vapor flow; as the drying front descends, this obstruction to vapor flow increases. If too much heat is applied, the sample will liquify. Figure 13 illustrates that, for this product, it was necessary to maintain a low drying temperature throughout the primary drying phase.

With the drying temperature set at 20°, the product temperature rose to within half a degree of the eutectic point. The drying temperature was lowered to 14°, and the product temperature dropped to −15°. The temperature of the product remained essentially constant for over 20 hr. before it began to rise gradually. However, it took over 45 hr. before the product passed through zero. The drying temperature was increased gradually to 60° and subsequently lowered to 40° when the product temperature passed through 40°. From the curves in Fig. 13, it was decided that the initial drying temperature should be no higher than 16°.

A full chamber load consisting of 1500 units, each containing 15 ml. of solution, was frozen to −35°, and the cycle proceeded as summarized in Table VI. With this product, it was necessary to run the cycle beyond 60 hr. to obtain a 100% acceptable yield. Moisture determination on the dried samples ranged from less than 0.1 to 0.25%.

An example for a poorly soluble drug has been omitted because eutectic temperatures for such medicinals are usually slightly below 0°, and the degree of supercooling is generally negligible. In such cases, the design of a cycle is dependent on the properties of the additive or bulking agent employed and not on the properties of the drug. A knowledge of the thermal conductivity of the frozen mass would be another refinement in the design of the freeze-drying cycles.

SUMMARY

A pilot plant lyophilizer was designed to permit versatility for the research and development of lyophilization cycles. The unit contains the necessary instrumentation to control temperatures accurately and to monitor temperature and pressure.

The equipment and instrumentation were described, and a procedure employed for evaluating the various systems comprising the unit was outlined. An explanation and examples were given to illustrate how the information obtained could be utilized to the greatest advantage in conducting preliminary studies and programming cycles.

Through the utilization of temperature–resistivity measurements obtained during preliminary screening and temperature–time and pressure–time curves from the initial freeze-drying runs on the pilot plant unit, a procedure was developed for programming drying cycles for production equipment.

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NOTES

Spectrophotometric Determination of Anthramycin

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Abstract □ A quantitative UV spectrophotometric method for the determination of a new antitumor antibiotic, anthramycin, is described. The method is shown to be applicable for the determination of anthramycin in fermentation beers of *Streptomyces refuineus* var. *thermotolerans* (NRRL 3143) and in various preparations containing this antibiotic.

Keyphrases □ Anthramycin—spectrophotometric determination □ *Streptomyces refuineus* var. *thermotolerans*—spectrophotometric determination of anthramycin in fermentation beers □ Fermentation beers—spectrophotometric determination of antibiotic, anthramycin

Anthramycin (I*a*), an active antitumor antibiotic, produced by *Streptomyces refuineus* var. *thermotolerans* (NRRL 3143), was recently isolated in the form of its crystalline methyl ether (I*b*) and as anhydroanthramycin (I*c*) (1, 2).

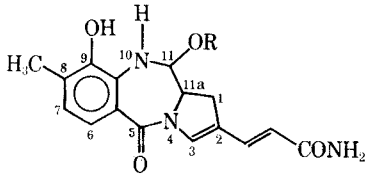
Anthramycin possesses a characteristic UV spectrum essentially identical with that of anthramycin methyl ether when measured in methanol (Fig. 1). Studies showed that the UV spectrum of anthramycin methyl ether conforms to Beer's law at concentrations of 2.0–10.0 mcg./ml. These data were used to establish an assay procedure for the quantitative determination of anthramycin based on the evaluation of UV absorption measurements.

Anthramycin can also be determined by *in vitro* biological assay, *e.g.*, *versus Bacillus* sp. TA (NRRL-B-3167).

