GC Determination of Tolazamide in Plasma

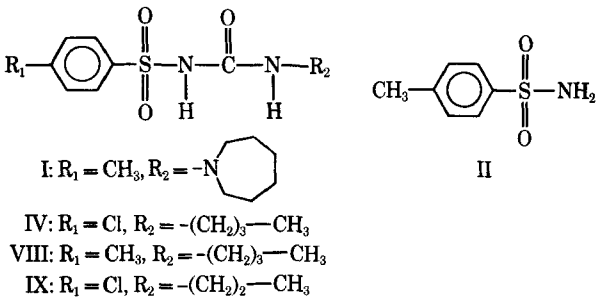
J. A. F. WICKRAMASINGHE* and S. R. SHAW

Abstract □ A highly specific, sensitive, GC method for the determination of tolazamide is described. The lower limit of detection sensitivity was 0.048 mcg. tolazamide "on column" or 0.7 mcg. tolazamide/ml. plasma. The chromatographic species was identified as *p*-toluenesulfonamide by GC–mass spectrometry. The method is based upon quantification of the sulfonamide. The utility of the procedure was demonstrated by its application to the determination of tolazamide in the plasma of drug-treated guinea pigs.

Keyphrases □ Tolazamide—GLC analysis, guinea pig plasma □ GLC—analysis, tolazamide in plasma

The sulfonylureas are widely used as oral hypoglycemic agents in the therapy of diabetes. Studies on the duration of drug action necessitate highly sensitive and specific analytical methodology for the determination of blood levels of the circulating drug. The need for such methodology becomes particularly important in the blood level determination of the more potent antidiabetic agents, *e.g.*, tolazamide[1] (I), encountered in biological samples at relatively lower concentrations. Most analytical procedures described in the literature (1–3) are colorimetric or spectrophotometric methods which lack high sensitivity and/or specificity. A GC method for the determination of tolbutamide and chlorpropamide was reported recently (4) (see *Results and Discussion* section).

The present article describes a specific and sensitive GC method for the determination of tolazamide (I).



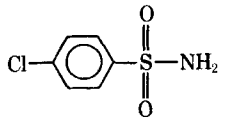
I: $R_1 = CH_3$, $R_2 = -N$

II

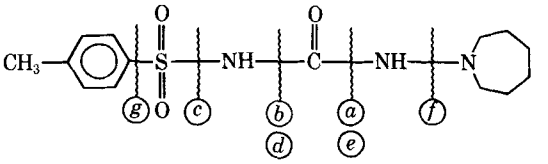
IV: $R_1 = Cl$, $R_2 = -(CH_2)_3-CH_3$

VIII: $R_1 = CH_3$, $R_2 = -(CH_2)_3-CH_3$

IX: $R_1 = Cl$, $R_2 = -(CH_2)_2-CH_3$



III



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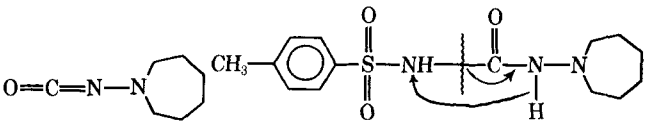

VI VII

EXPERIMENTAL

Biological—*Animals*—Male, Kuiper strain, albino guinea pigs (600–700 g. body weight) were used in the study. The animals were housed in holding cages. The guinea pigs were fasted for 16 hr. prior to dosing.

Dosing—Tolazamide was suspended in the sterile vehicle No. 100[2] and administered orally as a single dose of 15 mg./kg. body weight. Control animals received appropriate volumes (about 0.5 ml.) of the diluent by the same route. All animals were fasted for an additional 4 hr. after dosing. Food was then returned. The animals had access to water *ad libitum* at all times.

Blood and Urine Collection—Blood samples were drawn by heart puncture from the mildly anesthetized (ether) guinea pigs at 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 14, 24, 36, and 48 hr. One guinea pig per time interval was sacrificed. The blood samples, collected in heparinized tubes, were centrifuged for 10 min. at 2000 r.p.m. The plasma was harvested and stored at −18°.

