

IDENTIFICATION OF THE ANTIBIOTIC SUBSTANCE FROM CASSIA RETICULATA AS 4,5-DIHYDROXYANTHRA- QUINONE-2-CARBOXYLIC ACID*

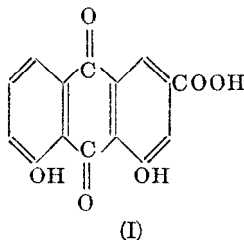
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Robbins, Kavanagh, and Thayer (1) recently reported the isolation from the leaves of *Cassia reticulata* Willdenow of a substance possessing antibiotic activity *in vitro* against *Bacillus mycoides*, *Bacillus subtilis*, *Staphylococcus aureus*, *Mycobacterium smegmatis*, and *Neisseria gonorrhoeae*. The compound was obtained in crystalline form by careful acidification of its dilute alkaline solution, and was called "cassic acid."

This substance has now been identified as rhein, a compound first isolated from Chinese rhubarb (2) and later from a species of cassia (*Cassia acutifolia*) (3). The structure of rhein has been established as 4,5-dihydroxyanthraquinone-2-carboxylic acid (I) (4-7).



For purposes of comparison, rhein was prepared both by isolation from rhubarb and by synthesis. Identity of the isolated and synthetic samples with the antibiotic substance and with each other was established by mixed melting points of the diacetates of the compounds, as well as by identity of the absorption spectra of the compounds and of their diacetates (Figs. 1 and 2). Rhein from rhubarb and synthetic rhein have the same antibiotic activity¹ as rhein from cassia.

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¹ Antibiotic activity refers to activity against *Staphylococcus aureus*, tested in nutrient broth near pH 7. For details of the method, see Kavanagh (8). The number of dilution units in a sample is given by the total volume (in ml.) to which it can be diluted with nutrient broth and still bring about complete inhibition of the growth of the test organism. The assays were carried out by a geometric serial dilution (2, 4, 8, etc.) and the values therefore lie between those given and the next higher power of 2.

Antibiotic activity of quinones has been reported frequently (9), but relatively few studies have been made in this connection with anthraquinone derivatives. Anthraquinone itself and chrysophanic acid have been found inactive (10), while it is interesting to note that anthraquinone and certain

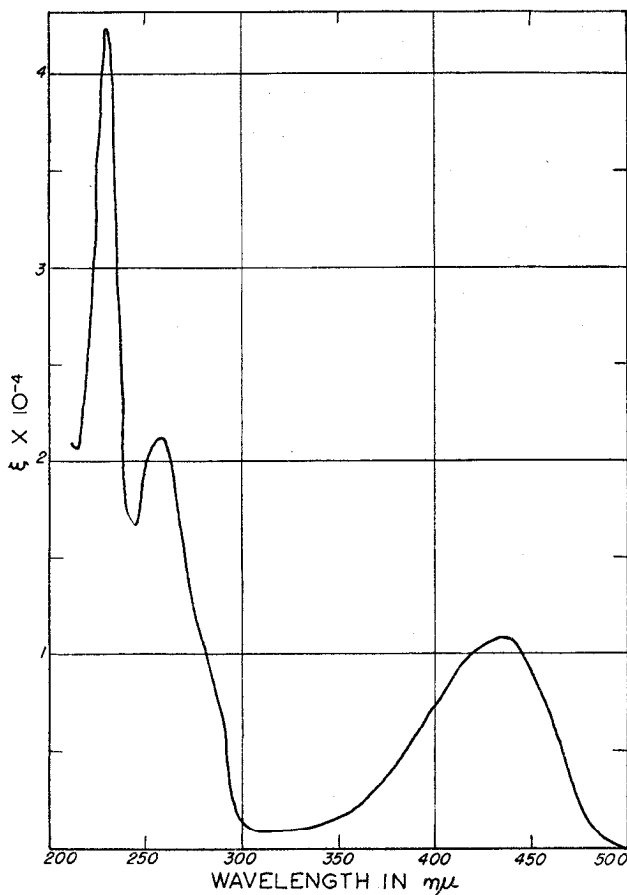


FIG. 1. Rhein in 95 per cent ethanol

of its derivatives, injected intraperitoneally, inhibit the growth of Twort carcinoma in mice (11). Emodin, which is present in rather large amounts in rhubarb, was found to be bacteriostatic under the conditions of the test employed.¹ The antibiotic activity of rhubarb extracts, therefore, is not due entirely to the presence of rhein.