EXPERIMENTAL AND RESULTS

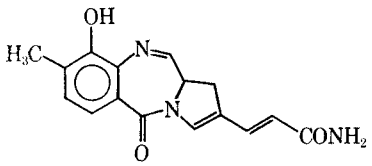
Determination of Standard Curve—Anthramycin methyl ether (10.0 mg.) was dissolved in methanol and brought to 100 ml. in a volumetric flask. This stock solution was then used to prepare 10 different concentrations of anthramycin methyl ether in the range of 2–10 mcg./ml. Absorbances were read in the Beckman DU spectrophotometer at 335 nm. Figure 2 shows UV absorption curves of anthramycin and its methyl ether in methanol.

Ethanol and *n*-butanol may also be used in place of methanol.



Ia: R = H, anthramycin

Ib: R = CH_3, anthramycin methyl ether



Ic: anhydroanthramycin

Method Precision and Recovery Experiments—With five standard solutions, prepared on consecutive days, absorbance values expressed as absorption of a 1% solution of anthramycin methyl ether with a cell length of 1 cm. (*a*) were 112.5 ± 0.38 (mean ± *SE*).

To establish the precision of the spectrophotometric method, a known quantity of anthramycin methyl ether was dissolved in water saturated with *n*-butanol (using 0.2 ml. of methanol as a primary solvent) and then extracted twice with equal volumes of *n*-butanol saturated with water. These combined *n*-butanol extracts were then diluted with methanol; the absorbance was read at 300, 335, and 370 nm. in a Beckman DU spectrophotometer. The concentration of anthramycin methyl ether in the combined *n*-butanol extracts were calculated using the absorbance value at 335 nm. and also by the modification of the baseline method (3) [$A = B - (C + D)/2$, where A = calculated absorbance, B = absorbance at 335 nm., C = absorbance at 300 nm., and D = absorbance at 370 nm.]. Both methods gave identical results when pure, crystalline anthramycin methyl ether was utilized. The starting material was 4.0 mg. of crystalline anthramycin methyl ether, and 3.89 ± 0.02 (mean value of six recovery experiments ±*SE*) was recovered (97.2%).

These results were essentially confirmed by repeating the recovery experiment with anthramycin methyl ether (20 mg.) labeled with ^{14}C in the methoxy group (1057 d.p.m./mg.) where, after two extractions with *n*-butanol saturated with water, 95.8% of the radioactivity (20,250 d.p.m.) was found in the *n*-butanol extract. All measurements of radioactivity were made in a Packard Tri-carb scintillation spectrophotometer at 20° with the following phosphor solution: PPO, 4 g./l.; and POPOP, 100 mg./l. toluene.

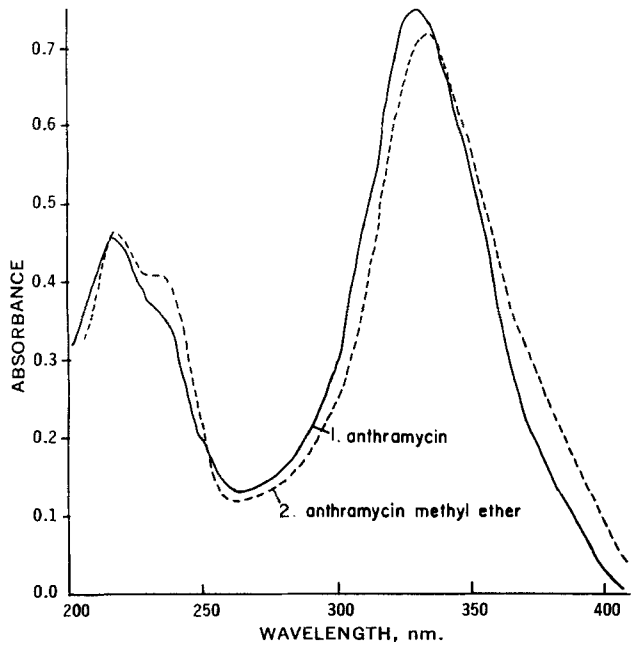


Figure 1—*UV spectra of anthramycin and anthramycin methyl ether at 0 hr. (in methanol). Key: 1, anthramycin, $\lambda_{max.}$ 333 nm., • = 37,000; and 2, anthramycin methyl ether, $\lambda_{max.}$ 334/5 nm., ϵ = 36,800.*

