EXPERIMENTAL[5]

5-Bromo-1-hydroxy-1,2,3-benzotriazole. To a solution of 2-nitro-5-bromophenylhydrazine (0.5 g.) in ethanol (20 ml.) was added hydrazine hydrate (2 ml. 50%). It was heated on a water bath for 0.5 hr., concentrated to a small volume, diluted with water and filtered. The filtrate on acidification with dilute hydrochloric acid gave 5-bromo-1-hydroxy-1,2,3-benzotriazole (0.3 g.) as colorless plates from ethanol, m.p. 220° dec. By adopting a similar procedure other hydroxybenzotriazoles were prepared. The data concerning the new compounds are listed in Table I. All of them explode above their melting points.

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(5) All melting points are uncorrected.

Identification of Caffeic Acid in Cigarette Smoke

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No previous report has been made of the presence of caffeic acid (3,4-dihydroxycinnamic acid) in cigarette smoke. Several groups of workers[1-3] however, have reported finding free caffeic acid in various cured tobaccos, but Roberts and Wood,[4] using fresh cigar tobacco, and Weaving,[5] using flue-cured tobaccos, could find none in their samples. Dieterman *et al.*[6] have recently pointed out that esculetin (6,7-dihydroxycoumarin) in tobacco may often be confused on paper chromatograms with caffeic acid. In the present study on tobacco in eight brands of cigarettes commonly smoked in the U. S., every sample tested was found to contain free caffeic acid. Also, in every case, the main stream smoke from the cigarette contained free caffeic acid.

In the purification of scopoletin (6-methoxy, 7-hydroxycoumarin) from cigarette smoke and from various tobacco extracts,[7,8] two or more interfering blue fluorescing compounds persisted with the scopoletin through several developments of paper chromatograms. Dieterman *et al.*[6] identified one of these interfering compounds as esculetin. The present identification establishes free caffeic acid as the other blue fluorescing compound.

During paper chromatography in certain acid solvent systems, such as 15% acetic acid–water, caffeic acid appears as two distinct zones. These have been shown to be *cis*- and *trans*- caffeic acid.

EXPERIMENTAL

Caffeic acid from cigarette tobacco. The tobacco obtained from one hundred and twenty cigarettes (three each from forty packs of the same brand) was mixed and ground to a powder. Six 5.5-g. samples of this powder were thoroughly extracted with 85% isopropyl alcohol, as previously described by Yang *et al.*[7] The combined extracts were concentrated under reduced pressure, and the concentrate was then subjected to separation by mass paper chromatography.[7] After the initial chromatography with Whatman 3MM paper, using the solvent system *n*-butyl alcohol–acetic acid–water (6:1:2 v./v.), each zone containing caffeic acid, still mixed with some esculetin and scopoletin, was cut off and then eluted with methanol. The eluates were combined and streaked on S & S No. 589, Red Ribbon, chromatography paper, and developed in the system chloroform–acetic acid–water (2:1:1 v./v., bottom layer). This solvent system proved to be superior to the nitromethane-benzene-water system (2:3:5 v./v., upper layer) used in our previous studies on scopoletin and esculetin. In the chloroform system, the scopoletin (R_f = 0.75) moves in a narrow zone quite removed from those of esculetin (R_f = 0.39) and of caffeic acid (R_f = 0.35). This was also the case with the benzene–propionic acid–water system (2:2:1 v./v., top layer) with R_f values: scopoletin (0.66); caffeic acid (0.32); and esculetin (0.26). The two top zones resulting from paper chromatography with the chloroform system contained primarily caffeic acid and esculetin. They were cut off from each chromatogram together; sewn onto a new sheet of paper; and then developed in ethyl acetate–formic acid–water (10:2:3 v./v.). Each zone containing caffeic acid, with a trace of esculetin still present, was cut off and eluted with the ethyl acetate solvent system. The eluates were combined and again streaked on S & S No. 589 paper and developed in 15% acetic acid–water. Although separation of caffeic acid from esculetin was completed by this chromatography with acetic acid, an isomer of caffeic acid now appeared as a separate, third zone.

