

The Isolation and Identification of Pyrocoll from Cigarette Smoke

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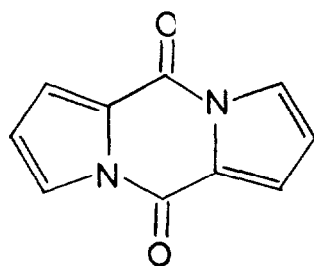
Studies to determine the nature and the relative amounts of the constituents of cigarette smoke have been undertaken with the object of gaining a better understanding of the processes which take place in the smoking of a cigarette. Results already reported from this and other laboratories have indicated that many of the components of tobacco are transferred to a greater or lesser extent, depending on their stability and volatility, by direct distillation into the smoke. For example, solanesol, a polyisoprenoid alcohol reported by Rowland (1956) to be present in flue-cured tobacco to the extent of 0.4%, was found in the smoke from cigarettes of a cased commercial blend of tobaccos (Mold and Booth, 1957) in an amount equivalent to 130 mg per thousand cigarettes. The partially hydrogenated acyclic di-terpene, neophytadiene, and several saturated aliphatic hydrocarbons, with C_{29} , C_{31} and C_{33} predominant, have similarly been found both in tobacco and in cigarette smoke. (Rowland, 1957; Gladding *et al*, 1959; Carruthers and Johnstone, 1959). Nicotine, a prime example of a compound, already present in tobacco, is transferred to the extent of approximately 35-50% into the main-stream smoke. This rather good yield is based on the tobacco consumed during puffing.

A second class of smoke components are those materials not present in tobacco but which are formed during smoking due to the pyrolysis or combustion of tobacco constituents. Dehydration, dehydrogenation, cracking, oxidation and condensation reactions occur with the large number of constituents in tobacco to yield a complex mixture of products in smoke which may well consist of tens or hundreds of thousands of components. Relatively few of these materials are present to an extent as great as 0.1% of the total smoke condensate. The majority of the compounds present in tobacco smoke in larger amounts have already been identified. The task of identifying the remainder becomes increasingly difficult because of the small amounts of materials present and the greater complexity of the structures.

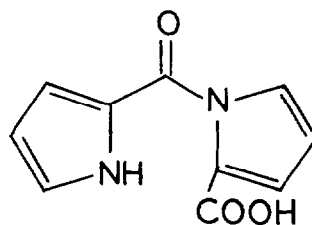
The compound described in this report, pyrocoll (I), dipyrrolo-[a,d]-pyrazine-5,10-dione, was isolated from the smoke of cigarettes made from a cased commercial blend of flue-cured, burley, Maryland and Turkish tobaccos. It was found to the extent of 0.006% of the smoke condensate, or 1.4 mg per 1000 cigarettes. Ultraviolet and infrared absorption spectra for pyrocoll are given in Figures 1 and 2. This material has been previously reported as arising from the pyrolysis of gelatin (Weidel and

Ciamician, 1880). A yield of 0.5-0.6 g of pyrocoll from 100 g of gelatin was reported by Hale and Hoyt (1916). Albumen, casein, or gluten apparently do not produce pyrocoll under similar conditions. The statement is made by Michelmann (1925) that pyrocoll has not been obtained from any of the substances known to yield pyrroles, other than gelatin, and scrap leather. Gelatin or collagen is notably high in proline (9.5%) and hydroxyproline (9.4, 14.1%) (Dakin, 1920; Levine, 1959). Plant proteins in general contain relatively larger amounts of proline than do animal proteins, but not, as a rule, as much as does gelatin (Onslow, 1931). Values of the proline content for the protein from several types of tobacco vary from 2.9-7.4% (Frankenburg *et al*, 1955, 1946; Zacharius and Frankenburg, 1953). Proline has been reported to constitute the sole amino end-group in the protein of tobacco mosaic virus (Schramm *et al*, 1954). Jean (1960) has reported it to be the major free amino acid present in flue-cured tobacco leaf. It was present to the extent of 0.4-1.3% of the dry leaf. No indication of hydroxyproline in tobacco protein has been reported and this amino acid is not a common constituent of plant protein (Steward and Pollard, 1958).

