High-Sensitivity Scanning Calorimetric Study of Mixtures of Cholesterol with Dimyristoyl- and Dipalmitoylphosphatidylcholines†

Susan Mabrey,‡ Pedro L. Mateo,§ and Julian M. Sturtevant*

ABSTRACT: A highly sensitive and stable scanning microcalorimeter is employed in a reinvestigation of the effect of cholesterol on multilamellar suspensions of dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC). Below 20 mol % cholesterol the DPPC mixtures give heat-capacity curves each of which can be resolved into a narrow and a broad peak, suggesting the coexistence of two immiscible solid phases; above 20 mol % only the broad peak is observed and this disappears at about 50 mol %. The DMPC mixtures show a more complicated behavior; from about 13.5 to 20 mol % cholesterol the observed curves appear to be the sum of three component peaks. As with the DPPC mixtures, only a single broad peak is observed above 20 mol % cholesterol, and this broad peak becomes undetectable above about 50 mol %. These results are discussed.

In view of the widespread occurrence of cholesterol in biological membranes, much attention has been devoted to the study of the effects of cholesterol incorporation on the behavior of relatively simple systems such as phospholipid bilayers (Oldfield and Chapman, 1972). In an earlier communication from this laboratory (Hinz and Sturtevant, 1972), it was concluded on the basis of scanning calorimetry performed on mixtures of cholesterol with dimyristoylphosphatidylcholine (DMPC)[1] and dipalmitoylphosphatidylcholine (DPPC) that the lipid phase transition from gel to liquid crystal disappears at 33 mol % cholesterol. This was interpreted as being due to the formation of a packing complex containing one molecule of cholesterol to two molecules of lipid. At the same time x-ray observations were interpreted in terms of the same apparent stoichiometry (Engelman and Rothman, 1972). More recent calorimetric evidence (de Kruyff et al., 1973, 1974) has also tended to support this stoichiometry. On the other hand, the results of spin-label studies (Shimshick and McConnell, 1973) of mixtures of cholesterol and DMPC have been interpreted as indicating the existence of two solid phases only up to 20 mol % cholesterol. This conclusion has been supported by freeze-fracture observations with a protein present as a marker (Kleeman and McConnell, 1976), and the 20 mol % mixture has been postulated to be a eutectic.

The availability of a scanning microcalorimeter (Privalov et al., 1975) having much improved sensitivity and baseline stability has prompted us to reexamine by this means mixtures of cholesterol with DMPC and DPPC, with results in better agreement with the spin-label studies than with our earlier calorimetric data. A preliminary report of this work has been made public (Mabrey et al., 1977).

Materials and Methods

Most of the experiments reported here were performed with L-α-dimyristoyl- and L-α-dipalmitoylphosphatidylcholine purchased from Calbiochem, San Diego, Calif., and used without further purification. The purity of the lipids was checked by high-sensitivity scanning calorimetry of multilamellar suspensions. Heat-capacity measurements provide a very general sensitive test of purity (Mastrangelo and Dornte, 1955; Sturtevant, 1971; Wunderlich, 1971) which is directly applicable to phosphatidylcholines in multilamellar suspension (Albon and Sturtevant, 1978), since they show what appears to be an isothermal first-order transition under these conditions. On the basis of comparisons with samples to which known amounts of other phosphatidylcholines or lysolecithin (from egg-yolk lecithin) were added, we estimate that the DMPC and DPPC used in this work contained no more than 0.5 mol % of impurities. Cholesterol from Matheson, Coleman and Bell was recrystallized twice from ethanol. The phospholipids and cholesterol lost no weight in a vacuum oven at 40–50 °C and were assumed to be anhydrous.

Suspensions for calorimetric study were prepared by dissolving weighed amounts of lipid and cholesterol in chloroform and evaporating to dryness first under nitrogen and then in a vacuum oven at 40–50 °C for several hours. The residue was then taken up in chloroform and again evaporated to dryness. An appropriate weighed amount of 0.01 M phosphate buffer, pH 7.0, was added and the mixture vortexed at about 60 °C in the presence of a few glass beads for several minutes. In a few cases at high cholesterol concentrations, the mixture was also sonicated in a Branson bath sonicator at room temperature for 1 min to increase the stability of the suspension. There was considerable tendency toward settling of the suspensions containing high concentrations of cholesterol but this appeared not to interfere with the calorimetric experiments.