The two zones of isomeric caffeic acid were cut from each chromatogram as a unit and sewn onto a new sheet of chromatography paper. Each such sheet was then developed in the ethyl acetate system to obtain one narrow blue zone for identification studies.

Identification of caffeic acid. The combined eluates containing the purified caffeic acid from each single zone obtained in the ethyl acetate system were then co-chromatographed with authentic caffeic acid purchased from California Foundation for Biochemical Research, using the *n*-butyl alcohol–acetic acid–water, chloroform–acetic acid–water, ethyl acetate–formic acid–water, benzene–propionic acid–water, and nitromethane-benzene-water systems already described, and *n*-butyl alcohol–benzene–pyridine–water (5:1:3:3 v./v., upper layer), isopropyl alcohol–pyridine–acetic acid–water (8:8:1:2 v./v.), and 15% acetic

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acid–water. Both the reference and isolated caffeic acid solutions gave the same R_f values in every test. In the 15% acetic acid system, both the reference and unknown caffeic acid samples gave two zones each, with corresponding R_f values.

The isolated and reference caffeic acids behaved similarly towards the chromogenic agents previously reported.[6] In addition, both gave the same color reaction with the Höfner reagent[4] (pink, changing to yellowish-brown) and with 2% alcoholic ferric chloride solution (green changing to gray).

The absorption spectra exhibited by the isolated caffeic acid in ethanol, and in buffer solutions at *p*H 3.5 and 6.8, checked in each case with the corresponding spectrum exhibited by the reference caffeic acid in ethanol and in buffer solutions at *p*H 3.5 and 6.8 in our laboratory and with those reported for these preparations by Sutherland.[9]

Caffeic acid in the mainstream smoke of cigarettes. The smoking of eight brands of cigarettes for caffeic acid analysis was performed by a procedure similar to the one already described for scopoletin in smoke by Yang *et al.*[8] Because caffeic acid, however, was present only in a trace amount in the smoke, samples representing smoke from forty packs of cigarettes were combined and concentrated to obtain sufficient caffeic acid for unambiguous studies by paper chromatography. The separation, purification, and identification of caffeic acid from the cigarette smoke condensates were carried out by mass paper chromatography in the same manner as already described above for determination of caffeic acid in tobacco. Cigarettes analyzed included Camel, Lucky Strike, Philip Morris, Old Gold Straights, Pall Mall, Winston, Viceroy, and Oasis.

Isomerization of caffeic acid. On paper chromatography with 15% acetic acid–water, the reference caffeic acid gave two distinct zones. The farther moving zone (R_f = 0.50) was called "CA-1," and the slower moving zone (R_f = 0.42) was called "CA-2." Each blue fluorescing zone was cut out separately; sewn onto separate new sheets of paper; and again developed in the 15% acetic acid. It was observed that from the slower moving zone (CA-2), the faster moving zone (CA-1) was produced every time that a separated CA-2 zone was rechromatographed in this acid system. If this procedure, involving separation by paper chromatography, cutting, sewing, and rechromatography of the CA-2 was repeated even five or more times, the slower moving zone of caffeic acid, would in every case, continue to change to give both isomers. The fluorescence of this slower moving zone would be weaker on each subsequent chromatogram. The CA-1 zone likewise gave both isomers on rechromatography of the faster moving zones, but produced only a relatively small amount of the CA-2 each time that the CA-1 was developed in the 15% acetic acid–water.

Both CA-1 and CA-2 co-chromatographed with the reference caffeic acid to give only one spot in all the solvent systems mentioned in this paper, except in the 15% acetic acid–water. In this latter system, the major spot from the reference caffeic acid on the first chromatograms was identical with CA-2, and the minor spot was the same as CA-1. Both CA-1 and CA-2 gave the same color reactions when tested with all the chromogenic agents described in this report.

