HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF NATURAL PRODUCTS.
II. DIRECT BIOLOGICAL CORRELATION OF COMPONENTS IN THE FERMENTATION BROTH*

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A portion of the eluent, during liquid chromatography of a fermentation broth, is applied directly to a paper strip at the same rate as the recording of the U. V. absorption. The paper is then placed on the agar inoculated with an appropriate test organism. This procedure permits direct correlation of biological activity with corresponding peaks in the U. V.

In an earlier communication from these laboratories we have shown that microparticulate bonded phases on silica provide an excellent stationary phase for the separation of various cephem derivatives directly from the fermentation broth of *Cephalosporium acremonium*. We now wish to report a new method of diverting a portion of the eluent from the column during liquid chromatography of the fermentation broth and applying it to an adsorbent paper at the same rate as the recording of the U.V. absorption. Following this step the paper is placed in the conventional manner over agar and the latter inoculated with an appropriate test organism. After incubation, the inhibition zones can be correlated with corresponding peaks in the U.V. recordings, and thus biological activity of pure components can be detected directly in fermentation broths.

Materials and Equipment

The stream-splitting unit was fabricated in our machine shop. A schematic drawing of the fluid diverting block is given in Fig. 2. All fluid carrying surfaces were made of 316 stainless steel. The remaining parts were manufactured using anodized aluminum or brass. The drum was driven by a Hurst synchronous motor and appropriately scaled gears to produce a 2.5 cm/min drive corresponding to the speed of the U.V. recorder. Bulk stream-splitting was controlled before its passage through the device using Nupro fine metering valves. Micrometers on these valves insured a proper resetting of experimental conditions between each run. Paper sheets used were Whatman #1 (40.5 × 17.5 cm).

All chromatograms were obtained using Waters M6000A pump, U6K septumless injector (Waters Assoc., Milford, Mass.) with Schoeffel Model 770 U.V. detector (Schoeffel Inst., Westwood, N.J.) and Fisher omniscribe recorder (Fisher Scientific, Cincinnati, Ohio). Solvents used were glacial acetic acid (J. T. Baker), glass-distilled methanol and acetonitrile (Burdick and Jackson, Muskegon, Mich.) and deionized water. The *d*-10-camphorsulfonic acid was reagent grade from Eastman Chemicals.

Results and Discussion

The problem of diversion of an appropriate amount of eluate from the column through the stream-splitter in order to insure collection of a sufficient amount of material on the paper for the visible

* For the first paper in this series see R. D. MILLER and N. NEUSS: Separation of cephalosporin C derivatives and cephalosporin antibiotics; Isolation of cephalosporin C from fermentation broth. J. Antibiotics 29: 902~906, 1976[1]