The protein content of cured tobaccos may constitute 6-10% of the total dry weight. If the pyrocoll found in cigarette smoke arises from proline in the tobacco, less than 0.1% yield would be required to provide the amount of pyrocoll found. The yield of pyrocoll from gelatin, under closer to optimum conditions, was about 2-3% based on the proline and hydroxyproline content (Hale and Hoyt, 1916). Pyrolysis of proline or glycylproline at 265-284°C failed to yield any pyrocoll while similar treatment of hydroxyproline resulted in a 0.7% yield of pyrocoll based on the ultraviolet absorption intensity. It



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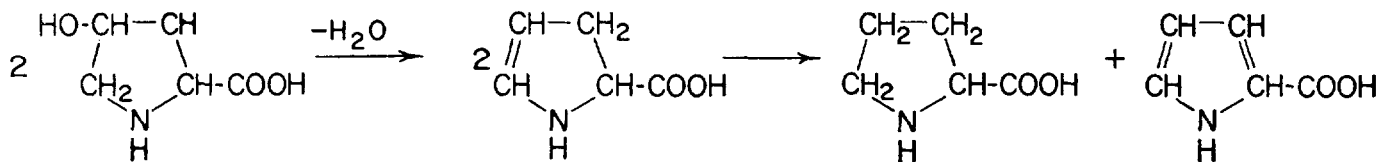
II

would be expected that hydroxyproline would provide a better source of pyrocoll than would proline, since dehydration of the former would lead to a pyrroline, one molecule of which might serve as a hydrogen acceptor for two hydrogen atoms from a second molecule.

(Buyske *et al*, 1956).

In the course of studies to identify the chemical structure of pyrocoll it was observed that, although this compound was reasonably stable in acid solution, it was extremely labile to alkali. This was evidenced by the

maximum at 257 m μ . The ease of alkaline hydrolysis of 2,5-diketopiperazine structures is well known, as is their stability toward acid (Karrer, 1950; Fisher, 1905). The spectrum for the second transformation product was identical to that for pyrrole-



Conversion of proline to pyrocoll would require the presence of some other compound to serve as a hydrogen acceptor. This could very well be the case in cigarette smoke since many hydrogen acceptors are present. On this basis it seems conceivable that the pyrocoll found in cigarette smoke could arise by pyrolytic processes from the proline present, free or combined, in tobacco. Other mechanisms of formation might prove equally tenable, e.g. from ammonia and mucic acid or via the lactam of glutamic acid, since glutamic acid has been reported present in cigarette smoke to the extent of 10 mg per thousand cigarettes

almost instantaneous change in the ultraviolet absorption spectrum to produce a single broad maximum at 295 m μ when alkali was added to the neutral compound in alcohol. This rapid change was irreversible upon immediate reacidification as was demonstrated by the failure to regain the original ultraviolet absorption spectrum. No shift in the position of the maximum was observed for the acidified sample. Upon longer exposure to alkaline solutions, a second transformation occurred. This could be detected by the gradual disappearance of the maximum at 295 m μ and the increase in intensity of a new

2-carboxylic acid. The conversion appeared to be nearly quantitative on the basis of the ultraviolet absorption intensity and the close similarity of the infrared absorption spectrum for the total product of the reaction with potassium hydroxide to that for potassium pyrrole-2-carboxylate (Figure 3).

The first transformation product is undoubtedly the open chain peptide, N-(pyrrole-2'-carboxy)-pyrrole-2-carboxylic acid (II). Equivalent weight and spectral data (Figures 4 and 5) are in accord with this structure.

