TABLE VIII

DETERMINATION OF PERCENTAGE P–Cl ESTERIFIED

Time, min.	Sample wt., g. Water–dioxane	Methanol	Ml. 0.995 *N* NaOH Water–dioxane	Methanol	Diff., ml. NaOH per 208.5 g.[a]	Equiv. unreacted P–Cl per 208.5 g.	P–Cl esterified, %
45	5.42	5.18	26.62	24.85	23.77	0.02375	97.62
75	5.49	5.43	26.78	26.00	16.52	0.01652	98.35

[a] 112.5 g. of dichloridate + 96.0 methanol = 208.5 g. of reaction product.

TABLE IX

CALCULATION OF CHLORIDE ION CONCENTRATION

Time, min.	Equiv. NaOH = equiv. total acid/208.5 g.	Ml. 0.995 *N* $AgNO_3$	Ml. 0.988 *N* KCNS	Chloride ion (equiv./208.5 g.)
45	1.020	30.00	4.50	0.977
75	1.011	30.00	4.30	0.972

were thermostated for specified times, diluted with 5–7 ml. of carbon tetrachloride and poured into 100 ml. of water. The test-tubes were rinsed with two 5–7-ml. portions of carbon tetrachloride and the washings combined with the quench solution. The hydrogen chloride liberated was titrated with standard alkali. Control runs with the dichloridate in the absence of alcohol indicated a negligible rate of hydrolysis by water in the time involved for quenching and titration. The use of carbon tetrachloride was intended in part to prevent or reduce solubilization of unreacted dichloridate in the presence of methanol which would be expected to accelerate hydrolysis in the quench solution.

Preparation of Dimethyl *m*-Tolyl Phosphate by Solvolysis.—One-half mole (112.5 g.) of distilled *m*-tolyl phosphorodichloridate was fed over 50 minutes at 15° to 96 g. (3.0 moles) of methanol with constant stirring and external cooling. Provision was made to absorb any hydrogen chloride which evolved. Under the conditions employed, no hydrogen chloride was lost from the reaction solution. After the completion of the dichloridate feed, the reaction temperature was maintained and samples were removed with a hypodermic syringe to determine the optimum time for quenching the reaction. Approximately 5-g. aliquots were added to each of two flasks, one containing 100 ml. of dry methanol, the other 50 ml. of water–dioxane (2:3). Using the procedure described elsewhere, the two solutions were diluted after 4 hr. with 30 ml. of distilled water and titrated with standard alkali to the phenolphthalein end-point. The difference in equivalents of base consumed is equal to the equivalents P–Cl solvolyzed (Table VIII).

From the titration of the water–dioxane solution, the total acid formed was calculated. The same solutions then were titrated by the Volhard procedure for chloride ion (Table IX). The yields were calculated as shown in Table X.

The reaction product remaining after 75 minutes of "cook" time was quenched in 300 ml. of water, extracted with 55 ml. of a 10% sodium hydroxide–10% sodium chloride solution, washed twice with 50-ml. quantities of 5% sodium chloride solution, freed of water under reduced pressure, filtered and weighed. The wash waters were extracted once with benzene and the ester recovered upon drying and evaporating the latter solution was added to the main portion of the product after the last wash. The yield data are summarized in Table XI.

TABLE X

CALCULATION OF YIELDS FROM SOLVOLYTIC RUN

Time, min.	Total acid, equiv.	Chloride ion, equiv.	Unreacted P–Cl, equiv.	PCl + Cl ion, equiv.	>POOH,[a] equiv.	PCl(equiv.[b]) from $ArOPOCl_2$ lost as unreacted PCl and as >POOH	PCl[c] converted to P–OMe ester, %
45	1.020	0.977	0.0238	1.0008	0.0192	0.0430	95.7
75	1.011	0.972	0.0165	0.9885	0.0225	0.0390	96.1

[a] >POOH (cleavage acid) = total acid − (PCl + chloride ion), equiv. [b] Columns 4 + 6. [c] Corrected for unreacted PCl and for cleavage.

