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# Studies on the Mode of Action of Excess of Vitamin A

## 5. THE EFFECT OF VITAMIN A ON THE STABILITY OF THE ERYTHROCYTE MEMBRANE*

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On the basis of experiments in which the intracellular enzymes of cartilage were released by treatment with hypo-osmotic solutions (Lucy, Dingle & Fell, 1961), it was proposed by Fell, Lucy & Dingle (1961) that the effects of excess of vitamin A on embryonic chick cartilage cultivated *in vitro* (Fell & Mellanby, 1952; Dingle, Lucy & Fell, 1961) could be explained by the hypothesis that the vitamin alters the permeability of intracellular particles. The liberation of one or more proteolytic enzymes, possibly cathepsins, from the lysosomes of chondrocytes was thought to be responsible for the degradation of the protein moiety of the polysaccharide–protein complex in embryonic cartilage that occurs when the tissue is cultivated *in vitro* with excess of the vitamin. This view was supported by the fact that vitamin A releases a proteolytic enzyme with an acid pH optimum from rat-liver lysosomes (Dingle & Lucy, 1961; Dingle, 1961).

In an attempt to discover whether the action of vitamin A is confined to the membrane of lysosomes, or whether it has a similar action on other membrane structures, the effect of the vitamin on the stability of the erythrocyte membrane has been investigated. The present paper presents observations which demonstrate that vitamin A alters the permeability of the cell membrane of the erythrocyte on incubation *in vitro*. This provides additional evidence for the hypothesis that the site of action of vitamin A is at the lipoprotein membranes of cells and their organelles. A preliminary communication has been published (Lucy & Dingle, 1962).

## EXPERIMENTAL

### *Materials*

Vitamin A alcohol (Roche) was dissolved in ethanol to give a 100 mg./ml. solution. (For convenience, 'vitamin A alcohol' is referred to as 'vitamin A' throughout the paper.) This was stored under nitrogen in the cold, and was stable for at least 3 weeks in the dark. Dilutions were made immediately before use. 4-Apo-β-carotenal and 3-apo-β-carotenal were given by Dr John Glover and Professor R. A. Morton, F.R.S., of the University of Liverpool. 4-Apo-β-carotenol and 3-apo-β-carotenol were obtained by reduction of the corresponding aldehyde in ether solution with sodium borohydride (sodium tetrahydroborate) at 0°. The methyl ether of vitamin A, β-ionylidene ethanol, β-ionylidene acetaldehyde, vitamin $A_2$ (alcohol) and vitamin $A_2$ aldehyde were gifts from Dr Philip L. Harris of the Biochemistry Laboratories, Distillation Products, Rochester, N.Y.; vitamin A acid was a gift from Dr T. Moore of the Dunn Nutritional Laboratory, Cambridge. Vitamin A acetate (Roche), vitamin A palmitate (British Drug Houses Ltd.) and all-*trans* vitamin A aldehyde (Eastman Organic Chemicals) were commercial preparations and were used without further purification. The other derivatives of vitamin A and related compounds were prepared as described by Fell, Dingle & Webb (1962). All other materials used were commercial preparations of the highest purity available.

Lysolecithin was a gift from Dr R. M. C. Dawson, of the Agricultural Research Council Unit for Animal Physiology, Babraham. This sample, which was free from venom, was prepared from ovolecithin by means of cobra venom.

The non-ionic detergent BRIJ 35 (Atlas Powder Co., Wilmington, Delaware, U.S.A.), an ether of poly(ethylene glycol), was stored as a 1% (v/v) solution in glass-distilled water and was diluted immediately before use.

The rabbit erythrocytes were obtained from six young adult males (mean weight 5 lb.). The animals were bled from the ear and the blood was collected in an approximately equal volume of Alsever's solution (Bukantz, Rein & Kent, 1946). The cells were centrifuged and washed twice with approximately 10 vol. of 0·9% sodium chloride solution. The packed-cell volume was then determined by centrifuging the cells in a small quantity of iso-osmotic sodium chloride solution at 220*g* for 5 min. in an angle-head centrifuge. Finally, the cells were suspended in 0·9% sodium chloride solution to give 1 ml. of packed cells/40 ml. of suspension. This was the standard suspension used in all experiments unless otherwise stated.

Bovine and pig blood were obtained from the slaughterhouse and rat blood from adult laboratory animals. The same anticoagulant and washing procedures were used for each species. All suspensions were kept at 5° until required.

### *Methods*

Since vitamin A and most of the other compounds tested were virtually insoluble in the suspending medium it was necessary to standardize the mode of addition of the test compound to the cells. Reproducible results were obtained when an ethanolic solution of the compound (0·05 ml.) was

* Part 4: Fell, Dingle & Webb (1962).

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pipetted into the bottom of a dry tube and then a measured volume (5 ml.) of the cell suspension added. An equal volume of ethanol was added in similar manner to the controls.

The suspension of erythrocytes in 0·9% sodium chloride solution was incubated with the test compound; the cold suspension of erythrocytes at 5° was placed directly in the water bath unless otherwise stated. At the end of the incubation period the suspension was immediately centrifuged for 4 min. and the supernatant removed. This was analysed for potassium with an EEL flame photometer, and the extinction at 500 mμ of the supernatant solution was determined as a measure of the release of haemoglobin. Total haemolysis was obtained by the addition of an excess of vitamin A (usually 1 mg./ml.).

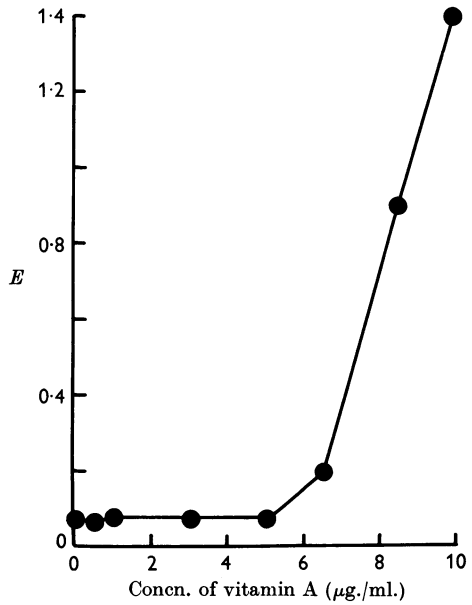


Fig. 1. Release of haemoglobin by vitamin A. The standard suspension of erythrocytes in 0·9% NaCl solution was incubated at 37° for 30 min. with the vitamin. The release of haemoglobin was followed by measurement of extinction at 500 mμ as described in the text.

