bach and Wald (*2*), however, have noted a blue-to-red change, using a Jena BG12 filter. It may be that the particular wavelength composition of the stimulus is more critical in the blue end of the spectrum. Our failure to confirm their observation may thus be a result of our not having that particular filter available.

The described color changes may be accounted for by photochemical adaptation, if it is assumed that there are at least two photopigments in the human retina, a "red" and a "green," with overlapping absorption spectra, and that the rate of regeneration of the "green" pigment is slightly greater than that of the "red." Equations basically derived from this hypothesis yield good qualitative agreement with our data. The verification of the hypothesis, however, must await the results of more extensive experiments.

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3. In preliminary experiments, subjects were simply instructed to report any color changes that occurred. All subjects reported the changes to yellow and green, in that order. However, the temporal variability of the judgments was smaller when the subjects were told, in advance, what color changes to expect.

3 July 1958

Low-Temperature Chromatography as a Means for Separating Terpene Hydrocarbons

Recently, a study of the citrus oils was undertaken in this laboratory for the purpose of determining flavor constituents. A gas-chromatographic apparatus was developed for the analysis of terpenes, but it was concluded that preliminary separations were necessary. Kirchner *et al.* (*1*) utilized various forms of silicic-acid chromatography for the separation of terpene hydrocarbons from the oxygenated constituents of citrus oils and for subsequent analysis of the oxygenated fraction. These procedures have been modified and utilized by other workers (*2*). In general, however, silicic-acid chromatography has been unsatisfactory for resolution of terpene hydrocarbons, which may constitute more than 90 percent of a citrus-peel oil.

It was concluded that the gas column would provide an excellent means for monitoring the effluents in a study of liquid-solid chromatographic procedures for separation of the terpenes. Since the results of the above-mentioned workers indicated slight differences in the migratory rates of certain terpene hydrocarbons on silicic acid, columns of various dimensions were prepared for a preliminary study. Synthetic terpene mixtures were then eluted with various organic solvents, and the effluents were analyzed by gas chromatography (as described below) to determine any tendencies toward resolution.

Table 1. Distribution of some terpene hydrocarbons in the effluent from a silicic-acid column.

Compound	Fraction
p-Menthane	3–4
α-Pinene	10–15
β-Pinene	16–21
d-Limonene	34–35

It was observed that results varied with pretreatment of the silicic acid. Silicic acid which was neither washed nor dried did not retain the hydrocarbons to any appreciable extent, and silicic acid which was dried at 105°C and stored over calcium chloride showed only a slight retentive capacity. Increased retention was observed when the silicic acid was washed free of fines (to facilitate packing), dried several hours at 150°C, and used immediately upon removal from the oven. Even after this process, however, the hydrocarbons moved rapidly and displayed only minor differences in migration rates.

On the assumption that migration would be retarded at lower temperatures, experiments were performed in which the columns were operated at the temperature of Dry Ice (−78.5°C). Results indicate that not only are the terpene hydrocarbons retarded at this temperature but that differential retarding occurs. As a result, complete resolution has been obtained in many instances. The example which follows suggests that the technique should be of considerable value in the study of terpenes, and perhaps in the study of hydrocarbons in general.

A special column was constructed for immersion in a vacuum flask, with details as shown in Fig. 1. Silicic acid (Mallinkrodt, 100-mesh) was washed free of fines by the method of Bulen *et al.* (*3*) and dried at 150°C for 48 hours or more. Approximately 5 g of the acid was cooled over calcium chloride (for from 2 to 3 minutes) and added to 50 ml of petroleum ether. A glass-wool plug was tamped into the lower end of the column, the column was half-filled with petroleum ether, and the silicic-acid slurry was added, to give a column 20 cm long. Pressure from a squeeze bulb was applied to the top of the column until no further packing occurred (a sufficient amount of solvent being added to maintain the level above the silicic acid). The column was then suspended in a bath of Dry Ice in acetone, with the level of the coolant well above that of the packing. After 1 to 2 hours, the terpene mixture (consisting of 30 mg each of *p*-menthane, α-pinene, β-pinene, and limonene in 0.5 ml of petroleum ether) was added to the top of the column, and pressure was applied until the solvent dropped to the level of the silicic acid. A glass-wool plug was inserted just above the packing, the solvent assembly was attached, and elution was started with petroleum ether. A flow rate of approximately 3 ml per hour was maintained, and fractions (2.5 ml) were collected manually. After collection of 30 fractions, the petroleum ether was withdrawn from the top of the column with a hypodermic syringe, and a 1:1 mixture of diethyl ether and petroleum ether was added (for elution of the limonene).