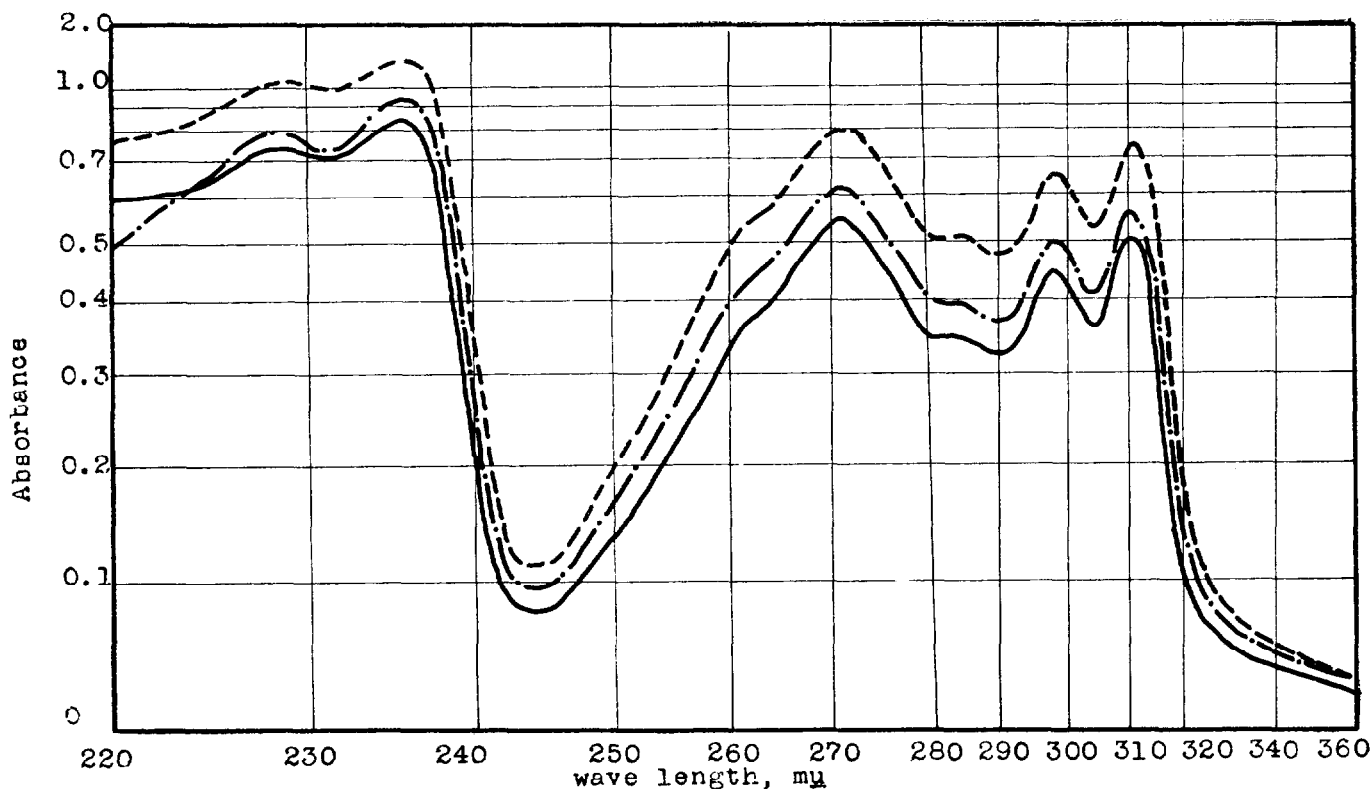


Figure 1. Ultraviolet Absorption Spectra of Pyrocoll in Cyclohexane (— isolated from smoke; --- synthetic; - · - · - from the pyrolysis of gelatin). Max. at 311.0 (4.22), 298.0 (4.16), 285.0 (4.05), 271.0 (4.25), 235.7 (4.44), 228.7 (4.38).

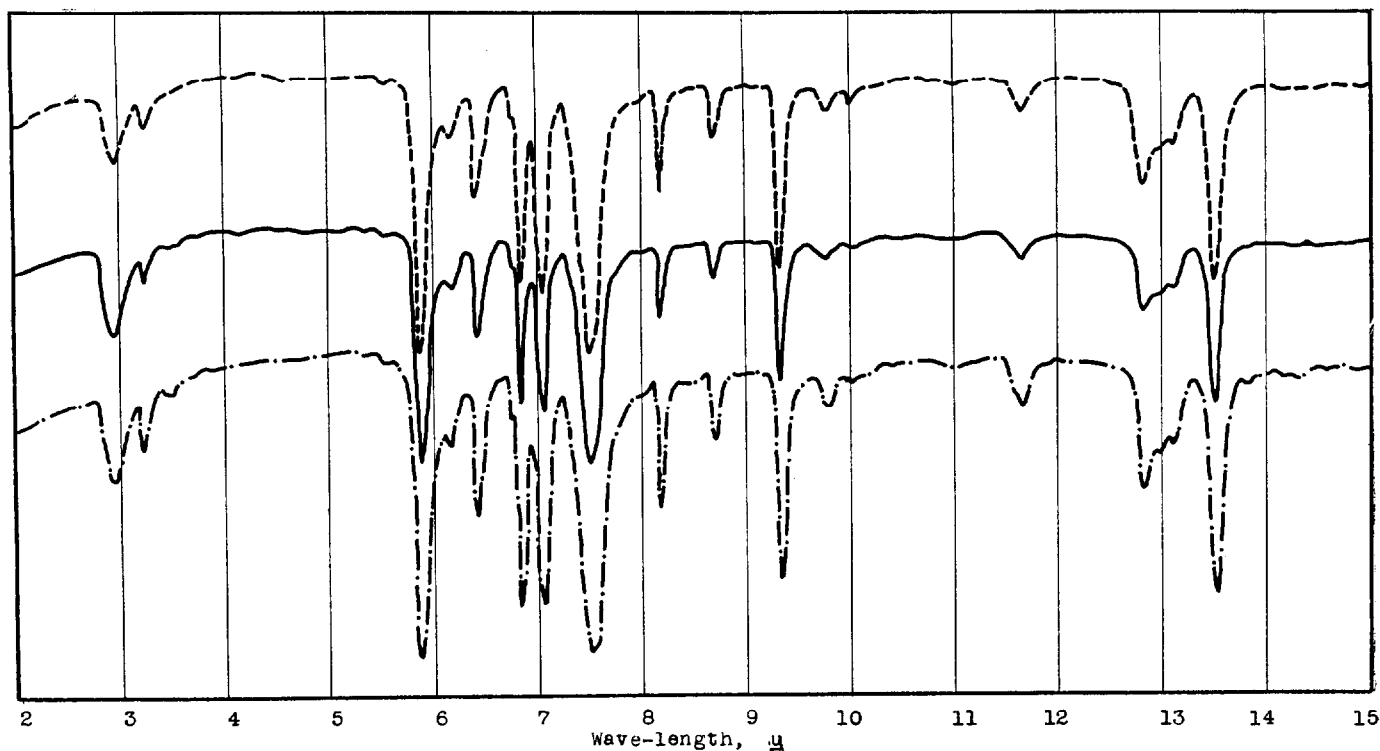


Figure 2. Infrared Absorption Spectra for Pyrocoll (— isolated from smoke; --- synthetic; -•-•- from the pyrolysis of gelatin).

