeared. The manganese dioxide was filtered off and the filtrate evaporated to 500 ml., acidified to *p*H 2.5 and cooled. The precipitate

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