Gas chromatography of the fractions was performed with a laboratory-built instrument, in the construction of which a 10-ft column of Apiezon-L or C-22 firebrick, packed in ¼-in. stainless-steel tubing, was utilized. Helium was used as the carrier gas, and samples were injected by means of a Fisher injection assembly. A four-filament conductivity cell (Gow-Mac No. 9285) served as a detector, and the response was recorded on a 10-mv recorder (Varian G-11).

The procedure was as follows: The solvent was evaporated from each fraction at reduced pressure, and the residue

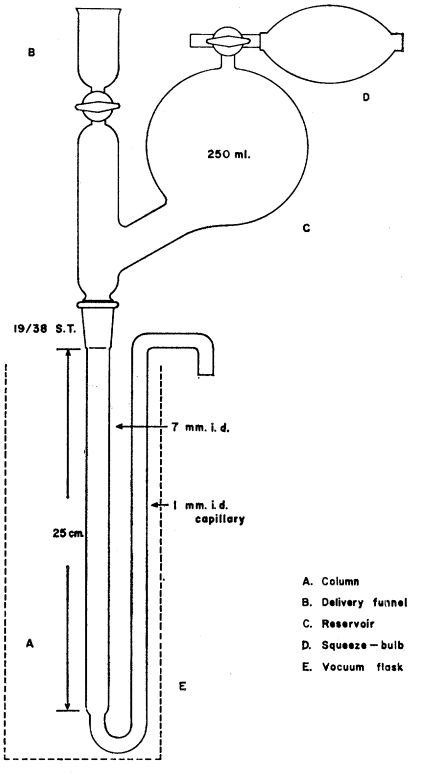

Fig. 1. Assembly for low-temperature chromatography.

was taken up in the injection pipet (0.01 ml). In those instances in which there was insufficient residue, petroleum ether was added to wash traces into the pipet. The sample was injected, with the column temperature maintained at 200°C and with a helium flow rate of 40 ml/min and a chart speed of 5 in. per hour. These conditions do not represent the optimum for resolution but were selected to provide characteristic but short retention times to facilitate rapid analyses.

Table 1 shows the distribution of the hydrocarbons in the effluent from the silica-gel column, as determined by retention times on the gas column. It may be noted that, under the conditions employed, the silicic-acid column provided complete resolution. These results have been verified by a number of replications. In addition, larger samples (0.2 g) have been separated by the same procedure. A mixture (0.15 g) containing 80 percent limonene, 10 percent α-pinene, and 10 percent β-pinene was resolved, indicating that high concentrations of limonene did not interfere with resolution of the more rapid moving components of the mixture.

It is highly improbable that the above-mentioned conditions represent the optimum for separation of the hydrocarbons studied. Moreover, it is quite likely that any procedure will be dictated by the particular mixture to be resolved. It is significant, however, that the pinenes were separated, since they differ only in the position of the double bond. The pronounced retention of limonene is of special interest where the citrus oils are concerned because of the occurrence of high concentrations of this compound in these oils.

It should be possible to adapt the procedure to a preparative scale by employing columns of larger diameter. Upon elution of the hydrocarbons, oxygenated components may be separated by continuation with suitable solvents. In addition, the low temperature should inhibit the chemical changes often observed in adsorption chromatography. In conjunction with gas chromatography the technique may be especially useful, since the gas column can provide additional resolution.

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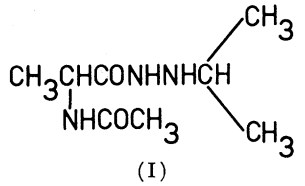
23 May 1958

Stereospecificity of Monoamine Oxidase Inhibitors

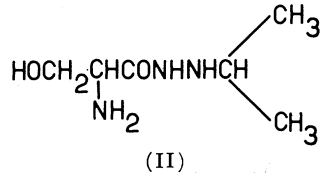
It has been found that isopropyl isonicotinic acid hydrazide (iproniazid, Marsilid) is a potent inhibitor of monoamine oxidase in vitro and in vivo. The alkylhydrazide group seems to be the active principle. Isonicotinic acid hydrazide (isoniazid, Rimifon), a compound similar to iproniazid but not containing the isopropyl group, is only a weak monoamine oxidase inhibitor. Furthermore, according to previous workers and to our own findings, N-isopropylhydrazine inhibits monoamine oxidase more markedly than iproniazid does (*1*).

