probably due to some effect of calcium ion on the red blood cell which made the cell more permeable to extracellular permeants. Calcium does not cross the red cell membrane under normal conditions (15). However, Davson and Danielli (16) have shown that calcium ion in concentrations greater than 0.01 *M* accelerates the penetration of extracellular potassium into the cell and increases loss of intracellular sodium from the cat erythrocyte. They also point out that unusual reponses of cell membranes take place in the presence of alkaline earth ions. Ruyssen and Croes (17) found that alkaline earths appear to thicken the wall of the red blood cell by forming insoluble salts with phosphatide acids. Dentzer (18) reported that calcium ion increased saponin hemolysis of human and cattle erythrocytes. These literature reports emphasize the fact that the calcium ion is capable of producing unusual responses in hemolysis studies.

The hemolysis behavior of the other divalent cation, magnesium, was similar to that of the majority of compounds employed in this investigation.

Jacobs, *et al.* (19), found that the rate of osmotic hemolysis of ox erythrocytes in solutions of penetrating nonelectrolytes (glycerin and ethylene glycol) were considerably increased by the addition of low concentrations of certain electrolytes. Salts with bivalent cations were more effective than those with univalent cations, while salts with bi- or trivalent anions usually had a retarding effect. In most instances, the effect of these ions on the rate of hemolysis are analogous to our present findings for the protective effect of similar ions against hemolysis of rabbit and human erythrocytes in concentrated propylene glycol solutions; calcium afforded the least protection while polyvalent anions gave the most protection.

REFERENCES

(1) Cadwallader, D. E., This Journal, **52**, 1175(1963).
(2) Grosicki, T. S., and Husa, W. J., *ibid.*, **43**, 632(1954).
(3) Zanowiak, P., and Husa, W. J., *ibid.*, **48**, 565(1959).
(4) Ansel, H. C., and Husa, W. J., *ibid.*, **48**, 516(1959).
(5) Eisenberg, P., *Zentr. Backteriol. Parasitenk.*, **69**, 173 (1913).
(6) Good, W., *Biochim. Biophys. Acta*, **44**, 130(1960).
(7) *Ibid.*, **48**, 229(1961).
(8) *Ibid.*, **50**, 485(1961).
(9) *Ibid.*, **52**, 545(1961).
(10) *Ibid.*, **53**, 549(1961).
(11) *Ibid.*, **57**, 104(1962).
(12) Stein, W. D., *ibid.*, **59**, 35(1962).
(13) Ponder, E., "Red Cell Structure and Its Breakdown," Springer Publishing Co., Vienna, 1955, pp. 59–61.
(14) Hober, R., "Physical Chemistry of Cells and Tissues," 1st ed., Blakiston Co., Philadelphia, Pa., 1945, pp. 246–248.
(15) Tullis, J. L., "Blood Cells and Plasma Proteins, Their State in Nature," Academic Press Inc., New York, N. Y., 1953, p. 202.
(16) Davson, H., and Danielli, J. F., "The Permeability of Natural Membranes," 1st ed., Macmillan Co., New York, N. Y., 1943, p. 156.
(17) Ruyssen, R., and Croes, R., *Mededel. Koninkl. Vlaam. Acad. Welenschap. Belg.*, **11**, 5(1949).
(18) Dentzer, G., *Pharmazie*, **10**, 734(1955).
(19) Jacobs, M. H., Parpart, A. K., and Corson, S. A., *J. Cellular Comp. Physiol.*, **9**, 177(1937).

Inclusion Compounds of α-Lipoic Acid Methyl Ester with Urea and Thiourea

By HIROYUKI MIMA and MASAO NISHIKAWA

Recently several works have been reported on stabilization of various pharmaceuticals by formation of urea, thiourea, or deoxycholic acid adducts. Methyl α-lipoate, a relatively unstable hepatotonic, was found to form adducts both with urea and thiourea. X-ray diffraction patterns and infrared spectra showed that these adducts were typical inclusion compounds. Methyl α-lipoate in the urea adduct was relatively stable under exposure to sunlight or to ultraviolet light, whereas it was not so stabilized in the thiourea adduct. It was also found that in these adducts methyl α-lipoate became its free radical under such irradiation and that this radical remained stable in these channels for several hours to one day even after the irradiation was stopped.