TABLE XI

CALCULATED AND RECOVERED YIELDS FROM SOLVOLYSIS

Time, min.	Ester yield based on titration data %	Wt., g.	Total yield, based on physical recovery and on 0.5 mole of dichloridate charged %	Wt., g.
45	95.7	103.6		
75	96.2	104.0	93.2	100.9

Considering the difference between actual and calculated yields to be only 3.1 g., which easily may be attributable to handling losses, the data confirm the validity of the titration technique. As isolated in this fashion, the resulting dimethyl *m*-tolyl phosphate contained a trace of the sodium phosphate salt, approximately 0.5% calculated as $(CH_3O)(CH_3C_6H_4O)POONa$. To remove this impurity the product was distilled and yielded a heart cut of 87.2 g., b.p. 114° (1 mm.).

Anal. Calcd. for $C_9H_{13}O_4P$: C, 50.0; H, 6.02; P, 14.35. Found: C, 49.9; H, 6.1; P, 14.3.

DETROIT 20, MICHIGAN

[CONTRIBUTION FROM THE KERCKHOFF LABORATORIES OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

Isolation of a New Pteridine from *Eremothecium ashbyii* and Some Observations on its Structure[1]

BY HUGH S. FORREST AND WALTER S. MCNUTT

RECEIVED APRIL 26, 1957

A new pteridine has been isolated in crystalline form from the mold *Eremothecium ashbyii*. Investigations of its structure have shown it to have certain similarities to riboflavin, but it is probably not an intermediate in the biosynthesis of this compound.

Although it is known from experiments on the mold *Eremothecium ashbyii* that the pyrimidine portion of riboflavin arises from adenine,[2] no information is available on the intermediate steps in this process. In experiments designed to investi-

(1) These investigations were supported by funds from the Rockefeller Foundation, the National Science Foundation, the Williams–Waterman Fund for the Combat of Dietary Diseases, a research grant from Merck and Co., Inc., and by funds from the Atomic Energy Commission administered through contract with the Office of Naval Research, contract No. N-6-onr-244, Task Order 5.

(2) W. S. McNutt, *J. Biol. Chem.*, **219**, 365 (1956).

gate the incorporation of radioactive guanosine, randomly labeled, into riboflavin in the same organism, it was noted that a compound with a brilliant blue fluorescence which occurs in very small quantity in the mold, also became radioactive.[3] It therefore seemed possible that this compound might be connected in some way with riboflavin biosynthesis, and it was decided to attempt to isolate it and to determine its structure. This paper reports the results of these investigations.

The amount of the blue fluorescent material (referred to hereinafter as Compound A) in the mold is very small and its isolation in quantity is therefore laborious. A more abundant source was sought and found in a "riboflavin feed,"[4] which is obtained by culturing the same organism. From this material, Compound A was isolated as an almost colorless crystalline compound, by successive chromatography of an aqueous acetone extract on "Filtrol Grade 76"[5] and filter paper or on "Filtrol" alone. The ultraviolet and infrared spectra of Compound A are shown in Figs. 1 and 2.

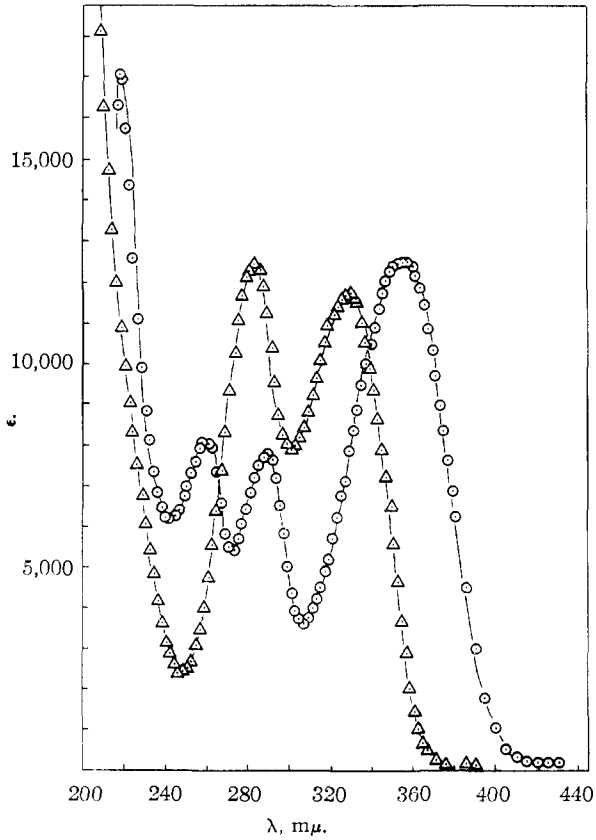


Fig. 1.—Ultraviolet absorption spectrum of Compound A: —△—△—, in 0.1 *N* hydrochloric acid; —⊙—⊙—, in 0.1 *N* sodium hydroxide.

