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SIMULTANEOUS INHIBITION OF ALTERNATIVE PATHWAYS OF CHOLESTEROL BIOSYNTHESIS BY TWO RELATED HYPOCHOLESTEREMIC AGENTS

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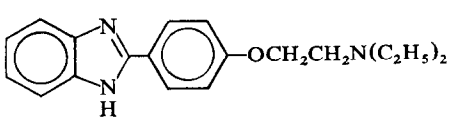
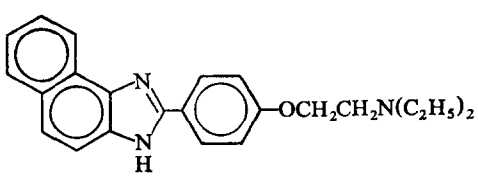
Abstract—Two related hypocholesteremic agents were administered, both singly and in combination, to rats and to monkeys. Analysis of the results in terms of chemical kinetic criteria provides, with more certainty than previously possible, a view of the mechanism by which the late stages of cholesterol biosynthesis are accomplished. Comparison of the structures and the different modes of action of the two agents illustrates the caution required in presuming that, when like structures exert like effects, they do so by the same mechanism.

WE HAVE described elsewhere[1] the properties of an orally effective hypocholesteremic agent (I_1) that acts by inhibiting the conversion of 7-dehydrocholesterol (**B** in Fig. 1) to cholesterol (**C** in Fig. 1) in the livers of rats and monkeys.* We now find that a related agent, I_2, is also a potent inhibitor *in vivo* of cholesterol biosynthesis in rat



I_1

2-[*p*-(2-diethylaminoethoxy)-phenyl]benzimidazole



I_2

2-{*p*-[2-(diethylamino)ethoxy]-phenyl}-3H-naphth[1,2-d]imidazole

liver, but because of the close structural similarity between I_1 and I_2, it is with some surprise that we find that I_2 acts by inhibiting, instead, the conversion of *desmosterol* (**D** in Fig. 1) to cholesterol. Evidence for this conclusion is presented in Fig. 2.

It is generally conceded[2–4] that **B** and **D** are terminal intermediates in alternative pathways of cholesterol biosynthesis. It seemed important, therefore, to study the

* I_1 is also active orally in the dog, though somewhat less so than in the monkey. Application of the methods described in our previous report[1] showed, for example, that **B** or other "fast-actors" represented approximately 16 per cent of the sterols from the liver of a female beagle treated orally for 2 days with a 25 mg/kg/day dose of I_1. There was, during the same period, a progressive fall of 28 per cent in plasma total sterol concentration relative to the pre-treatment control level.

effect of simultaneous administration of I_1 and I_2 to rats and monkeys for the additional mechanistic insight that might be gained from interpretation of the results in terms of chemical kinetic criteria. The results of these studies afford, in Fig. 1, a more definitive view than previously possible of the mechanism by which the terminal stages of cholesterol synthesis are accomplished.

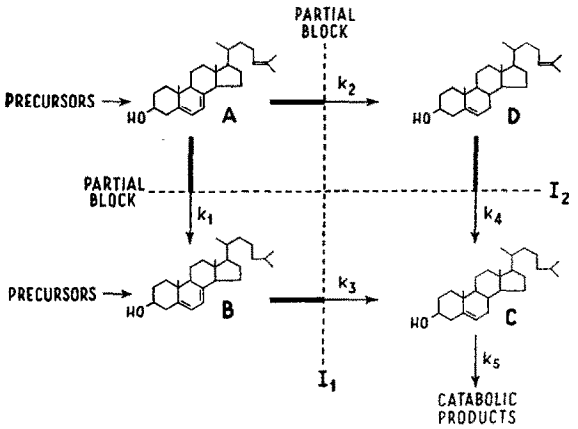


FIG. 1. Proposed mechanism for the terminal stages of cholesterol biosynthesis.