In order to decrease toxicity and to increase specificity of monoamine oxidase inhibitors in vivo, isopropylhydrazides of substances which occur naturally in the body—for example, the amino acids alanine (acetate)



(I)

and serine



(II)

were synthesized (*2*). It was observed that the derivatives of the *l*-amino acids behaved differently from those of the unnatural *d* isomers.

After administration of the *l* forms of I and II, a considerable increase in 5-hydroxytryptamine (5HT, serotonin) content occurred in the brain of rats, as measured by a spectrophotofluorometric method (*3*). The rise was much more marked than after equimolecular doses of iproniazid. The *d* forms of I and II had significantly less effect on the 5-hydroxytryptamine content of brain than the *l* forms ($p < 0.01$). In fact, with the *d* form of I, no significant increase in 5-hydroxytryptamine could be observed at all (see Table 1).

In vitro, there was also a significant difference between the *d*- and *l*-amino acid hydrazides ($p < 0.01$). However, no correlation could be found between the monoamine oxidase inhibition in vivo (as measured by the rise in 5-hydroxytryptamine in the brain) and in vitro (as measured in mitochondrial suspensions and supernatant). In fact, the *l* forms of I (*4*) and II showed markedly more activity in vivo than iproniazid did. In in vitro studies this was not the case. Thus, the *l* form of I caused significantly less monoamine oxidase inhibition in mitochondrial suspensions and liver supernatant than iproniazid ($p < 0.01$) did. The difference in activity between the *l* form of II and iproniazid was not significant ($p > 0.05$).

These results show that the steric configuration of the acyl moiety is an essential factor in the activity of the above-mentioned isopropylhydrazides on monoamine oxidase in vivo and in vitro. A similar stereochemical difference has been found for amino acid hydrazides without substitution in N_2, with respect to action on diamine oxidase preparations (*5*). This stereospecificity may be partly due to the fact that both amine oxidases contain an optically active center, too. The higher relative activity of isopropylhydrazides of natural amino acids, especially *l*-alanine, in vivo as compared to in vitro (see Table 1), is probably due to an additional factor. It

Table 1. Monoamine oxidase inhibition by amino acid hydrazides as compared with iproniazid.

Inhibitor	Rise of 5HT in rat brain (%) in vivo;* (iproniazid = 100 ±10)	Inhibition of monoamine oxidase (%) in vitro;† (iproniazid = 100 ± 10)	
		Mitochondria (rat brain)	Supernatant (guinea pig liver)
l-I	195 ± 10	55 ± 6	67 ± 11
d-I	6 ± 4	15 ± 8	15 ± 11
l-II	200 ± 9	142 ± 20	106 ± 12
d-II	144 ± 15	80 ± 14	76 ± 8

* Iproniazid in the amount of 100 mg/kg was injected intraperitoneally 16 hr prior to 5HT determination. † Brain and liver were homogenized in 0.25*M* sucrose. The mitochondria were isolated by centrifugation at 500*g* for 5 minutes and subsequently at 23,000*g* for 15 minutes, resuspended in 0.06*M* phosphate buffer (*p*H 7.3), and frozen. The supernatant was diluted 1/1 with 0.1*M* phosphate buffer (*p*H 7.3). The brain mitochondria as well as the liver supernatant were aged for 1½ hours at 37°C. Oxygen consumption was measured, tyramine (3×10^{-3} mole/liter) being used as substrate. Concentration of the inhibitors in the supernatant and of the mitochondrial suspensions were 5×10^{-5} and $3.3 \times ^{-5}$ mole/liter, respectively. At this concentration iproniazid caused monoamine oxidase inhibition of about 50 percent. The enzyme preparations were preincubated with the inhibitors for 15 minutes prior to dipping in the substrate.