The empirical formula and the absorption spectrum, as well as its relation to riboflavin, suggested that the compound was a dihydro pteridine. However, no fluorescent products could be detected after alkaline permanganate oxidation and so it was not possible to relate Compound A simply and directly to any known pteridine. All known, naturally occurring pteridines have the 2-amino-4-hydroxy structure, and production of guanidine when the pteridine is subjected to chlorine oxidation followed by hydrolysis, has been used as a diagnostic test for this grouping.[6]

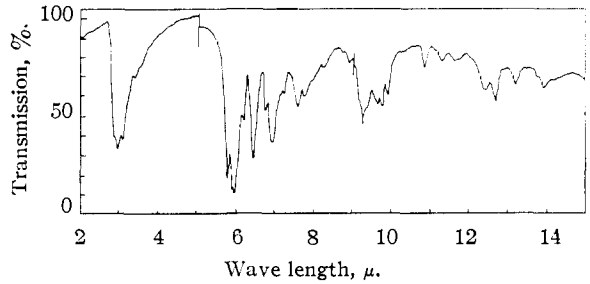


Fig. 2.—Infrared spectrum of Compound A: potassium bromide disc containing 0.119% of Compound A.

Under these conditions, when xanthopterin and leucopterin gave positive tests for guanidine, Compound A gave a negative reaction. Nevertheless Compound A was still considered to be a pteridine, and because of its presumed relationship to riboflavin, it seemed possible that it might be a 2,4-dihydroxy- or 2,8-dihydro-2-oxopteridine which would not give guanidine, but might be expected to yield urea when subjected to alkaline hydrolysis. Indeed, using conditions under which riboflavin is hydrolyzed to urea, a 52% yield of this substance was obtained from Compound A. Two conclusions can be deduced from this experiment. Firstly, good evidence was obtained that Compound A was indeed a 2,4-dihydroxy- or 2,8-dihydro-2-oxopteridine. Secondly, since only riboflavin, but not lumichrome, yields urea by this treatment, it is reasonable to suppose that there is a substituent on the N^8-position of the pteridine nucleus. When Compound A was submitted to catalytic hydrogenation, one mole of hydrogen (based on an equivalent weight of 342; see later) was absorbed. The hydrogenation product could not be isolated because it was very readily reoxidized to the original compound on exposure to air. This was not unexpected, since a reversible reduction with hydrosulfite and reoxidation in air already had been observed. It is not known whether this oxidation–reduction behavior has biological significance.

The possibility that the compound was a glycoside was tested by subjecting Compound A to vigorous acid hydrolysis. After 6 hr. hydrolysis at 100° with 6 *N* hydrochloric acid, most of the compound was recovered unchanged. A small amount of a second blue fluorescent compound with a higher R_f value in butanol–acetic acid–water was obtained but tests for sugars and amino acids on the evaporated and chromatographed hydrolysate were negative. The second constituent of the hydrolysis mixture had the same ultraviolet absorption spectrum as the original; therefore it probably differed from Compound A only in its side chain or chains. Since it was obtained in small yield, it was

(3) W. S. McNutt and H. S. Forrest, THIS JOURNAL, **80**, No. 4 (1958).

(4) Manufactured by Commercial Solvents Corp., Terre Haute, Indiana, to whom we are grateful for a generous supply of this material.

(5) Manufactured by the Filtrol Corporation, Los Angeles 23, California.

(6) E. L. R. Stokstad, *et al.*, THIS JOURNAL, **70**, 5 (1948).