Preparation and Extraction of Plasma Standards and Specimens from Dosed Animals—*Chloroform Standards*—Aliquots of a standard solution of tolazamide in chloroform containing 1.0, 2.0, 3.0, 4.0, 5.0, 7.0, 9.0, 10.0, and 12.0 mcg. were placed in glass-stoppered, 15-ml. centrifuge tubes. Aliquots (0.1 ml.) of the internal standard solution, containing 12 mcg. of IV[3], were added to each tube. All of the samples were evaporated to dryness under a gentle stream of nitrogen. Each sample was then reconstituted in 20 μl. of chloroform and analyzed by GC in the manner described later.

Guinea Pig Plasma Standards—Aliquots of a standard solution of tolazamide in chloroform containing 1.0, 2.0, 3.0, 4.0, 5.0, 7.0, 9.0, 10.0, and 12.0 mcg. were placed in glass-stoppered, 15-ml. centrifuge tubes. The samples were evaporated to dryness under a gentle stream of nitrogen. One-milliliter aliquots of control guinea pig plasma were added to each centrifuge tube and mixed well using a Vortex mixer. A control plasma blank was prepared by adding 1 ml. plasma to a 15-ml. centrifuge tube and processing it in the same manner as the other samples. The pH of all samples were adjusted to 2.5 using 1 *N* HCl and extracted with chloroform (5 ml./sample). Emulsions which formed were broken up by centrifugation at 1000 r.p.m. A measured volume of the chloroform extract (about 80%) from each extraction was pipeted out into fresh centrifuge tubes and evaporated to dryness under nitrogen. The residues were subjected to TLC in the manner described later.

Plasma Specimens from Dosed Guinea Pigs—One-milliliter aliquots of the plasma samples were placed in 15-ml. centrifuge tubes. The pH of each sample was adjusted to 2.5 using 1 *N* HCl, and the samples were processed in the same manner as the plasma standards.

TLC of the Guinea Pig Plasma Extracts—TLC was carried out on precoated TLC plates of silica gel F-254 of 250-μ thickness[4]. The plasma extracts were subjected to TLC in a solvent system composed of chloroform–methanol–formic acid (98.5:1.0:0.5). Reference spots of authentic tolazamide were used. The tolazamide zones

[1] 1-(Hexahydroazepin-1-yl)-3-*p*-toluenesulfonylurea.

[2] Vehicle No. 100 contains per milliliter: sodium carboxymethylcellulose (5 mg.), polysorbate 80 (4 mg.), sodium chloride (9 mg.), and benzyl alcohol (9 mg.) in distilled water. Supplied by The Upjohn Co., Kalamazoo, Mich.

[3] 1-(*n*-Butyl)-3-*p*-chlorobenzenesulfonylurea.

[4] Merck, Brinkmann Instruments, Inc., Westbury, N. Y.

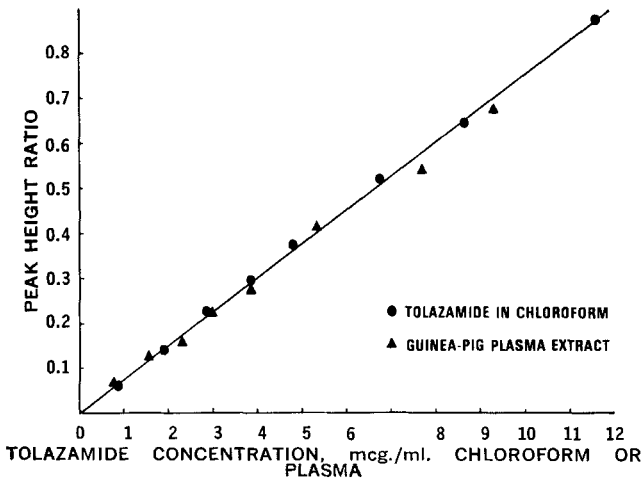

Figure 1—*Standard curves for tolazamide extracted from guinea pig plasma and in chloroform.*

were located by examination of the chromatograms using a UV lamp (254 nm.). The zones were removed separately and extracted out with methanol (2 × 1 ml.). To each extract was added 0.1 ml. of the internal standard solution containing 12 mcg. of IV, and the total was evaporated to dryness under nitrogen. The residue was reconstituted in 20 μl. of chloroform and analyzed by GC.