## RESULTS

*Haemolysis by vitamin A.* The addition of vitamin A to a suspension of rabbit erythrocytes in 0·9% sodium chloride solution caused rapid haemolysis. The degree of haemolysis was dependent on concentration, though changes in the extent of haemolysis in response to changes in concentration were found only over a very narrow range. Thus, under the conditions of the experiment shown in Fig. 1, 10 μg. of vitamin A/ml. caused extensive haemolysis, whereas half this concentration had no effect on the erythrocytes. (The *E* value for total haemolysis was 2·0.) The amount of vitamin per cell appeared to be the critical factor; thus, if the number of cells per unit volume of suspending medium was reduced, less vitamin A was required to give the same degree of lysis. If 50% lysis is taken as the standard, $2 \times 10^8$ cells required the addition of 7 μg. of vitamin A/ml. under the conditions of these experiments. If it is assumed that all the vitamin was taken up by the cells, a maximum of approx. $12 \times 10^{-11}$ μmoles of vitamin A per cell is obtained.

The absence of the normal ionic constituents of serum from the iso-osmotic sodium chloride solution

Table 1. *Effect of vitamin A on the morphology of rabbit erythrocytes*

Erythrocytes were suspended in 0·9% NaCl solution, and 5 ml. of this suspension was mixed with 0·05 ml. of vitamin A dissolved in ethanol (final concn. 35 μM). 4-Apo-β-carotenal was added in the same way to give the same final concentration. Ethanol only was added to the controls. The suspension was kept at room temperature (about 19·5°) and samples were withdrawn at intervals. These were placed on a silicone-treated slide and covered with a silicone-treated coverslip. At the end of the experiment the number of intact erythrocytes were counted.

| Time (min.) | Control | Vitamin A | 4-Apo-β-carotenal |
|---|---|---|---|
| 0 | Smooth disks | Smooth disks | Smooth disks |
| 1 | Smooth disks | Marked crenation of many cells | Crenated disks |
| 2–5 | Smooth disks | Crenations more numerous; some crenated spheres present | — |
| 5–11 | Smooth disks; a few slightly crenated cells | Crenated spheres | Crenated disks |
| 20 | Smooth disks; a few slightly crenated cells | Smooth spheres | Crenated disks |
| 25–30 | Smooth disks; a few slightly crenated cells | Some prelytic spheres | Crenated disks |
| 35 | Smooth disks; a few slightly crenated cells | Prelytic spheres; some stroma | Crenated disks |
| 64 | Smooth disks; a few slightly crenated cells | Stroma; some prelytic spheres | Crenated disks |
| Cell count at end of experiment | $141 \times 10^6$ cells/ml. | $1{\cdot}3 \times 10^6$ cells/ml. | $125 \times 10^6$ cells/ml. |

used had no influence on lysis of erythrocytes by vitamin A, since rabbit erythrocytes suspended in Hanks's saline (Paul, 1959) at pH 6·8 were lysed by the vitamin to an equal extent.

*Effect of vitamin A on the morphology of rabbit erythrocytes.* We are indebted to Dr M. Daniel for the microscopic observation of the cells during treatment with vitamin A and 4-apo-β-carotenal.

The changes observed after treatment with vitamin A (Table 1) were very similar to those after the addition of saponin (Ponder, 1952). The appearance of crenation was very rapid under the conditions of these experiments and may have been related to uptake of the vitamin by the cells. 4-Apo-β-carotenal, which had no haemolytic activity, also caused some crenation. The cell counts emphasized the difference between the control and treated suspensions at the end of the experiments. Treatment with ethanol at the same concentration as that used for the addition of vitamin A had no effect on the cells under these conditions.

*Effect of temperature on the release of haemoglobin.* Since the release of protease from rat-liver lysosomes treated with vitamin A is much affected by temperature (Dingle, 1961), the temperature-dependence of erythrocyte lysis by vitamin A was investigated to see if this phenomenon shows similar characteristics to those of lysosomal breakdown under the influence of the vitamin.

The release of haemoglobin was affected by the temperature of incubation with vitamin A. During a short incubation period, little release of haemoglobin from rabbit erythrocytes occurred below 25° (Fig. 2). Experiments with incubation periods of 5, 10, 30 and 60 min., however, showed that, as the time of incubation with the vitamin was lengthened, loss of haemoglobin from the cells occurred at progressively lower temperatures. During a period of 60 min., haemolysis was nearly complete at 25° and some haemoglobin was released from the treated cells even at 5° (Fig. 3). The rapid release of haemoglobin that occurred at 37°, as compared with that at 5°, closely resembled the effect of temperature on release of lysosomal protease under the influence of vitamin A (Fig. 4).

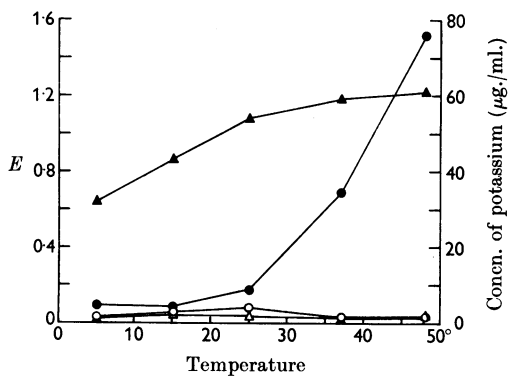


Fig. 2. Effect of temperature on haemolysis of erythrocytes by vitamin A (20 μg./ml.) during incubation for 5 min. ●, Release of haemoglobin by vitamin A; ○, release of haemoglobin by ethanol; ▲, release of potassium by vitamin A; △, release of potassium by ethanol.

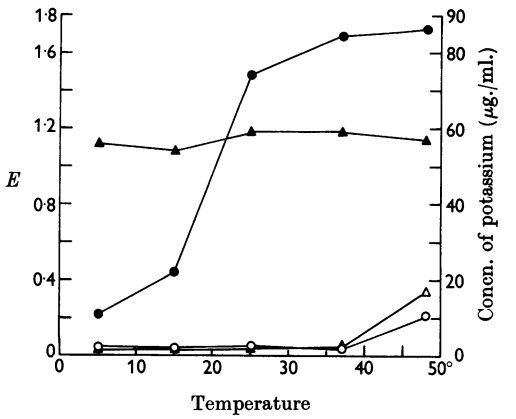


Fig. 3. Effect of temperature on haemolysis of erythrocytes by vitamin A (20 μg./ml.) during incubation for 60 min. ●, Release of haemoglobin by vitamin A; ○, release of haemoglobin by ethanol; ▲, release of potassium by vitamin A; △, release of potassium by ethanol.

