Isolation of an Antihistaminic Principle Resembling Tomatine from Crown Gall Tumors

Abstract. *Antihistaminic activity was demonstrated previously in extracts of crown gall tumors by assaying in vivo. A crystalline substance isolated from the crude extracts has now been found to possess a high degree of potency against histamine, acetylcholine, bradykinin, and serotonin when assayed in vitro. The chemical characteristics of the substance resemble closely those of tomatine. When tomatine was investigated, it was found to have biological properties similar to those of the crystalline substances isolated from crown gall tumors. It seems likely that the antihistaminic properties of crown-gall tumor extracts are due to tomatine or a substance which closely resembles it.*

It was first reported in 1950 that extracts of plant tumors (oak gall, crown gall) when injected into guinea pigs protected the animals against subsequent histamine aerosol (*1*). The experiments with oak galls were recently confirmed by Feldberg and Kovacs (*2*) and Berry *et al.* (*3*). It was subsequently shown (*4*) that a single injection of partially purified extracts of oak galls and crown galls brought about a protection which lasted for several days or weeks. None of these early extracts were active in vitro. In this report we describe a method for the isolation of an active principle found in crown gall extracts and its tentative identification with tomatine. It is also shown that the crystalline substance isolated from crown galls and the commercially available crystalline tomatine exert the same effects on the isolated guinea pig ileum preparation against histamine, bradykinin, serotonin, and acetylcholine induced contractions. Furthermore, data are presented showing that an intraperitoneal injection of crystalline tomatine protects the animals against the lethal effect of a 0.2-percent histamine aerosol.

Crown galls were induced on tomato stalks and extracted by methods previously described (*4*). Initially, biological activity of the various extracts was identified at each step by the histamine aerosol method (*4*). In the course of purification, however, this technique became a major obstacle to progress because of the large amounts of each fraction needed for testing. Further purification and eventual isolation of the active principle was achieved with relative ease after it had been found that the activity of the purified extracts could now be assayed on isolated organ preparations.

Crown gall tumors (350 g) were ground in a Waring blender and extracted three times with 350-ml portions of ethanol-ether (3:1, vol/vol). The pulp was filtered and the clear ethanol-ether supernatant was evaporated in an atmosphere of argon at reduced pressure and at 45° to 50°C. The residue was extracted with water (*p*H 5.6; 25 ml/100 g original tissue) and the aqueous extract was filtered. The clear filtrate was adjusted to *p*H 8 with dilute NaOH to give a precipitate of 175 mg. Unlike the more crude fractions, the precipitate was active both in vivo and in vitro. For further purification, 10 mg of precipitate was dissolved in 1 ml of dilute HCl (*p*H 3.2) and the brown-colored solution was applied to a column (0.9 × 30 cm) of Sephadex G-25. The gel had been equilibrated previously with dilute HCl (*p*H 3.2), and the same solvent was used to develop the chromatogram. Some light-colored component(s) appeared to remain firmly bound to the dextran gel. The effluent was obtained in 2-ml portions in a fraction-collector and assayed by the ninhydrin-colorimetric method of Hirs *et al.* (*5*). Each fraction was assayed also for antihistaminic activity on isolated ileum of the guinea pig. Inhibitory activity coincided with the single ninhydrin-positive peak obtained. From the assay, it was estimated that a 10- to 15-fold increase in specific activity had been obtained by the gel filtration procedure.

The peak fractions were pooled, brought to *p*H 8 with dilute NaOH, and extracted three times with 10-ml portions of chloroform-ethanol (4 : 1, vol/vol). The chloroform-ethanol extracts were dried at reduced pressure at 35°C and yielded a white, crystalline product.

The method and apparatus used for the guinea pig ileum preparation was the same as that described earlier (*6*). For assay, the various fractions were dissolved in water acidified to a *p*H of 3.2. The amount of the solution added to the bath varied from 0.1 to 0.3 ml since it was found that addition of 0.1 to 0.3 ml of water at the same *p*H did not influence the histamine-induced contraction.

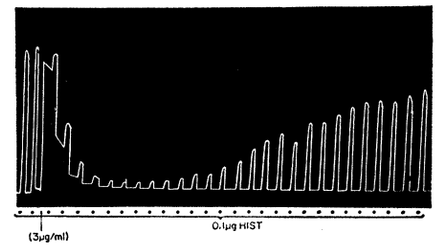

Fig. 1. Responses of a guinea pig ileum preparation to histamine before and after the addition of crystalline crown gall substance as marked at arrow, in a concentration of 3×10^{-6}. Intervals of 3 minutes elapsed between each addition of histamine, indicated by the black dots. The histamine was left in contact with the preparation for 20 seconds, and the crown gall substance for 2 minutes. The organ bath was washed out between each addition of histamine, the drum being stopped temporarily and restarted 20 seconds before the next addition.