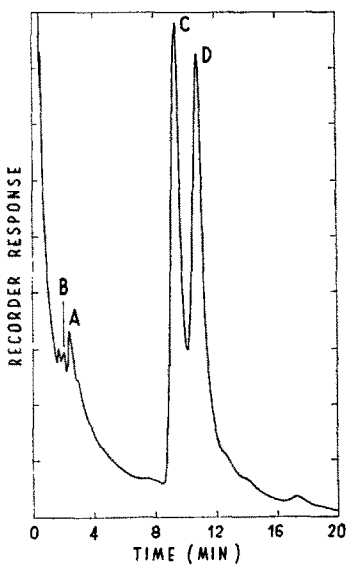


FIG. 2. Gas chromatogram of trifluoroacetates of digitonin-precipitated sterols from the livers of rats treated with I_2 alone. The drug treatment regimen for these animals is described in Table 2. The 7-dehydrocholesterol peak, **B**, is approximately equal in area to the corresponding peak in chromatograms of control liver sterol trifluoroacetates (see ref. 1). The A peak, however, is slightly larger in area than the control A peak, as one would expect from operation of the mechanism shown in Fig. 1. The liver sterol changes shown here were accompanied by a 60 per cent depression in total plasma sterols.

METHODS AND RESULTS

Preview of key observations

Experimental justification for the conclusions presented in Fig. 1 results from the following observations: a) considerable amounts of $\Delta^{5,7,24}$-cholestatrien-3β-ol (**A** in Fig. 1) accumulate in the livers of rats* treated simultaneously with $\mathbf{I}_1$ and $\mathbf{I}_2$, but not to any significant degree in the livers of animals treated with either inhibitor alone; b) the gas chromatographic technique that we have previously described[1] will detect the presence of a number of sterol intermediates in cholesterol biosynthesis in addition to those shown in Fig. 1; comparison of Figs. 3 and 4 will show, however, that $\mathbf{I}_1$ and $\mathbf{I}_2$, in the dose combinations studied, produced no net change in the concentration patterns of sterols other than those specifically identified in Fig. 1; c) variation of the $\mathbf{I}_1/\mathbf{I}_2$ dose ratio leads to variations in the liver concentrations of **A**, **B**, **C** and **D** in precise correspondence to the requirements of Fig. 1. These requirements can be developed from the following kinetic considerations.

Kinetic analysis of the reaction model shown in Fig. 1

The rates of change in sterol concentrations† required by Fig. 1 are,

$$dA/dt = A_f - k_1A - k_2A \quad (1)$$

$$dB/dt = B_f + k_1A - k_3B \quad (2)$$

$$dC/dt = k_3B + k_4D - k_5C \quad (3)$$

$$dD/dt = k_2A - k_4D \quad (4)$$

wherein A_f represents the rate of formation of **A**, and B_f represents the rate of formation of **B** from precursors‡ other than **A**. Point (b) in the previous paragraph emphasized that, over the combination dose ranges studied, $\mathbf{I}_1$ and $\mathbf{I}_2$ fail to change the accumulation patterns of any sterols earlier than **A** and **B** in the cholesterol synthetic sequence. This point, which will be defended in a later paragraph, implies that the rate of formation of **A** and the rate of formation of **B** from precursors other than **A** are unaffected by the inhibitors; the rate contributions, A_f and B_f, in eqs 1 and 2

* The same effect is seen, though to a considerably lesser degree, in monkey liver; the conclusions to be presented in subsequent paragraphs thus probably apply to this species as well as to the rat. The monkey's relative resistance to $\mathbf{I}_1$ and $\mathbf{I}_2$ leads, however, to such poorly resolvable changes in liver sterol concentration patterns that we cannot defend application of our subsequent arguments to the monkey with the same confidence with which we apply them to the rat.

† For typographic convenience, we forego the conventional brackets to denote the concentrations of the species enclosed. We will use, instead, italicized capital letters for the concentrations of the species in question, and boldface capitals as substitutes for the names of the species appearing in Fig. 1. The same convention will apply to $\mathbf{I}_1$ and $\mathbf{I}_2$, but in this case "concentration" becomes "dose." k_{1-5} will refer to the apparent rate constants for the reactions shown in Fig. 1. Italic type will be used in eqs 1–21 to denote variables, and roman type to denote constants or operators. It will at first appear that we have chosen to treat the reactions shown in Fig. 1 as first-order with respect to sterol concentractions and zero-order with respect to enzyme concentrations. The impression needs correcting before it leads to an apparent contradiction in a later paragraph. The need for correction arises from our further concern for typographic simplicity and from our consequent omission of enzyme concentration terms from all of our rate expressions. The presence of the appropriate concentration terms is nevertheless implied when we later perform a series of differentiations with respect to the apparent rate constants k_1–k_4, for we then acknowledge the presence of a hidden variable in each of them. It will be clear at that point that our model is in fact second-order, and that each of our apparent rate constants is actually the product of a true rate constant and an associated enzyme concentration variable. In concession to this point, we will write all but k_5 of the apparent rate constants in italic type as a reminder that they are to be considered variables in the sense explained in this footnote.