All calorimetric scans were performed with the Privalov calorimeter (Privalov et al., 1975), with a scan rate of 1 K min^{-1}, except at very low cholesterol concentrations when a scan rate of 0.5 K min^{-1} was used. In a number of cases the suspension was cooled after the first scan and then rescanned with satisfactory reproducibility, indicating essentially complete reversibility with no detectable hysteresis. Suspension concentrations ranged from 0.2 mg mL^{-1} for pure lipids to 6

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‡ Present address: Kraft, Inc., Glenview, Ill. 60025.

§ Present address: University of Granada, Granada, Spain.

[1] Abbreviations used: DMPC, DPPC, and DSPC, L-α-dimyristoyl-, L-α-dipalmitoyl-, and L-α-distearoylphosphatidylcholine; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy.

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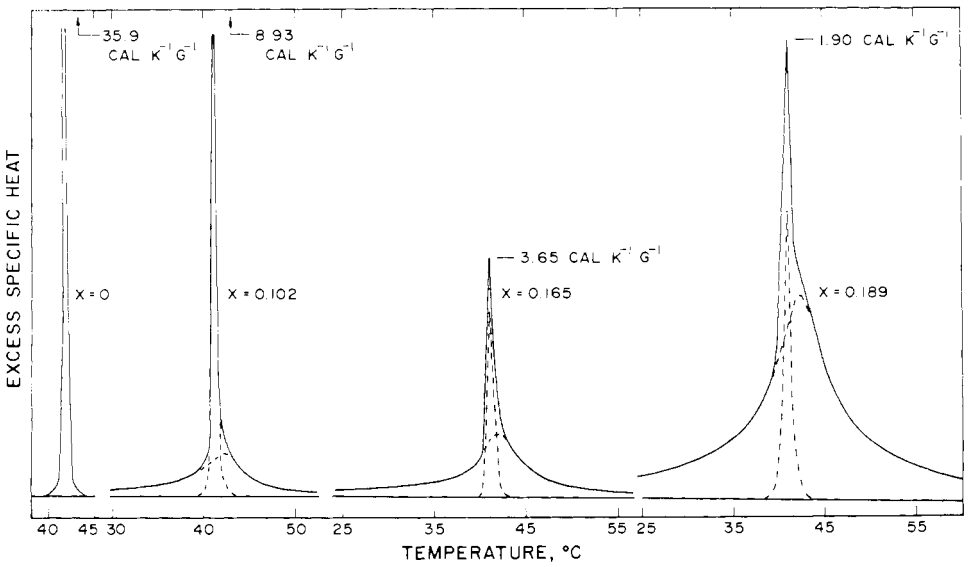


FIGURE 1: Calorimetric scans for various DPPC–cholesterol mixtures in multilamellar suspension. X is the mole fraction of cholesterol in the bilayer, and the maximum excess specific heat is given for each suspension. The solid curves are the observed curves and the dashed curves represent the decomposition of the observed curves into component curves.

mg of phospholipid mL^{-1} at high concentrations of cholesterol.

Detectable heat absorption was observed with cholesterol-containing suspensions over temperature intervals as large as 70 °C. Such behavior makes it difficult to obtain accurate data, even with the relatively stable baseline characteristic of the Privalov calorimeter, if a scan rate slow enough to ensure thermal and chemical equilibrium is employed. The errors in our previous work on cholesterol mixtures (Hinz and Sturtevant, 1972) arose from inability to distinguish small and very broad heat absorptions from the baseline of the less sensitive instrument used in that work.

Results

DPPC–Cholesterol Mixtures. At concentrations of cholesterol above about 5 mol %, the curve of excess specific heat vs. temperature can be analyzed as the sum of two approximately symmetrical peaks, a narrow one and a broad one. Although this analysis is convenient for the purpose of describing our observations, it is not necessarily a correct analysis. Typical calorimetric scans are shown in Figures 1 and 2. The difficulty of quantitative investigation of this system is well illustrated by the fact that the maximum excess specific heats, expressed in terms of the DPPC present, cover a range of almost 1000:1.