Fig. 1. System diagram: Components of the HPLC/ bioautographic system

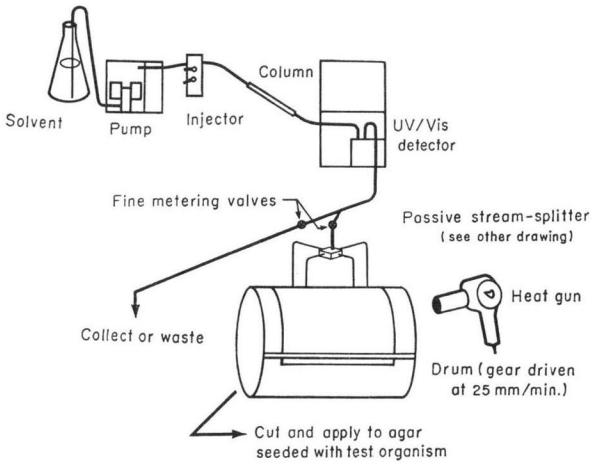


Fig. 2. Stream-splitter block schematic

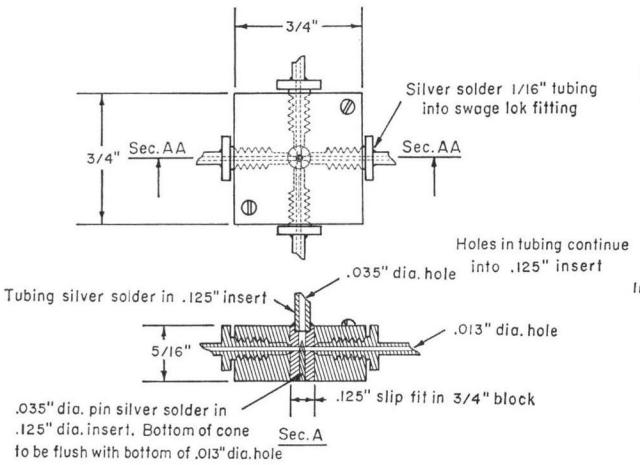
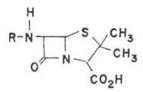
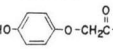
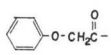


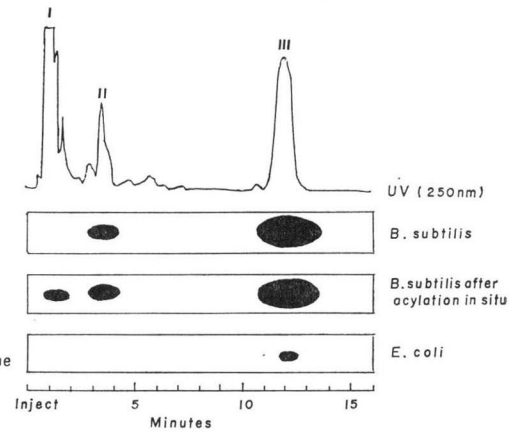
Fig. 3. *P. chrysogenum* broth precursed with phenoxyacetic acid (10 μl)
U.V. range: 254 nm, 2.0 Aufs.
Chart speed: 2.5 cm/min.
Flow rate: 1.5 ml/min.
Column size: 4×300 mm.
Packing: Microbondapak NH_2 (Waters).
Solvent: HOAc - CH_3OH - CH_3CN - H_2O (2: 4: 7.5: 86.5).



I. R = H: 6-Aminopenicillanic acid

II. R = HO-  -O-CH_2C- : Parahydroxy-penicillin V

III. R =  -O-CH_2C- : Penicillin V



growth inhibition was solved by passive stream-splitting or a free flow of eluate without any mechanical forcing. This permitted accumulation of a sufficient amount of eluate on the paper. The four part splitter was drilled with greatest care so that each path offered the same amount of resistance to the flow (Fig. 1 and Fig. 2).

The fermentation of penicillin using *Penicillium chrysogenum* and precursing with phenoxy acetic acid leads to the formation of three major metabolites in the broth: 6-amino penicillanic acid, *p*-hydroxy penicillin V, and penicillin V. Although they are present in different concentration, their activities are such that they can be seen as biologically active entities. In this example (Fig. 3) one can also see an additional option of acylating *in situ* on the paper before transferring components from the paper to the agar. Penicillin nucleus can easily be detected in this manner.

In another example the filtered broth from the fermentation of cephalosporin C using *C. acremonium* was subjected to this technique (Fig. 4). The presence of three components (penicillin N, desacetyl

Fig. 4. *C. acremonium* broth (25 μl)
U.V. range: 254 nm, 2.0 Aufs.
Chart speed: 2.5 cm/min.
Flow rate: 4 ml/min.
Column size: 4×300 mm.
Packing: Microbondapak NH_2 (Waters).
Solvent: HOAc - CH_3OH - CH_3CN - H_2O (2: 4: 7.5: 86.5).