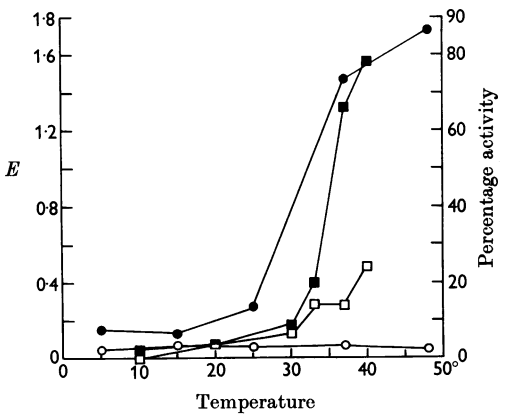


Fig. 4. Comparison of temperature characteristics of haemolysis and release of lysosomal protease by vitamin A. Erythrocytes were treated with vitamin A (20 μg./ml.) for 10 min. The results for the release of lysosomal protease are taken from Dingle (1961); the total activity was that obtained after suspending the lysosomal particles in water. ●, Release of haemoglobin by vitamin A; ○, release of haemoglobin by ethanol; ■, release of protease by vitamin A; □, release of protease by ethanol.

*Rate of release of haemoglobin.* Loss of haemoglobin from rabbit erythrocytes was rapid during treatment with vitamin A at 37°, and was virtually complete in 10 min. when the standard cell suspension was treated with 20 $\mu$g. of vitamin A/ml. When the same quantity of vitamin A was used at 25°, however, the kinetics of liberation could be followed easily since the release of haemoglobin occurred more slowly. Under these conditions, the rate of liberation of haemoglobin from the cells was linear with time during the first 60 min. (Fig. 5).

*Release of potassium: kinetics and temperature-dependence.* It was shown by Davson & Danielli (1938) that treatment of rabbit erythrocytes with subhaemolytic concentrations of pentanol or dihydric phenols caused loss of potassium from the cells. In our experiments with vitamin A, potassium was released much more rapidly than the haemoglobin. For example, at 25°, release of potassium reached a constant maximum value in 10 min.; during the same time haemoglobin was released only to the extent of 15–20 % of the total amount that can be liberated by vitamin A (Fig. 5).

Loss of potassium from erythrocytes treated with vitamin A also occurred quite rapidly in the cold, unlike the loss of haemoglobin. On treatment of the standard cell suspension with 20 $\mu$g. of vitamin A/ml., approximately 50 % of the total potassium was released from the cells in 5 min. at 5° when the release of haemoglobin was negligible (Fig. 2). Potassium loss, which was complete in 60 min. at 5° (Fig. 3), sometimes reached the maximum value in 30 min. under the same conditions.

*Temperature-dependence of haemolysis by BRIJ 35 and by lysolecithin.* Erythrocytes are easily lysed by a variety of chemical agents. It is of interest to know whether the lysis produced by other haemolysins has a temperature-dependence similar to that of vitamin A. The temperature-dependence of the lysis of rabbit erythrocytes by two other agents has therefore been investigated.

Rabbit erythrocytes were haemolysed by a neutral detergent, BRIJ 35. This was much less effective in producing lysis of the cells than vitamin A. For example, the standard suspension of cells was almost completely lysed in 10 min. by 20 $\mu$g. of vitamin A/ml. at 37°, whereas haemolysis was only 50 % complete under the same conditions with 100 $\mu$g. of BRIJ 35/ml. The effect of temperature on the release of haemoglobin and potassium from rabbit erythrocytes by BRIJ 35 is shown in Fig. 6. No loss of either potassium or haemoglobin occurred at 15° or 5°; both potassium and haemoglobin were released most rapidly at about 32° and lysis was less at 37° and at 48° than at 32°. Haemolysis by BRIJ 35 resembled that produced by vitamin A in that no haemoglobin was released in the cold. Haemolysis with the detergent, however, differed markedly from that with the vitamin in that with BRIJ 35 there was no release of potassium in the cold and both potassium and haemoglobin showed optimum liberation at 32° instead of progressively greater release with increasing temperature up to 48° as occurred with vitamin A (Fig. 2).

Lysolecithin caused a rapid release of both potassium and haemoglobin in the cold from rabbit erythrocytes. Lysis at 48° was not much greater than at 5°. A small peak of increased haemolysis was observed at about 32° (Fig. 7). Haemolysis of rabbit erythrocytes by lysolecithin is therefore virtually independent of temperature, in contrast with the effect of temperature on lysis produced by vitamin A (Fig. 2).

*Lysis of cells from different species.* Initial experiments on the effect of vitamin A on erythro-

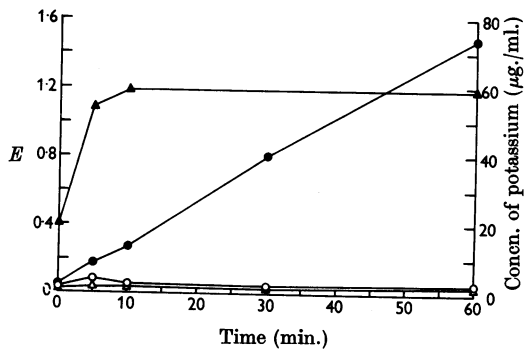


Fig. 5. Rate of haemolysis by vitamin A (20 $\mu$g./ml.) at 25°. ●, Release of haemoglobin by vitamin A; ○, release of haemoglobin by ethanol; ▲, release of potassium by vitamin A; △, release of potassium by ethanol.