‡ Dempsey *et al.*[2] have shown that the immediate 24,25-dihydroprecursor of **B** is Δ^7-cholesten-3β-ol, and[6] that $\Delta^{7,24}$-cholestadien-3β-ol is the probable precursor of **A**.

are thus properly considered constant. The rate of catabolism of cholesterol is not *directly* affected by the inhibitors; it is necessary, however, to include a contribution from this source in eq. 3, for the catabolic rate will vary with inhibitor-induced changes in the concentration of **C** in accordance with the mass-action requirement.

We now apply the conventional steady state assumption[5] to eqs 1–4 and get,

$$\mathrm{A_f} - k_1 A - k_2 A = 0 \tag{5}$$

$$\mathrm{B_f} + k_1 A - k_3 B = 0 \tag{6}$$

$$k_3 B + k_4 D - \mathrm{k_5} C = 0 \tag{7}$$

$$k_2 A - k_4 D = 0. \tag{8}$$

Solution of eqs 5–8 for the six possible concentration ratios then gives,

$$\frac{D}{A} = \frac{k_2}{k_4} \tag{9}$$

$$\frac{B}{A} = \frac{k_1}{k_3} + \frac{k_1 \mathrm{B_f}}{k_3 \mathrm{A_f}} + \frac{k_2 \mathrm{B_f}}{k_3 \mathrm{A_f}} \tag{10}$$

$$\frac{C}{A} = \frac{k_1}{\mathrm{k_5}} + \frac{k_1 \mathrm{B_f}}{\mathrm{k_5 A_f}} + \frac{k_2 \mathrm{B_f}}{\mathrm{k_5 A_f}} + \frac{k_2}{\mathrm{k_5}} \tag{11}$$

$$\frac{D}{B} = \frac{k_2 k_3 \mathrm{A_f}}{k_1 k_4 \mathrm{A_f} + k_1 k_4 \mathrm{B_f} + k_2 k_4 \mathrm{B_f}} \tag{12}$$

$$\frac{C}{D} = \frac{k_1 k_4}{k_2 \mathrm{k_5}} + \frac{k_1 k_4 \mathrm{B_f}}{k_2 \mathrm{k_5 A_f}} + \frac{k_4 \mathrm{B_f}}{\mathrm{k_5 A_f}} + \frac{k_4}{\mathrm{k_5}} \tag{13}$$

$$\frac{C}{B} = \frac{(\mathrm{A_f} + \mathrm{B_f}) k_1 k_3 + (\mathrm{A_f} + \mathrm{B_f}) k_2 k_3}{(\mathrm{A_f} + \mathrm{B_f}) k_1 \mathrm{k_5} + \mathrm{B_f} k_2 \mathrm{k_5}}. \tag{14}$$

We accept, in addition to the steady state assumption, the further assumption that an inhibitor-induced disturbance in the steady state pattern of *A*, *B*, *C* and *D* will be followed by a new steady state after a suitable period of adjustment of the enzyme system to the continuing presence of the inhibitors. It follows from this that, given sufficient time, a different steady state pattern will be associated with each new dose combination of the inhibitors, and that each new pattern that results will then be described by a new version of eqs 9–14.

Equations 9–14 relate the rate constants for the reactions shown in Fig. 1 to the concentration variables with sufficient simplicity to offer experimental means for testing the plausibility of the postulated mechanism. We must first, however, find a means of relating inhibitor dose ratios to the concentration ratios appearing in eqs 9–14. We can assume for this purpose that an increase in the dose of either inhibitor will be equivalent in its net effect to a depression in the two rate constants (see footnote that immediately precedes eq. 1) for the two reactions that each inhibitor is presumed to inhibit. The arguments to follow do not require, however, that we define the extent of this equivalence; they require only that the relationship be inverse.