Williams[10] has reported that cinnamic acid derivatives give two spots on paper chromatography with 2% acetic acid–water. He suggested that this was a case of *cis-trans* isomerization on paper. He did not, however, point out which spot corresponded to which isomer. Recently, Butler and Siegelman[11] have reported that the faster moving zone of caffeic acid during paper chromatography with 5% acetic acid–water is *cis*-, and the slower moving zone is *trans*-caffeic acid, on the basis that ultraviolet light converted a part of the slower moving zone into the faster moving one. Our slower moving zone behaved similarly, and based on their conclusions, it would appear that our CA-1 is *cis*- and our CA-2 is *trans*-caffeic acid. For further confirmation of these *cis* and *trans* configuration assignments to CA-1 and CA-2, we undertook ultraviolet and infrared spectral studies as described in following paragraphs.

Ultraviolet absorption spectra of the caffeic acids. Although the isomeric caffeic acids CA-1 and CA-2 could be readily obtained as completely separated zones on paper chromatograms, much difficulty was experienced in getting solutions of either isomer completely free of the other. During the preparation of such solutions by extraction or elution of the individual zones from the paper, and application of heat, isomerization was usually found to occur, and an equilibrium mixture was set up according to the temperature, solvent, etc. used. During such handling, except for the paper chromatography step itself, the CA-1 (*cis*) shifted more readily into the CA-2 (*trans*) than did CA-2 to CA-1. For the ultraviolet spectrophotometry, a solution consisting mainly of isomer CA-1 (but not entirely free of the other isomer) and another preparation consisting mainly of CA-2 were prepared as described in the next paragraph.

Each isomeric zone was cut from the chromatogram separately and eluted with 95% ethanol in an elution chamber. Each eluate was then evaporated to dryness, *in vacuo*, without application of heat. The residue, containing the caffeic acid isomer plus a filter paper impurity, was then dissolved by 1 ml. of hot distilled water, added drop by drop, while the container was kept rotating. A blank solution containing the filter paper impurity, but no caffeic acid, was prepared in exactly the same manner as just described, except that no caffeic acid was present on the sheet of chromatography paper. The aqueous solution of each isomer was then added to cold distilled water in separate cuvettes, drop by drop, with a capillary tube. To the cuvette used as a blank, approximately an equal amount of the blank solution containing the filter paper impurity, but no caffeic acid, was added. The absorption spectra of these CA-1 and CA-2 solutions were then measured with the Beckman spectrophotometer, Model DU. Results are shown in Fig. 1. The CA-1 preparation exhibited its high maximum

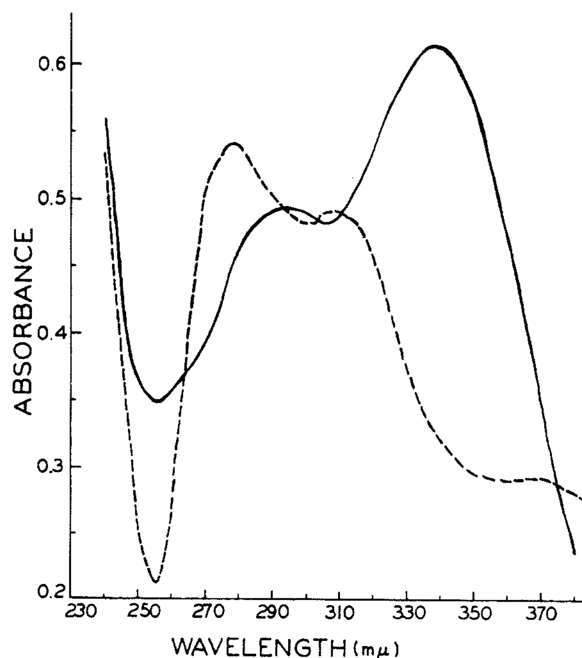

Fig. 1. Absorption spectra of aqueous solutions of caffeic acid prepared from zones CA-1 (- - - -) and CA-2 (———).

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at 278 mμ, whereas the CA-2 preparation had an even higher maximum at 340 mμ. Mixing of various amounts of CA-1 and CA-2 shifted the 340 mμ maximum of CA-2 to various corresponding lower wave lengths.

To interpret these results, one uses the working rule which states that when the absorption properties of the *cis-trans* isomers of a substance differ, "the more elongated isomer absorbs at somewhat longer wave lengths and more intensely."[12]

Haskins and Gorz[13] recently have found that such absorption data apply in their studies on *cis-* and *trans-o*-cinnamic acid. If this rule should also hold with caffeic acid, CA-1 would then appear to be the *cis* isomer and CA-2 the *trans* isomer of caffeic acid. These assignments of *cis* and *trans* to the caffeic acid isomers check with the designations in above paragraphs.

