over anhydrous sodium sulfate, filtering, and removing the chloroform *in vacuo*, a residue of 7.3 g. was obtained (Fraction II).

Fraction I (61 g.) was dissolved in benzene and added to the top of a chromatographic column filled with a benzene slurry of neutral alumina[1]. Elution was continued with benzene, and 500-ml. fractions were collected. Fractions 6–12 from the column were combined and taken to dryness (7.4 g.), and the residue was subjected to a gradient pH separation as previously described (2). The initial benzene extraction at pH 2.7 yielded 3.33 g. of alkaloid residue, which was chromatographed over a column of neutral alumina as previously described. Benzene was used as the eluent, and 200-ml. fractions were collected. Fractions 28–29 were combined, taken to dryness, and treated with hot methanol. On standing, crystals formed, which were dried *in vacuo* for 24 hr. Subsequently, these crystals were found to be identical in all respects with yohimbine, which was previously reported from this plant (1). Fractions 12–27 from the column were combined, taken to dryness *in vacuo*, and yielded 1.95 g. of alkaloid residue. This residue was rechromatographed over a column of neutral alumina as previously described. Benzene elution yielded a homogeneous material. Crystallization from ethanol yielded crystals that were identical in all respects with leurosine, which was previously isolated from the leaf alkaloid (A) fraction of this plant (1). A total of 0.255 g. of leurosine (base) was obtained.

Antitumor Testing—Extracts and alkaloids were assayed for activity against the P-1534 leukemia in DBA/2 mice according to protocols of the National Cancer Institute (3). The leaf crude alkaloid (C) fraction was inactive at doses ranging from 6.35 to 50.0 mg./kg. i.p., whereas leurosine was highly active against the same neoplasm at doses ranging from 0.375 to 3.0 mg./kg. i.p. (4).

[1] Woelm, activity grade III.

DISCUSSION

The anomalous isolation of an active antitumor alkaloid from a crude alkaloid fraction of *C. lanceus* prompted the thorough investigation of all alkaloid fractions from this plant, whether they are active or inactive against the P-1534 leukemia. The only speculation that can be offered at this time for this phenomenon is that the alkaloid (C) fraction contains, in addition to leurosine, a material that displays a delayed toxicity. Animals receiving the extract may be adversely affected only at about the time that the leukemic control group of animals die, *i.e.*, about 12–15 days from the time of infection. If this is indeed the reason, it might be profitable to subfractionate each crude alkaloid fraction and have each of these evaluated if the original fraction gives a negative antitumor test.

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Cycloheptaamylose–Barbiturate Inclusion Complexes: Solubility and Circular Dichroism Studies

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Abstract □ The interaction of cycloheptaamylose with some barbiturates was examined by solubility analysis and by circular dichroism. Complexation by inclusion of the barbiturate within the cavity of cycloheptaamylose leads to: (*a*) an enhancement in the aqueous solubility of the barbiturate, and (*b*) extrinsic Cotton effects in the circular dichroism spectra. Measurements from both methods yield formation constants for the 1:1 interactions. The relative strength of interaction with cycloheptaamylose is of the following order: phenobarbital > pentobarbital > amobarbital > barbital.

Keyphrases □ Cycloheptaamylose–barbiturate inclusion complexes—solubility and circular dichroism studies □ Barbiturate–cycloheptaamylose inclusion complexes—solubility and circular dichroism studies □ Circular dichroism—analysis of cycloheptaamylose–barbiturate inclusion complexes □ Interactions—cycloheptaamylose with phenobarbital, pentobarbital, amobarbital, and barbital □ Complexes, inclusion—cycloheptaamylose–barbiturate, solubility and circular dichroism studies

Cycloamyloses (cyclodextrins) are known to form inclusion complexes with drug molecules of a variety of structure types (1–5). The complexation of some barbituric acid derivatives with cycloheptaamylose was the subject of a preliminary report from this laboratory (6). Cycloheptaamylose was shown to complex with barbiturates by solubility and proton magnetic resonance techniques; the corresponding formation constants were obtained from solubility data. In the present report, details of the solubility measurements are given.

This paper is also concerned with an examination of the complexes by circular dichroism (CD), a technique that has been suggested (7–9) but not extensively applied before to cyclodextrin complexes. Inclusion of the barbiturate within the cavity of cycloheptaamylose results in extrinsic optical activity. The induced Cotton effects can be quantitatively treated to yield formation constants.