GC—GC was carried out on a gas chromatograph[5] equipped with a flame-ionization detector. Glass columns (U-shaped, 70 cm. × 3 mm.) packed with 0.5% Carbowax 20M on 80–100-mesh Chromosorb G were utilized throughout the study. Helium was used as the carrier gas at a flow rate of 80 ml./min. Oxygen and hydrogen flow rates were adjusted to give maximum response. The column oven was operated isothermally at 190°, the flash heater at 236°, and the detector at 220°. Under these conditions, the *p*-toluenesulfonamide fragment (II) and the *p*-chlorobenzenesulfonamide fragment (III) had retention times of ~2 and ~3.2 min., respectively. Aliquots (~1 μl.) of the TLC extracts of standard and dosed plasma specimens were analyzed by GC.

Internal Standard—A solution of IV in chloroform (0.12 mg./ml.) was employed as the internal standard for GC.

Calculations—Peak height ratios were calculated by dividing the height of the peak due to II (resulting from the thermal fragmentation of I) by the height of the peak due to III (resulting from the thermal fragmentation of IV). Standard curves for the recovery of tolazamide from guinea pig plasma were constructed by plotting the concentration of tolazamide (in micrograms per milliliter of plasma) against the peak height ratios. A typical standard curve is presented in Fig. 1.

Mass Spectrometry—Mass spectra were recorded in a high-resolution mass spectrometer[6].

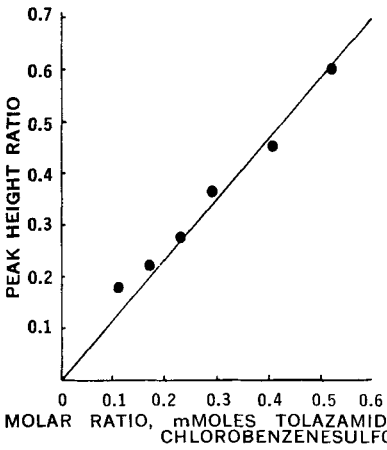

Figure 2—*Peak height ratio of tolazamide/1-(n-butyl)-3-p-chlorobenzenesulfonylurea, expressed as a function of concentration of tolazamide.*

[5] F&M model 402.
[6] Consolidated Electrodynamics Corp., model 21-110.

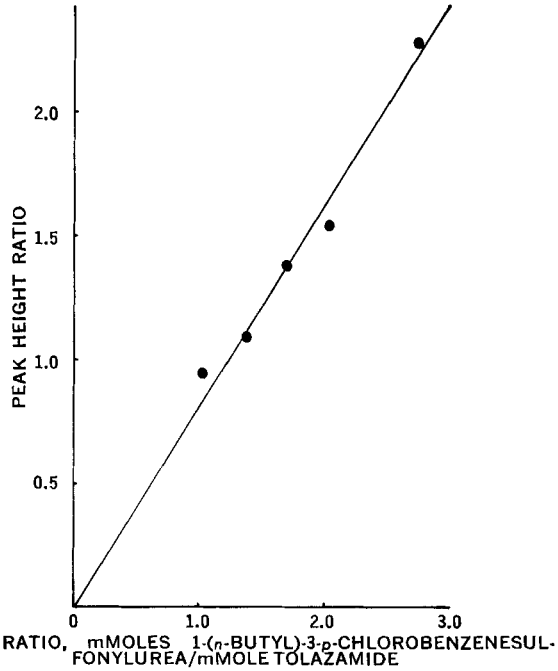

Figure 3—*Peak height ratio of 1-(n-butyl)-3-p-chlorobenzenesulfonylurea/tolazamide expressed as a function of concentration of 1-(n-butyl)-3-p-chlorobenzenesulfonylurea.*

Mass Spectrum of Tolazamide—At Low Temperature—A spectrum of tolazamide was recorded using the direct probe at 145° (70 ev.). This spectrum showed a molecular ion at *m/e* 311 and prominent peaks at: (*a*) 197 (M-114), (*b*) 171 (M-140), (*c*) 155 (M-156), (*d*) 140 (M-171), (*e*) 113 (M-198), (*f*) 98 (M-213), and (*g*) 91 (M-220), base peak; corresponding to a fragmentation of the type indicated in V.