The nature of our experimental design now requires that we rewrite eqs 9–14 in a form that will describe the differences in steady state patterns that will result from administration of the two inhibitors in different dose ratios to different groups of animals. For this purpose, we differentiate each of the expressions 9–14 with respect to the affected rate constants. The results

$$\mathrm{d}\left(\frac{D}{A}\right) = \frac{\mathrm{d}k_2}{k_4} - \frac{k_2\,\mathrm{d}k_4}{k_4^2} \tag{15}$$

$$\mathrm{d}\left(\frac{B}{A}\right) = \left(\frac{1}{k_3} + \frac{B_f}{k_3 A_f}\right)\mathrm{d}k_1 + \left(\frac{B_f}{k_3 A_f}\right)\mathrm{d}k_2 - \left(\frac{k_1}{k_3^2} + \frac{k_1 B_f}{k_3^2 A_f} + \frac{k_2 B_f}{k_3^2 A_f}\right)\mathrm{d}k_3 \tag{16}$$

$$\mathrm{d}\left(\frac{C}{A}\right) = \left(\frac{1}{k_5} + \frac{B_f}{k_5 A_f}\right)\mathrm{d}k_1 + \left(\frac{1}{k_5} + \frac{B_f}{k_5 A_f}\right)\mathrm{d}k_2 \tag{17}$$

$$\begin{aligned}\mathrm{d}\left(\frac{D}{B}\right) = &-\left(\frac{k_2k_3k_4A_f^2 + k_2k_3k_4A_fB_f}{[k_1k_4A_f + k_1k_4B_f + k_2k_4B_f]^2}\right)\mathrm{d}k_1 \\ &+\left(\frac{k_1k_3k_4A_f^2 + k_1k_3k_4A_fB_f}{[k_1k_4A_f + k_1k_4B_f + k_2k_4B_f]^2}\right)\mathrm{d}k_2 \\ &+\left(\frac{k_2A_f}{k_1k_4A_f + k_1k_4B_f + k_2k_4B_f}\right)\mathrm{d}k_3 \\ &-\left(\frac{k_1k_2k_3A_f^2 + k_1k_2k_3A_fB_f + k_2^2k_3A_fB_f}{[k_1k_4A_f + k_1k_4B_f + k_2k_4B_f]^2}\right)\mathrm{d}k_4\end{aligned} \tag{18}$$

$$\begin{aligned}\mathrm{d}\left(\frac{C}{D}\right) = &\left(\frac{k_4A_f + k_4B_f}{k_2k_5A_f}\right)\mathrm{d}k_1 - \left(\frac{k_1k_4A_f + k_1k_4B_f}{k_2^2k_5A_f}\right)\mathrm{d}k_2 \\ &+\left(\frac{k_1A_f + k_1B_f + k_2B_f + k_2A_f}{k_2k_5A_f}\right)\mathrm{d}k_4\end{aligned} \tag{19}$$

$$\begin{aligned}\mathrm{d}\left(\frac{C}{B}\right) = &-\left(\frac{k_2k_3k_5A_f^2 + k_2k_3k_5A_fB_f}{[k_1k_5A_f + k_1k_5B_f + k_2k_5B_f]^2}\right)\mathrm{d}k_1 \\ &+\left(\frac{k_1k_3k_5A_f^2 + k_1k_3k_5A_fB_f}{[k_1k_5A_f + k_1k_5B_f + k_2k_5B_f]^2}\right)\mathrm{d}k_2 \\ &+\left(\frac{k_1A_f + k_1B_f + k_2B_f + k_2A_f}{k_1k_5A_f + k_1k_5B_f + k_2k_5B_f}\right)\mathrm{d}k_3\end{aligned} \tag{20}$$

refer, in the strictest sense, to differences between already established steady states; they will apply with equal validity, however, as each of the different steady states is *approached*, but not yet reached, provided both patterns are examined after the two groups of animals have been exposed to the two inhibitor combinations for the same time period.

We now have in eqs 15–20 a set of testable consequences of Fig. 1, and it now remains only to strip them of their outward complexity and examine their essential meaning before matching them against our experimental results. The examination need only be qualitative, and can be limited to only one of the group, eqs 15–20, since they are identical in form and convey analogous meanings. We note in eq. 18, for example, that all of the individual terms within parentheses on the right-hand side are necessarily positive. We note next in eq. 18 that no subtractive operations are indicated within the same parentheses. It is obvious from these two observations that the signs of the parenthesized terms on the right-hand side are uniformly positive for all possible values of the terms that they contain. We now invoke our earlier assumption that the net effect of a change in inhibitor dose will be an inverse change in the two rate constants for the two reactions that each inhibitor is presumed to inhibit. Recalling further that $\mathbf{I}_1$ affects k_2 and k_3, while $\mathbf{I}_2$ affects k_1 and k_4, it then follows that a simultaneous increase in I_1 and decrease in I_2 will produce a decrease in k_2 and k_3, but an increase in k_1 and k_4. Hence, $\mathrm{d}k_2$ and $\mathrm{d}k_3$ in eq. 18 are both negative, while $\mathrm{d}k_1$ and $\mathrm{d}k_4$ are both positive. We may thus write a qualitative analog of eq. 18 in which the magnitudes of the terms are ignored, but their signs are acknowledged. The result