The narrow peak, centered at 41.4 °C (T_m) and about 0.3 °C wide ($\Delta T_{1/2}$) at half-maximum excess specific heat for pure DPPC, shifts to slightly lower temperatures (T_m = 40.9 °C at 10 mol %) and becomes somewhat broader as cholesterol is added. This broadening may be expressed in terms of the van't Hoff enthalpy evaluated from the calorimetric curve by the approximate equation

$$\Delta H_{vH} = 4RT_m^2 \left(\frac{c_{max}}{\Delta q}\right) \qquad (1)$$

where c_{max} is the maximum excess specific heat and Δq is the integrated area under the specific-heat curve. (It may be noted that the ratio $c_{max}/\Delta q$ has the dimension K^{-1}, and that no knowledge of the nature or amount of material undergoing transition is required in the evaluation of ΔH_{vH}.) The area under the narrow peak decreases as cholesterol is added, and the peak disappears at 20 ± 2 mol %.

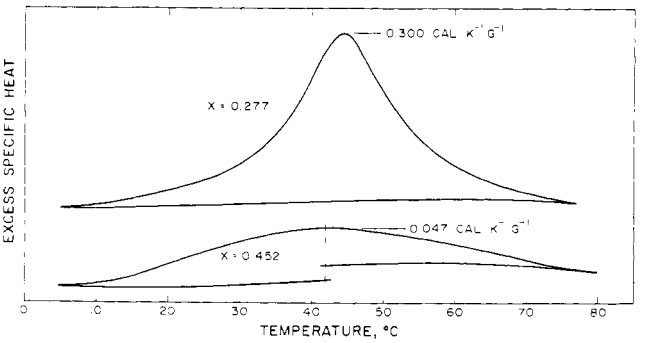


FIGURE 2: Calorimetric scans for two DPPC–cholesterol mixtures in multilamellar suspension. X is the mole fraction of cholesterol in the bilayer, and the maximum excess specific heat is given for each suspension.

The broad peak is centered at 41.0 to 41.6 °C up to 20 mol %, and then moves up to about 45.6 °C at 33 mol %. When it first becomes detectable at about 5 mol %, the broad peak appears to extend from 20 to 55 °C; at higher cholesterol contents it extends approximately from 5 to 80 °C. ΔH_{vH} for this peak ranges from 7.5×10^3 cal mol^{-1} at 5 mol % cholesterol to 2×10^3 at 45 mol %. It is only because of the stability of the baseline in the Privalov calorimeter that we can be sure that there is a transition at cholesterol contents as high as 45 mol %. The variation of T_m with cholesterol content for the narrow and broad peaks is shown in Figure 3, and the variation of $\Delta T_{1/2}$ of the broad peak is given in Figure 4.

The enthalpies corresponding to the areas of the two peaks are given in Figure 5. Only those experiments in which the DPPC concentration was 2 mg mL^{-1} or more are included in the figure; experiments at lower DPPC concentrations gave results in general agreement with those shown but with considerably larger scatter. The enthalpies of the narrow peak (filled circles) are calculated on the basis of the total DPPC present and fit the least square line with a standard deviation of ±0.58 kcal mol^{-1}. If we assume the narrow transition to be due to nearly pure DPPC, we can estimate the amount of DPPC involved from the known transition enthalpy (Mabrey and Sturtevant, 1976) and by subtraction from the total DPPC

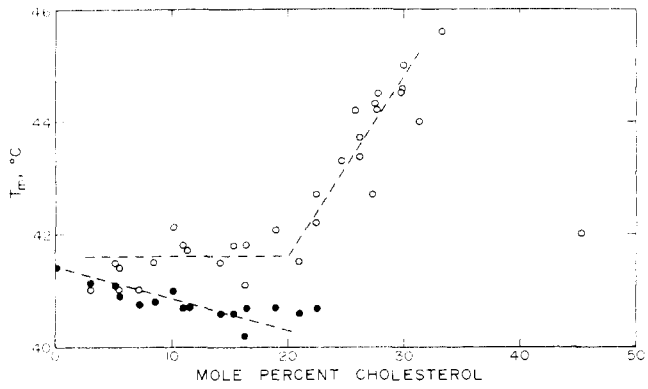


FIGURE 3: The variation with cholesterol content of the temperature of maximum excess specific heat for the narrow peak (●) and the broad peak (○) into which the observed curves for DPPC–cholesterol mixtures can be resolved.

