LXXXIV. THE OXIDATION OF VITAMIN A *IN VITRO*. INFLUENCE OF THE SOLVENT.

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(Received April 26th, 1932.)

THE problem of the oxidative destruction of vitamin A attracted the attention of the earliest workers upon the fat-soluble vitamins and has retained its interest on account of its bearing upon methods of handling and preserving materials containing vitamin A. Observations on the oxidation of vitamin A have generally been made at high temperatures, with the result that at first some confusion arose between the effects of heat and of oxygen upon the vitamin. A wide divergence of opinion among the early workers resulted [*cf.* McCollum and Davis, 1915; Osborne and Mendel, 1916; Steenbock, Boutwell and Kent, 1918], some holding that heat alone and others that oxidation alone was the cause of the observed destruction of the vitamin. This confusion was cleared up on the simultaneous appearance of papers by Hopkins [1920] and Drummond and Coward [1920]. Hopkins confirmed the findings of Osborne and Mendel [1916] that the vitamin A contained in butter is "relatively resistant to heat...but this factor is rapidly destroyed by exposure to atmospheric oxygen at temperatures from 15° to 120°." The same conclusion was reached by Drummond and Coward using different experimental methods. Thus it was definitely established that under certain conditions high temperatures have little destructive effect upon vitamin A in the absence of oxygen, and that oxidation actively destroys the vitamin both at high and low temperatures.

It was not possible at that time to obtain more than a qualitative demonstration of the oxidative destruction of vitamin A because the only available method for the estimation of the vitamin was the biological assay, and to follow the course of the oxidation quantitatively by this assay would require very large numbers of animals. The first quantitative data concerning the oxidation were in consequence not obtained until a chemical method for the estimation of vitamin A had been introduced. Wokes and Willimott [1927], employing the arsenic trichloride and antimony trichloride reagents for the estimation, demonstrated the rapid destruction of the vitamin A of cod-liver oil when the oil was aerated at temperatures from 88° to 125°, and obtained a figure for the temperature coefficient of the oxidation. They also showed, in accord with the results of Hopkins and of Drummond and Coward described above, that the destruction of the vitamin at these temperatures

was very much slower when the cod-liver oil was kept in tubes loosely plugged with cotton wool, without aeration or agitation.

Since the appearance of the paper by Wokes and Willimott little mention of the oxidation of vitamin A in solution is to be found in the literature, but the oxidation of the vitamin when adsorbed by fine powders has attracted attention. Dunn [1924] observed that the vitamin A of cod-liver oil was destroyed by mixing the oil with granulated starch and storing it in dark corked bottles. Marcus [1931] extended this observation by mixing a vitamin A concentrate with various fine powders and following the destruction of the vitamin by the antimony trichloride estimation. His results were in harmony with the idea that the destruction observed was due to oxidation of the vitamin by occluded oxygen at the surface of the particles, although the evidence is by no means conclusive.

At the present time it is generally agreed that although vitamin A is not destroyed by heat alone it is very labile and very readily oxidised. The writer has already shown in a preliminary communication [Dann, 1931] that this opinion requires strict qualification. Full details of the observations leading to this view are presented below.

Evidence that vitamin A is not always labile and easily oxidised. Smith and Hazley [1930] during an investigation of the antimony trichloride reaction of vitamin A were led to state that "It appears that the chromogen is more stable than has sometimes been supposed," although they failed to realise that it can resist the action of oxidising agents under certain conditions. There has however long been evidence of the considerable stability of vitamin A under some conditions in the recognition that saponification of liver oils followed by ether extraction of the soap solutions in the preparation of vitamin A concentrates causes little or no destruction of the vitamin. This demonstrates its stability (*a*) in alkaline alcoholic solutions at high temperatures, and (*b*) when dissolved in ether, which is an active oxidising agent unless specially purified immediately before use.

Cady and Luck [1930] found that the vitamin A of cod-liver oil shows marked stability and is not rapidly oxidised when treated with hydrogen peroxide. They emulsified cod-liver oil in an aqueous solution of hydrogen peroxide and observed that after 18 days at room temperature only partial oxidation of the vitamin A had occurred. Evidence of the stability of the vitamin A contained in butter is afforded by the work of Scheunert [1931] who demonstrated that a large fraction of the vitamin A remains intact during the ordinary processes of baking and roasting.