$$\text{Sign of } \mathrm{d}(D/B) = -[(+)(+)] + [(+)(-)] + [(+)(-)] - [(+)(+)]$$

predicts, as a consequence of the mechanism shown in Fig. 1, that a simultaneous increase in I_1 and decrease in I_2 will produce a decrease in the D/B response ratio. Application of similar logic to eqs 15, 19 and 20 will make it clear that the same change in the inhibitor dose ratio will also produce an increase in C/D, but decreases in D/A and C/B. Analysis of eq. 17 is equally straightforward if care is taken in the choice of inhibitor doses to make the increase in I_1 contribute more to the total response differential than the decrease in I_2, for eq. 17 will then require a decrease in C/A.* Analysis of eq. 16 also requires special attention, since the direction of change of B/A likewise depends on a further logical circumstance. The dependence becomes clear when eq. 5 is differentiated and the result

$$\mathrm{d}k_1 = -(\mathrm{A_f}/A^2)\,\mathrm{d}A - \mathrm{d}k_2$$

is substituted into eq. 16. The new result

$$\mathrm{d}\left(\frac{B}{A}\right) = -\left(\frac{\mathrm{A_f}}{k_3A^2} + \frac{\mathrm{B_f}}{k_3A^2}\right)\mathrm{d}A - \frac{\mathrm{d}k_2}{k_3} - \left(\frac{k_1}{k_3^2} + \frac{k_1\mathrm{B_f}}{k_3^2\mathrm{A_f}} + \frac{k_2\mathrm{B_f}}{k_3^2\mathrm{A_f}}\right)\mathrm{d}k_3$$

is equivocal about the direction of change in B/A when an *increase* in A results from an increase in I_1/I_2. It obviously requires, however, that B/A increase when a *decrease* in A results, and comparison of Figs. 3 and 4 will show that our increase in dose ratio did indeed produce a decrease in the absolute value of A.

* We did not determine directly the hypocholesteremic responses to each drug alone at the precise dose levels at which the drugs were administered together (see Table 2). Our choice of dose combinations to fit this condition was dictated instead by interpolation from the single drug dose-response curves. By this criterion, the response differential attributable to the increase in the $\mathbf{I}_1$ dose was approximately 1·5 that attributable to the decrease in the $\mathbf{I}_2$ dose in the inhibitor combination experiments.

Correspondence between experimental results and the kinetic requirements of the mechanism shown in Fig. 1

The extent to which our experimental data are consistent with the requirements of the preceding paragraph will be seen in Table 1 in which we have entered the areas under the **A**, **B**, **C** and **D** peaks in the gas chromatograms shown in Figs. 3 and 4.

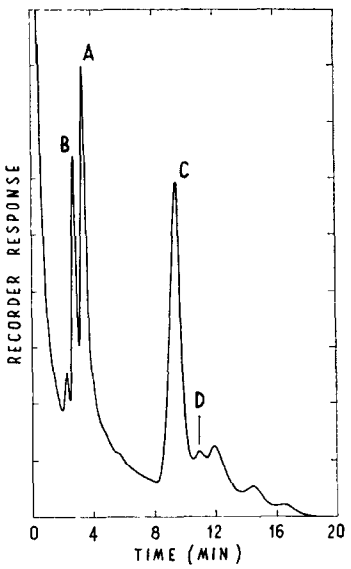


FIG. 3. Gas chromatogram of trifluoroacetates of digitonin-precipitated sterols from the livers of rats treated with a 1·5/50·0 dose ratio of I_1 and I_2 (see Table 2).

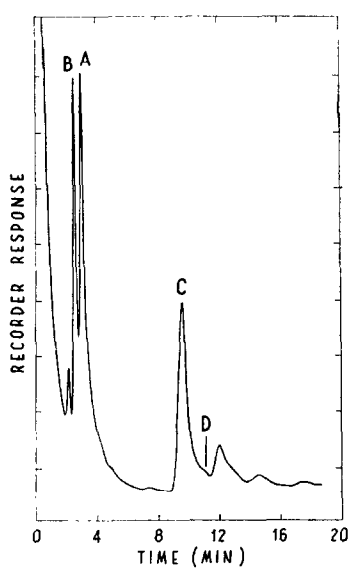


FIG. 4. Gas chromatogram of trifluoroacetates of digitonin-precipitated sterols from the livers of rats treated with a 5/30 dose ratio of I_1 and I_2 (see Table 2).