Other anthraquinone derivatives are being prepared for further study.

Compounds of the type of the acetate which are gradually hydrolyzed under physiological conditions (Fig. 3)² may also prove of interest.

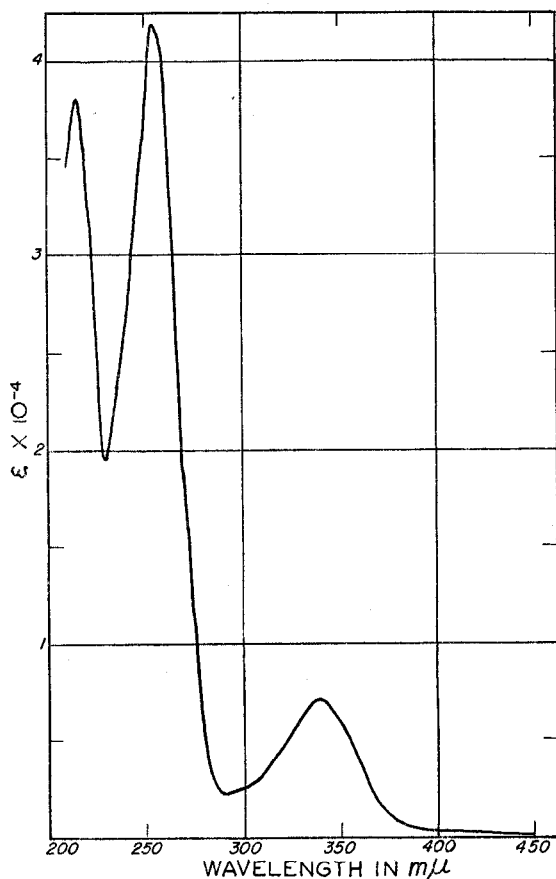


FIG. 2. Rhein diacetate (or its methyl ester) in 95 per cent ethanol

EXPERIMENTAL³

Isolation Procedure—A batch of 100 gm. of coarsely ground leaves of *Cassia reticulata* Willd. was placed loosely in a fluted filter paper over a

² Rhein diacetate shows about half the antibiotic activity of rhein itself. However, it is difficult to determine whether this activity is actually due to the diacetate or whether it results from the slow splitting of the diacetate to free rhein which occurs under the conditions of the test. It is apparent from the curves in Fig. 3 that, after 20 hours of incubation, the acetate has been in large part hydrolyzed.

³ All melting points are corrected.

wad of glass wool in an extractor of the type described by Clarke and Kirner (12), and the active material was extracted for three periods of 1, 1.5, and 1.5 hours, with 750 ml. of water⁴ for each extraction. (Extraction by suspension of the leaves in boiling water was unsatisfactory, since a muddy solution resulted with which it was very difficult to work.) The

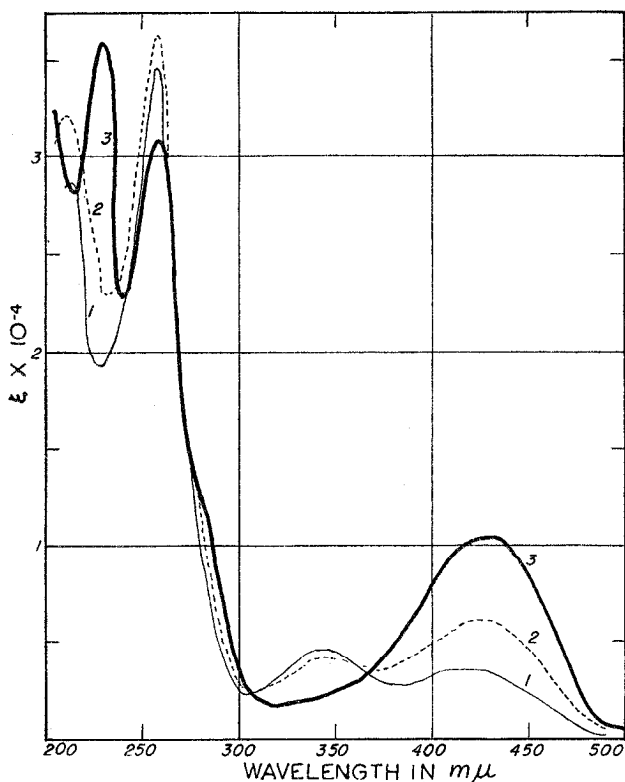


FIG. 3. Rhein diacetate in aqueous solution at a pH near 7. (The substance was dissolved by neutralization with NaOH.) Curve 1, within 1 hour of solution; Curve 2, after 2 hours incubation at 37°; Curve 3, after 20 hours incubation at 37°.