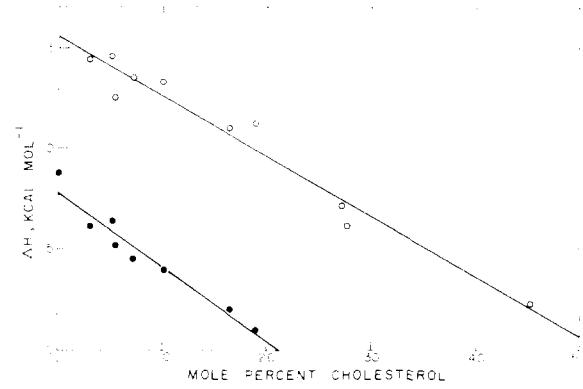


FIGURE 5: The variation with cholesterol content of the enthalpies obtained by integration of the narrow peak (●) and the broad peak (○) into which the observed excess specific heat curves for DPPC–cholesterol mixtures can be resolved.

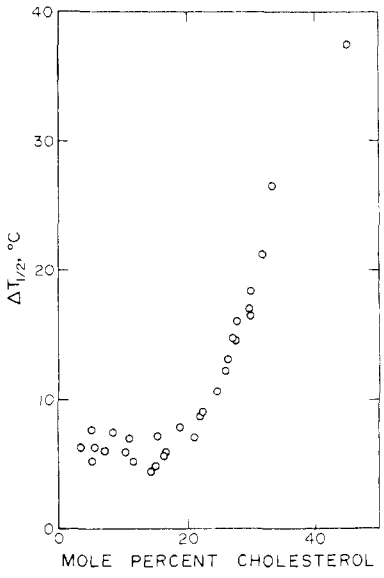


FIGURE 4: The variation with cholesterol content of the peak width at half-maximum specific heat for the broad peak obtained by resolution of the observed curves for the excess specific heat of DPPC–cholesterol mixtures.

present obtain the amount of phospholipid involved in the broad peak. The enthalpies for this peak (unfilled circles) were calculated on the basis of the reduced DPPC contents, on the arbitrary assumption that cholesterol makes no direct contribution to the observed enthalpies. The calculated enthalpies fit the least-square line in the figure with a standard deviation of ±0.84 kcal mol^{-1}.

The so-called pretransition of DPPC, for which T_m = 35.3 °C and ΔH = 1.83 kcal mol^{-1} (Mabrey and Sturtevant, 1976), is not visible at cholesterol contents above about 6 mol %. The T_m of this transition is more affected by the addition of cholesterol than is that of the main transition; for example, the T_m drops to 30 °C in the presence of 5.2 mol %.

Results similar to those obtained by us have recently been reported by Estep et al. (1977).

DMPC–Cholesterol Mixtures. As in the case of DPPC–cholesterol mixtures, addition of cholesterol leads to the initiation of detectable excess enthalpy absorption at temperatures well below the main transition of DMPC, with such absorption extending to temperatures as high as 80 °C at 40 mol % cholesterol. At low cholesterol contents, the observed specific-heat curves can again be viewed as the sum of a narrow and a broad peak, with the T_m of the former only slightly below that of pure DMPC (23.9 °C; Mabrey and Sturtevant, 1976). In contrast with the DPPC mixtures, at a rather sharply defined cholesterol content it becomes obvious that a summation of at least three peaks is necessary for an adequate reproduction of the observed specific-heat curve. Whereas at 11.6 mol % (Figure 6, curve A) the observed curve can be decomposed with adequate accuracy into a narrow peak with T_m = 23.5 °C and a broad peak with T_m = 24.8 °C, at 13.3 mol % there is a slight shoulder on the high-temperature side of the narrow peak (T_m = 23.2 °C), and at 14.3% there is a moderately narrow peak at 22.2 °C, a somewhat broader one at 24.6 °C, and a very broad one centered at about 26 °C. Figure 7 shows a possible decomposition of the 14.3 mol % curve into three approximately symmetrical peaks. A similar situation is seen at 16.3 mol % (Figure 6, curve B). At 18.1 mol %, the first peak appears smaller than the second one, the two of them together constituting only a small fraction of the total enthalpy change. As with the DPPC mixtures, only a single broad peak is seen above 20 mol % (Figure 6, curve C), with gradually decreasing area with increasing cholesterol content. Since decomposition of the observed curves into three component peaks would be much more arbitrary than the decomposition into two peaks adopted for the curves for the DPPC mixtures, no extensive analysis in these terms has been attempted. It should be noted that a similar complexity in the DPPC–cholesterol system could have been overlooked if the two narrower peaks happened to have values of T_m closer together than in the DMPC mixtures.