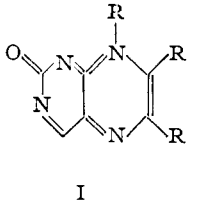
not further investigated. Thus, it seems likely that Compound A is not a glycoside and this conclusion was further substantiated by a consideration of the products of oxidation with sodium metaperiodate, which indicate an open chain polyhydroxy compound (see later).

From the empirical formula and assuming a 2,4-dihydroxypteridine or 2,8-dihydro-2-oxopteridine structure, part of the evidence for which has been presented above, a fragment C_6H_{10} or $_{12}O_6$ remains to be accounted for. It already has been postulated that Compound A has a side-chain on N^8. The existence of a second side chain can be inferred from the following experiments. Compound A is a monobasic acid, and has an equivalent weight of 348—in good agreement with the molecular weight from the empirical formula and the hydrogenation results. Treatment of Compound A with sodium metaperiodate resulted in the consumption of 2 moles of the oxidant, with the liberation of formaldehyde and formic acid. A probable formula for this side chain based on the evidence given above would therefore be $-CH_2CHOHCHOHCH_2OH$.

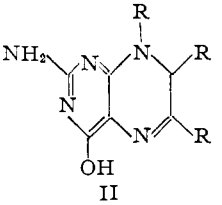
Degradation of the trihydroxybutyl side chain postulated above, also could be effected by exposing Compound A, dissolved in dilute sodium hydroxide (0.1 *N*), to sunlight. Another new compound was obtained (in 10–20% yield) which again was still acidic and had spectral characteristics similar to Compound A but which did not react with sodium metaperiodate nor with 2,4-dinitrophenylhydrazine. Its infrared spectrum however differed from Compound A in having no bands in the primary and secondary alcohol regions, but a new band was present at 7.3 μ. This might be attributed to a C-methyl group and the photolysis to a degradation of the polyhydroxyalkyl chain to methyl.

Discussion

From the evidence given above it is not possible to deduce a structure for Compound A. The compound has properties consistent with a reduced pteridine ring with hydroxy (or oxo) substituents at C_2 and C_4, as deduced from the absorption spectrum, the production of urea on alkaline hydrolysis, and the consumption of one mole of hydrogen. There are probably two side chains, one containing a trihydroxybutyl group and the second a carboxyl group not in direct conjugation with the chromophoric system of the ring. The spectrum, however, differs markedly from those of 2,8-dihydropteridines such as I, or 7,8-dihydropteridines such as II, both of which types have been synthesized recently.[7,8] In both cases the extinction coefficients of the maxima at longer wave lengths are consistently lower than those of the maxima below 300 mμ (in contrast to Compound A, *cf.* Fig. 1) and in the 2,8-dihydro compounds the E_M's are much higher than those of Compound A. Boothe, *et al.*,[9] synthesized a "5,8-dihydropteridine" but again the absorption spectrum is quite unlike that of Compound A.

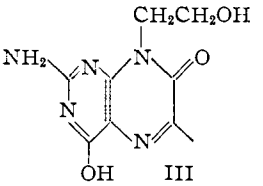


I



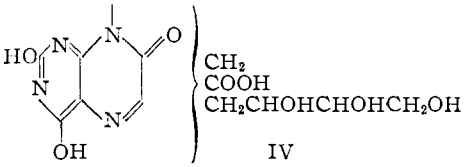
II

On the other hand, the absorption spectrum of III[10] resembles that of Compound A quite closely.



III

Attempts to hydrolyze III to the 2,4-dihydroxy derivative which would be more closely related to Compound A have so far been unsuccessful. A structure based on this analogy (IV) leaves unexplained the apparent absence of properties to be expected from the —C(OH)=C— group, which should manifest itself in the infrared spectrum (Fig. 2), and in the titration data.[11] A possible explanation of this might be that the 4-hydroxy group could be masked by hydrogen bonding with the trihydroxybutyl side chain if this was attached at position 6. Further work is in progress to test these hypotheses and to locate the side chains on the ring.