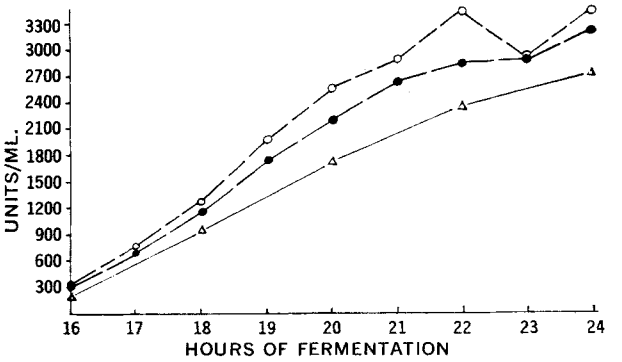


Figure 2—*Determination of anthramycin in fermentation beer. Key: ●, spectrophotometric assay of beer filtrate; ○, microbiological assay of beer filtrate; and △, microbiological assay of whole beer.*

In a separate experiment to determine the distribution coefficient (K) of anthramycin methyl ether in a *n*-butanol–water system containing 1% of methanol, 85.5% of the anthramycin methyl ether was extracted by *n*-butanol saturated with water. Total recovery of the antibiotic was 99.0%, and the distribution coefficient K = 6.3. On the basis of the K-value, two successive extractions with equal volumes of *n*-butanol saturated with water should give a total extraction of anthramycin methyl ether of 97.9%.

Determination of Anthramycin in Fermentation Beers—The determination of anthramycin in fermentation beers of *Streptomyces refuineus* var. *thermotolerans* (NRRL 3143) was conducted essentially as described previously.

A fermentation beer of *Streptomyces refuineus* var. *thermotolerans* (NRRL 3134) was sampled every hour during fermentation. The cells from these samples were separated by centrifugation, and the aqueous supernatant was used for the spectrophotometric and microbiological analyses.

For UV spectrophotometric analysis, the following procedure was used. To 4.2 ml. of each supernatant (fermentation beer filtrate with pH adjusted between 6 and 7, if necessary) in a 15-ml. centrifuge tube, 0.8 ml. of *n*-butanol was added to saturate the aqueous phase with *n*-butanol. Each sample was then extracted twice with 5.0 ml. of *n*-butanol saturated with water; the phases were separated after each extraction by centrifugation and the butanol extracts were combined. The butanol extracts were transferred to 100-ml. volumetric flasks and diluted to the mark with methanol. Flasks were heated for 3 min. at 60° (to transform anthramycin into anthramycin methyl ether), and the UV absorbance was read at 300, 335, and 370 nm. in a Beckman DU spectrophotometer.

In all experiments, a corresponding blank (*n*-butanol saturated with water) and an anthramycin methyl ether standard control were run simultaneously. The amount of the anthramycin expressed as anthramycin methyl ether in micrograms per milliliter of fermentation beer filtrate was calculated as follows: anthramycin methyl ether (mcg./ml. of beer filtrate) = $(100 \times a)/(b \times c \times d)$, where a represents the baseline value of the sample, b = baseline value for anthramycin methyl ether at a concentration of 1 mcg./ml. = 0.067, c = recovery of anthramycin methyl ether = 0.97, and d = volume of fermentation beer filtrate in milliliters; in this case = 4.2.

To determine the validity of the technique applied to the fermentation beer filtrate, a known quantity of anthramycin methyl ether standard (0.5 mg. in 50 ml. of methanol) was added to the fermentation beer filtrate. Determination of anthramycin was then accomplished by the previously described procedure; it was found from the six determinations that the average recovery was 97.7% with extremes ±1.8%.

