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On the mechanism of salicylate-induced hypothrombinaemia

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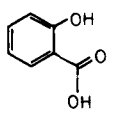
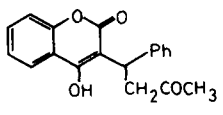
The mechanism of salicylate-induced hypothrombinaemia has been investigated in the rabbit. Administration of methyl salicylate produced a significant decrease in prothrombin complex activity, in the activity of clotting factors II, VII and X but no significant change in factor V activity. Metabolic studies with [^{3}H]vitamin K_1 showed that salicylate increased the plasma concentration ratio of [^{3}H]vitamin K_1-epoxide: [^{3}H]vitamin K_1. The results are consistent with the concept that salicylate produces its anticoagulant effect, like the coumarin anticoagulants, by interruption of the physiologically important vitamin K_1-epoxide cycle at the epoxide reductase.

There have been several reports of hypothrombinaemia in patients and volunteers given large (ca 6 g doses of salicylates (Quick & Clesceri 1960; Barrow et al 1967; Fausa 1970; Goldsweig et al 1976). Salicylates interfere with the synthesis of the vitamin K_1-dependent clotting factors VII, IX and X (Rothschild 1979) and the effect may be reversed by administration of vitamin K_1 (Fausa 1970). It has been suggested (Barrow et al 1967) that the mechanism of salicylate-induced hypothrombinaemia might be similar to that of the structurally related coumarin anticoagulants which are thought to act by interfering with the normal metabolism of vitamin K_1 (Bell 1978). Salicylates also prolong prothrombin time in vitamin K_1-deficient rats (Link et al 1943) in the rabbit but not in the dog (Rapoport et al 1943).

Salicylic acid



Warfarin



We have therefore investigated the mechanism of salicylate-induced hypothrombinaemia by studying the effect of salicylate on the relationship between clotting factor synthesis and vitamin K_1 metabolism in the rabbit using an established experimental protocol (Park et al 1979).

MATERIALS AND METHODS

Male New Zealand white rabbits (2·5 kg) were used. [5-^{3}H]vitamin K_1 (spec. act. 21 Ci mmol^{-1}) was a gift from Hoffman-La Roche, Basle. Methyl salicylate, salicylic acid and vitamin K_1 (phylloquinone) were obtained from Sigma. Vitamin K_1 epoxide was synthesized by the method of Tishler et al (1940). Factor II deficient substrate plasma was obtained from DADE American Hospital suppliers. Factor X deficient substrate plasma and Russell's Viper Venom/Cephalin for use in the assay of factor X were obtained from Diagnostic Reagents Ltd., Thame, Oxon, England. Platelet substitute with kaolin for use in the assay of factor IX was obtained from Diamed Diagnostics, Liverpool, England. Thromboplastin was obtained from the National (U.K.) Reference Laboratory for Anticoagulant Reagents and Control, Manchester and beagle dog factor VII deficient plasma was a gift from Dr. L. Poller.

Prothrombin complex activity (PCA) was determined as previously described (Park et al 1979). The assays for clotting factors II, V, VII and X will be described elsewhere (Leck & Park 1981). Plasma salicylate concentrations were determined by the fluorometric method of Rowland & Reigelman (1967) and had a 0·89% coefficient of variation. Methyl salicylate did not interfere with the assay. The concentration of [^{3}H]vitamin K_1-epoxide and [^{3}H]vitamin K_1 in rabbit plasma was determined as previously described (Park et al 1979).

Investigation of the relationship between plasma salicylate concentrations and clotting factor synthesis

The control PCA and the control activities of clotting factors II, V, VII and X were determined for each animal. Methyl salicylate (1 g kg^{-1}) was injected intramuscularly into the thigh of four rabbits in a split dose. Blood samples (3 × 0·9 ml)

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were then collected from the marginal ear vein into 3·8% citrate (0·1 ml) at regular intervals for the determination of plasma salicylate concentrations, prothrombin complex activity and the activity of the individual clotting factors. For clotting factor analysis, plasma was stored frozen at −40 °C until assayed, except for factor V which was measured immediately.

In a separate experiment vitamin K_1 (2 mg kg^{-1}) was administered to rabbits, similarly dosed, when prothrombin complex activity had fallen below 10% of control activity. Thereafter prothrombin complex activity was measured at regular intervals as described above.