IV

It is apparent, however, from a consideration of these formulas, that some doubt is thrown on the original supposition that Compound A is an intermediate in riboflavin biosynthesis (see following paper for further discussion of this point). Neither would it appear to be the Compound X postulated by Nathan, Hutner and Levin[12] as an intermediate between pteridines of the folic acid and biopterin groups and riboflavin. The observation that its concentration in the mold fluctuates with the riboflavin content, may only be a reflection of its origin from a common precursor, adenine, through similar biochemical pathways. Recently Masuda[13] has isolated an N^4-substituted derivative of 2,4-dihydroxy-6,7-dimethylpteridine ("G substance") from the mycelium of *Eremothecium ashbyii*, and a second crystalline material ("V substance") which appears to be similar to Compound A (it should be

(7) G. P. G. Dick, W. E. Fidler and H. C. S. Wood, *Chemistry & Industry*, 1424 (1956).

(8) W. E. Fidler and H. C. S. Wood, *J. Chem. Soc.*, in press. We are grateful to these authors for allowing us to read their manuscripts before publication.

(9) J. H. Boothe, *et al.*, THIS JOURNAL, **70**, 27 (1948).

(10) G. B. Elion and G. H. Hitchings, *ibid.*, **75**, 4311 (1953). We are grateful to these authors for a small sample of III supplied as the ethyl ester of the -6-carboxylic acid.

(11) *Cf.* the data given by E. L. Rickes, L. Chaiet and J. Keresztesy, *ibid.*, **69**, 2749 (1947), for rhizopterin and xanthopterin.

(12) H. A. Nathan, S. H. Hutner and H. L. Levin, *Nature*, **178**, 741 (1956).

(13) T. Masuda, *Pharm. Bull. (Japan)*, **4**, 71, 375 (1956).

noted at this point that we have not been able to find Masuda's "G substance" in the mycelium of our culture of *E. ashbyii*). So far as is known, these compounds represent the first pteridines with the 2,4-dihydroxy structure to be isolated from natural sources. The possibilities that they represent a new class of natural compounds and that they may play a role in the mold as co-enzymes by virtue of their oxidation–reduction properties remain to be investigated.

Experimental

Isolation of Compound A.—Riboflavin feed[4] (500 g.) was extracted with a total of 5 l. of 50% aqueous acetone at room temperature. The resulting dark solution was evaporated *in vacuo* to a volume of about 500 ml. and this was then filtered and the filtrate adjusted to *p*H 1 with hydrochloric acid. The solution was then passed through a column (7 × 35 cm.) of washed "Filtrol Grade 76"[5] which adsorbed most of the fluorescent materials. After thorough washing with 2% acetic acid (3 l.) the blue fluorescent material, along with riboflavin 5'-phosphate and flavin adenine dinucleotide, was eluted with 30% aqueous acetone. This eluate was evaporated to small bulk and chromatographed on large sheets of paper (Munktells' No. 20/150), using butanol, acetic acid, water (4:1:5, alcoholic phase) as the irrigating solvent. The blue fluorescent band (R_f, 0.26) after elution was rechromatographed in *n*-propyl alcohol water, concd. NH_4OH (40:20:1). The fluorescent band (R_f, 0.49) was eluted and the eluate was evaporated *in vacuo* to 1 ml. Glacial acetic acid (0.4 ml.) was then added, followed by methanol (1 ml.). The slight precipitate which formed was collected by centrifugation and discarded. The supernatant, on addition of more methanol (4 ml.), slowly deposited clumps of pale yellow crystals of the ammonium salt of Compound A.

Anal. Calcd. for $C_{12}H_{19}N_5O_8$: C, 39.9; H, 5.3; N, 19.4. Found: C, 40.2; H, 5.5; N, 19.2.

An alternative procedure was to evaporate the eluate from the "Filtrol" column and to pass the concentrated solution through a second "Filtrol" column, washing this thoroughly with water, and finally eluting the blue fluorescent material with 10% aqueous acetone. The eluate was then evaporated and the product crystallized by the method given below.

The free acid was obtained by dissolving the above material (180 mg.) in water (1 ml. and adding hydrochloric acid (2 *N*; 0.3 ml.) and methanol (5 ml.). Pale buff colored clumps of needles separated over a period of 2–3 days; m.p. 256–258°; $\alpha^{25}D$ +8.7 ± 0.5° (*c* 2.8, 1 dcm., 0.1 *N* sodium hydroxide), pK_a 3.9.

