SEPARATION AND FORMATION OF RYANODINE FROM DEHYDRORYANODINE. PREPARATION OF TRITIUM-LABELED RYANODINE.

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SUMMARY

The commercially available preparation of the naturally occurring diterpene ester ryanodine contains several compounds in addition to ryanodine. In the present study these compounds were separated and purified using high performance liquid chromatography. The two major components, ryanodine and dehydroryanodine represented 90% of the material present. A method for the efficient reduction of dehydroryanodine to ryanodine was developed and used to produce ryanodine having tritium atoms at positions 19 and 20 and a specific activity of 60.8 Ci/mmole.

Key Words: Ryanodine, Dehydroryanodine, Tritium, Catalytic Reduction

INTRODUCTION

Ryanodine, an alkaloid isolated from the bark of Ryania speciosa Vahl (1,2), causes significant alterations of muscle function when present in submicromolar concentrations (3,4). Of the subcellular processes tested in vitro thus far, only calcium movements across sarcoplasmic reticulum membranes have been found to be affected by this agent (3,4). The specificity and potency of the actions of ryanodine suggest that it may prove useful as an experimental probe of sarcoplasmic reticulum calcium metabolism.

The ryanodine preparations used to date are mixtures of two major (2,5) and several minor components (see below). Consequently, in characterizing the mechanism of action of this agent it will be important to identify which is the

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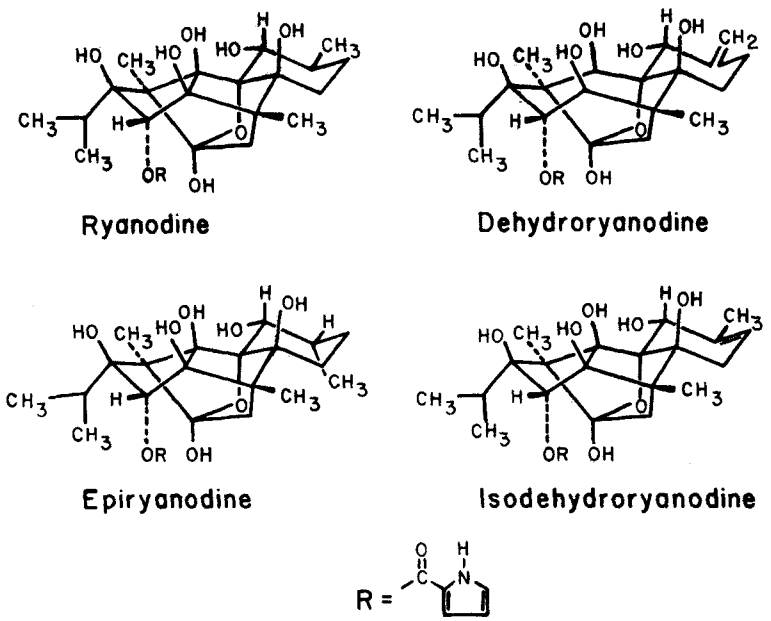

Fig. 1. Chemical structures of ryanodine and its analogs, dehydroryanodine, epiryanodine and isodehydroryanodine.

the active principle. To this end we have separated and purified the two major constituents, ryanodine and dehydroryanodine (Figure 1) (2,9).

A second step in elucidating the mechanism and site of action of a drug having the potency of ryanodine is to make it traceable. Fairhurst made [^{3}H]-ryanodine by introducing bromine atoms across the double bonds of the pyrrole moiety (see Figure 1) and then tritiating the compound through a halogen-tritium exchange reaction (6). In the present report we describe an alternative procedure through which tritium may be introduced into a different part of the ryanodine molecule.

The present work was based on the results of extensive chemical characterization and identification of the compounds present in extracts of the bark of Ryania speciosa conducted by Ruest, Taylor and Deslongchamps (2,9). They identified dehydroryanodine (Δ^{9-20}ryanodine) as the other major component present and determined its structural and chemical characteristics. While this report

was being prepared the tritiation of ryanodine was achieved by another laboratory (7) using the general approach described here, but different reaction conditions which resulted in a lower yield of ryanodine than was obtained in the presently reported studies.

MATERIALS AND METHODS

Partially purified preparations of ryanodine were either purchased from S.B. Penick and Co. (Lot no. 704-RWP-1, Lyndhurst, NJ) or prepared by extracting the bark of Ryania Speciosa (1,2,9). All other chemicals used were of reagent grade or better. The experimental procedures used are described in detail below.