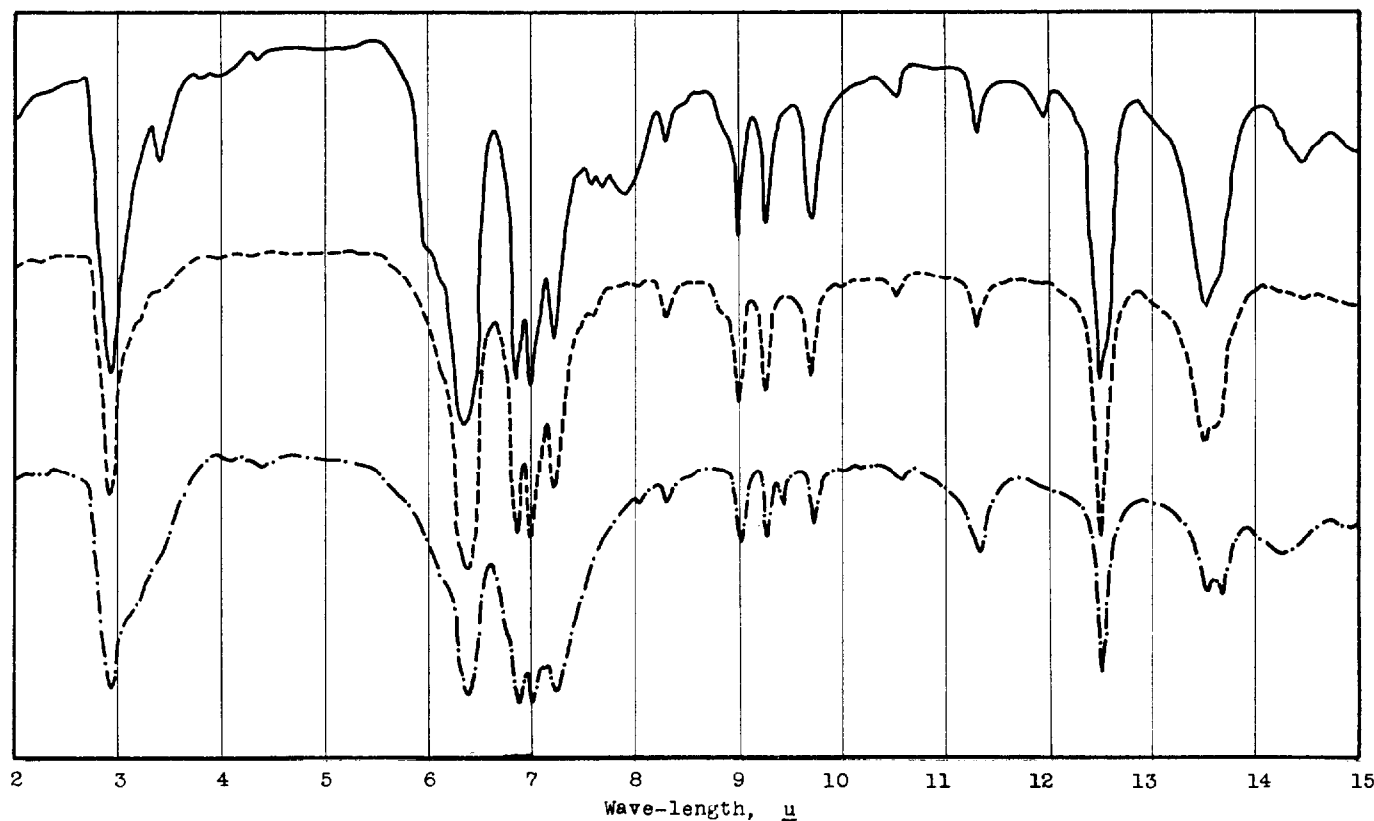


Figure 3. Infrared Absorption Spectra of the Potassium Salt of Pyrrole-2-Carboxylic Acid (— from alkali treatment of pyrocoll from smoke; --- from alkali treatment of pyrrole-2-carboxylic acid; -•-•- synthetic potassium pyrrole-2-carboxylate).