Anal. Calcd. for $C_{12}H_{16}N_4O_8$: C, 41.9; H, 4.7; N, 16.3; neut. equiv., 344. Found: C, 41.6; H, 4.8; N, 16.6; neut. equiv., 348.

Chlorine Oxidation of Compound A.—The crystalline material (10.3 mg.) dissolved in a saturated aqueous solution of chlorine was allowed to stand at 40° for 15 min. The solution was evaporated *in vacuo*, the residue redissolved in water and the solution was again evaporated. After heating the residue at 100° for 30 min. with hydrochloric acid (0.1 *N*; 4 ml.), the acid was removed over potassium hydroxide, and the residue dissolved in water (1 ml.). An aliquot (0.075 ml.) of this solution was treated with the guanidine reagent.[14] The characteristic orange color which was obtained from a like aliquot of solutions obtained from leucopterin (5.3 mg.) or xanthopterin (5.3 mg.) treated similarly, was absent.

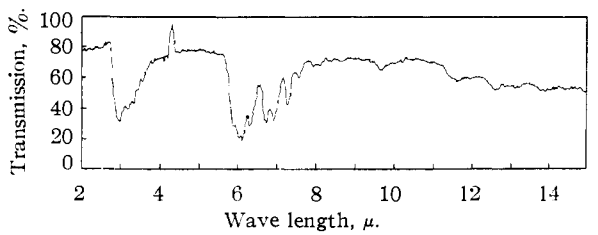


Fig. 3.—Infrared spectrum of the photodegradation product from Compound A.

Degradation to Urea.—Compound A (2.3 mg.) was heated at 80–90° in a nickel container with sodium hydroxide (0.5 *N*; 2 ml.) for 6–8 hr. The volume in the open container was maintained by addition of water from time to time. Finally, the solution was saturated with carbon dioxide and evaporated to dryness in a desiccator. The residue was extracted with a small volume of hot ethanol, and the resulting solution was used for paper chromatography using *n*-propyl alcohol 1% ammonia (2:1) as the irrigating solvent. An authentic sample of urea, detected after chromatography as its mercury salt,[2] was used to locate the appropriate area of the paper. This band was then cut out and the urea in it measured by the method of Conway.[15] The quantity obtained was 209 μg. or 52% of the theoretical. The urea was further identified by paper chromatography in two different solvents, *n*-pentanol–formic acid–water (50:10:40, upper phase), R_f, 0.22, and *t*-amyl alcohol–formic acid–water (65:30:5), R_f, 0.61.

At least five different fluorescent products were produced in this hydrolysis, but none of them were obtained in sufficient quantity for identification.

Hydrogenation of Compound A.—The crystalline acid (10.869 mg.) was hydrogenated in glacial acetic acid (5 ml.) using Adams catalyst. The uptake of hydrogen which ceased sharply, was 0.712 ml. (S.T.P.). Based on a molecular weight of 342, this corresponds to 1 mole of hydrogen/mole. Attempts to isolate the hydrogenation product or even to determine its absorption spectrum were unsuccessful, since it reoxidized rapidly as soon as it was removed from the reaction vessel.

Acid Hydrolysis.—The pteridine (1 mg.) was heated at 100° for 6 hr. with hydrochloric acid (6 *N*; 1 ml.). The acid was then removed in a desiccator over sodium hydroxide and the residue, dissolved in water, was chromatographed on paper using butanol–acetic acid–water (4:1:5) as solvent. There were two main fluorescent bands, the larger of which (R_f, 0.17) was identified as due to the original compound. The material recovered by elution from the second band (R_f, 0.29) was found to have an absorption spectrum corresponding exactly to that of the original compound (maxima at 278 and 325 mμ; minima at 250 and 302 mμ in 0.1 *N* hydrochloric acid; maxima at 252, 285 and 352 mμ; minima at 245, 270 and 308 mμ in 0.1 *N* sodium hydroxide).

Two further very faint fluorescent bands R_f's 0.38 and 0.55) were detectable on the chromatogram, which however showed no reaction when sprayed with aniline hydrogen phthalate reagent (for sugars) or ninhydrin reagent (for amino acids).

