The distribution ratio for Am has been shown to have an approximate third-power dependence on the tributyl phosphate concentration, expressed as volume per cent. In this, also, Am behavior parallels that of Pm.

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Coprogen, the Isolation of a New Growth Factor Required by *Pilobolus* Species

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The complex nutrition of the genus *Pilobolus* has been described by several investigators.[1,2] These workers found it necessary to add dung or dung extracts to their culture medium. In a general survey of the distribution of the factor(s) that was essential for the growth of *Pilobolus kleinii*, it was found that the dung extracts could be replaced by the fermentation liquors of a number of species of bacteria and fungi. The isolation of a crystalline, biologically-active compound from such sources has been announced.[3] This substance has been designated "Coprogen" because of its ability to stimulate the growth of the *Coprophyllic* fungi. The method of assay and the general cultural and nutritional characteristics of the genus *Pilobolus* has also been described.[3,4]

Hesseltine and co-workers[4] listed a number of microörganisms that had been tested for their ability to produce the factor essential for *Pilobolus kleinii*. Culture *Penicillium* sp. appeared to be the most feasible organism for large scale production of the growth substance and was, therefore, used to produce adequate amounts of the compound for isolation.

The purification of the compound was initially done by solvent extraction, adsorption and elution from florisil and partition chromatography. Later procedures eliminated the use of the florisil adsorption step.

Crystallization of the active compound was effected by dissolving the active lyophilized fraction from the partition chromatogram in absolute ethanol. On standing a brick-red, crystalline compound separated. If only a slight trace of moisture was present, the compound tended to hydrate and precipitated in an amorphous form. The crystalline material is practically insoluble in ethanol and, for recrystallization, the compound must be dissolved in water, lyophilized and then again crystallized from ethanol.

Elemental analysis of the compound indicated the presence of carbon, hydrogen, nitrogen, oxygen and iron. The organo-iron nature of the compound and the fact that certain metallo porphyrins are growth factors for microörganisms[5-8] suggested that perhaps Coprogen was related to the porphyrin compounds. The absorption spectra and chemical properties of the isolated compound ruled against this possibility.

Coprogen exhibited a broad absorption maximum at 440 mμ with an $E^{1\%}_{1\ cm.}$ of 36.6 in 50% ethanol. The Soret band which is characteristic of heme compounds was clearly missing.

When Coprogen was dissolved in dilute alkali, ferric hydroxide precipitated and the ultraviolet absorption spectrum of the compound was destroyed. Concomitantly, the biological activity of the compound also was destroyed.

Experimental

Assay Methods.—Initially, the activity of the various fractions was determined by the plate assay method. In subsequent work, *Pilobolus kleinii* was cultured in liquid media and the weight of the mycelia was used as a measure of growth.[4]

Fermentation.—Culture *Penicillium* sp. was cultured in large scale fermentation apparatus equipped for agitation, aeration and temperature control. The medium contained 1% Bacto peptone, 0.1% ammonium sulfate, 0.1% sodium acetate and 0.1% potassium dihydrogen phosphate. Tap water was used throughout. After inoculation the fermentation was continued for three days at a temperature of 26–28° and with an aeration rate of 1 volume of air per volume of medium per minute. Filter-cel (0.1%) was added and the mycelium and Filter-cel was removed in a filter press.

Solvent Extraction.—To the clear filtrate was added 500 g. of ammonium sulfate per liter. The solution was then extracted twice with one-quarter volumes of butanol. The butanol extracts were combined and then concentrated under reduced pressure with the slow, constant addition of water. The distillation was continued until the butanol was completely removed and only a small volume of water remained.

The solution was filtered to remove any suspended matter and then extracted with approximately four one-quarter volumes of benzyl alcohol. The benzyl alcohol extracts were combined and then extracted with a one-tenth volume of water which removed inorganic salts and other impurities. Two volumes of ether or ethyl acetate were added to the combined benzyl alcohol extracts and the solution was then extracted with several one-quarter volumes of water. The activity and color were almost quantitatively extracted into the aqueous phase. The aqueous extracts were combined, concentrated to remove solvents and then lyophilized. The dried product was orange-brown in color, stable for storage and very convenient to handle.