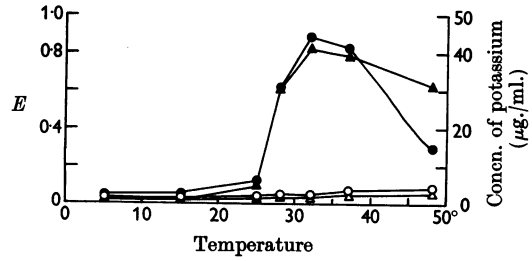


Fig. 6. Effect of temperature on haemolysis of erythrocytes by BRIJ 35 (100 $\mu$g./ml.) during incubation for 10 min. Cell suspension (5 ml.) was added to an aqueous solution of BRIJ 35 (0·05 ml.); the usual incubations with ethanol (0·05 ml.) were also carried out. ●, Release of haemoglobin by BRIJ 35; ○, release of haemoglobin by ethanol; ▲, release of potassium by BRIJ 35; △, release of potassium by ethanol.

cytes *in vitro* were done with cells from the rabbit. To determine whether the lysis of erythrocytes caused by vitamin A is peculiar to the rabbit, single samples of erythrocytes from a number of other species were tested. Erythrocytes from human, ox, pig and rat were lysed on incubation *in vitro* with vitamin A. The sensitivity of the single samples tested from the different species varied; that from the human was least and that from the rat most affected by a dose of 10 $\mu$g. of vitamin A/ml. of suspension.

The sensitivity of these cells to lysis by lysolecithin was also investigated; except for the rat, ease of lysis by lysolecithin occurred in the reverse order to that with vitamin A (Table 2).

*Resistance of cells to haemolysis by vitamin A.* The experiments with rabbit erythrocytes were all done with erythrocytes obtained from six rabbits that were bled in rotation at intervals for 3 months. At the end of this period, cells were occasionally found to be partially or wholly resistant to haemolysis by 10 $\mu$g. of vitamin A/ml. during incubation for 15 min. at 37°. In these instances, the cells were discarded. Treatment of 'resistant' cells with a slightly larger quantity of vitamin A (15 $\mu$g./ml.) caused extensive lysis during incubation for 15 min. at 37°. Incubation of 'resistant' cells for 30 min. at 37° before treatment with the vitamin frequently reduced the resistance of the cells to lysis by 10 $\mu$g. of vitamin A/ml.

The phenomenon of resistance to haemolysis by vitamin A may be associated with the frequent repeated bleeding of animals, and the problem is being investigated further. Our observations may be related to the suggestion of Pollard & Bieri (1958) that reticulocytes are largely responsible for the destruction of vitamin A by haemolysed blood.

*Inhibition of haemolysis by serum.* No observations appear to have been recorded in the literature on the haemolysis of erythrocytes *in vivo* under conditions of hypervitaminosis A. This may indicate that the lysis of erythrocytes by excess of the vitamin is prevented *in vivo* by the presence of serum proteins that bind the vitamin and thereby render it inactive in lysing erythrocytes. The effect of treating erythrocytes with vitamin A *in vitro* in the presence of serum was therefore investigated to see if serum inhibits lysis under these conditions.

Vitamin A was added to a series of dilutions of rabbit serum immediately before the addition of a suspension of erythrocytes from the same animal. In a second experiment, the serum solutions were added to the cell suspensions before the addition of the cells to vitamin A. The cell suspensions were then incubated at 37° for 10 min. The presence of 2 %, 8 % and 17 % of serum in the final volume of 6 ml. inhibits completely the release of haemoglobin from cells treated with 8·3 $\mu$g. of vitamin A/ml. of suspension. With 0·2 % and 0·8 % of serum, inhibition occurs but is not complete (Fig. 8). After the cells had been treated with vitamin A in the presence of 2 % of serum and then removed, the supernatant solution contained only one-fifth of the quantity of potassium found when the cells were treated with vitamin A in the complete absence of serum. (The addition of larger quantities of serum, 8 % and 17 %, increased the final concentration of potassium by virtue of the potassium already present in the added serum, and caused a slight increase in the extinction of the supernatant solution.) These observations therefore demonstrate that the release of both potassium and haemoglobin from rabbit erythrocytes treated with vitamin A *in vitro* is inhibited by homologous serum.

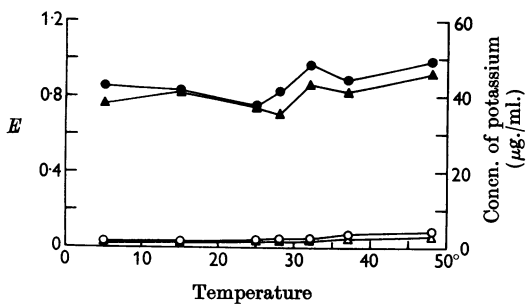


Fig. 7. Effect of temperature on haemolysis of erythrocytes by lysolecithin (35 $\mu$M) during incubation for 10 min. ●, Release of haemoglobin by lysolecithin; ○, release of haemoglobin by ethanol; ▲, release of potassium by lysolecithin; △, release of potassium by ethanol.

Table 2. *Lysis of erythrocytes from different species by vitamin A (alcohol) and by lysolecithin*

The values represent percentage of total haemolysis.

| Treatment | Human erythrocytes | Ox erythrocytes | Pig erythrocytes | Rabbit erythrocytes | Rat erythrocytes |
|---|---|---|---|---|---|
| Incubation for 10 min. at 37° with vitamin A (35 $\mu$M) | 4 | 4 | 16 | 34 | 54 |
| Incubation for 30 min. at 37° with vitamin A (35 $\mu$M) | 13 | 13 | 22 | 47 | 63 |
| Incubation for 15 min. at 37° with lysolecithin (35 $\mu$M) | 82<br>84 | 80<br>73 | 49<br>56 | 21<br>23 | 65<br>47 |

*Effect of preincubation on the release of haemoglobin.* The release of haemoglobin from rabbit erythrocytes under the influence of vitamin A is much more rapid at 37° than at 5°. Temperature-dependence of this kind may indicate that an enzymic mechanism is involved in the action of vitamin A on the erythrocyte membrane. If this is so, preincubation and treatment of the cells with the vitamin at 48° might result in heat-inactivation of the enzyme system concerned.

To test this possibility, standard suspensions of rabbit erythrocytes were incubated at 48° and 37° for 30 min. before treatment with vitamin A at 48° and 37° respectively. Cells that had been preincubated at 37° for 30 min. and then incubated with vitamin A at 37° for 30 min. released the same quantity of haemoglobin as cells treated with the vitamin in the same way but not preincubated. Further, cells preincubated at 48° and treated with vitamin A at 48° released more haemoglobin than cells that were preincubated at 37° and then treated with the vitamin at 37°. It is thus unlikely that a heat-labile enzyme is involved in the action of vitamin A on the erythrocyte membrane, but the possibility that heat-insensitive enzymes may participate in the process is not excluded.