At High Temperature—A spectrum of tolazamide was recorded using the heated inlet system at 220° (70 ev.). The spectrum showed no molecular ion. Prominent peaks were present at *m/e* 171 (M-140), 155 (M-156), and 140 (M-171).

GC–Mass Spectrometry—GC–mass spectra were recorded on an LKB-9000 gas chromatograph–mass spectrometer. The column packing utilized for the GC analysis was 3% OV-17 on Gas-Chrom Q (80–120 mesh). The column was maintained at a temperature of 200° and the flash heater at 220°. Helium was used as the carrier gas at a flow rate of ~45 ml./min.

Tolazamide—Three GC peaks were observed. The first GC peak was of low retention time, ~0.2 min. The mass spectrum of this material showed a prominent peak at *m/e* 140, which may be attributed to the isocyanate fragment (VI). The mass spectrum also showed peaks at *m/e* 112 (M-28) and 84 (M-56). The second GC peak was the most intense and had a retention time of ~1 min. The mass spectrum of this material showed a molecular ion at *m/e* 171 corresponding to *p*-toluenesulfonamide (II). The spectrum also showed prominent peaks at *m/e* 155 (M-16), 139 (M-32), and 123 (M-48). The third GC peak was of low intensity and rather diffuse. It had a retention time of ~6 min. The mass spectrum showed peaks at *m/e* 256, 171, 156, and 139. The nature of this material is obscure.

1-(n-Butyl)-3-p-chlorobenzenesulfonylurea (IV)—Only one major GC peak was observed under the conditions employed. This peak had a retention time of ~2 min. The mass spectrum of this material showed a molecular ion at *m/e* 191, which may be attributed to *p*-chlorobenzenesulfonamide (III). The spectrum showed other prominent peaks at *m/e* 175 (M-16), 127 (M-64), and 111 (M-80).

RESULTS AND DISCUSSION

GC of tolazamide using column packings of 1% SE-30 on 80–100-mesh Gas-Chrom Q (column temperature, 200°; flash heater, 275°) or 1% OV-17 on 80–100-mesh Gas-Chrom Q (column temperature, 150°; flash heater, 192°) revealed a major sharp peak of ~1-min. retention time and a minor peak of very low retention time (~0.2

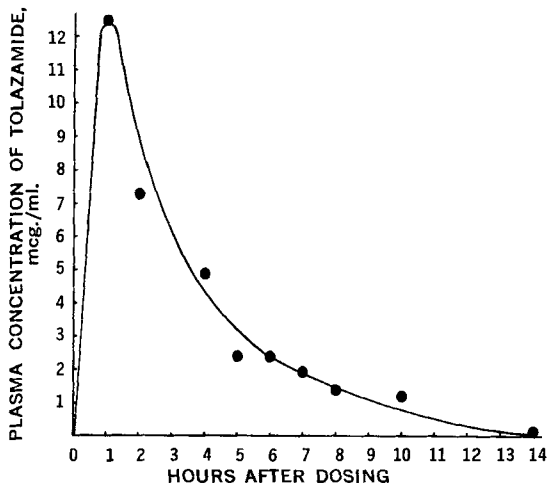

Figure 4—*Plasma concentrations of tolazamide* versus *time in guinea pigs after single-dose oral administration of 15 mg./kg. body weight.*