Partition Chromatography.—"Celite 545"[9] (50 g.) was mixed with 25 ml. of the aqueous phase of a mixture of butanol:ethyl acetate:0.01 *N* hydrochloric acid (2:1:1). The moist "Celite" was packed into a column 2 cm. i.d. × 60 cm. Five hundred mg. of the crude lyophilized material was dissolved in 25 ml. of the solvent phase. This solution was placed in the column and immediately followed by fresh solvent.

If the column was extruded after the solvent front had reached the end of the column, several well-defined zones were evident. The first zone was purple and gave a strong $FeCl_3$ test. The second zone was orange, reacted strongly positive with $FeCl_3$ and contained Coprogen. The third and fourth zones were white and pale purple, respectively, and both zones gave a strong $FeCl_3$ test.

A flowing chromatogram was used and the fraction containing the orange band was collected, neutralized with sodium hydroxide and then concentrated under vacuum.

(1) A. H. R. Buller, "Researches on Fungi," Vol. VI, Longmans, Green and Co., New York, N. Y., 1934.

(2) E. A. Bessey, *Mich. Acad. Sci.*, **32**, 15 (1948).

(3) C. W. Hesseltine, C. Pidacks, A. R. Whitehill, N. Bohonos, B. L. Hutchings and J. H. Williams, THIS JOURNAL, **74**, 1362 (1952).

(4) C. W. Hesseltine, A. R. Whitehill, C. Pidacks, M. Ten Hagen, N. Bohonos, B. L. Hutchings and J. H. Williams, *Mycologia*, **45** [1] 7 (1953).

(5) A. Lwoff, *Compt. rend. soc. biol.*, **122**, 1041 (1936).

(6) M. S. Rao, *Ind. J. Med.*, **27**, 75 (1939).

(7) J. F. Jiminez, *Proc. Soc. Expil. Biol. Med.*, **45**, 402 (1940).

(8) J. D. Liberman and M. J. Pickett, *Bact. Proceedings*, P, 54 (1952).

(9) Johns-Manville Co., Lompoc, California.

The residue was dissolved in water and thence extracted into benzyl alcohol and finally partitioned into water as described above. The aqueous solution was lyophilized to yield a chromatographically pure product. The amorphous compound was readily soluble in water, methanol, ethanol, *n*-propanol, benzyl alcohol and the various cellosolves but was insoluble in ether, ethyl acetate, chloroform, benzene or the cellosolve esters.

For crystallization the amorphous compound was dissolved in absolute ethanol. On standing Coprogen separated as clusters of fine, dark brick-red needles. After drying at 110° under reduced pressure, the following analytical values were obtained.

Anal. Found: C, 50.96; H, 6.83; N, 10.26; Fe, 6.61.

Tests for halogen, phosphorus and sulfur were negative. When the compound was dissolved in dilute sodium hydroxide, ferric hydroxide precipitated. Assay with *P. kleinii* indicated that the alkali-treated solution was devoid of growth-promoting activity.

Ultraviolet Absorption Spectra.—Coprogen was dissolved in 50% ethanol at a concentration of 50 mc. per ml. and the ultraviolet absorption spectra was determined with a Beckman Model DU spectrophotometer.

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Synthesis of Cyclobutane by the Dehydroxymethylation Method

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The synthesis of cyclobutane and its intermediates has been widely studied. One of the procedures used in the preparation of cyclobutane involves the oxidation of methylenecyclobutane with performic acid to 1-(hydroxymethyl)-1-cyclobutanol which on cleavage with lead tetraacetate forms cyclobutanone[3]; the latter is then reduced to cyclobutane. The cyclobutanone also was prepared in 30–36% yield by ozonization of methylenecyclobutane.[4] Another method of synthesizing cyclobutanone in good yields consisted of treating ketene with diazomethane.[5] Other procedures of preparation of cyclobutane utilize cyclobutanecarboxylic acid as a starting material. This acid is converted by a series of known reactions to cyclobutylamine, which, by exhaustive methylation and decomposition, yields cyclobutene.[6] The latter is then hydrogenated to cyclobutane.[7] Cason and Way[8] synthesized cyclobutane by bromination of cyclobutanecarboxylic acid to cyclobutyl bromide and then converted the latter to cyclobutane *via* the Grignard reaction. The over-all yield from cyclobutanecarboxylic acid was about 39%.

(1) Universal Oil Products Company Fellow, 1951.