EXPERIMENTAL

Materials—Recrystallized amobarbital, m.p. 156–158°, recrystallized barbital, m.p. 190–192°, recrystallized pentobarbital, m.p. 129–130°, and phenobarbital USP were used as received. Cyclohepta-

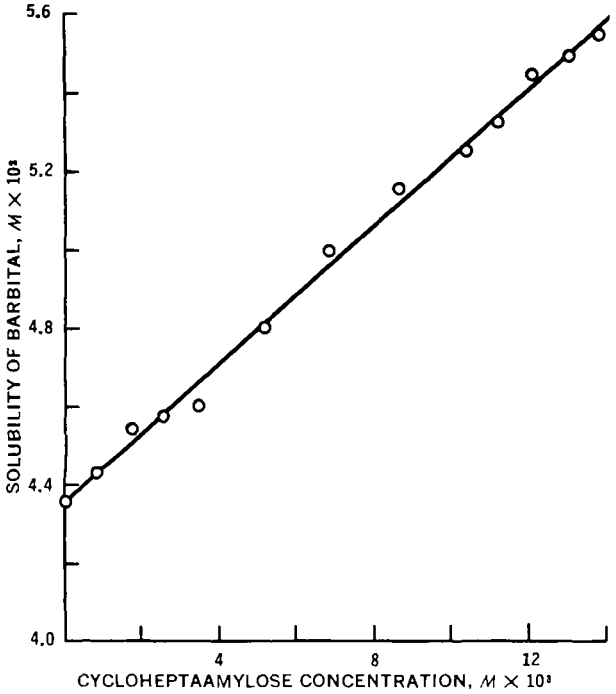

Figure 1—*Solubility of barbital as a function of cycloheptaamylose concentration in water at 30°.*

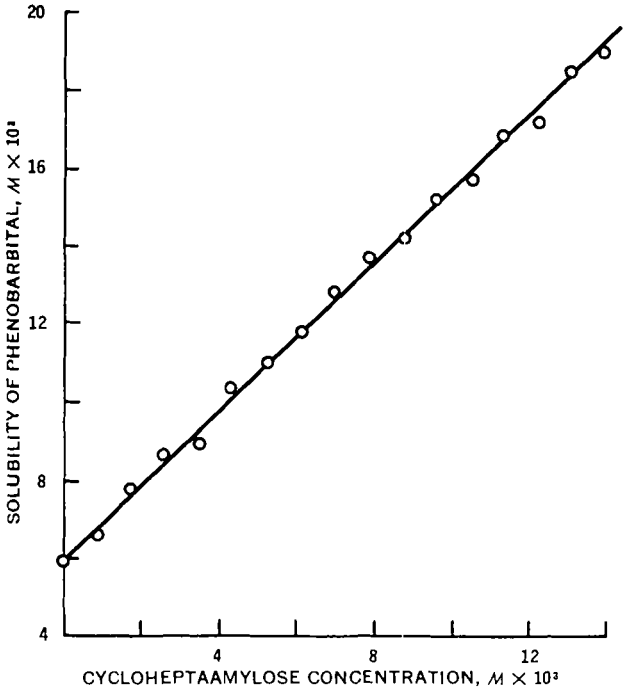

Figure 2—*Solubility of phenobarbital as a function of cycloheptaamylose concentration in water at 30°.*

amylose[1], $[\alpha]_D^{25}$ in water, 162.0 ± 0.5°, was recrystallized from water. The water of hydration of cycloheptaamylose, determined by Karl Fischer titration, was taken into consideration when recording its correct weight.

Solubility Studies—The solubility method of Higuchi and Lach (10) was followed. The aqueous barbiturate–cycloheptaamylose systems were equilibrated with constant agitation at 30° for 48–96 hr. This time was initially found to be more than sufficient to ensure equilibrium. Upon equilibration, the solutions were filtered through Millipore filters (0.45-μ pore size). Aliquots of the filtrates were suitably diluted with pH 9.5 buffer and were analyzed spectrophotometrically at ~238 nm. The presence of trace amounts of cycloheptaamylose did not interfere with the spectrophotometric assay.

Since the barbiturates used are known to exhibit polymorphism (11, 12), it was necessary to make sure that the crystalline form of the excess of barbiturate remaining after equilibration with aqueous cycloheptaamylose was the same as that equilibrated with water alone. X-ray diffraction patterns (Debye–Scherrer) of the various samples confirmed that no change in crystalline form occurred.