Infrared absorption spectra of the caffeic acids. To prepare samples of CA-1 and CA-2 for infrared studies, caffeic acid solution was streaked onto S & S No. 589 paper and developed in 15% acetic acid-water. The CA-1 and CA-2 zones were cut out and separately eluted with methyl alcohol. The eluate containing CA-1 was extracted with *n*-hexane, which is supposed to favor solution of the *cis* isomer.[14] The hexane was removed *in vacuo* at room temperature in the dark, and crystals of CA-1 were obtained. The methyl alcohol eluate CA-2 was concentrated *in vacuo* almost to dryness, in the dark at room temperature, and the residue was extracted several times with ethyl ether. Crystals of CA-2 were obtained after evaporation of the ether. Two milligrams of each of the crystalline CA-1 and CA-2 were mixed with 400 mg. of potassium bromide and made into pellets. These were studied with the Perkin-Elmer recording infrared spectrophotometer, Model 21.

At 814 cm.$^{-1}$, the absorption of the compound from CA-2 (*trans*) showed stronger intensity than did the absorption from compound CA-1 (*cis*). Bellamy[15] states that conjugation of the double bond with carbonyl groups has a very marked effect, and that the group —CH=CHCOOR (*cis*) absorbs near 820 cm.$^{-1}$ with sufficient regularity for this to be a useful assignment. He continues by stating that this absorption from the *cis* form is usually much weaker in intensity than that from the *trans* series. Also, at 1640 cm.$^{-1}$, CA-2 showed stronger absorption than did CA-1. Thus, the infrared data confirmed the previous indications that the CA-2 fraction was primarily the *trans* isomer, and the CA-1 fraction was mainly the *cis* isomer of caffeic acid.

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Halogenation of Glycoluril and Diureidopentane

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The literature reveals the preparation of 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (I),[1,2] 1,3,4,6-tetrachloro-3a,6a-dimethylglycoluril (II),[2,3] and of 1,3,4,6-tetrachloro-3a-methyl-6a-phenylglycoluril (III)[2] but does not disclose 1,3,4,6-tetrachloroglycoluril (IV). This paper deals with the preparation of IV and some related compounds.

```
          R
          |
X—N———C———N—X
  |       |       |
O=C   (CH2)n   C=O
  |       |       |
X—N———C———N—X
          |
          R'
```

	R	R'	X	n
I	C_6H_5	C_6H_5	Cl	0
II	CH_3	CH_3	Cl	0
III	CH_3	C_6H_5	Cl	0
IV	H	H	Cl	0
V	H	H	Br	0
VI	H	H	I	0
VII	H	H	H	0
IX	CH_3	CH_3	H	1
X	CH_3	CH_3	Cl	1

We found that chlorination of aqueous suspensions of glycoluril (VII),[4–6] under a variety of conditions, gave IV. Excellent yields were obtained when the chlorination mixture was kept neutral or slightly alkaline (*p*H 7–8) by the addition of various basic materials either as solids or as solutions. Although a wide variety of alkaline materials was successfully used, a 1 to 6*N* sodium hydroxide solution was the most convenient alkali to add.

Bromination of glycoluril to 1,3,4,6-tetrabromoglycoluril (V) required somewhat more alkaline conditions (*p*H 8–11). The use of analogous techniques failed to give tetraiodoglycoluril (VI).

A clear solution resulted on treatment of an aqueous suspension of VII with half the theoretical amount of chlorine required for the preparation of IV. Further chlorination of this solution caused the precipitation of IV. Concentration of the clear solution resulted in the isolation of a dichloroglycoluril (VIII). No attempt was made to separate or characterize the possible isomers.

No material corresponding to a mono- or a trichloroglycoluril was found. Chlorination of VII to a theoretical trichloroglycoluril stage gave a solid which was readily separated into IV and VIII by extraction with water. The water solubility, at

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