min.) poorly resolved from the solvent. These GC peaks were investigated on a gas chromatograph–mass spectrometer analyzer unit, using a column packed with OV-17 (3%) on Gas-Chrom Q at a column temperature of 200° and a flash heater temperature of 220°. A mass spectrum of the major GC peak showed a molecular ion at *m/e* 171 and a fragmentation pattern (see *Materials and Methods* section) which indicated that the material corresponding to the peak was *p*-toluenesulfonamide (II). A mass spectrum of the minor GC peak of low retention time showed a molecular ion at *m/e* 140 and a fragmentation pattern (see *Materials and Methods* section) which indicated that this peak was due to the isocyanate (VI). The two fragments, II and VI, obviously resulted from the thermal degradation of tolazamide (see VII).

The thermal fragmentation property of tolazamide was clearly demonstrated by mass spectrometry of the sulfonylurea at low and high temperatures. A mass spectrum of tolazamide, recorded on the high-resolution mass spectrometer at 145° using the direct probe, showed a distinct molecular ion at *m/e* 311 and fragments derived from it by further fragmentation (see *Materials and Methods* section) in the manner indicated in V.

On the other hand, a mass spectrum of tolazamide, recorded using the heated inlet system at 220°, showed no molecular ion. The fragmentation pattern was markedly different. Prominent peaks were observed at *m/e* 171 (base peak), corresponding to a toluenesulfonamide fragment, and at *m/e* 140, corresponding to the isocyanate fragment (see *Materials and Methods* section). Most of the other peaks observed could be attributed to further fragmentation of these ions.

To improve the GC resolution of the *p*-toluenesulfonamide from the solvent to facilitate working at higher sensitivities, several column packings were examined. The best results were obtained with a column packed with 0.5% Carbowax 20M on Chromosorb G (80–100 mesh). Such a column packing was employed by Liliedahl (5) for the separation and determination of *o*- and *p*-toluenesulfonamides.

Prior to examining the consistency of the GC thermal fragmentation of tolazamide, it was necessary to select a satisfactory internal standard. Several structurally related sulfonylurea compounds were screened. Compound IV was deemed to be the most satisfactory. GC–mass spectrometric analysis under the conditions already described showed only one major GC peak. A mass spectrum of the material corresponding to this peak showed a molecular ion at *m/e* 191 and a fragmentation pattern (see *Materials and Methods* section) which indicated that it was indeed III. The latter is obviously derived from IV by a thermal process analogous to that by which II is produced from tolazamide (see VII). The isocyanate fragment is probably lost with the solvent or it may undergo further degradation. The TLC mobility of IV was found to be identical with that of tolazamide on silica gel in the solvent system of chloroform–methanol–formic acid (98.5:1:0.5). The thermal fragmentation of tolazamide (I) and the internal standard (IV)

Table I—Recovery of Tolazamide Added to Guinea Pig Plasma as Measured by GC Assay

Tolazamide Added, mcg./ml.	Tolazamide Found, mcg./ml.	Percent Recovery
0.771	0.867	112
1.542	1.660	107
2.314	2.082	90
3.085	3.024	100.5
3.856	3.631	94.2
5.398	5.512	102.1
7.712	7.166	92.9
9.254	9.005	97.3

[a] Mean ± standard deviation = 99.6 ± 7.64%.

to yield *p*-toluenesulfonamide (II) and *p*-chlorobenzenesulfonamide (III), respectively, as measured by GC response was found to be linear over the concentration ranges examined—*viz.*, that of ~0.1 to ~0.9 mcg. tolazamide and ~0.6 to ~3.2 mcg. IV "on column," respectively. The concentrations, expressed as molar ratios, were plotted *versus* the peak height in Figs. 2 and 3. The "on column" lower limit of detection sensitivity was 0.048 mcg. tolazamide.