Investigation of the relationship between clotting factor synthesis and [^{3}H]*vitamin* K_1 *metabolism*

Eight hours after the administration of methyl salicylate (1 g kg^{-1}), as described above, four rabbits were dosed with [^{3}H]vitamin K_1 (100 μCi) in 5% Tween-0·9% NaCl (saline) intravenously into the marginal ear vein. Blood samples (4 ml) were taken from the other marginal ear vein into heparinized tubes 1, 2, 3, 4, 5 and 6 h after administration of [^{3}H]vitamin K_1 for determination of [^{3}H]vitamin K_1 and [^{3}H]vitamin K_1-epoxide concentrations. A similar experiment was carried out to determine the concentrations of [^{3}H]vitamin K_1 and its epoxide in four control rabbits. PCA was monitored throughout the experiment to ensure that clotting factor synthesis was inhibited during the metabolism of [^{3}H]vitamin K_1.

Statistical analysis

For comparative purposes, PCA and clotting factor activity for each animal was expressed as a percentage of its own control, taking 100 per cent as the beginning of each experiment. The data was analysed using Student's *t*-test for grouped data. Results are presented as the mean ±s.e.m.

RESULTS

The effect of methyl salicylate (1 g kg^{-1}) on PCA in the rabbit is shown in Fig. 1. The rate of loss of PCA was 7·59 ± 0·34 h. The plasma PCA began to recover after approximately 30 h. The corresponding salicylate concentrations are shown in Fig. 2. The relationship between plasma salicylate concentrations and the appearance of PCA was determined according to Nagashima et al (1969) and gave a typical dose–response curve. The concentration of salicylate in the plasma which apparently totally suppresses the synthesis of prothrombin

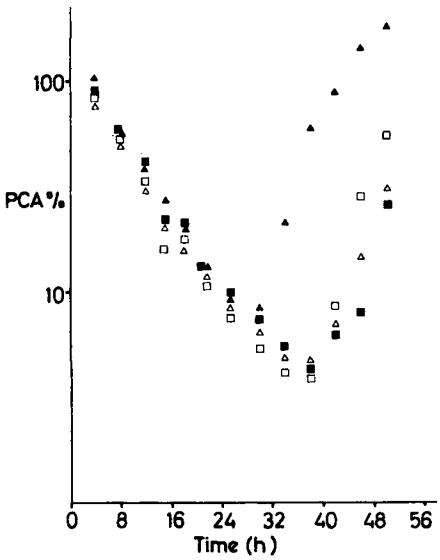

FIG. 1. Prothrombin complex activity in four rabbits (▲, ■, △, □) at various times after administration of methyl salicylate (1g kg^{-1}).

complex activity was found to be 355 ± 12 μg ml^{-1}. The activities of clotting factors II, V, VII and X corresponding to the minimum PCA observed in the individual animals are shown in Table 1. In a separate experiment it was shown that PCA returned from less than 10% to normal within 2 h of administration of vitamin K_1 (2 mg kg^{-1}).

The effect of salicylate on the plasma concentration ratio [^{3}H]vitamin K_1-epoxide[^{3}H]vitamin K_1 is shown in Fig. 3. Salicylate produced a significant ($P < 0·01$) increase in the [^{3}H]vitamin K_1-epoxide: [^{3}H]vitamin K_1 plasma concentration ratio at 2, 3, 4,

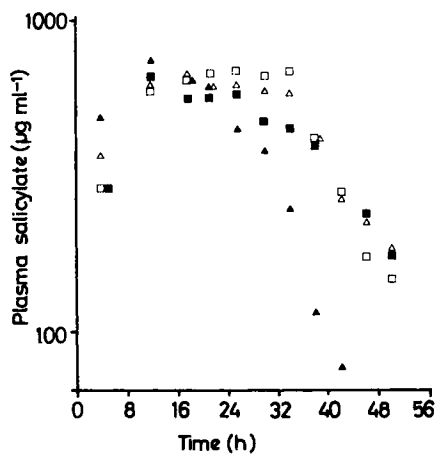

FIG. 2. Plasma salicylate concentrations (μg ml^{-1}) in four rabbits (▲, ■, △, □) at various times following administration of methyl salicylate (1 g kg^{-1}).

Table 1. The effect of methyl salicylate on the activity of clotting factors II, V, VII and X and on PCA in the rabbit.

Clotting factor	Minimum activity (% of control)
II	*29.75 ± 6.21
V	98·75 ± 4·26
VII	*<3
X	*16·08 ± 4·94
PCA	5·50 ± 0·96

Results are expressed as means (n = 4) ± s.e.m. *$P < 0.001$.