Factors influencing the oxidation of vitamin A in vitro. In addition to the well-recognised factors affecting the oxidation of vitamin A there is at least one other which appears to have escaped recognition: namely, the solvent effect. When for instance observations concerning the oxidation of the vitamin A contained in cod-liver oil have been made, it appears generally to have been assumed implicitly that the oxidation would follow the same course

had the vitamin A been contained in any other natural foodstuff or laboratory solvent, or even had it been separated in the pure state. Little attention has been paid to the influence exerted on the oxidation of the vitamin A by the complex media containing the vitamin, presumably owing to the implicit assumption mentioned above. As most of the media containing vitamin A are complex mixtures of fats which themselves undergo autoxidation, they may *a priori* be expected to exert an effect on the oxidation of the vitamin itself [cf. Moureu and Dufraisse, 1926].

For the related compound carotene Olcovich and Mattill [1931] found that when it occurs in vegetable foodstuffs its stability towards oxygen "does not depend upon the carotene itself or on the presence of oxygen, primarily, but is conditioned by the presence of other substances." It may be expected that the same will hold true for vitamin A.

As pure vitamin A is not yet available, data concerning the effect of chemical environment upon the oxidation may be obtained by dissolving a vitamin A concentrate in different solvents and following the oxidation of the vitamin in the solutions under similar conditions. When dilute solutions of the vitamin concentrate are used, it is to be expected that the effect of the impurities in the concentrate upon the course of the oxidation will be outweighed by the effect of the solvent, and that differences in the oxidation of the vitamin in different solvents can be referred to the effects of the solvents themselves.

EXPERIMENTAL.

A. OBSERVATIONS ON THE OXIDATION OF VITAMIN A BASED ON COLORIMETRIC DETERMINATION¹.

1. *The oxidation of vitamin A by aeration in different solvents.*

The source of vitamin A for all the experiments described in this section was a crude cod-liver oil concentrate which gave a colour reaction of about 100 blue units per mg. with the antimony trichloride reagent. In following the course of oxidation of the vitamin in a given solvent the following procedure was adopted throughout. About 150 mg. of the concentrate were transferred to a 100 cc. round-bottomed flask and weighed. The calculated weight of solvent was added to produce a solution containing 1 % by weight of the concentrate, and the flask warmed and shaken until the concentrate had dissolved. A 1 cc. sample of the solution was withdrawn by pipette and its vitamin A content measured colorimetrically. The flask was fitted with a stopper bearing a reflux condenser and a narrow inlet tube reaching to the bottom of the flask; the condenser was clamped in position over a water-bath so that the boiling water reached the neck of the flask. Through the inlet tube was passed a quick current of air which had been dried in a calcium chloride tube and filtered through glass-wool. At intervals a 1 cc. sample of the solution

¹ The results of the colorimetric determination of vitamin A are expressed in terms of "blue units" as defined by Moore [1930].

was removed by pipette and its vitamin content estimated. All experiments were performed with the aeration flask in a boiling water-bath and observation showed that the temperature of the solutions remained at 98°, except when using ethyl alcohol and ethyl acetate as solvents, when the solutions remained at 78° and 66° respectively.

In order to estimate the vitamin content of each sample of solution, the vitamin was separated from the solvent as described below and dissolved in 2 cc. of chloroform. An aliquot part of the chloroform solution was diluted to 0.5 cc. with chloroform, 2 cc. of the antimony trichloride reagent were added and the colour of the fluid was matched against the standard glasses of the Lovibond tintometer. A standard tube, 1 cm. diameter, was used to

Fig. 1. The oxidation of vitamin A when dissolved in esters and aerated at 98°. A. Ethyl acetate and triacetin. (Ethyl acetate at 66°.) B. *n*-Amyl acetate. C. Tributyrin. D. Triolein. E. Ethyl laurate. F. Ethyl oleate. G. Ethyl stearate.