Inclusion compounds are described as crystalline compounds which consist of two or more distinct components and in which one of the components fits into cavities provided by the other. The component which forms the cavity is designated as the host molecule, and the component which is included in the cavity as the guest molecule.

Urea and thiourea form such adducts or inclusion compounds with various other organic molecules such as hydrocarbons, acids, and esters. These interesting properties, found by Bengen (1), Angla (2), and Schlenk (3) in the 1940's, have been the subject of many investigations. X-ray studies have revealed the general details of the structure of these adducts. Ordinary urea has a tetragonal crystal structure but when crystallized from methanol containing normal paraffins, normal fatty acids, or other straight-chain molecules, it adopts a hexagonal crystal

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structure. In this structure, long hexagonal channels are formed with the lattice of urea molecules and straight-chain molecules are included in these channels. The diameter of urea channels is about 5 Å. Consequently branched molecules or cyclic molecules are too large to be included in urea channels.

On the other hand, ordinary thiourea has a rhombic structure. On inclusion of cyclohexane, carbon tetrachloride, or other molecules of similar sizes, it changes its crystal lattice to a rhombohedral form. The principle of the adduct formation of thiourea and urea is the same but the diameter of thiourea channels is a little larger. Though branched-chain molecules or cyclic molecules can be included, straight-chain molecules cannot form stable adducts with thiourea because they are too thin to remain in the channels.

The compounds included in urea or thiourea channels will be stabilized, as there is no room to react within channels with other molecules such as oxygen. When adducts are dissolved in water or extracted with ether, they decompose into their component host and guest molecules. Therefore, these adducts will be utilized as "molecular coating" of otherwise unstable pharmaceuticals. With these ideas, one of us has studied the stabilization and extractive crystallization of some pharmaceuticals (4–6). For example, 9-oxocamphor is used as a cardiotonic in Japan but it is extremely unstable and loses its potency almost completely on standing overnight in the air. However, its thiourea adduct protects 9-oxocamphor from oxidation for as long as one year and by dissolving the adduct in water, effective 9-oxocamphor is readily regenerated at any time (4).

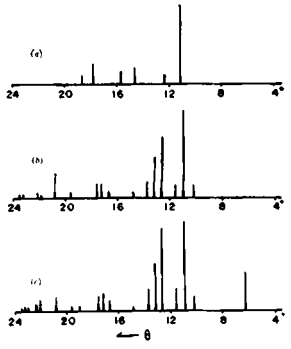


Fig. 1.—X-ray powder diffraction patterns of methyl α-lipoate urea adduct and its related compounds. (*a*) Urea (tetragonal); (*b*) methyl α-lipoate urea adduct; (*c*) methyl stearate urea adduct.

In the present work, we tried to stabilize methyl α-lipoate by the same technique. Methyl α-lipoate is a widely used hepatotonic in Japan but it is also unstable and polymerizes rapidly on standing in the air, especially under sunlight or ultraviolet light. The polymerization would proceed through the mechanism as shown below, which was assumed from the studies conducted by Barltrop, *et al.* (7) and Thomas and Reed (8).

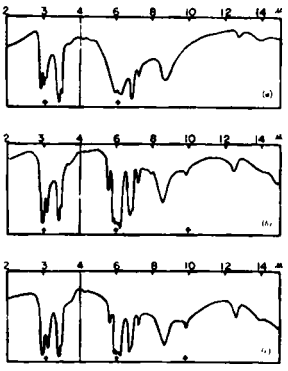
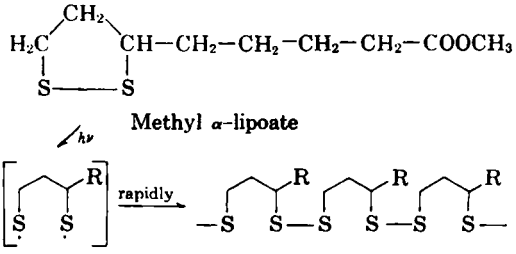


Fig. 2.—Infrared spectra of methyl α-lipoate urea adduct and its related compounds. (*a*) Urea (tetragonal); (*b*) methyl α-lipoate urea adduct; (*c*) methyl stearate urea adduct.