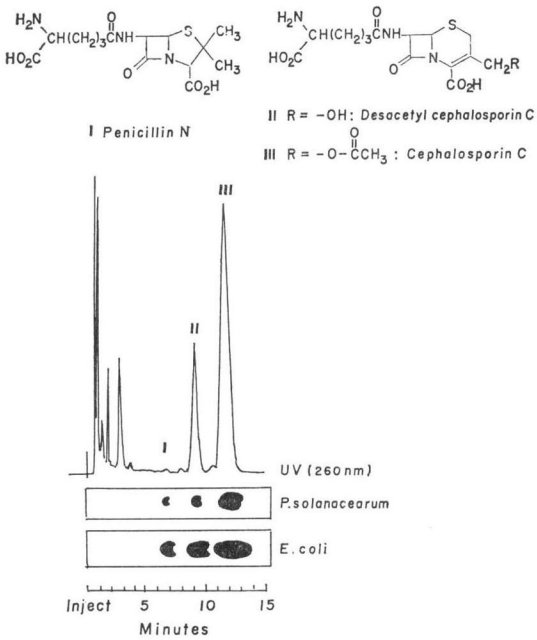


Fig. 5. *Streptomyces clavuligerus*[3] broth (200 μl) (ATCC NRRL 3585).
U.V. range: 254 nm, 1.0 Aufs.
Chart speed: 2.5 cm/min.
Flow rate: 3 ml/min.
Column size: 4×300 mm.
Packing: Microbondapak NH_2 (Waters).
Solvent: HOAc - CH_3OH - CH_3CN - H_2O (2: 4: 7.5: 86.5).

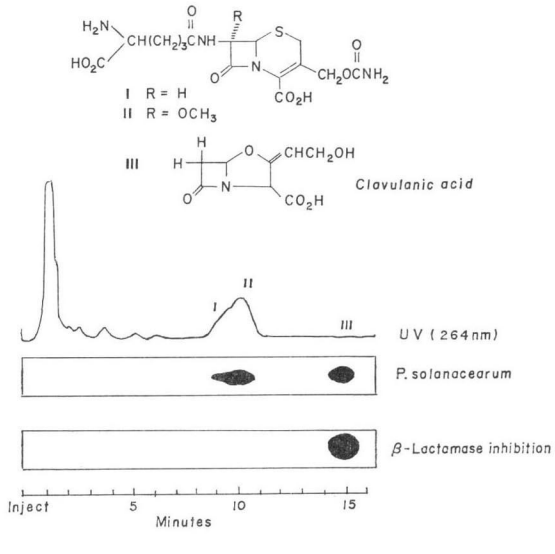


Fig. 6. Isolate* from *Nocardia uniformis tsuyamanensis* fermentation (ATCC 21806) (500 μg).
U.V. range: 254 nm, 0.4 Aufs.
Chart speed: 2.5 cm/min.
Flow rate: 2.5 ml/min.
Column size: 4×300 mm.
Packing: Microbondapak NH_2 (Waters).
Solvent: HOAc - CH_3OH - CH_3CN - H_2O (2: 4: 7.5: 86.5)

*provided by Dr. K. KOCH and Mr. S. NASH of these laboratories.

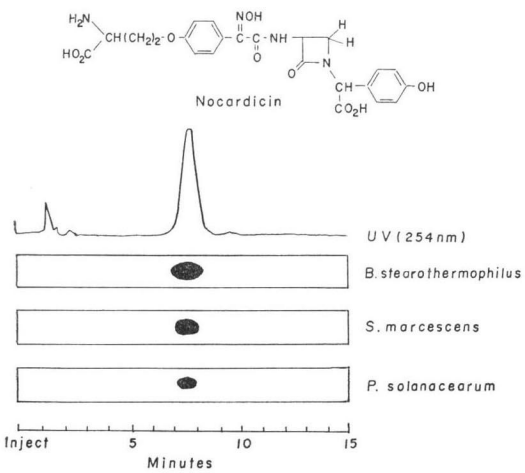
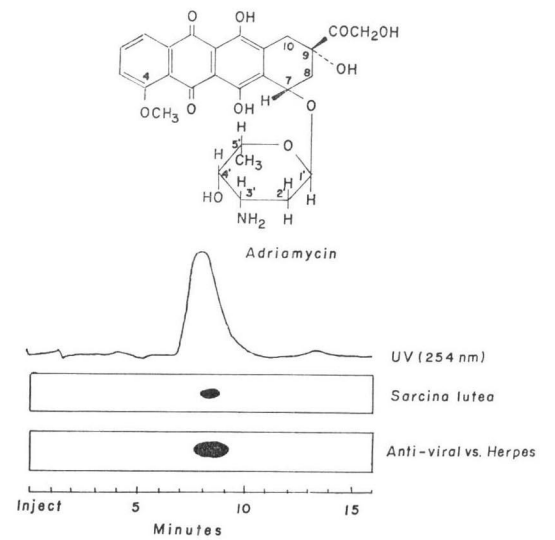