hold the reaction mixture, and the dilution of the solution of vitamin arranged so that the resultant colour with antimony trichloride was matched by 3–5 Lovibond blue units. The procedure adopted to separate the vitamin from the solvent varied for different samples according to the solvent. (a) For solutions in esters, the sample was run into 10 cc. of 20 % alcoholic potash and boiled for 3 minutes. The resultant solution was poured into water and the vitamin extracted from the soap solution in the usual way with ether. (b) For solutions in acids, the sample was run into 40 cc. 5 % aqueous potash, shaken to dissolve the acid, and the vitamin separated by ether extraction. (c) For solutions in alcohols, the sample was diluted with chloroform and the antimony trichloride test applied direct, after it had been found by trial that the alcohols used had no effect upon the colour test. For solutions in ethyl alcohol and alcoholic potash the samples were run into 40 cc. water and the vitamin extracted by ether.

The experimental results are presented graphically. Unsmoothed curves

obtained in single experiments are given as it was found possible to repeat them within the limits of experimental error. In each figure the percentages of the initial vitamin contents of the solutions remaining undestroyed during aeration are plotted as ordinates against the times of aeration as abscissae.

Fig. 2. The oxidation of vitamin A when dissolved in acids and aerated at 98°. A. Caproic acid. B. Acetic acid. C. Stearic, oleic and lauric acids.

Fig. 3. The oxidation of vitamin A when dissolved in alcohols and aerated at 98°. A. Ethyl, *n*-butyl and cetyl alcohols. (For ethyl alcohol the temperature was 78°.) B. *n*-Amyl alcohol.

In Fig. 1 are collected the results obtained with a number of esters as solvents, and it is evident that there is a great variation in the rate of oxidation of vitamin A when aerated under similar conditions in solutions of different esters, from an oxidation too slow to be measured during 4 hours in ethyl acetate and triacetin to an oxidation so rapid as to be almost complete within 2 hours in ethyl oleate and ethyl stearate. The variation in the rate of oxida-

tion of the vitamin when aerated in different acids is much less; oxidation is relatively rapid in all the acids (Fig. 2). In the alcohols examined, on the contrary, the rate of oxidation is always relatively slow; for three out of the four no measurable oxidation occurred in 4 hours (Fig. 3). For the miscellaneous solvents, no measurable oxidation took place in 20 % alcoholic potash

Fig. 4. The oxidation of vitamin A when dissolved in various solvents and aerated at 98°. A. 20 % alcoholic potash. B. Cod-liver oil. C. Coconut oil. D. Arachis oil.

Fig. 5. The oxidation of vitamin A dissolved in different solvents and aerated at 98°. (For ethyl alcohol at 78°.) A. Ethyl alcohol and ethyl alcoholic potash. B. Cetyl alcohol.

within 4 hours, and fairly rapid oxidation in the natural oils (Fig. 4). The observations upon ethyl alcohol, cetyl alcohol and 20 % alcoholic potash were repeated over an extended period of 24 hours; no oxidation of vitamin A could be measured after this time when aerated in ethyl alcohol or alcoholic potash, but a considerable oxidation occurred in cetyl alcohol (Fig. 5).

The observations recorded above and included in Figs. 1-5 were all made with a single specimen of vitamin A concentrate of rather low potency (1 mg. gave 100 blue units with antimony trichloride) which was chosen as it had remained unchanged in strength over a period of months. These experiments were almost all repeated using a second specimen of concentrate of approximately the same potency but obtained from a different source, with results which were identical within the limits of experimental error.

Some observations of oxidation by aeration were also made using high potency concentrates as source of vitamin A, but the number of experiments performed was limited by the small amount of the concentrates available, and they were therefore confined to the use of one solvent. Ethyl alcohol was chosen on account of the interest of the earlier results with this solvent. Two concentrates were treated, one prepared from pig-liver oil (1 mg. gave 1000 blue units) and the other from turbot-liver oil (1 mg. gave 1250 blue units), and with each specimen no oxidation occurred when the vitamin was dissolved in ethyl alcohol and aerated at the boiling-point for 24 hours.