RESULTS AND DISCUSSION

1. Separation of the components of ryanodine. High performance liquid chromatography (HPLC) was used to separate the components present in commercially available ryanodine. Two simple systems based on existing thin layer chromatographic procedures gave equivalent separations. A HPLC system developed at S.B. Penick and Co. (R. Harmetz and R. Puchalski, personal communication) involves preparing ryanodine in 25% acetonitrile in water and the isocratic elution of the components from a reversed phase C_{18} column with this same solvent. In a second system, 50% methanol in water was used as the solvent. The concentration of methanol is not critical; 40% methanol gave an equivalent, but more lengthy separation. A typical chromatogram is shown in Figure 2 and complete procedural details are given in the legend to this figure. The peaks designated as A and B in Figure 2 have been identified as dehydroryanodine and ryanodine, respectively, using standards prepared as described previously (2,9). The identity of the standards with authentic samples was established by spectroscopic analyses (Mass, IR, NMR) and by chemical conversion of dehydroryanodine to ryanodine by catalytic hydrogenation (2,9). In addition, these compounds can be easily distinguished by the formation of typical red (dehydroryanodine) and brown (ryanodine) chromophores upon exposure

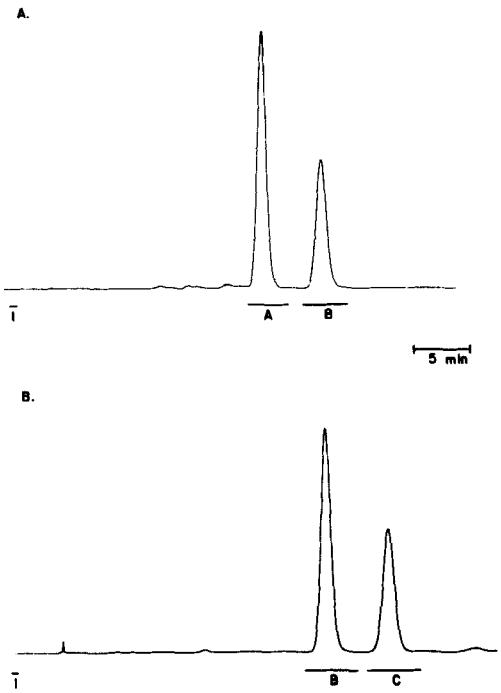

Fig. 2. HPLC chromatograms of ryanodine obtained from S.B. Penick and Co. (Panel A) and of the products of the catalytic reduction of dehydroryanodine (Panel B). The peaks designated as A and B in Panel A are dehydroryanodine and ryanodine, respectively; while those labeled B and C in Panel B represent ryanodine and epiryanodine. The separations were achieved using 50% methanol in water as both the sample and elution solvents. The samples were applied to a Waters μBONDAPAK C_{18} column (P/N 84176, Waters Associates, Milford, MA) and eluted at a flow rate of 2 ml/minute. The peaks were detected at 254 nm using a detector sensitivity of 0.2 AUFS. The time of sample injection is noted by the letter I.

roryanodine prepared in this manner were used in the studies described below.

to sulfuric acid (50%) with slight heating (1,2). The eluted peaks containing ryanodine and dehydroryanodine were collected and the solvent was removed by evaporation and lyophilization. Approximately 90% of the ryanodine mixture applied to the column was recovered in these two peaks. The purified compounds are stable for at least several months, either in aqueous solution stored at 0-4 °C, or as a powder kept at room temperature. Purified ryanodine and dehyd-

Ryanodine and dehydroryanodine are equipotent in their effects on the mechanical performance of intact cardiac muscle and on ATP-dependent accumulation

of calcium by skeletal and cardiac muscle sarcoplasmic reticulum membranes[1]. This suggests that the C-ring of the ryanodine molecule (8) is relatively unimportant for these activities and may provide sites at which this compound can be chemically modified.

2. Reduction of dehydroryanodine to ryanodine and epiryanodine. Taylor (2) demonstrated that dehydroryanodine was converted to ryanodine, epiryanodine and isodehydroryanodine in a ratio of 1:2:1 in a catalytic hydrogenation reaction. In these studies the starting material, dehydroryanodine was present at a concentration of 1.8 mg/ml in ethanol; the catalyst, 10% palladium on charcoal was present at a concentration of 2.67 mg/ml and the reaction was allowed to proceed for 1.1 hours under atmospheric pressure. These reaction conditions were used in the present study with the exceptions that the catalyst concentration was increased to 3.0 mg/ml and the reaction was allowed to proceed for 20 hours. These changes were an attempt to convert the isomer of dehydroryanodine, isodehydroryanodine, formed during this reaction to either ryanodine or epiryanodine. This was at least partially accomplished, since a greater yield of ryanodine was obtained. Ryanodine and epiryanodine, the major products formed under the modified reaction conditions, represented approximately 50 and 25% of the starting material, respectively. The remaining 25% was present as several minor unidentified components (Figure 2). The formation of greater amounts of ryanodine than epiryanodine in the present studies suggests that isodehydroryanodine may be converted preferentially to ryanodine. The yield of ryanodine could thus be increased if dehydroryanodine were isomerized prior to the hydrogenation step. We also conducted the hydrogenation reaction under a pressure of 60 psi, but found no change in the products obtained.