Discussion

In our earlier study (Hinz and Sturtevant, 1972) of the DMPC- and DPPC–cholesterol systems, we failed to recognize the presence of the broad peaks and concluded that the transition enthalpies went to zero at 33 mol % cholesterol. These errors, which are related, were both the result of employing a calorimeter having a less stable baseline than that characteristic of the Privalov calorimeter and of using insufficiently large concentrations of the lipids.

Mabrey and Sturtevant (1976) reported that some mixtures of DMPC and DSPC show transition curves with two peaks. In these cases, analysis into separate peaks appears to be incorrect, since the fact that the initiation temperature of the transition increases with increasing DSPC content indicates that only one gel phase is present at all compositions. In the DPPC–cholesterol mixtures, on the other hand, this situation is not observed and the observed results may be interpreted in

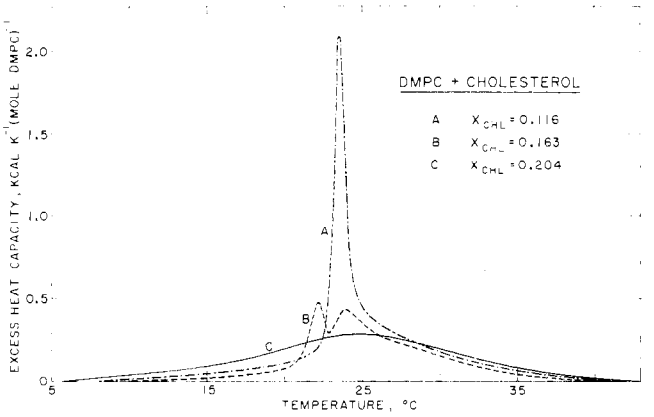


FIGURE 6: Representative excess heat-capacity curves for DMPC-cholesterol mixtures: (A) mole fraction of cholesterol equals 0.116; (B) mole fraction equals 0.163; (C) mole fraction equals 0.204.

terms of two immiscible gel phases. In this interpretation the conclusion seems unavoidable that the narrow peak observed at cholesterol contents below 20 mol % represents nearly pure DPPC. The observed values of ΔH_{vH} are approximately half the large values observed with the pure lipid and correspond to cooperative unit sizes which suggest purities of the order of 99%. The linear decrease in apparent transition enthalpy as cholesterol is added indicates that material is removed from the pure phospholipid gel phase by association with cholesterol in the molar ratio of approximately 4:1 phospholipid–cholesterol. This new species, whatever its nature, has three properties which appear evident: it is essentially insoluble, at least in the solid state, in DPPC; it exhibits an extremely broad phase transition; and it in turn is converted by addition of cholesterol above 20 mol % to one or more additional species which show no detectable phase transitions in the temperature range studied.

It has been pointed out by Martin (R. B. Martin, personal communication) that several different packing complexes of DPPC with cholesterol can be predicted on the basis of model building, depending on whether lipid chains can or cannot be shared by cholesterol molecules and whether monomeric or dimeric cholesterol is involved. The complex of monomeric cholesterol with shared lipid chains is the 33 mol % complex described by Engelman and Rothman (1972). If unshared lipid chains are required, the composition is about 22 mol %. The complex indicated by the present data may be this one. The unusual breadth of its transition may reflect a very imperfect crystalline structure, with lattice forces too weak to lead to a highly cooperative phase transition, as is also indicated by the increased mobility of the hydrocarbon chains in the presence of cholesterol below the phase-transition temperature (Oldfield and Chapman, 1972). It might also be suggested that this is an unusual solid phase showing a continuous transition similar to that of a liquid–vapor transition just above the critical point. The transition breadth increases as the complex is broken up by addition of excess cholesterol.

The fact that the apparent enthalpy of the broad peak decreases linearly with increasing cholesterol concentration, starting at 0 mol %, is puzzling. This, together with the steadily decreasing cooperativity, would seem to indicate a solid phase of continuously varying composition even below 20 mol %, but this is inconsistent with the disappearance of the sharp peak at the composition corresponding to the complex containing 20 mol % of cholesterol.

The high transition enthalpy indicated for the broadly melting species at low cholesterol concentrations is of interest.