A typical example of the effect obtained by the use of crystalline crown gall substance on contractions induced by histamine is shown in Fig. 1. The addition of the substance in a concentration of 3×10^{-6} to the bath itself elicited an initial contraction similar to that of histamine. However, the responses to the subsequent standard dose of 0.1 μg of histamine, with repeated washings, became progressively smaller. The sensitivity of the ileum remained much reduced for periods in excess of 1 hour. The initial exciting and the subsequent very long-lasting depressive effect is a very characteristic

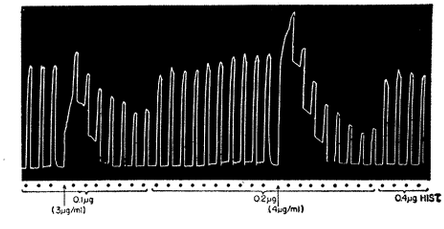

Fig. 2. Responses of guinea pig ileum preparation to histamine (added to the bath at black dots) before and after the addition of crystalline tomatine as marked at arrows, in two different concentrations (left, 3×10^{-6} and right, 4×10^{-6}). Intervals of 3 minutes elapsed between each addition of histamine. In each instance the drum was stopped temporarily after washing out the organ bath and restarted 20 seconds before the next addition. The histamine was left in contact with the preparation for 20 seconds, and the tomatine for 2 minutes.

feature of the crystalline substance, and both effects show a direct correlation with the amounts added to the bath—that is, the higher the dose the stronger the initial contraction and the subsequent depression.

From its chromatographic behavior with Sephadex G-25 it appeared that the active principle had a molecular weight in the range 1000 to 2000. It was therefore of interest to compare the crystalline crown gall substance with tomatine, a known constituent of tomato plant of similar molecular weight (1035).

The crystalline crown gall product and tomatine (*7*) were spotted on Whatman No. 1 paper and chromatographed according to the method of Tukalo (*8*). The chromatograms were dried at room temperature in a draft of air. Spots were developed with ninhydrin and phosphomolybdate reagents (*9*), the latter to locate steroid-containing component(s). The results indicated that similar, if not identical, steroid-containing components were present in both samples. It was therefore of interest to study the biological effects of tomatine.

Figure 2 shows that the effect of crystalline tomatine on histamine-induced contractions, when administered in concentrations of 3×10^{-6} and 4×10^{-6} to the bath, is very similar to the effect of crystalline crown gall substance. To reduce the time required for each assay, it was necessary to double the original dose of histamine so as to obtain responses similar to those of the standard controls. When tested against contraction induced by bradykinin, serotonin, and acetylcholine, the efficacy of both tomatine and the crystalline crown gall substance was the same as against histamine.

The antihistaminic activity of crown-gall tumor extracts up to the stage of Sephadex fractionation has been firmly established in vivo. However, we have not yet accumulated a sufficient quantity of the crystalline product for testing in vivo. Three batches of crystalline commercial tomatine have been tested by the histamine aerosol technique, on 40 guinea pigs. When given intraperitoneally, in a dose of 10 mg/kg, only one batch showed definite protection against the lethal effect of 0.2 percent histamine aerosol.

From these findings it appears that the principle(s) found in crown gall tumors is unique in its properties since it inhibits equally four major smooth-muscle contracting agents for a long period of time. This prolonged effect is also apparent in vivo, as evaluated against histamine aerosol. Although crystalline, there may be more than one principle, and it seems quite probable that one of them is tomatine or something similar. We are aware of the implications of a naturally occurring antihistaminic with a steroid nucleus.

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Chloramphenicol in Blood: Simple Chemical Estimations in Patients Receiving Multiple Antibiotics

Abstract. *A simple method is described which obviates the difficulties of estimating the concentration of chloramphenicol in the blood in the presence of other antibiotics. This is of importance in avoiding hematologic depression from chloramphenicol toxicity.*

Chloramphenicol has proved itself an extremely valuable antibiotic, but its use has been occasionally associated with blood dyscrasias. The commonest form of hemopoietic toxicity is transient erythropoietic depression, which appears to be closely related to the concentration of the drug in the plasma (*1*), and measurements of such concentrations in the blood may therefore be of value in predicting its harmful effects.

After the administration of chloramphenicol, the free or biologically active form is converted into two major products (*2*), the glucuronide and aryl amines, both of which are biologically inert. When chloramphenicol succinate (*3*) or palmitate (*4*) is employed, the esters are first hydrolyzed, liberating free chloramphenicol. The free form of the drug assumes importance in evaluating both its therapeutic and toxic effects.

In the past, there have been disadvantages with both the chemical and biological methods of assaying chloramphenicol when the patients have been receiving multiple antibiotics, as in cases of meningitis. The biological assay, though measuring free chloramphenicol, requires the use of aseptic techniques and is of limited value when the patient is receiving multiple antibiotics. Chemical procedures (*3*, *5*) dependent on extraction with ethyl acetate, followed by evaporation, reduction of the nitro compound, and diazotization and coupling, are more sensitive, but may be time-consuming and are rendered useless when sulfonamides and procaine penicillin are also being administered, owing to gross interference by aryl amine compounds (*3*). In addition, when chloramphenicol succinate is used, the ethyl acetate solution has to be washed with sodium bicarbonate in order to extract the unhydrolyzed ester (*3*).

Kakemi *et al.* (*6*) recently described a chemical method for assaying chloramphenicol in which the drug is ex-

Table 1. Concentrations of chloramphenicol in the plasma of two patients, estimated by the methods of Kakemi *et al.* (*6*) and Levine and Fischbach (*5*).

Patient	Unconjugated chloramphenicol (μg/ml)	
	Kakemi *et al.*	Levine and Fischbach
A	14	22
B	9.5	15