Experimental

Isolation of Pyrocoll from Cigarette Smoke.—Three hundred and sixty-six thousand regular length

(70 mm) cigarettes of a cased commercial blend of flue-cured, burley, Maryland and Turkish tobaccos were smoked mechanically on a constant

puff-volume type cigarette smoking machine (E. J. Hackney, Liggett and Myers Tobacco Co., unpublished). Thirty-five milliliter puffs, each of

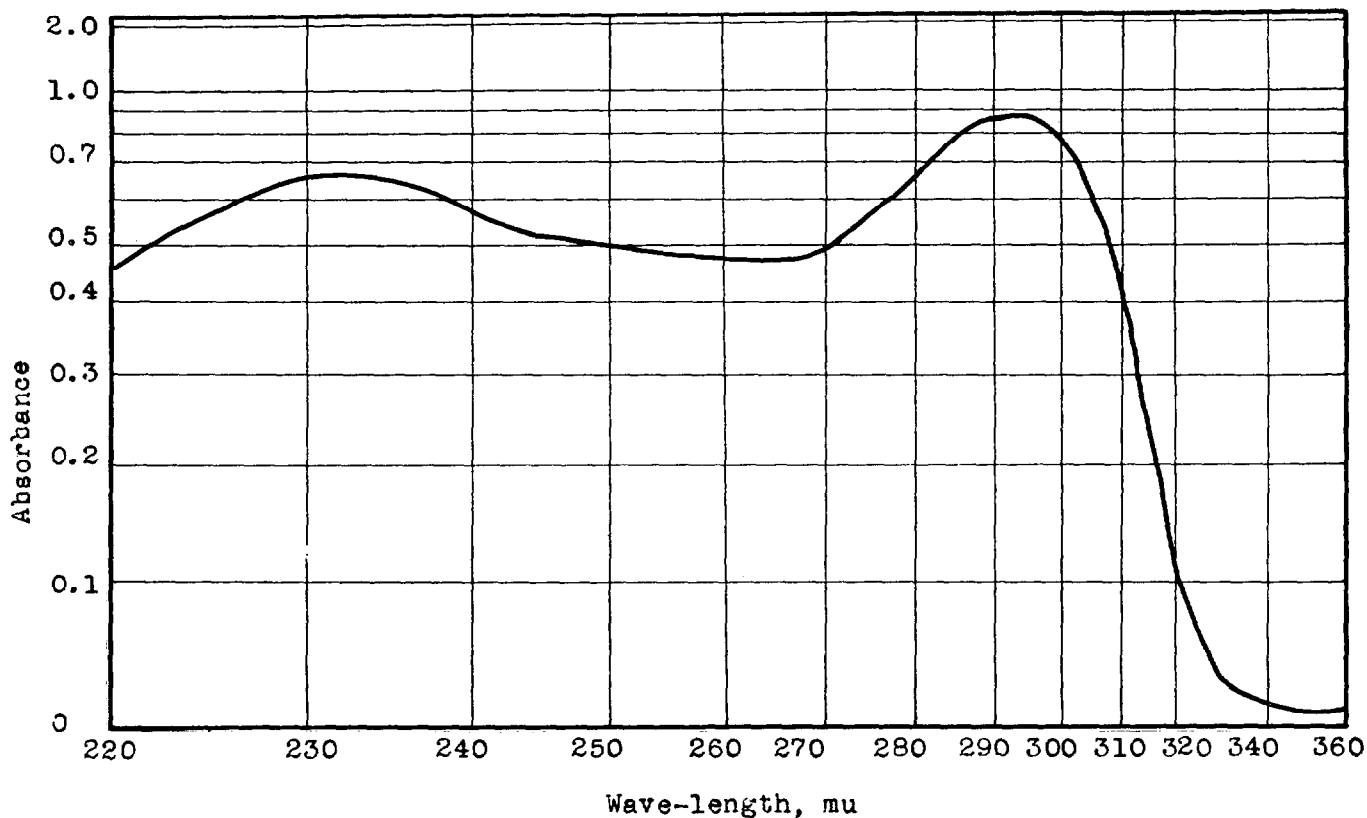


Figure 4. Ultraviolet Absorption Spectrum of N-(Pyrrole-2'-carboxy)-pyrrole-2-carboxylic acid in Absolute Ethanol. Max. at 294 (4.27), 232 (4.15).