TABLE 1. AREAS OF THE PRINCIPAL PEAKS IN FIGS. 3 AND 4 AND CHANGES IN THEIR INTERRELATIONSHIPS RESULTING FROM CHANGES IN THE DOSE RATIO OF I_1 AND I_2*

Fig. No.	Peak areas as % of total area measured						Differences in area ratios, e.g. $\left(\frac{B_4}{A_4} - \frac{B_3}{A_3}\right)$						Ratio of area differences, e.g. $\left(\frac{D_4 - D_3}{B_4 - B_3}\right)$		
	A	*B*	*C*	*D*	Δ^7	Sum	*B/A*	*C/D*	*D/A*	*D/B*	*C/B*	*C/A*	*B/C*	*D/B*	*C/D*
3	25	17	44	4	8	98					Found experimentally:				
							+ 0·12	+ 155·7	— 0·16	— 0·23	— 1·48	— 0·88	— 0·72	— 0·38	+ 3·67
4	34	27	30	0·18	8	99·18									
											Signs required by theory:				
							+	+	—	—	—	—			+

* All but one of the entries in the table were obtained by resolution of the total curve envelopes of Figs. 3 and 4 into Gaussian components by use of the DuPont Model 310 Curve Resolver. The exception, the *D* component in Fig. 4, appeared impossibly broad because of poor resolution in the **D** region. We therefore ignored the instrumental value for the area of the **D** peak and evaluated its upper limit by independent and more acceptable means. We did so by preparing and derivatizing a series of solutions of accurately known concentrations of authentic cholesterol and desmosterol together in tetrahydrofuran. The solutions were identical in cholesterol concentration, but variable in desmosterol concentration from a *D/C* ratio of 0·0003 to a *D/C* ratio of 0·0496. Gas chromatography of the resulting solutions showed that desmosterol can be separated as a point of inflection distinct from the foot of the cholesterol peak when $D/C \geqslant 0{\cdot}0062$, but not when $D/C \leqslant 0{\cdot}0031$. The **D** peak in Fig. 4 is not a distinct inflection, and we are thus able to set the value that corresponds to it in the table as the upper limit to its area relative to the area of the **C** peak. The peaks to the immediate right of the **D** peaks in Figs. 3 and 4 correspond to Δ^7-cholestenol; their areas have been included in the table for the added support that their equivalence provides for the specificity of I_1 and I_2.

We have also included in Table 1 a numerical value for each of six area ratios from Fig. 4 minus the corresponding area ratios from Fig. 3. These values, which appear in the middle panel of Table 1, will be seen to correspond in meaning with the left-hand sides of eqs 15–20. Advertence to the previous paragraph will show, however, that the mechanistic tests described there are purely qualitative and that only the proper distribution of signs among the quantities in the center panel of Table 1 is needed for confirmation of the mechanism shown in Fig. 1. Comparison of the signs of these quantities with those required by the previous paragraph will indicate that all six of the mechanistic criteria provided by eqs. 15–20 have indeed been met.

A further test of the mechanism proposed in Fig. 1 is available by noting that the identity

$$\frac{dC}{dD} \cdot \frac{dD}{dB} \cdot \frac{dB}{dC} = 1 \tag{21}$$

is satisfied when eqs 2–4 are combined in the manner indicated. It is obvious from the rightmost panel of Table 1 that the analogs of dB/dC and dD/dB are both negative. Equation 21 then requires that dC/dD be positive, as is found experimentally. The further qualitative test provided by eq. 21 thus brings to a total of seven the number of kinetic consequences of the presumed mechanism that have been verified experimentally. It should be noted, however, that the test provided by eq. 21 is conceptually different from those provided by eqs 15–20.

Drug treatment procedure

Our previous report[1] describes the drug treatment procedure used in the present work. $\mathbf{I}_1$ was always administered as the hydrochloride salt. $\mathbf{I}_2$ was available to us as the free base and as the hydrochloride salt; we used both forms of $\mathbf{I}_2$ interchangeably, but Table 2, which relates our drug treatment protocols to Figs. 2–4, reports dose schedules in terms of free-base equivalents for both drugs.