(2) Deceased, November 29, 1952.

(3) J. D. Roberts and C. W. Sauer, THIS JOURNAL, **71**, 3925 (1949).

(4) C. P. Krimmel, Ph.D. Thesis, Pennsylvania State College, 1945.

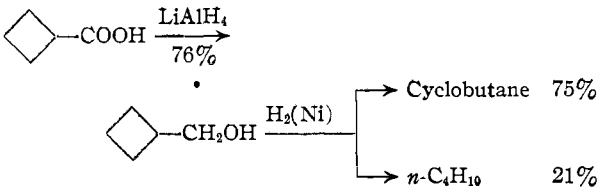
(5) (a) P. Lipp and R. Koster, *Ber.*, **64**, 2823 (1931); (b) Sj. Kaarsemaker and J. Coops, *Rec. trav. chim.*, **70**, 1033 (1951).

(6) R. Willstätter and W. U. Schmaedel, *Ber.*, **38**, 1992 (1905).

(7) R. Willstätter and J. Bruce, *ibid.*, **40**, 3979 (1907).

(8) J. Cason and R. L. Way, *J. Org. Chem.*, **14**, 31 (1949).

It has now been found that the preparation of cyclobutane from cyclobutanecarboxylic acid can be achieved in a 57% yield by a two-step synthesis which involves the reduction of the acid to cyclobutylmethanol and the catalytic dehydroxymethylation of the latter to cyclobutane. This can be presented by the equations



n-Butane is the only by-product of the dehydroxymethylation reaction and the formation of this compound could probably be suppressed by adjusting the experimental conditions. The cyclobutane formed is 99–100% pure.

The dehydroxymethylation method, which has been used previously for the preparation of various hydrocarbons,[9] could very well lend itself to the synthesis of various alkylcyclobutanes.

Experimental Part

Cyclobutylmethanol.—It was prepared by the reduction of cyclobutanecarboxylic acid[10,11] according to the general procedure described in the literature[12] with the modification that a slurry of 9.2 g. (0.24 mole) of lithium aluminum hydride in 350 ml. of ethyl ether was added to a solution of 27 g. (0.27 *M*) of cyclobutanecarboxylic acid. The cyclobutylcarbinol distilled at 142–143.5°, $n^{20}D$ 1.4450, yield 76%.

Cyclobutane.—The dehydroxymethylation was made in a 450-ml. capacity rotating autoclave. Cyclobutylmethanol, 17.2 g., and 1.8 g. of UOP nickel-kieselguhr catalyst[13] were placed in the autoclave which was then pressured with 100 atmospheres of hydrogen and heated at 154° for 5 hours. The final pressure at room temperature was 87 atmospheres. The non-condensable gases consisted of 89% hydrogen and 10% methane. The remainder of the product which was distilled on a low temperature Podbielniak column[14] consisted of 75% cyclobutane and 21% *n*-butane and the residue consisted of material boiling above 130°.

The cyclobutane was analyzed on a Consolidated Engineering Corporation Mass Spectrograph Type 21-103.[15] The remarkable agreement with the spectrum of 99–100% purity cyclobutane published in the American Petroleum Institute's Research Project 44 catalog of mass spectra, serial 416, indicates that the cyclobutane prepared was of similar purity.

The infrared spectrum of the cyclobutane was identical with that reported in the literature.[3]

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(9) V. N. Ipatieff, W. W. Thompson and H. Pines, THIS JOURNAL, **73**, 553 (1951); V. N. Ipatieff, G. Czajkowski and H. Pines, *ibid.*, **73**, 4098 (1951).

(10) G. G. Heisig and F. H. Stodola, *Org. Syntheses*, **23**, 16 (1943).

(11) The yield of diethyl cyclobutanedicarboxylate can be raised to 60% by modifying the experimental conditions: J. Cason and C. F. Allen, *J. Org. Chem.*, **14**, 1036 (1949).

(12) R. F. Nystrom and W. G. Brown, THIS JOURNAL, **69**, 1197 (1947).

(13) V. N. Ipatieff and B. B. Corson, *Ind. Eng. Chem.*, **30**, 1039 (1938).

(14) The distillation was made by M. J. Stross, Universal Oil Products Company.

(15) The mass spectrographic analysis was made by J. B. Grutka, Physics Laboratory, Universal Oil Products Company.