Treatment of Compound A with 2,4-Dinitrophenylhydrazine.—The pteridine (0.5 mg.) was allowed to stand with a saturated solution of 2,4-dinitrophenylhydrazine in hydrochloric acid (2 *N*; 0.3 ml.) for 14 hr. The clear solution was then chromatographed on paper using butanol–acetic acid–water (4:1:5) as the solvent. The pteridine (R_f, 0.19) was recovered unchanged.

Action of Light on Compound A.—A solution of the compound (1 mg.) in sodium hydroxide (0.1 *N*; 0.3 ml.) was allowed to stand in sunlight in a capillary tube (bore, 1.5 mm.) for 3 hr. The solution was streaked on paper (Whatman No. 3) and the chromatogram developed with dilute acetic acid (*N*) as the solvent. Two blue fluorescent bands were obtained, the faster moving being unchanged starting material. The slower one was eluted from the dried paper with dilute ammonia, the eluate was evaporated to dryness, and the residue was crystallized from a small amount of dilute acetic acid. From a total of 60 mg. of Compound A, about 7 mg. of this compound was produced. Its absorption spectrum was similar to that of Compound A (maxima at 277 and 323 mμ, minima at 243 and 297 mμ in 0.1 *N* hydrochloric acid; maxima at 250, 275 and 334 mμ, minima at 242, 268 and 295 mμ in 0.1 *N* sodium hydroxide). The compound was still acidic (paper electrophoresis) but was no longer oxidized by sodium metaperiodate. Its infrared spectrum showed a typical C-methyl absorption band at 7.3 μ. Its R_f values in various solvents were: in *n*-propyl alcohol, 1% ammonia, 0.37; in 4% sodium citrate, 0.30; in butanol, acetic acid, water (4:1:1), 0.29.

(14) J. E. Andes and V. C. Myers, *J. Biol. Chem.*, **118**, 137 (1937).

(15) E. J. Conway, "Micro-Diffusion and Volumetric Error," 3rd Edition, D. Van Nostrand Co., Inc., New York, N. Y., 1950, p. 152.

Action of Sodium Metaperiodate.—A solution (1 ml.) of the compound (0.732 mg.) in water was oxidized in the usual way with sodium metaperiodate (0.02 *M*) and after 30 min. (when the reaction was found to be complete), excess oxidant was estimated volumetrically with sodium thiosulfate. Using a value of 340 for the molecular weight, the uptake of periodate was calculated as 2 moles/mole. A check on this figure was obtained by following the oxidation spectrophotometrically,[16] when the same value was obtained, and the reaction was shown to be virtually complete after 20 min.

A qualitative test for formaldehyde using chromotropic acid on the periodate oxidized material was strongly positive. The compound (2 mg.) was oxidized with sodium metaperiodate in a Conway microdiffusion dish and after 30 min. sulfuric acid (10%; 1 ml.) was added and then anhydrous sodium sulfate (3 g.). The outer chamber contained water (0.5 ml.). The volatile constituents were allowed to diffuse into this during 24 hr., when the aqueous solution was carefully neutralized and then evaporated. After standing for 4 days in a desiccator, the residue was dissolved in water (1 ml.) and the solution divided into two parts. One sample gave a very weak reaction with chromotropic acid; the second was treated with hydrochloric acid and magnesium ribbon,[17] and then tested with chromotropic acid. It gave a strong positive reaction, indicating that formic acid was produced during the oxidation.

(16) J. S. Dixon and D. Lipkin, *Anal. Chem.*, **26**, 1092 (1954).

(17) W. M. Grant, *ibid.*, **20**, 267 (1948).

PASADENA 4, CALIFORNIA

[CONTRIBUTION FROM THE RESEARCH DIVISION, PARKE, DAVIS & COMPANY]

The Isolation and Characterization of Three Crystalline Antibiotics from *Streptomyces plicatus*

BY THEODORE H. HASKELL, ALBERT RYDER, ROGER P. FROHARDT, SALVATORE A. FUSARI, ZBIGNIEW L. JAKUBOWSKI AND QUENTIN R. BARTZ

RECEIVED AUGUST 26, 1957

Three crystalline basic antibiotics have been isolated from fermentation broths of an actinomycete designated as *Streptomyces plicatus*. One of these antibiotics is amicetin, while the other two, designated as plicacetin and bamicetin, are novel. The three entities readily can be differentiated from each other by paper chromatographic and solvent extraction procedures. Comparison of physical and chemical properties indicates that the three antibiotics are closely related in structure.