*Molecular structure and biological activity.* The structural requirements at the molecular level for the action of vitamin A in animals are highly specific, and this specificity was also found both in the action of the vitamin on chick-limb-bone rudiments in culture and on isolated rat-liver lysosomes (Fell *et al.* 1962). It was therefore desirable to compare these requirements with those for activity towards the rabbit erythrocyte. Table 3 shows that the action of the vitamin in releasing haemoglobin and potassium from the erythrocyte was closely dependent on molecular structure, and, with certain exceptions, this dependence was very similar to that observed in the systems *in vivo* and *in vitro* mentioned above. Of the substances tested, no compound without both a β-ionone ring and a conjugated double-bond system had any effect on the erythrocytes when used at a concentration of 35 μM. The chain length is of great importance since compounds having shorter or longer chains than vitamin A were much less active than the $C_{20}$ vitamin A alcohol and aldehyde compounds (Table 4). β-Ionone and other low-molecular-weight terpenes were also without activity (Table 3).

The activity of the vitamin A molecule is greatly reduced in all systems after oxidation or hydrogenation. Oxidized vitamin A is without effect on animals, isolated lysosomes or erythrocytes, and has only a very slight effect upon the growth of chick-limb-bone rudiments. The hydrogenated form of the vitamin, though ineffective in the animal and on lysosomes, had a slight action both in organ culture and on the erythrocyte (Table 3). This action of hydrogenated vitamin A on erythrocytes had the same dependence on temperature as vitamin A itself.

The low activity in the erythrocyte system of anhydrovitamin A, the methyl ether of vitamin A, and the acetate and palmitate esters, would seem to indicate that the nature of the end of the side chain is of importance (Table 3). Vitamin A aldehyde is as effective in the lysis of erythrocytes as vitamin A, but vitamin A acid had only a slight action.

*Effect of low ionic strength.* In an attempt to obtain more information about the reason for the very different activities of vitamin A and vitamin A acid, the effect on haemolysis of replacing sodium chloride by sucrose, in a series of solutions ranging from 0·154 M-sodium chloride solution to 0·25 M-sucrose solution, was investigated. Equal quantities of cells were suspended in the different media and treated with vitamin A (10 μg./ml.) for 15 min. at 25°, or vitamin A acid (10 μg./ml.) for 15 min. at 37°.

The release of haemoglobin and potassium by vitamin A and by vitamin A acid was greater from cells suspended in media composed of equal parts of iso-osmotic sodium chloride solution and iso-osmotic sucrose than from cells suspended in iso-osmotic sodium chloride solution alone (Figs. 9 and 10). The stepwise increase in the release of haemoglobin by vitamin A acid on reducing the ionic strength was found to be reproducible. Cells

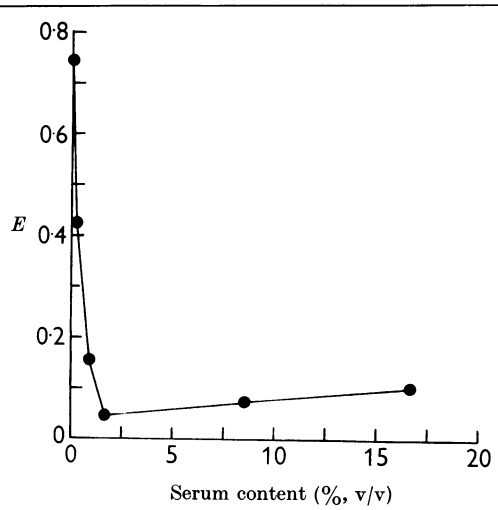


Fig. 8. Inhibition of haemolysis by homologous serum. Vitamin A (8·3 μg./ml.) was added to a series of dilutions of rabbit serum in 0·9% NaCl solution immediately before the addition of a suspension of erythrocytes from the same animal. The suspension was then incubated at 37° for 10 min.

Table 3. *Comparison of molecular structure and activity in various systems*

The percentage activity of each compound is expressed relative to that of vitamin A (alcohol). The effect on the growth of chick-limb-bone rudiments was measured as the difference in length between the treated and control cultures after 8 days in culture; test compounds were added to the media to give a final concentration of 10 $\mu$M. Isolated lysosomes were suspended in 0·25 M-sucrose made 0·7 mM with respect to each test compound for 40 min. at 37°; the released enzyme was measured in the supernatant fluid after centrifuging. Rabbit erythrocytes, suspended in 0·9 % sodium chloride, were treated for 15 min. at 37°, centrifuged, and the released haemoglobin and potassium measured in the supernatant fluid; the test compounds were used at a final concentration of 35 $\mu$M.

| | Molecular structure | | Action on erythrocytes | | Biological action: Action on rat-liver lysosomes (Fell, Dingle & Webb, 1962) | Biological action: Growth of limb rudiments in organ culture (Fell, Dingle & Webb, 1962) | Biological action: In animals (Moore, 1957) |
|---|---|---|---|---|---|---|---|
| Compound | No. of conjugated double bonds | End group | Haemoglobin loss | $K^+$ loss | | | |
| Vitamin A (alcohol) | 5 | $-CH_2 \cdot OH$ | 100 | 100 | 100 | 100 | 100 |
| Vitamin A aldehyde | 5 | $-CHO$ | 102 | 100 | — | — | 91 |
| Vitamin A acid | 5 | $-CO_2H$ | 8 | 8 | 92 | 94 | 100* |
| Hydrogenated vitamin A | 0 | $-CH_2 \cdot OH$ | 10 | 16 | 0 | 25 | 0 |
| Oxidized vitamin A alcohol | 4 (?) | $-CH_2 \cdot OH$ | 1 | 1 | 0 | 11 | 0 |
| Anhydrovitamin A | 6 | $=CH_2$ | 3 | 3 | 0 | 0 | 0·4 |
| Vitamin A methyl ether | 5 | $-CH_2 \cdot O \cdot CH_3$ | 2 | 4 | — | — | 100 |
| Vitamin A acetate | 5 | $-CH_2 \cdot O \cdot CO \cdot CH_3$ | 1 | 6 | 11 | — | 100 |
| Vitamin A palmitate | 5 | $-CH_2 \cdot O \cdot CO \cdot C_{15}H_{31}$ | 2 | 0 | 0 | — | 100 |
| Vitamin $A_2$ alcohol | 6 | $-CH_2 \cdot OH$ | 9 | 22 | — | — | 40† |
| Vitamin $A_2$ aldehyde | 6 | $-CHO$ | 17 | 28 | — | — | 33‡ |
| $\beta$-Ionone | 3 | $-CO \cdot CH_3$ | 0 | 0 | 25 | 2 | — |
| Geraniol | 0 | $-CH_2 \cdot OH$ | 0 | 0 | 27 | — | — |
| Citral | 0 | $-CHO$ | 0 | 0 | 0 | 17 | — |
| Phytol | 0 | $-CH_2 \cdot OH$ | 4 | 4 | 7 | 0 | — |
| Dodecanol | 0 | $-CH_2 \cdot OH$ | 1 | 1 | 0 | — | — |
| Ethanol | 0 | $-CH_2 \cdot OH$ | 0·4 | 0 | 0 | 0 | — |

* Inactive in the retina and reproductive tissues of the rat.
† Shantz & Brinkman (1950).
‡ Sundaresan & Cama (1961).