combined solution usually contained about 23,000 dilution units¹ for *Staphylococcus aureus*, of which about two-thirds were obtained in the

⁴ Methods involving carbonate or bicarbonate extraction of the leaves yielded mixtures from which the active substance could not satisfactorily be isolated. In one of the earlier large batches (2 kilos) of leaves extracted with carbonate and subjected to a complicated fractionation, a biologically inactive, pale yellow crystalline material was obtained, in amounts roughly corresponding to the expected amount of rhein, while none of the latter could be obtained. The structure of this substance is being investigated.

first extract. The solution was concentrated under reduced pressure to about 100 ml., and the syrupy concentrate was extracted with methyl isobutyl ketone in a continuous extractor until the extract was practically colorless. The organic solution was then shaken in a separatory funnel with small portions (10 to 25 ml.) of 5 per cent sodium bicarbonate solution, as long as the typical reddish color appeared in the extracts. Usually a total of about 50 to 100 ml. was used. The iced bicarbonate extract was acidified to about pH 2 with cold, dilute hydrochloric acid, and the tan amorphous precipitate was centrifuged, washed with water, and dried *in vacuo*. The yield varied from 215 to 348 mg., with an average, in twenty-three runs, of 310 mg., having a potency of 64 dilution units¹ per mg. This amounts to about 20,000 dilution units, or 87 per cent of the 23,000 in the original aqueous extracts.

On recrystallization from acetic acid, the yields varied widely, depending on the length of time the material was in contact with the hot solvent. Previous removal of dark pigment with cold acetic acid, or with acetone followed by cold acetic acid, was necessary for subsequent successful crystallization. The active substance crystallized in pale yellow hair-like needles, mostly in rosettes or sheaves. Under certain conditions, usually too concentrated a solution or too sudden crystallization from the hot solution, an orange powder precipitated, which consisted of microscopic diamond-shaped spicules. This had the same antibiotic activity as the yellow needles. The melting point was not characteristically different. Exact conditions for production of each form were not determined, but seeding the solution with the yellow needle form usually resulted in crystallization of this type. Likewise, local cooling usually started crystallization in the yellow form, which then continued. The recovery of activity varied from roughly 100 per cent to as low as 6 per cent, and the potency of the once crystallized material from 128 to 256 dilution units per mg. The substance is difficult to obtain pure. Samples, recrystallized several times and dried *in vacuo* at 100°, melted at 326–329° with decomposition, and gave the following analyses:

$C_{15}H_8O_6$.	Calculated.	C 63.37, H 2.81
234	Found.	" 62.78, 63.73; H 3.22, 3.28

The absorption spectra in 95 per cent ethanol and in 0.1 N sodium hydroxide are shown in Figs. 1 and 4 respectively. The ethanol solution shows maxima at 230, 260, and 430 $m\mu$, and minima at 215, 245, and 307 $m\mu$. In alkali the spectrum changes gradually with time, as shown in Fig. 5. The measurements for the sodium hydroxide curve in Fig. 4 were completed within half an hour after the sample was dissolved, and show maxima at 240 and 500 $m\mu$, and a minimum at 370 $m\mu$. Between 265 and 280 $m\mu$, there is a distinct shoulder.

The crystalline material gives the tests described as characteristic for rhein (13).

Diacetate of Rhein Isolated from Cassia (4,5-Diacetoxyanthraquinone-2-carboxylic Acid)—A solution of 142 mg. (0.5 mm) of crystalline rhein and 200 mg. of dry sodium acetate, in 25 to 50 ml. of acetic anhydride, was