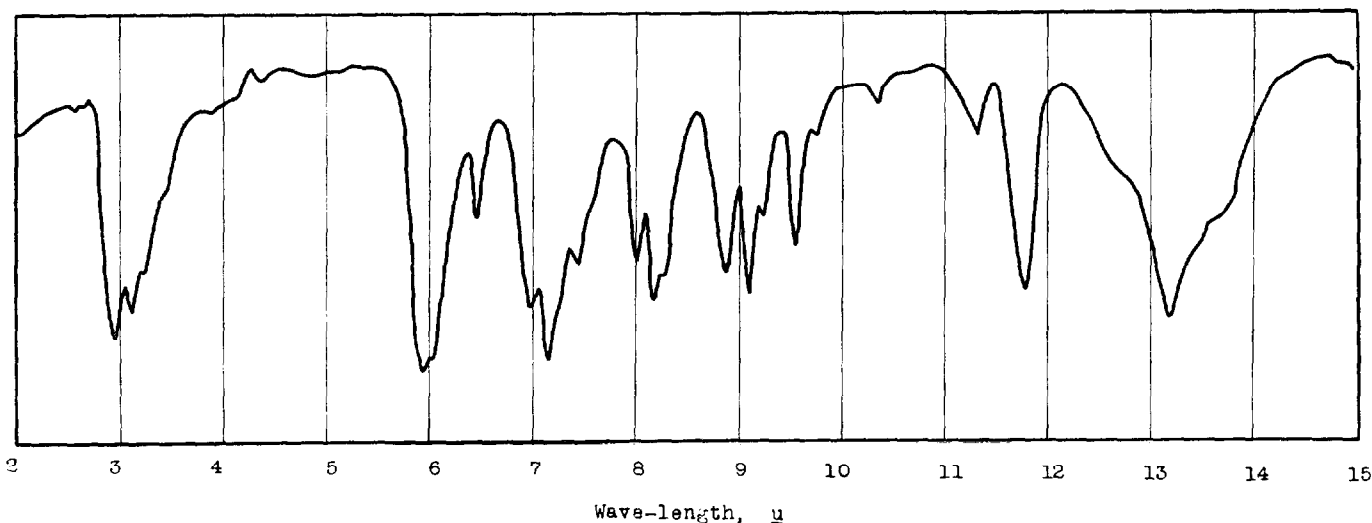


Figure 5. Infrared Absorption Spectrum for N-(Pyrrole-2'-carboxy)-pyrrole-2-carboxylic Acid.

two seconds duration, were taken at one minute intervals from each cigarette. Eight puffs were required to smoke each cigarette to an average butt length of 30 mm. The smoke was condensed in a series of three all-glass traps cooled in liquid air. The condensate was rinsed out of the traps with ethyl ether, water and hexane. A total yield of 20.9 g of condensate, non-volatile at 25°C and 25 mm Hg, was obtained, on an

average, from 1000 cigarettes. The solvents were combined, concentrated to remove the ether, and the volume adjusted to 200 ml each of water and hexane per thousand cigarettes. Each 200 ml layer was then washed in a countercurrent fashion with three 50-ml portions of hexane and water. The filtered hexane layers were combined, and the solution evaporated *in vacuo* at less than 50° to a concentration of 200 mg per

ml. This solution, containing 7.25 g. of total solids per thousand cigarettes, was extracted ten times with volumes of 84% (W/W) methanol (aq) equal to the volume of hexane solution. The aqueous methanol was equilibrated with hexane prior to its use.

The combined aqueous methanol extracts, containing 3.26 g of non-volatile materials per thousand cigarettes, were concentrated *in vacuo*

at 50° to remove the methanol, and the aqueous suspension remaining was then extracted with ether until the final ether extract was nearly colorless. The ether extracts were evaporated to dryness. The residue was redissolved in acetone and evaporated onto an eight-fold (W/W) portion of alumina (100-200 mesh, Merck acid-washed, activated at 110° for 16 hrs.). Final traces of acetone were removed by drying *in vacuo* over CaCl₂ overnight. The dried material was applied as a slurry in hexane to the top of a column prepared with an additional amount of hexane-wet alumina, twenty times the weight of the total solids in the fraction to be chromatographed. Elution was carried out with ten ml of benzene per g of alumina. The solvent was evaporated *in vacuo* to yield 0.39 g of solids per thousand cigarettes.

The residue was next distilled at 50° for 24 hrs. at 0.1-0.3 mm Hg, 18 hrs. at 0.1-0.2 mm Hg, and 24 hrs. at 1-35 microns Hg, in a short-path (ca 2 cm) distillation device. Ethylene glycol chilled mechanically to -10° was circulated through the condenser. The pot was maintained at 50° by circulating the appropriate mixture of hot and cold water. Thirty-five percent of the recovered solids was found in the first distillate, 7.0% in the second, and 7.2% in the third. The majority of each of these fractions was readily soluble in acetone or cyclohexane; however, a small portion of crystalline material failed to dissolve completely in either solvent. This was present to the greatest extent in the second distillate but was also found in the first and third distillates. Successive crops of the crystals were collected from cyclohexane solution. The combined crude material was recrystallized once from ethyl acetate and once from benzene. It was then decolorized with active charcoal and sublimed *in vacuo* at 135°. The sublimate was recrystallized finally from benzene and dried at 25 mm Hg at 45° for analysis. The pale yellow crystalline product melted at 272.5° (corr) in a sealed tube. It sublimed too readily to permit an accurate determination in an open tube. The melting point for an authentic sample of pyrocoll was 272.5° (corr) and a mixture of the two showed no depression in the melting point. The molecular weight was determined by measurement of the diffusion rate in benzene through a sintered glass disc.¹

¹ The analysis was performed by R. J. Randall according to the technique described by R. J. Randall, W. W. Bates and J. F. Williams at the Southeastern Regional Meeting of the American Chemical Society, Durham, N. C., Nov. 14-16, 1957.