Table 4. *Relative activity in haemolysis of vitamin A (alcohol) and vitamin A aldehyde, as compared with derivatives having side chains of differing length*

| Compounds | No. of C atoms per molecule | Percentage activity |
|---|---|---|
| $\beta$-Ionylidene-ethanol | 15 | 8 |
| $\beta$-Ionylidene-acetaldehyde | 15 | 3 |
| Vitamin A (alcohol) | 20 | 100 |
| Vitamin A aldehyde | 20 | 100 |
| 4-Apo-$\beta$-carotenol | 25 | 49 |
| 4-Apo-$\beta$-carotenal | 25 | 4 |
| 3-Apo-$\beta$-carotenol | 27 | 7 |
| 3-Apo-$\beta$-carotenal | 27 | 4 |

suspended in a solution of 6 % (w/v) dextran containing 5 % (w/v) of glucose were also lysed by vitamin A more readily than cells in 0·154 M-sodium chloride solution. In control incubations loss of haemoglobin from cells suspended in 0·25 M-sucrose (Figs. 9 and 10), or in dextran–glucose solution, and then treated with ethanol was negligible under the conditions of the experiments. Considerable loss of potassium occurred, however, when cells were incubated at 37° for

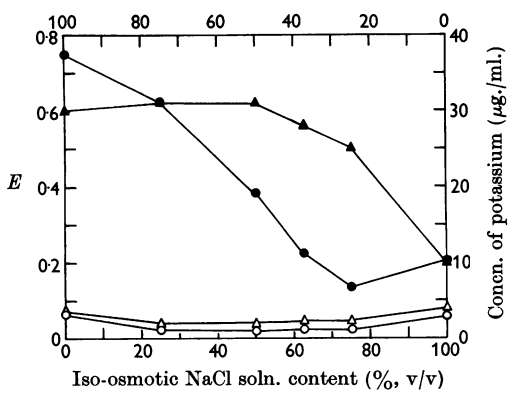


Fig. 9. Relationship of haemolysis of erythrocytes by vitamin A to the salt concentration of the iso-osmotic suspending medium. Erythrocytes were suspended in media composed of differing proportions of iso-osmotic NaCl solution and iso-osmotic sucrose solution. The cells were incubated with vitamin A (10 $\mu$g./ml.) for 15 min. at 37°. ●, Release of haemoglobin by vitamin A; ○, release of haemoglobin by ethanol; ▲, release of potassium by vitamin A; △, release of potassium by ethanol.

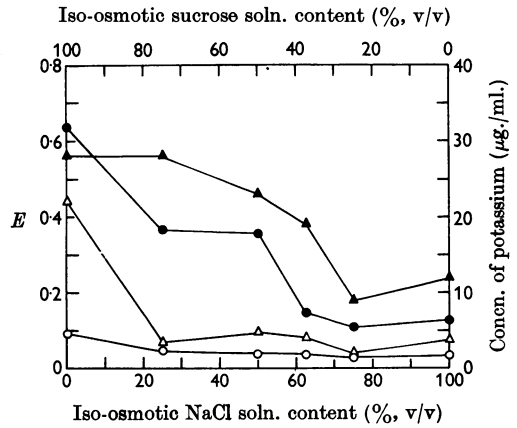


Fig. 10. Relationship of haemolysis of erythrocytes by vitamin A acid to the salt concentration of the iso-osmotic suspending medium. Erythrocytes were suspended in media composed of differing proportions of iso-osmotic NaCl solution and iso-osmotic sucrose solution. The cells were incubated with vitamin A acid (10 μg./ml.) for 15 min. at 37°. ●, Release of haemoglobin by vitamin A acid; ○, release of haemoglobin by ethanol; ▲, release of potassium by vitamin A acid; △, release of potassium by ethanol.

15 min. in iso-osmotic sucrose and in the presence of ethanol (Fig. 10), but this was not observed at 25° (Fig. 9).

## DISCUSSION

The experiments reported in this paper show that vitamin A has a profound effect on the stability of the erythrocyte membrane when erythrocytes are incubated *in vitro* with the vitamin, and the cells are rapidly destroyed when treated at 37° with 10 μg. or less of vitamin A/ml. It is clear, therefore, that vitamin A can alter the permeability of a cell membrane as well as that of lysosomal subcellular particles.

Haemolysis of erythrocytes may be prevented in animals suffering from hypervitaminosis A by the protective action of serum proteins in the blood. Once the erythrocyte is removed from the influence of serum, however, it is readily lysed by the vitamin. Inhibitor systems of this kind may provide a means of protecting tissues and controlling the action of vitamin A. T. Moore & I. M. Sharman (personal communication) have recently found that erythrocytes from rats that had been treated with large doses of vitamin A were more sensitive to haemolysis by dialuric acid than erythrocytes from normal animals.

The quantity of vitamin A that will cause 50% haemolysis is related linearly to the number of erythrocytes in suspension. This finding indicates that the addition of the cell suspension to vitamin A dissolved in ethanol successfully overcomes the difficulty of bringing cells in contact with the fat-soluble vitamin.