Methyl α-lipoate

If methyl α-lipoate is included in urea or thiourea channels, its trimethylene disulfide rings will be forced to be separated from each other so that such polymerization cannot take place and therefore the compound will be stabilized.

METHODS

Preparation of Adducts.—Methyl α-lipoate was prepared by esterification of commercially available α-lipoic acid with diazomethane in ether. On several careful redistillations under a reduced pressure, a yellow liquid with low viscosity was obtained, the boiling point ranging from 137 to 140° under 0.4 mm. Hg (9).

Preparation of its urea adduct was conducted as follows: 5 Gm. of freshly distilled methyl α-lipoate was dissolved (by heating if necessary) in 50 ml. of methanol containing 15 Gm. of urea. The resulting clear solution was then allowed to stand

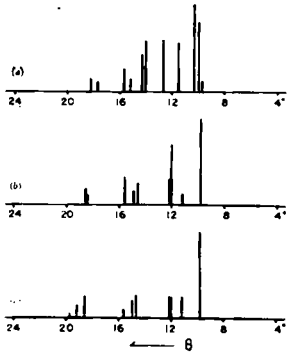


Fig. 3.—X-ray powder diffraction patterns of methyl α-lipoate thiourea adduct and its related compounds. (*a*) Thiourea (rhombic); (*b*) methyl α-lipoate thiourea adduct; (*c*) cyclohexane thiourea adduct.

overnight at room temperature. About 10 Gm. of the needle crystals separated were filtered, washed with isooctane to eliminate excess methyl α-lipoate, and dried on filter paper. This adduct decomposed at 63° and melted at 130°.

The preparation method of the thiourea adduct was almost the same as that of the urea adduct. Nine grams of thiourea and 8 Gm. of methyl α-lipoate in 40 ml. of methanol gave about 8 Gm. of the yellow adduct crystals. Acetone was used as washing solvent in this case. The decomposition point of the thiourea adduct was 106° and the melting point was 176°. Further purification was not conducted in both cases since these adducts partly decomposed into their components on recrystallization.

Stability Test.—About 50 mg. each of liquid methyl α-lipoate and the crystals of its urea and thiourea adducts was spread on a watch glass. A mere mechanical mixture of methyl α-lipoate and ordinary urea was also prepared and was spread in the same way. As a source of ultraviolet light, 15 W Blacklite blue (Sylvania) was used. The watch glasses were placed at the distance of 5 cm.

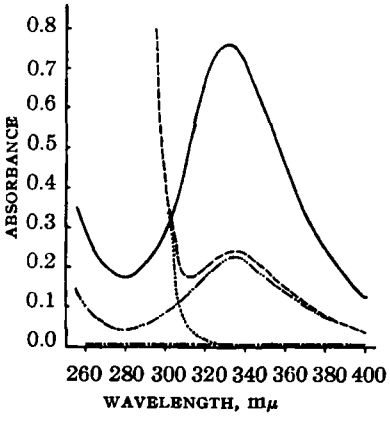


Fig. 4.—Ultraviolet spectra at methyl α-lipoate adducts and their related compounds. Key: ———, methyl α-lipoate; –·–·–·, urea; –··–··, methyl α-lipoate urea adduct; ....., thiourea; - - - - -, thiourea adduct.

from the light source and exposed to the light for 9 hours. The amount of unpolymerized methyl α-lipoate was determined by measurement of the intensity of the ultraviolet absorption at 334 mμ in methanolic solution and by the height of the reduction wave of polarography due to the —S—S— bond at pH 6.5.

Tests of stability under the exposure to sunlight were conducted by a similar method. In this case, the intensity of the sunshine was measured by a Matsuda integrating heliograph model IL-1-A.

RESULTS AND DISCUSSION

For confirmation that the crystals obtained as above are really urea and thiourea inclusion compounds, X-ray powder diffraction patterns and infrared spectra are very useful. As shown in Fig. 1, the X-ray pattern of methyl α-lipoate urea adduct is different from that of urea and is almost the same as that of methyl stearate urea adduct, one of the typical urea inclusion compounds.

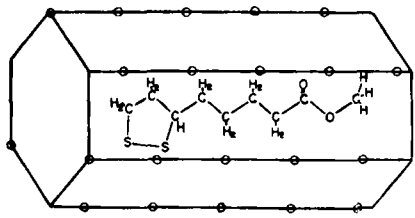


Fig. 5.—A possible structure of methyl α-lipoate in a urea channel. Key: O, urea molecules.