3. Preparation of [^{3}H]ryanodine. [^{3}H]ryanodine can now be prepared by two procedures which introduce tritium into different parts of the ryanodine molecule. First, using the bromination method (6), the pyrrole moiety can be tritiated. We have repeated this procedure using hydrogen instead of tritium and confirmed the formation of bromoryanodine and its subsequent hydrogenation to biologically active ryanodine. Fairhurst (6) obtained [^{3}H]ryanodine with a

[1]Sutko et al. Unpublished Observations.

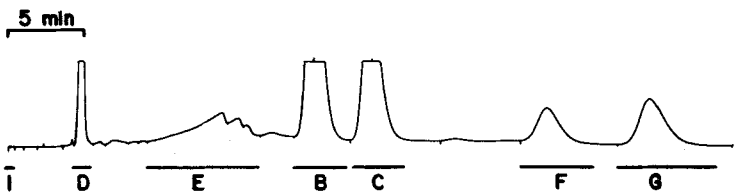

Fig. 3. HPLC chromatogram for the products of the tritiation reaction. The peaks labeled B and C are ryanodine and epiryanodine, respectively. Those designated as D, E, F and G are at present unidentified. The letter I indicates the point of sample injection. Separation conditions were as described for Figure 2.

specific activity of 2.85 Ci/mmole using this procedure, but presumably much higher specific activities can now be achieved using current radiochemical techniques.

Second, as described in this report tritium can be incorporated into the C-ring of the molecule as dehydroryanodine is reduced to ryanodine. We contracted New England Nuclear (Boston, MA) to conduct this reaction and [^{3}H]ryanodine and [^{3}H]epiryanodine with specific activities of 60.8 and 45.8 Ci/mmole, respectively, were obtained. These compounds were quantitated using the absorption of the pyrrole moiety at 270 nm.

Ryanodine and epiryanodine may be susceptible to radiation-induced structural modification. As can be seen in Figure 3, four peaks labeled D, E, F and G were prominent in the chromatograms obtained for the products of the tritiation reaction, but much less obvious in those obtained for the products of the hydrogenation reaction (Figure 2). The formation of these compounds reduced the yield of [^{3}H]ryanodine and [^{3}H]epiryanodine to 22 and 17%, respectively. In the present case, delays in shipping and handling of the radiolabeled compounds were incurred due to the Christmas holiday season. More expedient purification and dilution of the tritiated material may result in yields which more closely approximate those obtained in the hydrogenation studies.

The conditions under which the labeled material is stored is also an important determinant of its stability. As can be seen in Figure 4, [^{3}H]ryanodine

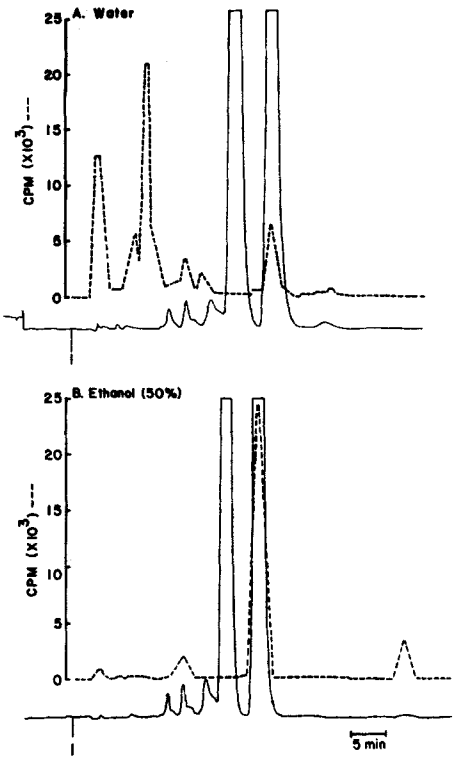

Fig. 4. HPLC chromatogram (solid lines) and distribution of radioactivity (dashed lines) obtained for samples of [^{3}H]ryanodine which had been stored at -20 °C for 6 months in either aqueous solution (Panel A), or in 50% ethanol in water (Panel B). Separation conditions were as described in Figure 2. A small aliquot of [^{3}H]ryanodine was injected with nonlabeled ryanodine to facilitate spectrophotometric detection.

stored at -20 °C in 50% ethanol in water was much more stable than that kept in aqueous solution. After six months of storage in the former solution, between 72-80% of the labeled ryanodine remained (results of three determinations). The [^{3}H]ryanodine which was present after this period of time exhibited the same specific activity as when initially produced.

The availability of radioactive ryanodine will facilitate the use of this compound to probe the molecular mechanisms involved in sarcoplasmic reticulum calcium metabolism. Recently, a preliminary characterization of the [^{3}H]ryanodine binding sites present in this organelle has been reported (10).

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