Anal. Calcd. for C₁₀H₆N₂O₂: C, 64.51; H, 3.25; N, 15.05; mol. wt., 186.2. Found: C, 64.52, 64.68; H, 3.21, 3.31; N, 14.78; mol. wt., 189.

Pyrocoll is soluble in water to the extent of about 0.0026 mg/ml at 25°. It is not very soluble in acetone or cyclohexane (0.072 mg/ml cyclohexane at 25°) but is somewhat more soluble in ethyl alcohol (0.15 mg/ml), ethyl acetate, or benzene. The ultraviolet absorption spectrum, determined in water, gave maxima at 322 (log_ε 4.18), 308 (log_ε 4.14), 276.7 (log_ε 4.20) and 235.7 mμ (log_ε 4.42). In 95% ethanol the corresponding maxima and intensities were: 316.0 (4.19), 303.2 (4.16), 273.0 (4.26), 235.7 (4.49). The spectrum in cyclohexane is given in Figure 1.² The infrared spectrum is given in Figure 2.³

On the basis of recovered pure pyrocoll and ultraviolet absorption intensities for the less pure fractions, a total of 298 mg was present in the distillates from the smoke condensate representative of 366,000 cigarettes (0.81 mg per 1000 cigarettes). A determination was carried out with a smaller number of cigarettes (33,000) in which 15 mg of pyrocoll was isolated from the distillates (0.45 mg per 1000 cigarettes) and an additional 30 mg from the residue on further fractionation (0.91 mg per 1000 cigarettes) for a total of 1.4 mg per 1000 cigarettes. The amounts are, of course, minimum figures since the isolation techniques would not be expected to be quantitative. It is likely that additional material was also present in the distillation residue from the larger batch of smoke but this has not been determined.

Alkaline Hydrolysis of Pyrocoll Isolated from Cigarette Smoke.—(A) A 3.56-mg sample of pyrocoll, isolated from cigarette smoke, was dissolved in 25.0 ml of absolute ethanol. This solution was made 0.046 M in potassium hydroxide, the solution stoppered, and aliquots of 0.100 ml were withdrawn at intervals and diluted for the determination of the ultraviolet absorption spectrum. After 19 hours, the solution was neutralized by the addition of an equivalent amount of aqueous hydrobromic acid. The ultraviolet absorption maximum shifted slightly

² Ultraviolet absorption spectra were determined with a Warren Spectracord, Warren Electronics Co., Bound Brook, New Jersey.

³ Infrared absorption spectra were determined by E. Sivas using a Perkin-Elmer Model 21 infrared spectrophotometer with NaCl prism (Perkin-Elmer Corp., Norwalk, Conn.). Samples were ground or lyophilized with KBr and pressed into transparent discs for the measurement.

from 257 to 259.5 mμ upon neutralization, with no marked change in intensity (log_ε 4.12). An aliquot of this solution was mixed with potassium bromide, diluted with water, and lyophilized for the determination of the infrared spectrum in a potassium bromide pellet (Figure 3).

A sample of authentic pyrrole-2-carboxylic acid was dissolved in alcohol, 0.05 M in potassium hydroxide, and allowed to stand 24 hours at 25°. No change in the ultraviolet absorption spectrum was observed indicating the stability of this compound under these conditions. The pH of this solution was adjusted and the infrared absorption spectrum determined as described above. This is compared in Figure 3 with the infrared absorption spectrum of a sample of pyrrole-2-carboxylic acid to which an equivalent amount of potassium hydroxide has been added.

Pyrrole-2-carboxylic acid.—Pyrryl magnesium iodide was prepared and reacted with dry carbon dioxide gas according to the directions of McCay and Schmidt (1926) to yield the crude pyrrole-2-carboxylic acid. This was purified as described by Blicke and Blake (1930) and finally recrystallized from 95% ethanol and dried at 45° and 25 mm Hg. This material decomposed at 193.5-4.5° (corr) (lit m.p 192°; 196-7°; Maxim *et al*, 1938; Smissman *et al*, 1956).