Comparison of the infrared spectra (Fig. 2) would also lead to the same results. Therefore, methyl α-lipoate urea adduct was definitely identified as an inclusion compound. Similarly, methyl α-lipoate thiourea adduct was also identified as a thiourea inclusion compound (Fig. 3). This would be the first time that a molecule which forms stable inclusion compounds both with urea and thiourea at room temperature was found, at least in the case of such a small molecule, since molecules to be included in urea and thiourea channels have been thought to be essentially different owing to the different diameters of the two channels (4).

Ultraviolet spectra of methyl α-lipoate urea and thiourea adducts in methanolic solution are shown in Fig. 4. Since these adducts dissociate into their components on dissolution in solvents, their spectra will be just the superposition of those of methyl α-lipoate and urea or thiourea. From the extinction coefficient of the absorption at 334 mμ which arises from the trimethylene disulfide ring in methyl α-lipoate, the molar ratios of [urea]:[methyl α-lipoate] and [thiourea]:[methyl α-lipoate] were determined to be 8.8:1 and 6.2:1, respectively. The molar ratios were also determined by elementary analysis and polarography and the values of 8.2:1 and 8.4:1 were given to the urea adduct and those of 5.9:1 and 6.2:1 were given to the thiourea adduct. Some discrepancy of these data may be attributed to the difficulty in purification of the adduct crystals. How methyl α-lipoate is included in urea or thiourea channels will be elucidated from these molar ratios without difficulty. Taking the value of 8.5:1 for the urea adduct as an average, the number of the atoms which form a zigzag chain in urea channels was calculated to be 10.2 by Smith's equation (10). If a methyl α-lipoate molecule is assumed to be enclosed in urea channels in such a state as suggested in Fig. 5, the number of the atoms in the zigzag chain including three carbon atoms in the trimethylene disulfide ring will be 10, which is in good accordance with the value calculated above. In the case of the thiourea adduct the molar ratio of about 6:1 indicates that one methyl α-lipoate molecule is enclosed only in one unit cell or, in other words,

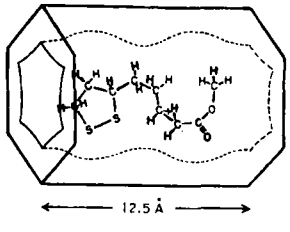


Fig. 6.—A possible structure of methyl α-lipoate in a thiourea channel.

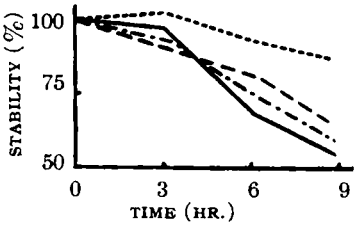


Fig. 7.—Stability of methyl α-lipoate and its adducts under the exposure to ultraviolet light. Key: ———, methyl α-lipoate; - - - -, urea adduct; – – – –, thiourea adduct; –·–·–·, mechanical mixture of methyl α-lipoate and urea.

in two relatively wide parts of thiourea channels (3). Therefore, the methyl α-lipoate molecules may be twisted to some extent in channels as suggested in Fig. 6.

The results of stability tests of methyl α-lipoate in its urea and thiourea adducts under the exposure to ultraviolet light are shown in Fig. 7 in comparison with those of liquid methyl α-lipoate itself. From the figures it is clear that the urea adduct stabilizes methyl α-lipoate considerably whereas the thiourea adduct has no such effect. As the adducts exist as fine crystals, scattering of light on the surface may be stronger than liquid methyl α-lipoate itself and this may make α-lipoate in the adduct stable. However, this effect seems to be insignificant since the mere mechanical mixture of methyl α-lipoate and ordinary urea powder does not stabilize α-lipoate as shown in Fig. 7. Stabilizing effect of the urea adduct must be, therefore, attributed to the suppression of polymerization reaction of methyl α-lipoate molecules by the hexagonal crystal lattice of urea. In the case of the thiourea adduct, such effect became apparent if sunlight was used as an irradiation source, although it was small compared with that of the urea adduct (Fig. 8).