TABLE 2. DRUG TREATMENT PROTOCOLS CORRESPONDING TO FIGS. 2–4

Fig. No.	No. rats	$\mathbf{I}_1$ dose (mg/kg/day × 7)	$\mathbf{I}_2$ dose (mg/kg/day × 7)
2	10		45
3	30	1·5	50
4	30	5·0	30

Sterol isolation and gas chromatographic identification procedures

Our previous report also describes our sterol isolation and gas chromatographic procedures. That report illustrates the liver sterol pattern found in control rats and in rats treated with $\mathbf{I}_1$ alone. The same report also illustrates the degree to which **B**, **C** and **D** can be resolved by gas chromatographic means when the three components are present in comparable amounts.

The relative gas chromatographic retention times of **B**, **C** and **D** are in part the basis for the criteria offered by Clayton[7] for predicting retention times for other

members of the cholestenol series. Given the retention times of these and other reference sterols in our system, we are then able by use of Clayton's criteria to assign the retention time to **A** that appears in Figs. 3 and 4.

Despite the usefulness of Clayton's criteria for assigning the retention time of **A** relative to **B**, we learned early in our gas chromatographic experience that, at least under the conditions we employ, relative retention time alone is a poorly reproducible indication of identity when one or more of the following circumstances prevail: (a) when the peaks appear superimposed on the falling solvent front, as **A** and **B** do in Figs. 3 and 4; (b) when resolution is not complete, as in the **A-B** and early post-**C** regions of Figs. 3 and 4; c) when the incompletely resolved species are not present in comparable amounts, as also in the early post-**C** region of Figs. 3 and 4. It will be noted that as a consequence measurable discrepancies in relative retention times appear in Figs. 2, 3 and 4. For this reason, we have continued the practice that we previously used[1] for the confirmation of suspected peak identities: namely, the serial addition of authentic sterol samples in very small amounts to a given sterol isolate, derivatization of each resulting mixture, and examination of each resulting chromatogram for the position and integrity in shape of the accentuated peak. Some of the variation in retention times seen in Figs. 2, 3 and 4 is, of course, also the result of the slight but unavoidable variation in day-to-day operating conditions that we referred to earlier.[1] It will be obvious to the reader who refers to our previous report[1] that the retention times reported there are considerably longer than those reported here. The difference is the result of our present preference for a shorter analysis time for the partial insurance that it provides against pyrolytic fragmentation of **A** and **B**. The shorter analysis time has been achieved by use of a carrier gas flow rate of 35 ml/min instead of 26–30 ml/min, and by use of a column preconditioned for 24 hr at 212° instead of 240°.

The post-**D** peaks in Figs. 3 and 4 are more prominent than the corresponding peaks in the control liver chromatogram in our previous report. This difference, which suggests that $\mathbf{I}_1$ and $\mathbf{I}_2$ may not be as specific as Fig. 1 implies, may be real or it may be an artifact resulting from the change in chromatographic conditions that we referred to in the preceding paragraph. The difference, whatever its origin, is nevertheless immaterial to our present case, for our present case rests entirely upon a kinetic analysis of the response differential between two groups of treated animals relative to each other, but without reference to the control animals. The relevant observation is therefore the close similarity in prominence of the post-**D** peaks in Figs. 3 and 4, but without reference to the control liver chromatogram. Table 1 shows, for example, that Δ^7-cholestenol, represented by the first post-**D** peak, is present in approximately equal concentrations in the sterol isolates from the two groups of treated animals.

Isolation of **A** *and* **B** *as epiperoxides from total liver sterols*

Confirmation of the presence of **A** and **B** in the livers of rats treated simultaneously with $\mathbf{I}_1$ and $\mathbf{I}_2$ was based on photolytic peroxidation[2, 8] of the semi-pure total liver sterols, chromatographic isolation of the **A** and **B** 5,8-α-epiperoxides that result, and comparison of the melting points of these materials with the melting points of authentic **A** and **B** peroxides. The oxidation was essentially complete in 3 hr, as judged by disappearance of the 281·5 mμ band in the u.v. spectrum. On three occasions, the

reaction mixture was evaporated to dryness and the residue was extracted with benzene. The benzene extract was filtered, washed several times with water, dried over Drierite and applied to a 1 × 8 cm column of chromatographic grade silicic acid (Mallinckrodt, 100 mesh). Continued washing of the column with benzene and evaporation of fractions of the eluate gave a series of oily residues. Treatment of each of these residues with a drop of methanol induced partial to complete crystallization in several of the late fractions. Combining the crystalline and near-crystalline fractions and recrystallizing from ethanol–water (4:1) gave nearly pure **A** peroxide, m.p. 138–140°. A further recrystallization from methanol gave pure material melting at 139–140°, in correspondence with the value reported by Dvornik *et al.*[9] The column was then stripped with methanol. Evaporation of the total eluate and recrystallization of the resulting residue from ethanol–water (4:1) gave, on one occasion, 8·5 mg **B** peroxide, m.p. 151–152·5°, from 50·3 mg of total sterols. A mixed melting point of this material with authentic **B** peroxide[2, 8] was undepressed. Repetition of the peroxidation procedure on 133 mg of total sterols from the livers of rats treated with $\mathbf{I}_1$ alone gave 83 mg **B** peroxide.