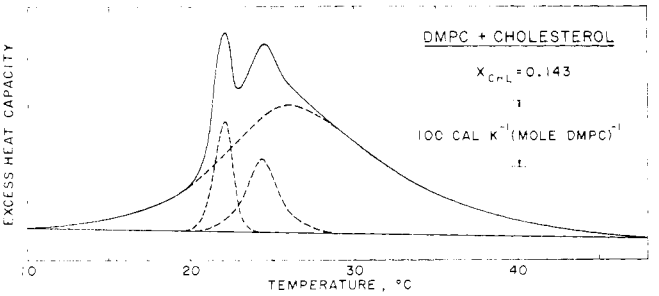


FIGURE 7: Decomposition of the observed excess heat-capacity curve for 14.3 mol % cholesterol in DMPC into three component curves.

This enthalpy would be somewhat lower if the cholesterol content were added to the phospholipid content with an extrapolated value of 12.6 kcal mol^{-1} instead of 15.6 kcal mol^{-1}. If packing complexes are indeed formed, it may be assumed that at least at low cholesterol contents the only one present is the 22 mol % complex and that the extrapolated enthalpy is characteristic of this complex. It appears that cholesterol–lipid interactions involve a significantly larger enthalpy than lipid–lipid interactions.

Shimshick and McConnell (1973), using the spin-label Tempo, presented phase diagrams for mixtures of cholesterol with DMPC and DPPC showing horizontal solidus curves, indicative of immiscibility in the solid state, up to 20 mol % cholesterol. These diagrams cannot account for our observations, since in each case we clearly observe the start of heat absorption at temperatures much below the T_m of pure phospholipid and continuing heat absorption to temperatures higher than indicated by these diagrams. Kleemann and McConnell (1976) have recently proposed a modified phase diagram with a eutectic at 20 mol % cholesterol and a fluid plus cluster phase. This diagram would predict inception of "melting" at a constant temperature only slightly below the T_m of the pure lipid, in disagreement with the calorimetric observations. A recent ^{2}H NMR study (Haberkorn et al., 1977) gave results interpreted in terms of phase boundaries at both 20 and 33 mol % cholesterol.

It appears that 20 mol %, at least, is a composition of special importance. Since throughout the composition range the maximum of the broad peak occurs at a temperature above the T_m of the phospholipid, compound formation seems more likely than eutectic formation. It is unlikely that the polymorphic phase transition of pure cholesterol at 35 °C (Spier and van Senden, 1965) has any influence on our measurements, since no pure cholesterol phase exists at compositions up to at least 50 mol %.

The interpretation given here for DPPC mixtures is somewhat weakened by the fact that it cannot be easily applied to the DMPC–cholesterol system. There is no obvious reason to expect any fundamental difference between the behavior of DPPC and DMPC in the presence of cholesterol. The resolution of the DMPC curves into three peaks cannot, according to the Gibbs phase rule, correspond to the coexistence of three solid phases over a composition range in a system of only two components. (If water is added as a third component, the aqueous phase must be added as an additional phase, and the above statement still holds.) Essentially identical triple-peaked scans have been observed at scan rates of 0.46 and 0.95 K min^{-1}, which suggests that kinetic limitations are not of any significance. It is conceivable that in this case the 20 mol % complex is sufficiently soluble in the phospholipid to lead to a double peaked transition curve similar to those observed in other systems (Mabery and Sturtevant, 1976).

References

Albon, N., and Sturtevant, J. M. (1978), *Proc. Natl. Acad. Sci. U.S.A.* (in press).

de Kruyff, B., Demel, R. A., and Rosenthal, A. F. (1973), *Biochim. Biophys. Acta 307*, 1–19.

de Kruyff, B., van Dijk, P. W. M., Demel, R. A., Schuijff, A., Brants, F., and van Deenen, L. L. M. (1974), *Biochim. Biophys. Acta 356*, 1–7.

Engelman, D. M., and Rothman, J. E. (1972), *J. Biol. Chem. 247*, 3694–3697.

Estep, T. N., Mountcastle, D. B., Biltonen, R. L., and Thompson, T. E. (1978), *Biochemistry 17*, 1984.

Haberkorn, R. A., Griffin, R. G., Meadows, M. D., and Oldfield, E. (1977) *J. Am. Chem. Soc. 99*, 7353–7355.

Hinz, H.-J., and Sturtevant, J. M. (1972), *J. Biol. Chem. 247*, 3694–3697.