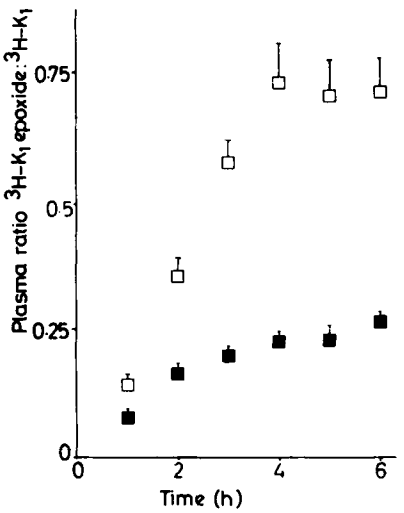

FIG. 3. The effect of methyl salicylate (□) on the plasma [^{3}H]vitamin K_1-epoxide: [^{3}H]vitamin K_1 ratio concentration in rabbit plasma 1 – 6 h after injection of [^{3}H]vitamin K_1, compared with controls (■).

5 and 6 h. The disappearance of [^{3}H]vitamin K_1 from plasma was monoexponential over the period studied and corresponds to the terminal plasma half-life of [^{3}H]vitamin K_1 in the rabbit (Park et al 1980a). There was no significant difference in the *plasma half-life of [^{3}H]vitamin K_1 between animals dosed with salicylate (2·22 ± 0·54 h) and control* animals (1·90 ± 0·39 h) indicating that salicylate does not alter the uptake of [^{3}H]vitamin K_1 into the liver.

DISCUSSION

Vitamin K_1 is essential for normal blood coagulation because it is a cofactor for the postribosomal γ-carboxylation of glutamic acid residues in clotting factors II, VII, IX and X (Jackson & Suttie 1977). It is thought that during the carboxylation process vitamin K_1 is converted into its 2,3-epoxide and that it must be regenerated by the enzyme epoxide reductase for continued clotting factor synthesis. It has been suggested that interruption of the vitamin K_1-epoxide cycle, at either the epoxidase step or reductase step, may result in a reduction in clotting factor synthesis (Bell 1978). Consistent with this hypothesis, it has been shown that warfarin produces an increase in [^{3}H]vitamin K_1-epoxide concentrations in rabbit plasma after administration of [^{3}H]vitamin K_1 while 2-chloro-vitamin K_1, in contrast, produces a decrease (Park et al 1979).

We have now used a similar experimental protocol to investigate the mechanism of salicylate-induced hypothrombinaemia in the rabbit. Salicylic acid was administered as the methyl ester, according to Rapoport (1943), which is rapidly hydrolysed to the free acid in the liver (Davison et al 1961), the major site of clotting factor synthesis (Esnouf 1977). Methyl salicylate produced the same maximum rate of loss of PCA as warfarin and Cl–K (Park et al 1979) indicating the complete inhibition of clotting factor synthesis. Inhibition of PCA was related to plasma salicylate concentrations according to the mathematical model derived for warfarin by Nagashima et al (1969). The minimum concentration of salicylate which corresponded to maximum inhibition of clotting factor synthesis was 355 μg ml^{-1}. In man, prothrombin times are rarely prolonged unless salicylate concentrations exceed 300 μg ml^{-1} (Rothschild 1979).

Despite the high dose of methyl salicylate required for this investigation, clotting factor synthesis eventually recovered. The recovery could be accelerated by intravenous administration of vitamin K_1 indicating that we were not observing a non-specific hepatotoxic effect. Furthermore it was found that while the activity of the vitamin K_1-dependent clotting factors were significantly depressed, the activity of factor V was not affected.

The effect of salicylate on [^{3}H]vitamin K_1 metabolism is similar to that produced by warfarin and other coumarin anticoagulants in the rabbit (Park et al 1979, 1980a, b). After administration of [^{3}H]-vitamin K_1 there was a significant increase in the plasma concentration ratio [^{3}H]vitamin K_1-epoxide: [^{3}H]vitamin K_1 but no change in the rate of elimination from plasma of [^{3}H]vitamin K_1. Therefore, it would appear that salicylate produces its hypothrombinaemic effect by interrupting the physiologically important vitamin K_1-epoxide cycle at the

epoxide reductase, thus preventing the regeneration of vitamin K_1.

The present work, together with our earlier studies (Park et al 1979, 1980a) would suggest that oral anticoagulants possess two functionally distinct groups: an aromatic nucleus required for intrinsic activity and a lipophilic side-chain which provides additional affinity for the receptor. Hence the order of anticoagulant potency salicylate < warfarin < brodifacoum correlates with increasing size and lipophilicity of the side-chain. Although salicylate does not produce hypothrombinaemia at normal therapeutic doses, it may do so when taken in overdose or when used in conjunction with other drugs which affect vitamin K concentrations in the liver.

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