The method described was applied to several fermentation runs of *Streptomyces refuineus* var. *thermotolerans* (NRRL 3143), and results of the determination of anthramycin in two particular runs (one with a high concentration of antibiotic and the other with a much lower concentration) are presented in Figs. 2 and 3, respectively. In the same figures, along with the spectrophotometric determinations, are presented results of the biological assays. A plate-cup

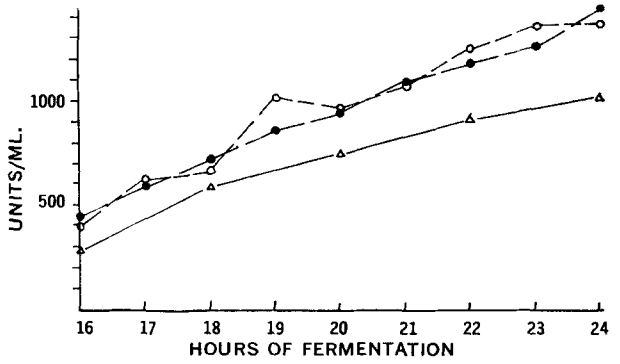


Figure 3—*Determination of anthramycim in fermentation beer. Key: ●, spectrophotometric assay of beer filtrate; ○, microbiological assay of beer filtrate; and △, microbiological assay of whole beer.*

assay against *Bacillus* sp. TA (NRRL B-3167) was simultaneously run with the spectrophotometric assay on aliquots of the fermentation beers and fermentation beer filtrates. One milligram of pure crystalline antibiotic anthramycin methyl ether was arbitrarily assigned 16,000 units of activity *versus Bacillus* sp. TA (NRRL B-3167).

It is evident from Figs. 2 and 3 that both determinations, spectrophotometric and microbiological, agree very well and that the quantity of anthramycin in fermentation beer filtrates can be directly, accurately, and rapidly determined by the presented UV spectrophotometric method.

Some discrepancy between the results of the spectrophotometric and microbiological determinations at different hours of fermentation can be explained by the fluctuation in microbiological assay rather than by inaccuracies of the spectrophotometric method.

The identical procedure can be applied for the determination of anthramycin methyl ether as a bulk drug and in pharmaceutical preparations. However, in these cases, the samples should first be suspended in a very small amount of methanol to which is added a volume of *n*-butanol (saturated with water) adequate to bring the concentration of methanol to 1%, followed by the same volume of water (saturated with *n*-butanol). After shaking, both phases should be separated, extraction with *n*-butanol (saturated with water) repeated, the butanol extracts combined, and then treated as described under *Determination of Anthramycin in Fermentation Beers.*

DISCUSSION

The quantitative determination of the antibiotic anthramycin as its methyl ether (I*b*) in pharmaceutical preparations appears to be a straightforward procedure; however, application of the same method for the determination of this antibiotic in the fermentation beer requires additional comment.

Anthramycin methyl ether is not present in the fermentation beer as such. It was established previously that the methoxy group at Position 11 is derived from methanol during crystallization of the crude antibiotic from this solvent (1). When the crude antibiotic was crystallized from acetone, anhydroanthramycin (I*c*) was obtained. On crystallization of this substance from methanol–water, anthramycin methyl ether was fomed. Anthramycin, which most likely exists as such in fermentation beers, was obtained only synthetically in a pure state by addition of water to anhydroanthramycin. Anthramycin, when dissolved in methanol (Fig. 1), is transformed into anthramycin methyl ether; the rate is slow at room temperature with incomplete reaction even after 24 hr. However, anthramycin is transformed quantitatively into anthramycin methyl ether by crystallization from hot methanol–water.

Differences in wavelength of maximum absorption ($\lambda_{max.}$) and molar absorptivity (ϵ) between anthramycin and anthramycin methyl ether are of such a low order that, regardless of the structure in which the antibiotic exists during quantitative determination, there is no appreciable effect on the accuracy of the method. The validity of the method is further confirmed by concordance of UV spectrophotometric determination with microbiological assay.

The concentration of the antibiotic is lower in the fermentation beer than in the beer filtrate. This can be explained by the fact that the whole fermentation beer contains cells that are inactive and comprise 11% of the total volume, consequently yielding a lower antibiotic content in the whole beer than in the corresponding beer filtrate.

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