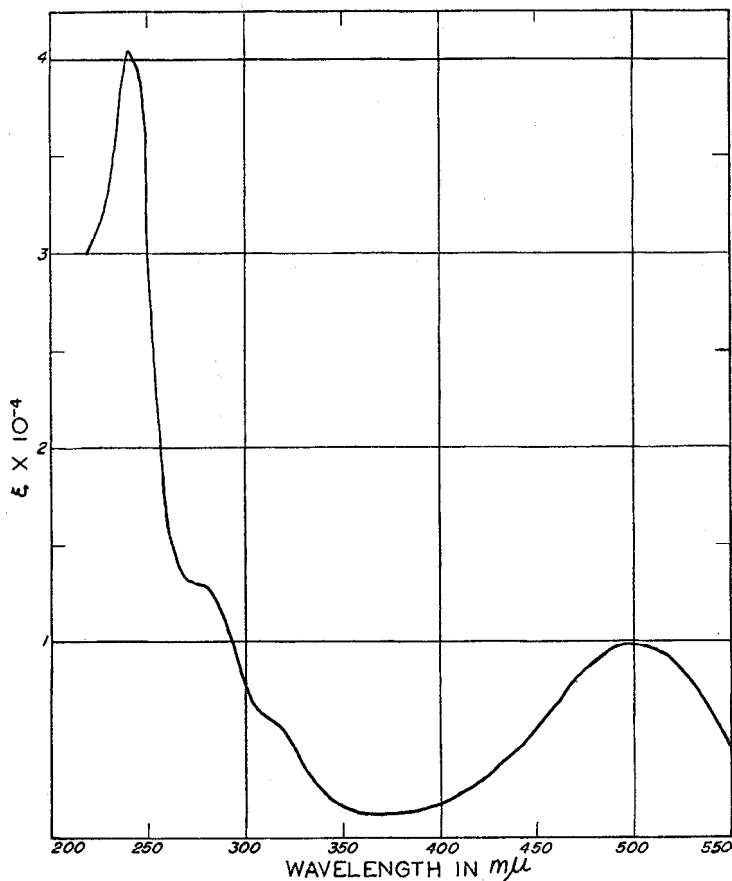


FIG. 4. Rhein in 0.1 N NaOH at room temperature. (The "blank" cell of the Beckman spectrophotometer contained 0.1 N NaOH.)

boiled under a reflux for 15 minutes, centrifuged clear of sediment, and poured into 250 ml. of ice water. The precipitate, a pale yellow powder, melted unsharply around 236°. After one recrystallization from acetic acid, 110 mg. (60 per cent of the theoretical) were obtained, melting at 247–252°. After several recrystallizations, the material melted at 250–

251°, and gave no depression with the synthetic product melting at 250–253°.

The absorption spectrum in 95 per cent ethanol is shown in Fig. 2. Maxima are present at 215, 255, and 340 $m\mu$, and minima at 230 and 295 $m\mu$. The curve for the synthetic diacetate is superimposable.

The solution for the curves shown in Fig. 3 was prepared by neutralization of the diacetate with sodium hydroxide. While the diacetate is much

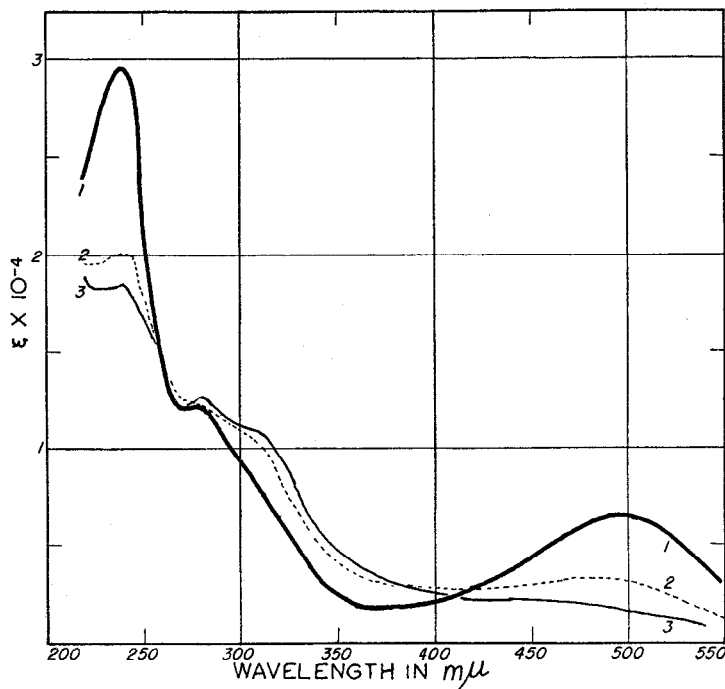


FIG. 5. The effect of alkali on rhein. (The conditions are the same as those for Fig. 4.) Curve 1, after 4 hours; Curve 2, after 48 hours; Curve 3, after 4 days.

more readily crystallized than rhein itself, a good analysis could not be obtained. A summary of the difficulties described by previous authors is given by Tutin and Clewer (3), who believed that they had the pure compound, but remarked on its peculiar behavior; *i.e.*, it dissolved completely in hot xylene and reprecipitated immediately. In a later paper (14), Tutin attributes this behavior to the presence of acetic anhydride of crystallization.