Three weakly basic antibiotics active against experimental mouse tuberculosis have been isolated in crystalline form from the filtrates of an actinomycete arbitrarily designated as *Streptomyces plicatus*. One of these antibiotics is amicetin,[1-3] while the other two, designated as plicacetin[4] and bamicetin, are novel. During the course of the investigation of these antibiotics at least two others were shown to be present in culture broth filtrates of this *Streptomyces*. However, since these occurred in small and variable quantities, their isolation was not attempted and only the isolation of plicacetin, amicetin and bamicetin and the further characterization of plicacetin and bamicetin will be described in this paper.

The three antibiotics named above were extractable from filtered beer with 1-butanol at slightly alkaline *p*H values. They could then be extracted from butanol into water acidified to *p*H 2.0. Furthermore, these weakly basic substances could be precipitated from aqueous solutions with various aromatic azosulfonic acid dyes, and they could be successfully adsorbed on and eluted from weak cation-exchange resins such as Amberlite IRC-50. However, since it was found that these substances could be isolated by simple solvent extraction techniques, the aforementioned procedures are not included in this publication. The three antibiotic substances readily can be differentiated from one another by paper strip chromatography and Craig countercurrent extraction techniques.

Plicacetin was the first of these antibiotics to be isolated by us in pure form. This compound, which can exist in three different crystalline modifications, was isolated from shaker flask and stirred-jar fermentor filtrates. Structural studies[5] of this substance showed it to be identical with the amicetin molecule minus the α-methylserine moiety.[6] This conclusion was reached from an alkaline hydrolysis experiment, the only products formed being cytosamine[6] and *p*-aminobenzoic acid, and finally by synthesis as described in a subsequent paper.[5,7] Thus plicacetin is, in all probability, a precursor to amicetin. Since the *in vitro* and *in vivo* biological activity of plicacetin *vs. Mycobacterium tuberculosis* H37Rv was less than amicetin and bamicetin, our interest was devoted primarily to the latter. The ultraviolet absorption spectra of plicacetin in acidic, basic and neutral media are shown in Fig. 1. The *a* values obtained were 36.8 and 29.2 at λ_{max} 257 and 311.5 mμ, respectively, in 0.1 *N* hydrochloric acid; 26.6 and 53.5 at λ_{max} 249 and 321 mμ, respectively, in *p*H 7.0 phosphate buffer; 63.9 at λ_{max} 329 mμ in 0.1 *N* sodium hydroxide. Potentiometric titration showed three ionizable groups with pK_a' values of 2.2, 7.0 and 10.9. The first value is the ionization constant of the aryl amine. Molecular weight values of 520 and 552 were obtained by titration and the Rast procedure,

(1) (a) C. DeBoer, E. L. Caron and J. W. Hinman, THIS JOURNAL, **75**, 499 (1953); (b) J. W. Hinman, E. L. Caron and C. DeBoer, *ibid.*, **75**, 5864 (1953).

(2) M. H. McCormick and M. M. Hoehn, *Antibiotics & Chemotherapy*, **3**, 718 (1953).

(3) Y. Hinuma, M. Kuroya, T. Yajima, K. Ishihara, S. Hamada, K. Watanabe and K. Kikuchi, *J. Antibiotics (Japan)*, **A8**, 148 (1955).

(4) Parke, Davis & Co., British Patent 707,332 (Apr. 14, 1954); [*cf. C. A.*, **48**, 13173 (1954)].

(5) T. H. Haskell, THIS JOURNAL, **80**, 747 (1958).

(6) E. H. Flynn, J. W. Hinman, E. L. Caron and D. O. Woolf, Jr., *ibid.*, **75**, 5867 (1953).

(7) After these two papers were written a manuscript by P. Sensi, A. M. Greco, G. G. Gallo and G. Rolland of Lepetit S.p.A., Milan (Italy) was received in which they described the isolation and structure of an amicetin-like antibiotic (amicetin B) which is identical with plicacetin; *Antibiotics & Chemotherapy*, in press.