Fig. 7. Isolate** from *Streptomyces peucetius caesius* fermentation (adriamycin) (ATCC 27952) (300 μg).
U.V. range: 254 nm, 1.0 Aufs.
Chart speed: 2.5 cm/min.
Flow rate: 2.5 ml/min.
Column size: 4×300 mm.
Packing: Microbondapak C_{18} (Waters)
Solvent: 0.010 M *d*-10-camphor sulfonic acid, 4% (v/v) HOAc, 50% (v/v) CH_3OH, q.s. with water

** We thank Dr. F. ARCAMONE of Laboratories Farmitalia, Milano, Italy for the sample of adriamycin.



cephalosporin C, and cephalosporin C) has been established by spiking samples of the broth with authentic reference compounds. The results shown do not require any additional comments.

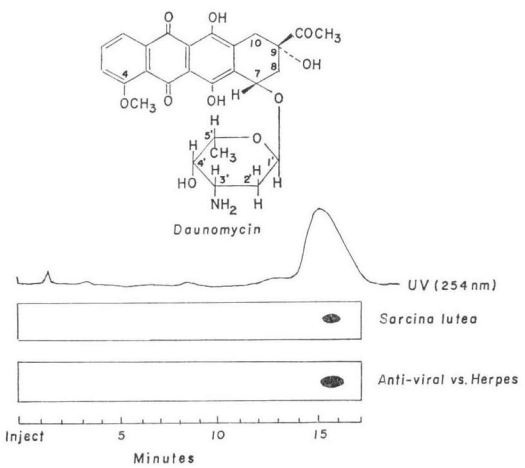
Examination of the broth of *S. clavuilgerus*[3] offers an interesting example of the utility of this method. This fermentation produces, in addition to 7-OCH_3-cephalosporins, a new metabolite, clavulanic acid.[4] Although not visible using U.V. detection at 254 nm, its presence is readily detected by β-lactamase inhibition[5] (Fig. 5.).

The fermentation of *Nocardia uniformis tsuyamanensis* (ATCC 21806) produces among other metabolites the novel compound, nocardicin,[6] a monocyclic β-lactam. The stream splitting device was used in conjunction with the isolation of this antibiotic in our laboratories. Fig. 6 is a representation of a run on a fermentation isolate demonstrating retention and sensitivity of the method.

Adriamycin[7] and daunomycin[8] are important medicinal agents produced by *Streptomyces peucetius caesius* (ATCC 27952) and *S. peucetius* (ATCC 29050), respectively (Figs. 7 and 8). Since these compounds require ion pairing* for their chromatography, it is important to note that the presence of the counterion (0.005 M camphor sulfonate) does not interfere with the growth of the test organisms. This method can be also applied directly to the examination of crude fermentation broths producing these types of compounds.

Fig. 8. Isolate*** from *Streptomyces peucetius* fermentation (daunomycin) (ATCC 29050) (300 μg).
U.V. range: 254 nm, 1.0 Aufs.
Chart speed: 2.5 cm/min.
Flow rate: 2.5 ml/min.
Column size: 4×300 mm.
Packing: Microbondapak C_{18} (Waters).
Solvent: 0.010M *d*-10-camphorsulfonic acid, 4% (v/v) HOAc, 50% (v/v) CH_3OH, q.s. with water

*** We thank Dr. F. ARCAMONE of Laboratories Farmitalia, Milano, Italy for the sample of daunomycin.



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