CD Studies—Barbiturate solutions were prepared in an aqueous solution of cycloheptaamylose of known concentration. The amount of barbiturate in each solution was determined spectrophotometrically following appropriate dilution of an aliquot with pH 9.5 buffer.

The CD spectra were recorded on a spectropolarimeter[2] with a slit programmed for a half bandwidth of 15 Å. Silica cells of 1-, 5-, and 10-mm. pathlengths were used.

RESULTS AND DISCUSSION

Solubility Studies—Figures 1–3 show the solubility of barbiturates as a function of cycloheptaamylose concentration in water. Solubility enhancement with increasing cyclodextrin concentration is similar to that observed with several other drugs (1–5). The plateau in the amobarbital plot indicates that a complex of limited solubility is formed. This amobarbital–cycloheptaamylose complex was isolated and found to be a 1:1 complex with about 8 moles of water of hydration. The stoichiometric relationship was determined: (*a*) by elemental analysis, (*b*) by spectrophotometric analysis of the crystalline complex for its amobarbital content, and (*c*) from the ratio of the anomeric protons of cycloheptaamylose to the methyl protons of amobarbital in the integrated NMR spectrum of a solution of the complex in deuterated dimethyl sulfoxide.

In view of the experimentally determined 1:1 stoichiometry for the amobarbital complex and the NMR evidence suggesting a similar mode of interaction for the various barbiturates (6), similar stoichiometries were assumed for cycloheptaamylose complexes

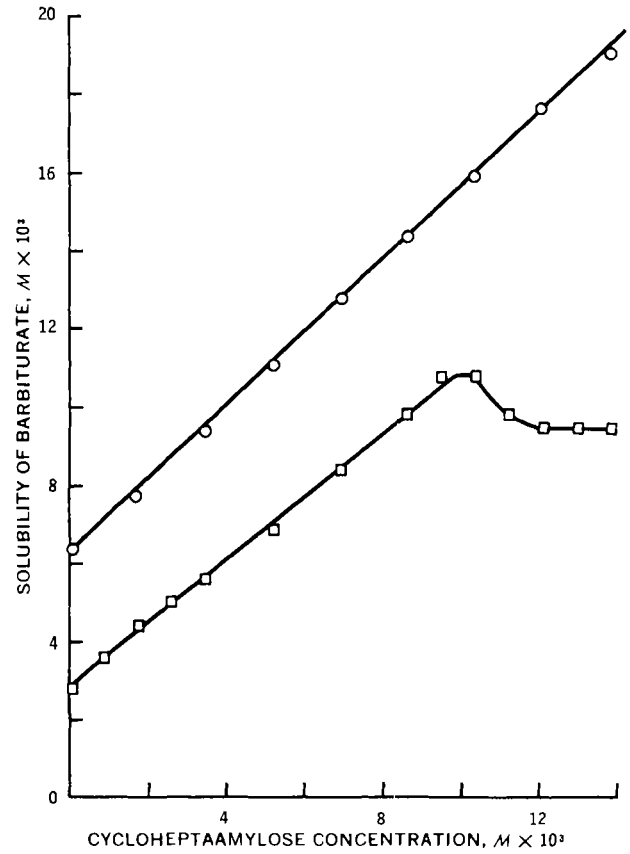

Figure 3—*Solubility of amobarbital* (□) *and pentobarbital* (○) *as a function of cycloheptaamylose concentration in water at 30°.*

[1] Supplied by Corn Products Sales Co., New York, N. Y.
[2] Cary 60 with a 6002 attachment.

Table I—Formation Constants of Cycloheptaamylose–Barbiturate Complexes

Barbiturate	Formation Constant: Solubility Method	Formation Constant: CD Method[a]
Barbital	1.51×10^2	—
Amobarbital	1.24×10^3	1.42×10^3 (±15%)
Pentobarbital	1.82×10^3	1.72×10^3 (±10%)
Phenobarbital	3.60×10^3	4.09×10^3 (±5%)

[a] Duplicate measurements were used. The K values lie within the indicated ± range.

of the other barbiturates. The apparent formation constants for the complexes were then calculated by the method of Thoma and Stewart (13). The formation constants are listed in Table I. Values from the CD method are also listed in the table. The rather large values of the formation constants, which compare well with the formation constants for other cycloamylose complexes (1–5) are indicative of stable complexes.