On four other occasions, the isolation of **A** as the epiperoxide proved more troublesome, owing to the persistence of intractable oils throughout the entire silicic acid fractionation attempt. Whenever this happened, we recombined the oily fractions from silicic acid chromatography, redissolved them in benzene and applied the benzene solution to a 1 × 10 cm column of Florisil (Fisher, 60–100 mesh). We then continued to wash the column with benzene until the eluate was free of oil. The column was then stripped with methanol, the total methanol eluate was evaporated to dryness and the residue was recrystallized from methanol. On two such occasions, the product was sufficiently pure (m.p. 139–140°). On the remaining successful occasion, however, it was necessary to purify the material further by preparative TLC on 5 × 7·5 cm plates coated with 1 mm of hydrated silica gel with $CaSO_4$ binder (Camag). This procedure included the use of a benzene–methanol (10:1) solvent system and treatment of a monitor plate with iodine vapor as a means of locating the principal band. Mechanical removal of the major band and extraction of the silica gel carrier with warm methanol then gave, on concentrating and cooling the methanol extract, pure **A** peroxide (m.p. 139–140°). Further crystallization of this material had no effect on the melting point. The peroxide of **B** was never recovered when the Florisil or Florisil/silica gel procedure was used, owing presumably to its elution from Florisil before **A** peroxide and loss in the initial oily fractions. Yields of **A** peroxide from our seven isolation attempts are summarized in Table 3.

Except for one inexplicable failure in which methanol failed to strip anything at all from the Florisil column, the entries in Table 3 indicate that the liver sterols in our $\mathbf{I}_1 + \mathbf{I}_2$ treated animals contain at least 10 per cent **A**. Our manipulative losses must have been rather serious, however, for Table 1 indicates concentrations of **A** more than twice as great.

DISCUSSION

Several authors[6, 9, 10] have been attracted by the mechanism discussed here as a possible explanation for the accumulation of **A** in tissue homogenates and in the tissues of intact animals exposed simultaneously to Δ^7- and Δ^{24}-reductase inhibitors. The novelty of our work thus lies not in the proposal shown in Fig. 1 in itself, but

rather in the firmness with which that proposal is now supported by kinetic criteria. As a result, we are also able to support an obvious and previously drawn[4, 9, 11] corollary of Fig. 1: that Δ^7-reduction and Δ^{24}-reduction in the cholesterol synthetic sequence can each occur without regard for the redox state of the rest of the sterol molecule, and that only two enzymes, a Δ^7-reductase and a Δ^{24}-reductase, are thus required to convert **A** to **C** via the alternative intermediates, **B** and **D**.

TABLE 3. SUMMARY OF YIELDS OF **A** PEROXIDE FROM VARIOUS ISOLATION PROCEDURES

I_1/I_2 dose ratio	Semi-pure total sterols (mg)	Yield of A peroxide (mg)	Isolation method
5/30	50·3	6·0	Silicic acid
5/30	75·0	6·3	Silicic acid
5/30	322·0	0·0	Florisil
5/30	365·0	16·0	Florisil/silica gel
1·5/50	105·4	13·3	Silicic acid
1·5/50	100·0	9·0	Florisil
1·5/50	20·0	2·0	Florisil

We have emphasized in this report the usefulness of multiple inhibition in elucidating, with the help of kinetic principles, the mechanism by which a metabolic sequence occurs. As a result of this emphasis, the reader may have overlooked a point that deserves repetition because of its practical significance for the medicinal chemist. It is, of course, one of the basic premises of medicinal chemistry that, when like structures produce the same net biologic effect, one first assumes that they do so by the same mode of action. The value of this premise remains beyond question, but its potential for occasional surprise could not, in our opinion, be better illustrated than by the present case in which different modes of action must be attributed to two inhibitors whose structural similarity would have suggested to the reader, as it did to us, *identical* modes of action.

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