Kleemann, W., and McConnell, H. M. (1976), *Biochim. Biophys. Acta 419*, 206–222.

Mabrey, S., Mateo, P. L., and Sturtevant, J. M. (1977), *Biophys. J. 17*, 82a.

Mabrey, S., and Sturtevant, J. M. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73*, 3862–3866.

Mastrangelo, S. V. R., and Dornte, R. W. (1955), *J. Am. Chem. Soc. 77*, 6200–6201.

Oldfield, E., and Chapman, D. (1972), *FEBS Lett. 23*, 285–297.

Privalov, P., Plotnikov, V. V., and Filimonov, V. V. (1975), *J. Chem. Thermodynam. 7*, 41–47.

Shimshick, E. J., and McConnell, H. M. (1973), *Biochem. Biophys. Res. Commun. 53*, 446–451.

Spier, H. L., and van Senden, K. G. (1965), *Steroids 6*, 871–873.

Sturtevant, J. M. (1971), in Physical Methods of Chemistry, Vol. I, part V, Weissberger, A., and Rossiter, B. W., Ed., New York, N.Y., Wiley-Interscience, pp 374, 396.

Wunderlich, B. (1971), in Physical Methods of Chemistry, Vol. I, part V, Weissberger, A., and Rossiter, B. W., Ed., New York, N.Y., Wiley-Interscience, pp 483–487.

CORRECTIONS

Adenosine 3′:5′-Monophosphate Dependent Protein Kinase from Bovine Heart. Characterization of the Catalytic Subunit, by Kristine A. Peters, Jacques G. Demaille, and Edmond H. Fischer,* Volume 16, Number 26, December 27, 1977, pages 5691–5697.

On page 5694, column 2, several lines are missing from the paragraph starting on line 11. The paragraph should read: Graphical determination (not illustrated)[2] shows that a first group reacts very rapidly ($t_{1/2}$ = 0.36 min, k = 1.9 min^{-1}) while another one reacts approximately 10 times slower ($t_{1/2}$ = 3.5 min, k = 0.2 min^{-1}). The third SH group reacts very slowly ($t_{1/2}$ = 110 min, k = 0.006 min^{-1}), and its substitution . . . .

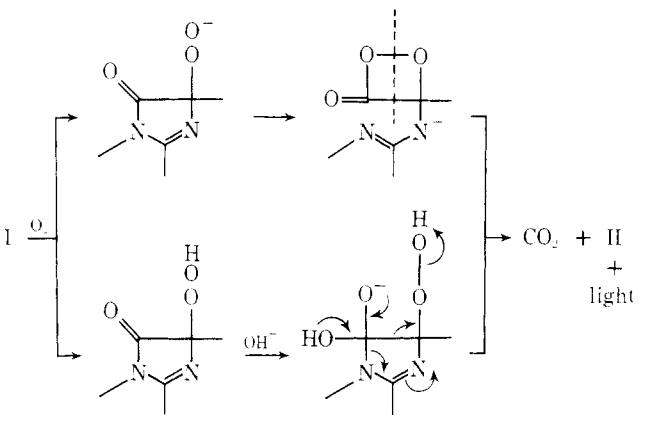
Multiple Thymine Dimer Excising Nuclease Activities in Extracts of Human KB Cells, by Kem H. Cook and Errol C. Friedberg,* Volume 17, Number 5, March 7, 1978, pages 850–857.

On page 856, Table IV, the first entry in column six should read 14.3 instead of 4.3.

Properties and Reaction Mechanism of the Bioluminescence System of the Deep-Sea Shrimp *Oplophorus gracilorostris*, by Osamu Shimomura,* Takashi Masugi, Frank H. Johnson, and Yata Haneda, Volume 17, Number 6, March 21, 1978, pages 994–998.

The structural formula in the upper right-hand part of Scheme I, page 997, has an error consisting of five valence bonds on one of the carbons. The corrected scheme should appear as follows.

SCHEME I



Two Human Trypsinogens. Purification, Molecular Properties, and N-Terminal Sequences, by Odette Guy,* Dominique Lombardo, Diana C. Bartelt, Jean Amic, and Catherine Figarella, Volume 17, Number 9, May 2, 1978, pages 1669–1675.

In Table III, page 1673, the value in line 2 of column 2 should read 4.10, not 14.10 as printed.