A study was conducted to determine the extent of fragmentation of tolazamide (I) and the internal standard (IV) under the GC conditions employed. A plot of the ratios of the peak heights of the fragmentation products, II and III, expressed as a function of the corresponding molar ratios, was assumed to represent 100% fragmentation of I and IV. From a comparison of the slope of this plot with the slope of a plot of the ratios of peak heights of I and III *versus* the corresponding molar ratios, the fragmentation of I was estimated to be 30%. Similarly, the fragmentation of the internal standard (IV) was estimated to be 20% from a comparison of the slope of a plot of the peak height ratios of II and the internal standard (IV) expressed as a function of the corresponding molar ratios, with the slope of the plot representing 100% fragmentation of I and IV.

Determination of Tolazamide in Guinea Pig Plasma—The procedure for the determination of tolazamide in guinea pig plasma consists of: (*a*) chloroform extraction of the acidified plasma samples (pH 2.5), (*b*) TLC of the chloroform extract, and (*c*) analysis of the recovered tolazamide by GC using a flame-ionization detector. With this procedure, tolazamide "spiked" plasma standards were extracted, and a standard curve for the recovery of tolazamide from guinea pig plasma was constructed (Fig. 1). The recovery of tolazamide from guinea pig plasma under these conditions was quite satisfactory (average recovery of 99.6%; standard deviation ±7.64%), as may be seen from Fig. 1 and Table I. The lower level of detection sensitivity for tolazamide in guinea pig plasma was 0.70 mcg./ml. (plasma blank is equivalent to ~0.6 mcg. tolazamide/ml.).

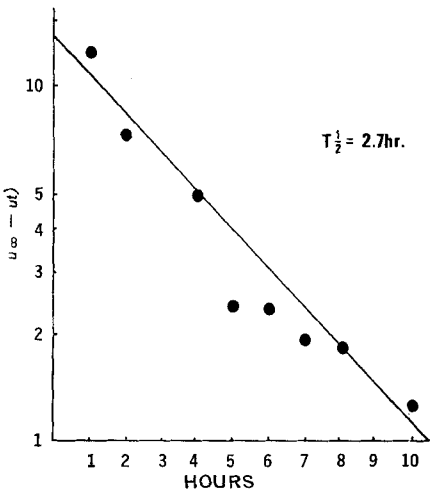

Figure 5—*Plasma drug disappearance half-life of tolazamide in guinea pigs after single-dose (15 mg./kg. body weight) oral administration of tolazamide.*

Specimens of plasma from tolazamide-treated guinea pigs (single oral dose of 15 mg. drug/kg. body weight) were extracted and analyzed in the same manner as described for the plasma standards. The concentration of tolazamide was determined from the standard curve. A peak plasma tolazamide concentration of 12.5 mcg./ml. was observed at 1 hr. after oral drug administration, indicating rapid drug absorption. A plot of the plasma concentrations of tolazamide (expressed in micrograms per milliliter) *versus* time (in hours) is presented in Fig. 4. By using the data between 1 and 14 hr., the plasma drug disappearance half-life was graphically estimated to be 2.7 hr. (Fig. 5).

During the course of this work, Sabih and Sabih (4) published a GC method for the determination of tolbutamide (VIII) and chlorpropamide (IX). These authors claimed to have analyzed the intact methyl derivatives of these drugs by GC. However, they did not establish the identity of the chromatographic species either by collecting a sample of the effluent or by GC–mass spectrometry. Considering the high operating temperatures reported (4) (flash heater at 330° and column temperature at 210°), the thermal fragmentation of the methyl derivatives cannot be excluded.

The present GC method described for the determination of tolazamide (I) in guinea pig plasma is better than the previously reported methods (1–3, 5) since: (*a*) it has greater detection sensitivity [the lower limit being 0.7 mcg. tolazamide/ml. plasma rather than 20 mcg./ml. plasma (2) or ~3 mcg./ml. plasma (3)], and (*b*) it has greater specificity on account of the preliminary TLC purification step which is capable of resolving intact tolazamide from its metabolites as well as from plasma contaminants. The method could be conveniently adapted for determination of other related sulfonylureas in pharmaceutical preparations as well as a variety of biological specimens.

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ACKNOWLEDGMENTS AND ADDRESSES

Received April 23, 1971, from the *Research Laboratories, The Upjohn Company, Kalamazoo, MI 49001*

Accepted for publication July 26, 1971.