These results may be explained as follows. Methyl α-lipoate may be excited by a wide range of ultraviolet and visible light, since it has absorption maximum in the near ultraviolet region. Thiourea also absorbs ultraviolet light having wavelengths shorter than 300 mμ and therefore it can react with methyl α-lipoate under exposure to such short wavelength light. However, light with wavelengths longer than 300 mμ may have little power to give rise to such reaction. On the other hand, urea does not absorb light up to 260 mμ, and thus it has a stronger stabilizing effect.

In the course of this work, we observed that the

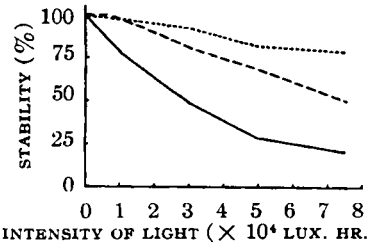


Fig. 8.—Stability of methyl α-lipoate and its adducts under the exposure to sunlight. Key: ———, methyl α-lipoate; - - - -, urea adduct; – – – –, thiourea adduct.

original crystals of urea or thiourea adducts of methyl α-lipoate were yellow, but when they were irradiated with sunlight or ultraviolet light, the color gradually changed to pink. This pink remained unchanged for several hours to 1 day even after the irradiation was stopped and then returned to the original yellow or a slightly faded color. Besides, the color disappeared immediately on dissolving in solvents. For the investigation of the origin of this pink, visible absorption spectra of these colored crystals were measured by rearranging the crystals side by side on a thin NaCl plate. Nujol was used as a binder. As shown in Fig. 9, the irradiated crystals of the urea adduct have an absorption maximum at 530 mμ. This region is transparent for the nonirradiated yellow crystals.

The spectrum of the irradiated thiourea adduct was completely the same as that of the irradiated urea adduct. This fact suggests that the origin of the pink has nothing to do with host molecules and may be attributed to a certain excited state of methyl α-lipoate. Barltrop, *et al.* (7) have found that if trimethylene disulfide was photolyzed at the

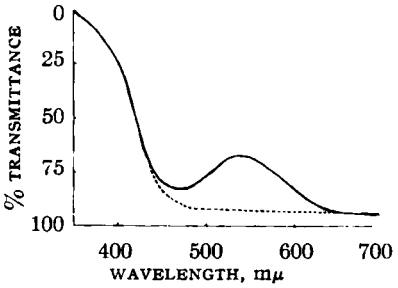


Fig. 9.—Ultraviolet spectra of crystals of methyl α-lipoate urea adduct. Key: - - - -, initial, nonirradiated crystals; ———, irradiated crystals.

temperature of liquid nitrogen a clear pale salmon color appeared. This color was attributed to free radicals of trimethylene disulfide since the colored solution faded the color of diphenylpicrylhydrazyl. As the pink observed in our experiments resembles this salmon, the origin of the pink would be attributable to free radicals of the trimethylene disulfide ring of methyl α-lipoate which are formed by the irradiation with sunlight or ultraviolet and as these radicals are restricted in the space of the crystal lattice of urea or thiourea they would exist stable without affecting further reactions.

In this way free radicals which otherwise do not exist in a stable state except at extremely low temperature can be isolated in the channel of inclusion compounds even at room temperature.

REFERENCES

(1) Bengen, M. F., *German Patent Application O. Z.*, 12, 438(1940); *Angew. Chem.*, 63, 207(1951).
(2) Angla, B., *Compt. Rend.*, 224, 402, 1166(1947); *Ann. Chim.*, 4, 639(1949).
(3) Schlenk W., *Ann.*, 565, 204(1949); *ibid.*, 573, 142 (1951).
(4) Mima, H., *Yakugaku Zasshi*, 77, 1196(1957).
(5) *Ibid.*, 79, 891(1959).
(6) Mima, H., Asahi, Y., Okuto, H., and Kanzawa, T., *ibid.*, 80, 1233(1960).
(7) Barltrop, J. A., Hayes, P. M., and Calvin, M., *J. Am. Chem. Soc.*, 76, 4348(1954).
(8) Thomas, R. C., and Reed, L. J., *ibid.*, 78, 6148(1956).
(9) Grunsalus, I. C., Barton, L. S., and Gruber. W., *ibid.*, 78, 1763(1956).
(10) Smith, A. E., *Acta Cryst.*, 5, 224(1952).