The results of analyses of samples prepared from isolated rhein are presented in Table I. The analytical figures suggest retention of the solvent.

Methyl Ester of Diacetate of Isolated Rhein (4,5-Diacetoxyanthraquinone-2-carboxylic Acid, Methyl Ester)—A suspension of 55.2 mg. (0.15 mm) of the diacetate in dry ether was treated with an ethereal diazomethane solution until no further reaction occurred. The ether was removed, and the residue was taken up in 10 ml. of 95 per cent ethanol and a small amount of solid removed by centrifugation. When the alcoholic solution was concentrated to about 3 ml. and allowed to cool, the product crystallized in beautiful clusters of pale yellow needles. Yield, 39 mg. (68 per cent of the theoretical), melting around 184°. After several recrystallizations from 95 per cent ethanol, the product melted at 194–195°. It was dried *in*

TABLE I

Analyses of Diacetate of Samples of Rhein Isolated from Cassia

Sample solvent	Melting point °C.	Dried at	C	H	COCH ₃
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Calculated, C ₁₉ H ₁₂ O ₈	368		61.93	3.26	23.36
Acetic acid	250–251	78°, 1hr., <i>in vacuo</i>	61.10	3.81	25.35
95% ethanol	252–254	Room temperature, 2 wks. <i>in vacuo</i>	60.85	3.28	21.82

vacuo at 100° for 1 hour, for analysis. The absorption spectrum in 95 per cent ethanol is the same as that of the diacetate, shown in Fig. 2.

C ₂₀ H ₁₄ O ₈	Calculated.	C 62.82,	H 3.69,	OCH ₃ 8.10
382	Found.	“ 62.61,	“ 3.64,	“ 8.35

Synthesis of Rhein—Synthetic rhein was obtained from chrysophanic acid synthesized according to the method of Eder and Widmer (7). The chrysophanic acid was converted to rhein through the acetate, by chromic acid oxidation, as described by Fischer, Falco, and Gross (4). The synthetic rhein melted at 325–330°. Its diacetate melted at 250–253°, and, when mixed with the acetate of the isolated product melting at 250–251°, it gave no melting point depression. Absorption spectra of the rhein samples and of their diacetates were identical (Figs. 1 and 2). Both the synthetic and isolated rhein samples showed antibiotic activity of 256 dilution units per mg.

Isolation of Rhein from Rhubarb Root—A 50 gm. batch of dry, coarsely ground root of *Rheum officinale* (rhubarb root, U. S. P., Chinese, Penick) was extracted as described for cassia leaves for four 1.5 hour periods, with one 1 liter and three 750 ml. portions of water. The combined extracts contained 46,400 dilution units. The solution was concentrated under reduced pressure to a volume of 400 ml., made 0.25 N with respect to hydro-

chloric acid, boiled under a reflux for 1 hour, and extracted with methyl isobutyl ketone in a continuous extractor for about 12 hours. The organic layer was shaken with 5 per cent sodium bicarbonate solution. On acidification, 204 mg. of crude rhein were obtained, with a potency of 64 dilution units per mg. After purification, the potency was 256 dilution units per mg. The acetate of this material melted at 249–251°, and gave no melting point depression with the acetate of rhein isolated from cassia leaves. The absorption spectra of the two compounds were identical with those of the isolated material and its diacetate, shown in Figs. 1 and 2 respectively.

SUMMARY

Simple methods for the isolation of the antibiotic principle from the leaves of *Cassia reticulata* Willd., and from rhubarb root, are described. The antibiotic substance, previously named "cassic acid," is identified as 4,5-dihydroxyanthraquinone-2-carboxylic acid (rhein). A new derivative of rhein, the methyl ester of the diacetate, is described, and absorption spectra are presented for this compound as well as for rhein and its diacetate.

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