CD Studies—Azo dyes (7) as well as benzoic acid and iodine (8, 9) have been shown to generate extrinsic Cotton effects upon binding to cyclodextrins. In these earlier reports, no attempt was made to obtain any quantitative information regarding the complex formation from the generated CD curves. The binding of barbiturates to cycloheptaamylose produces large extrinsic Cotton effects which can be quantitatively treated to give binding constants.

Let us consider the reaction $A + B \rightleftarrows C$. If A and B denote the initial concentrations of cyclodextrin and barbiturate, respectively, and if C denotes the equilibrium concentration of the complex, then K, the binding constant, is given by:

$$K = \frac{C}{[A - C][B - C]} \qquad \text{(Eq. 1)}$$

Upon rearrangement:

$$C = \frac{1 + K(A + B) \pm [1 + 2K(A + B) + K^2(A + B)^2 - 4K(KAB)]^{1/2}}{2K} \qquad \text{(Eq. 2)}$$

In dilute solutions, the observed ellipticity is proportional to the concentration of complex at any fixed wavelength, so $C = E/\epsilon$, where E is the observed ellipticity and ϵ is the proportionality constant for a given pathlength of cell at the particular wavelength of measurement. Equations 1 and 2 yield the following:

$$E = [\epsilon] \cdot \frac{1 + K(A + B) \pm [1 + 2K(A + B) + K^2(A + B)^2 - 4K(KAB)]^{1/2}}{2K} \qquad \text{(Eq. 3)}$$

A nonlinear regression analysis was used, with the aid of a digital computer, to estimate the parameters ϵ and K.

Ellipticities of a series of solutions of known barbiturate to cycloamylose ratio were measured. From these ellipticity values, the binding constants were determined. Ellipticities at at least three wavelengths were used to calculate the constants shown in Table I. Figure 4 shows the CD curves for various concentrations of phenobarbital at a fixed concentration of cycloheptaamylose in water. These curves have peaks or shoulders at 270, 265, and 258 nm., whereas the curves for pentobarbital and amobarbital show single positive peaks at 261 nm.

The CD spectrum of barbital in aqueous cycloheptaamylose showed a weak negative peak at 278 nm. and a slightly stronger positive peak at 260 nm. These peaks were too weak, even at the highest barbital concentrations, to allow quantitation. The apparently anomalous negative peak at 278 nm. possibly arises from a dissociation of drug molecules. Barbiturates in dilute or alkaline solutions show UV absorption near this wavelength; the peak has been associated with the enolic form (14–16).

Induced optical activity upon interaction of the barbiturates with cycloheptaamylose is probably the result of the inclusion of barbiturate side chains within the cavity of cycloheptaamylose. With phenobarbital, this is almost certainly the case since the induced CD curve is characteristic of an asymmetrically perturbed aromatic chromophore. NMR data (6) have also shown that the phenyl ring of phenobarbital is included within the cyclodextrin cavity upon complexation. In the case of the other nonaromatic barbiturates, their nonpolar side chains get included within the hydrophobic cavity of cyclodextrin. Such inclusion provides a certain rigidity to the complex and places the barbiturate nucleus in close physical proximity to the primary hydroxyl side of the cyclodextrin. Evidently, the extrinsic Cotton effects with amobarbital and pentobarbital arise as a consequence of a specific, hydrogen-bond-type interaction between the barbiturate nucleus and the primary hydroxyl groups of cycloheptaamylose.

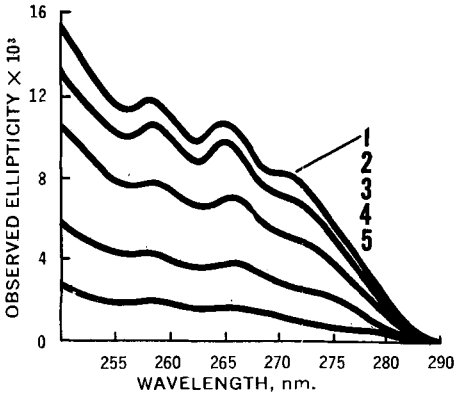

Figure 4—*Induced CD curves for phenobarbital–cycloheptaamylose complexes (1-mm. cells). Cycloheptaamylose concentration = 8.815 × 10⁻³ M. Phenobarbital concentrations = (1) 1.345 × 10⁻² M, (2) 8.743 × 10⁻³ M, (3) 5.380 × 10⁻³ M, (4) 2.690 × 10⁻³ M, and (5) 1.345 × 10⁻³ M.*

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