The temperature-dependence of erythrocyte lysis, as measured by release of haemoglobin, is similar to that of the release of a proteolytic enzyme from lysosomes treated with vitamin A *in vitro* (Dingle, 1961). In both systems there is little release of protein in the cold as compared with that between 30° and 40°. One possible explanation of the observed kinetics and temperature characteristics of haemoglobin and potassium liberation from treated erythrocytes may be that the rates of release are determined by diffusion of the haemoglobin and potassium through a cell membrane that had been damaged or altered by the vitamin. The small $K^+$ ion might then be expected to be liberated from the cell more easily than the bulky haemoglobin molecule. Although there is no direct evidence to show that the effect of vitamin A on the surface of the lysosome is closely related to its effect on the membrane of the erythrocyte, the similarity of the temperature characteristics for release of protein in the two systems would be consistent with the operation of similar mechanisms. On the other hand, it seems reasonable to conclude that the mechanism of the lytic action of vitamin A on erythrocytes is different from that of either BRIJ 35 or lysolecithin, since the effect of temperature on haemoglobin release by vitamin A differs from the temperature characteristics of the lysis caused by the other two compounds. Confirmation of this hypothesis is provided, with lysolecithin, by the fact that the sensitivity of the samples of erythrocytes from human, ox, pig and rabbit towards lysis by vitamin A increased progressively on passing from human cells to rabbit cells, whereas the sensitivity to lysis by lysolecithin increased in the reverse order. The temperature-dependence of the action of vitamin A on erythrocytes is also different from that of the rapid phase of haemolysis produced by anionic detergents in which rising temperature decreases the rate of haemolysis (Rideal & Taylor, 1957).

At a concentration of 35 μM vitamin A acetate and vitamin A palmitate are unable to lyse erythrocytes. Similar observations have been made with these compounds in a study of the effect of vitamin A on the stability of rat-liver lysosomes (Fell *et al.* 1962). It is possible that the efficacy of the esters that is normally observed in nutritional experiments and with whole tissues may be dependent on esterase activity. This might apply to vitamin A acetate, for example, both under physiological conditions and in studies of the action of excess of vitamin A on skin made by Fell & Mellanby (1953) in which the acetate was used. Lysosome preparations from rat liver might be

unable to hydrolyse vitamin A acetate to the alcohol during short-term experiments. The inactivity of the esters towards rat-liver lysosomes may explain the fact that vitamin A is stored in the liver principally in an esterified form. The very specialized erythrocyte may not possess the required esterase. Although Krause & Alberghini (1950) reported hydrolysis of esterified vitamin A by blood, no hydrolysis could be demonstrated by the cellular elements of the blood. Vitamin A acetate may be inactive towards lysosomes and erythrocytes because of steric hindrance that occurs when the chain length of the molecule is increased by the addition of the acetate group. The absence of an alcohol or aldehyde end group in the acetate is thought to be a more likely reason for inactivity, however, since anhydrovitamin A is also inactive and this molecule, unlike the acetate, has a chain length similar to that of vitamin A and thus cannot be sterically hindered. The hypothesis that the nature of the end group is important for activity towards the erythrocyte membrane is also supported by the finding that the methyl ether of vitamin A, which is active *in vivo* (Moore, 1957), is inactive with erythrocytes.

Another requirement for activity concerns the properties of the chain. For the molecule to be active, the chain must apparently be rigid since hydrogenated vitamin A, which has a very flexible side chain, is only weakly active. An alternative explanation of the inactivity of the hydrogenated form may lie in the absence of the conjugated double-bond system. The weak activity of the hydrogenated vitamin A has the same dependence on temperature as the activity of vitamin A itself, despite the fact that the hydrogenated material used was completely free from vitamin A as shown by spectrophotometric measurements. The length of the side chain is also of great importance in determining the activity of the molecule as a haemolysin. Thus decreasing the number of carbon atoms in the side chain from 9 to 5 (excluding methyl groups), or increasing the number to 13, results in greatly decreased haemolytic activity. The reason for the greater activity of the $C_{15}$ and $C_{25}$ alcohols, as compared with the corresponding aldehydes, is at present unknown.

Although vitamin A acid is able to replace vitamin A in the functions of growth and general tissue maintenance, it cannot do so in the retina of the rat (Dowling & Wald, 1960) or in the reproductive processes of male and female rats (Thompson, Howell & Pitt, 1961*a*, *b*). In our experiments, vitamin A acid was relatively inactive in causing haemolysis of rabbit erythrocytes. It is possible that the rate of haemolysis is partly governed by interaction of the negative charge on the surface of the erythrocyte with the charge on the haemolysin. Bangham & Dawson (1962) showed that *Penicillium notatum* phospholipase requires lecithin micelles to possess a minimum net negative $\zeta$-potential before activity begins, whereas phospholipase C of *Clostridium perfringens* hydrolyses lecithin only when it possesses a positive $\zeta$-potential, achieved by adding long-chain bases. Ionization of vitamin A acid, with the production of a negative anion, might be responsible for its low activity as compared with the much more weakly ionized vitamin A alcohol. Although the presence of sugars has an inhibitory action on the lysis of erythrocytes by saponin (Ponder, 1952) and on the slow lysis by anionic detergents (Rideal & Taylor, 1957), the reduced concentration, or absence, of sodium chloride in the media of high sucrose concentration used in our experiments may have been the important factor in causing increased lysis of erythrocytes by both vitamin A alcohol and acid. Thus the increased lysis at low salt concentrations may be related to the decrease in the net negative electrokinetic charge density of the surface of the erythrocyte observed with decreasing ionic strength (Seaman & Heard, 1960). The finding that vitamin A acid is relatively inactive in lysing erythrocytes is significant, since it indicates that the membrane structure of the tissue concerned (retina, placenta, erythrocyte) may determine whether or not vitamin A acid is active.

Fell & Mellanby (1952) showed that excess of vitamin A has a drastic effect on the matrix of embryonic chick cartilage during cultivation *in vitro*. The changes produced in the matrix are similar to those observed in rabbits under conditions of hypervitaminosis A (Thomas, McCluskey, Potter & Weissmann, 1960). Lucy *et al.* (1961) demonstrated that an effect on normal cartilage similar to that of vitamin A can be produced by liberating the intracellular enzymes by treatment with hypo-osmotic solutions and then incubating the cartilage for 2 hr. at pH 3–5. In the light of these and other experiments (Dingle *et al.* 1961), it was suggested that vitamin A acts on chick cartilage by altering the permeability of the lysosomes of the chondrocytes, thereby liberating hydrolytic enzymes (Fell *et al.* 1961). This view was supported by the fact that vitamin A releases a proteolytic enzyme from isolated rat-liver lysosomes (Dingle & Lucy, 1961; Dingle, 1961). In speculations about the mechanism of action of excess of vitamin A on membranes, two possible modes of action of the vitamin are immediately apparent. First, the compound may activate enzyme systems which then attack the components of the membranes of cells and particles; degradation of the structural framework of the membrane in this way would then lead to release of the materials inside the cell or particle. On the other

hand, vitamin A may actually become attached to the structural components of membranes either in an addition reaction or in a displacement reaction whereby the normal molecular components of the membrane are displaced. The intrusion of vitamin A in this way may result in mechanical weakening of the membrane, or the vitamin may interfere with reactions that are essential to the maintenance of the membrane, with the result that the structure is no longer stable.