Anal. Calcd. for C₅H₅NO₂: C, 54.05; H, 4.54; N, 12.61; equiv. wt. 111.1. Found: C, 54.02, 54.00; H, 4.62, 4.72; N, 12.85, 12.87; equiv. wt., 110.⁴

The absorption maximum at 262.2 mμ in 95% methanol had an intensity of log_ε 4.14. Andrisano and Pappalardo (1955) report a maximum at 258 mμ (log_ε 4.10) and Scrocco and Nicolaus (1957) 262 mμ (log_ε 4.08) for alcoholic solutions of the pyrrole-2-carboxylic acid. The infrared spectrum of the potassium salt is given in Figure 3.³

Pyrocoll (I). Pyrrole-2-carboxylic acid was heated for one hour at 135° with an excess of acetic anhydride, then distilled *in vacuo* to remove the excess reagent (Ciamician and Silber, 1884). A cold finger condenser was inserted into the flask and the temperature was increased to 165°, whereupon crude pyrocoll sublimed out of the mixture. A total of 20% of the theoretical yield was obtained. This crude product was recrystal-

⁴ The equivalent weight was determined by R. Doughton by potentiometric titration in pyridine with tetrabutylammonium hydroxide dissolved in benzene-methanol (9:1).

lized from ethyl acetate and from benzene. It was then treated with charcoal and crystallized again from benzene. After drying at 45° and 25 mm Hg it melted at 272.5° (corr). Weidel and Ciamician (1980) reported 268°.

Anal. Calcd. for $C_{10}H_6N_2O_2$: C, 64.51; H, 3.25; N, 15.05. Found: C, 64.82, 64.71; H, 3.27, 3.03; N, 14.92, 15.03.

The ultraviolet absorption spectrum is given in Figure 1.² The infrared absorption spectrum is given in Figure 2.³

Pyrocoll from the pyrolysis of gelatin. Seven grams of Knox unsweetened, unflavored gelatin was introduced into a Pyrex tube, preheated to 300°. A slow stream of nitrogen was introduced at one end of the tube to direct the flow of products. After two hours, a thick, dark brown syrup and yellow crystals had collected in the cooler portion of the tube. Heating was discontinued. The distilled tar and crystals were scraped from the tube and suspended in water. Extraction of the crude pyrocoll was accomplished with ethyl acetate. It was crystallized from benzene and ethyl acetate, decolorized with charcoal, sublimed at 135°, and the sublimate recrystallized from benzene. After drying at 45° and 25 mm Hg, the pale yellow crystals melted at 271° (corr). A mixture with pure synthetic pyrocoll melted at 271.5° (corr). The ultraviolet spectrum is given in Figure 1.² The infrared spectrum is given in Figure 2.³

N-(Pyrrole-2'-carboxy)-pyrrole-2-carboxylic acid (II).—Pyrocoll (40.2 mg 0.216 mmole) was dissolved in 275 ml of absolute ethanol. To this solution was added 2.75 ml of 4.4 N KOH (12.1 mmole) to give a final concentration of 0.044 N in alkali. The solution was mixed thoroughly, and within one minute, an equivalent amount of 0.25 N HBr was added to prevent further hydrolysis from occurring. The alcohol was evaporated *in vacuo* and the aqueous solution was extracted three times with redistilled ethyl acetate. The ethyl acetate extracts were washed five times with water, the solvent evaporated, and the residue crystallized from a benzene-methanol mixture after decolorizing with a small amount of active charcoal. The crude product was further purified by chromatography on silica gel (100-200 mesh, containing 6% H_2O) with 20% ether in hexane. Traces of pyrocoll were eluted first. Its elution can be easily followed by its pale green-blue fluorescence under ultraviolet light. Pyrrole-2-carboxylic acid

was eluted next. This material was associated with a yellow band on the column when illuminated with ultraviolet light. Following this, the desired product was eluted. Recrystallization from benzene-methanol gave white crystals of N-(pyrrole-2'-carboxy)-pyrrole-2-carboxylic acid melting at 162.5-3.5°C (corr). Aqueous titration indicated an equivalent weight of 199 (theoretical for $C_{10}H_8N_2O_3$, 204.2). The ultraviolet absorption spectrum is given in Figure 4.² The infrared absorption spectrum is given in Figure 5.³ The relative instability of this compound made it necessary that analyses be performed shortly after the sample had been purified. Two samples of identical melting point and absorption spectra gave erratic values when sent out for carbon, hydrogen and nitrogen analysis.

Summary

Pyrocoll (I), the diketopiperazine derivative of pyrrole-2-carboxylic acid, has been found in cigarette smoke to the extent of 1.4 mgm per thousand cigarettes. Proline present in the tobacco, free and combined, has been proposed as a possible precursor. The open-chain dipeptide of pyrrole-2-carboxylic acid, N-(pyrrole-2'-carboxy)-pyrrole-2-carboxylic acid (II), has been prepared and partially characterized.

Acknowledgment

Analyses for carbon, hydrogen, and nitrogen were performed by Schwarzkopf Microanalytical Laboratory, Woodside 77, New York.

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Reprinted from 'Tobacco' New York, Vol. 151, No. 1, pps. 22-28 (Tobacco Science, Vol. 4, pps. 130-136) July 1, 1960