The authors thank Drs. A. A. Forist and D. G. Kaiser for their interest and encouragement and Dr. M. F. Grostic and Mr. R. J. Wnuk for their assistance with mass spectrometric analysis.

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Opium Alkaloids X: Biosynthesis of 1-Benzylisoquinolines

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Abstract □ Radioactively labeled norreticuline was incorporated into papaverine in *Papaver somniferum* to an extent of about 5% without prior demethylation to norlaudanosoline. 1,2-Dehydronorreticuline does not participate in the biosynthesis of this alkaloid. (+)-Laudanosine is derived from (+)-reticuline *via* (+)-laudanidine, while (+)-codamine plays only a minor role. Radiodilution studies indicated that tetrahydropapaverine and papaveroline 6,3′,4′-trimethyl ether (pacodine) occur in the opium poppy. Based on the results of a series of tracer experiments, a scheme was proposed for the biosynthesis of benzylisoquinoline alkaloids in *P. somniferum*.

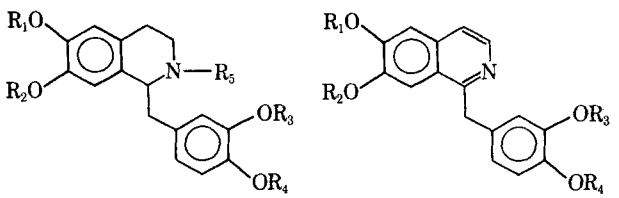
Keyphrases □ 1-Benzylisoquinolines—proposed biosynthesis in *P. somniferum* □ Biosynthesis—1-benzylisoquinolines in *P. somniferum*, proposed □ *Papaver somniferum*—proposed biosynthesis of 1-benzylisoquinolines □ Radioactive labeling—norreticuline, biosynthesis in *P. somniferum* □ Opium alkaloids—proposed biosynthesis of 1-benzylisoquinolines

The benzylisoquinoline alkaloids are widely distributed in the *Papaveraceae* family, occurring most extensively as the 1,2,3,4-tetrahydro derivatives. Four alkaloids of this general structure have been isolated from the opium poppy or from its dried latex, namely, codamine (I), laudanidine (II), laudanosine (III), and reticuline (IV). Two of these are present in both enantiomeric forms (II and IV) with an excess of one of the optical isomers [(−)-laudanidine and (+)-reticuline]. Norreticuline (V) has not yet been isolated but was shown to be an effective precursor of morphine and codeine (1).

The oxidized, fully aromatic benzylisoquinolines are not as common; two such alkaloids, papaverine (VIII) and palaudine (IX), were isolated from opium (2).

PREVIOUS WORK

In 1910, Winterstein and Trier (3) proposed that the benzylisoquinoline system is derived biosynthetically from two units of 3,4-dihydroxyphenylalanine (dopa) which, by decarboxylation and oxidative deamination, were believed to give rise to 3,4-dihydroxyphenethylamine (dopamine) and 3,4-dihydroxyphenylacetaldehyde, respectively. Formation of a Schiff's base and ring closure would give the substance known as norlaudanosoline (tetrahydropapaveroline) (VI). Modern theories (4) consider the reacting units to be



I: $R_1 = R_3 = R_4 = R_5 = CH_3$, $R_2 = H$
II: $R_1 = R_2 = R_4 = R_5 = CH_3$, $R_3 = H$
III: $R_1 = R_2 = R_3 = R_4 = R_5 = CH_3$
IV: $R_1 = R_4 = R_5 = CH_3$, $R_2 = R_3 = H$
V: $R_1 = R_4 = CH_3$, $R_2 = R_3 = R_5 = H$
VI: $R_1 = R_2 = R_3 = R_4 = R_5 = H$
VII: $R_1 = R_2 = R_3 = R_4 = CH_3$, $R_5 = H$
VIII: $R_1 = R_2 = R_3 = R_4 = CH_3$
IX: $R_1 = R_2 = R_4 = CH_3$, $R_3 = H$