It may be thought that information obtained on the mechanism of action of vitamin A used in concentrations greater than the normal physiological level has little relation to the mechanism of action of the vitamin under physiological conditions. Experimental observations on the molecular structural requirements for degradation of cartilage *in vitro*, liberation of protease from lysosomes (Fell *et al.* 1962), and haemolysis of erythrocytes show, however, that, with certain exceptions discussed above, the relationships between structure and activity in these systems are the same as for the prevention of vitamin A deficiency in animals. Further, the present experiments have demonstrated that both vitamin A alcohol and aldehyde ($C_{20}$) are 10 times more effective in producing lysis of erythrocytes than the related $C_{15}$ and $C_{27}$ alcohols and aldehydes that have shorter and longer side chains respectively. Haemolytic activity is therefore a property of vitamin A alcohol and aldehyde which is possessed in only a small degree by quite closely related molecules. It it suggested, therefore, that alterations in the structure of cellular and intracellular membranes with concomitant changes in permeability may be a common feature underlying the differing actions of vitamin A in many different systems. This type of action of vitamin A on the membranes of cells and intracellular particles is probably not limited to vitamin A alone but may be characteristic of fat-soluble vitamins. For instance, vitamin E deficiency has been shown by Heard, Moore & Sharman (1952) to increase the ease of chemical lysis of erythrocytes, and there is evidence that this deficiency may also be associated with increased catheptic activity in muscle, probably of lysosomal origin (Zalkin, Tappel, Desai, Caldwell & Peterson, 1961). Vitamin D is important in maintaining the structure of mitochondria (DeLuca, Reiser, Steenbock & Kaesberg, 1960), though it has yet to be established if it plays a part in maintaining the structure of either the lysosome or the erythrocyte.

The function of vitamin A may be regarded as the control of membrane permeability, particularly in relation to large molecules, and it is possible that the behaviour of the vitamin under physiological conditions is similar to its action when present in excess except that the membranes affected become excessively unstable in the latter situation. Further, the effect of excess of vitamin A on the erythrocyte membrane may be regarded as a model of the way in which vitamin A may act on membrane structures in general.

## SUMMARY

1. The addition of a suspension of erythrocytes from rabbit, pig, ox, rat or human to vitamin A alcohol, dissolved in ethanol, results in rapid lysis of the cells and production of erythrocyte stroma on incubation at 37°. Homologous serum inhibits lysis. Erythrocytes suspended in a medium containing sucrose and sodium chloride are more easily lysed than those in sodium chloride solution alone.

2. The temperature-dependence of the release of haemoglobin by vitamin A is similar to that of the release of protease from rat-liver lysosomes treated with the vitamin.

3. Haemolysis produced by vitamin A has been compared with that caused by a neutral detergent and by lysolecithin.

4. Only weak haemolytic activity is possessed by hydrogenated vitamin A, oxidized vitamin A, anhydrovitamin A, vitamin $A_2$ alcohol and vitamin $A_2$ aldehyde. $\beta$-Ionone, geraniol, citral, phytol and dodecanol are without activity at the concentrations tested.

5. Changes in the nature of the end group or the chain length of the molecule result in diminished haemolytic activity. The molecular specificity for lysis is similar to that for biological activity and for action on isolated lysosomes.

6. The hypothesis is presented that the site of action of the vitamin is at the lipoprotein membranes of cells and their organelles and that its function is the control of the permeability of biological membranes.

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# The Effect of Thiouracil on the Metabolism of L-Thyroxine

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Thiouracil not only blocks the formation of thyroxine in the thyroid gland (Franklin, Lerner & Chaikoff, 1944; Astwood, 1945), but also appears to alter its peripheral utilization. Andik, Balogh & Donhoffer (1949) found that the action of thyroxine was inhibited in thyroidectomized rats which had been given methylthiouracil for 4 weeks. Stasilli, Kroc & Edlin (1960) found that the administration of thyroxine to rats which had been given thiouracil for 6–8 weeks produced only 7% of the calorigenic action observed in untreated rats.

The decreased excretion of radioactive iodide in urine from administered L-[3′- or 5′-$^{131}$I]thyroxine, which has been found in rats given propylthiouracil, indicates decreased deiodination of the phenolic ring of thyroxine. This is accompanied by an increased secretion of $^{131}$I in the bile (Van Arsdel & Williams, 1956) and excretion in faeces (Hogness, Wong & Williams, 1954; Jones & Van Middlesworth, 1960; Escobar del Rey & Morreale de Escobar, 1961).

In the metabolism of L-[3′- or 5′-$^{131}$I]thyroxine in the dog partial deiodination occurs by removal of one iodine atom from the 3′- or 5′-position of the phenolic ring with the excretion of radioactive iodide in the urine and the formation of calorigenic 3,3′,5-tri-iodothyronine. Small amounts of conjugated tri-iodothyronine are excreted in the bile. Partial deiodination occurs in the dog also by removal of one iodine atom from the 3- or 5-position with the formation of non-calorigenic 3,3′,5′-tri-iodothyronine (Flock, Bollman, Grindlay & Stobie, 1961).

The quantity of 3,3′,5′-tri-iodothyronine glucuronide excreted in the bile or in the urine after removal of the liver is sufficiently great to indicate that this type of deiodination is an important mechanism in the inactivation of L-thyroxine. Partial deiodination proceeds further with the formation of 3,3′-di-iodothyronine which is also non-calorigenic.

A search has been made for these partially deiodinated metabolites in the rat. In addition the effect of thiouracil on the peripheral utilization of thyroxine has been studied.

A preliminary account of this work has been published (Flock & Bollman, 1961).

## METHODS

Rats of the Sprague–Dawley strain, weighing 200–340 g. and maintained on Friskie dog food, were used. Some rats were given powdered Friskie dog food, to which thiouracil (0·1% or 0·3%, w/w) had been added, for 16–56 days. A biliary fistula was made in each rat, under ether anaesthesia, by cannulation of the bile duct 1 cm. below the