A further observation with alcohol as solvent was made at a lower temperature using a rat-liver oil concentrate (1 mg. gave 2150 blue units) as source of the vitamin. 100 mg. of the concentrate were dissolved in 10 cc. ethyl alcohol and the solution was stored in a conical flask loosely plugged with cotton-wool, kept at 0°. At intervals 1 cc. samples of the solution were withdrawn for estimation of the vitamin concentration and it was found that after 4 months 22 % of the vitamin A had disappeared. Reasons are given below, however, for believing that the observed destruction of vitamin A in this experiment was not oxidative.

2. *The aeration of solutions of cholesterol.*

It has been shown [Takahashi *et al.*, 1925] that the rather ill-defined substance called oxycholesterol responds somewhat like vitamin A to a number of chemical tests, and in particular [Rosenheim, 1927] it reacts with antimony trichloride to give a blue coloration. As the vitamin A concentrate used in most of the experiments described above contained some cholesterol, the question of possible interference by oxycholesterol was investigated. If oxycholesterol were formed during the aeration of a solution of vitamin A, the observed rate of oxidation of vitamin A would be less than the true rate of oxidation. Blix and Löwenhielm [1928] found that pure cholesterol is not transformed into oxycholesterol when dissolved in ethyl alcohol and aerated for a few hours at 60°-70°, but it was thought desirable to extend this observation.

0.2 g. cholesterol was dissolved in 10 cc. ethyl alcohol and aerated at the boiling point of the solution for 24 hours. 5 cc. of the solution were evaporated to dryness and the residue was dissolved in 2 cc. chloroform and tested by adding 0.5 cc. of the solution to 2 cc. of antimony trichloride reagent. No colour was produced. Thus although the test sample contained at least a

hundred times as much cholesterol as any of the samples taken in the oxidation experiments described in the last section, no perceptible amount had been transformed into oxycholesterol. This experiment was repeated with 1 % of ethyl oleate dissolved in the alcohol together with the cholesterol, with identical results. As further evidence, the observations upon aeration of vitamin A dissolved in ethyl alcohol, *n*-butyl alcohol and ethyl acetate were repeated with 1 % cholesterol added to the solutions. The results were identical with those obtained without the added cholesterol.

Thus the observed stability of vitamin A when aerated in certain solvents cannot be ascribed to oxidation of the cholesterol to oxycholesterol capable of exerting a compensatory effect in the colorimetric estimation.

3. *The effect of hydrogen peroxide upon vitamin A in alcohol.*

The remarkable stability of vitamin A in alcohol to atmospheric oxygen suggested the extension of the observations to some other oxidising agent. Cady and Luck [1930] showed that the vitamin A of cod-liver oil is attacked slowly by hydrogen peroxide in the cold, but a possible criticism of their results may be directed at the ill-defined conditions under which the reaction was studied. The vitamin and the hydrogen peroxide were present in separate liquid phases whose degree of dispersion was unknown and changing. Alcohol as solvent has the advantage that it dissolves both vitamin A and hydrogen peroxide and observations can therefore be made on a homogeneous solution. The following experiment was performed. 137 mg. of a vitamin A concentrate were dissolved in 9 cc. of alcohol and 1 cc. of aqueous hydrogen peroxide (30 % H_2O_2 by weight) was added. After stirring, 1 cc. of the reaction mixture was withdrawn by pipette and run into 25 cc. ether. The ethereal solution was washed with water, dried and evaporated; the residue was dissolved in chloroform and its vitamin content estimated. The reaction mixture was allowed to stand at 15° and at intervals further samples were taken. At the end of 24 hours the vitamin content of the solution remained unchanged. Therefore under the conditions described no oxidation of vitamin A by hydrogen peroxide takes place.

B. OBSERVATIONS ON THE OXIDATION OF VITAMIN A BASED ON THE BIOLOGICAL ASSAY.

1. *The stability of vitamin A in alcohol verified by biological tests.*

Although it is now realised that for dealing with concentrates the colorimetric estimation of vitamin A with the antimony trichloride reagent is quite trustworthy, it is still desirable to check any new result by applying the biological assay for confirmation. In the present study it was out of the question to use the biological assay to follow the oxidation of vitamin A in more than one or two solvents even had this been thought necessary, owing to the large number of animals which would be required. The more important

results obtained with the colorimetric estimation were therefore selected for confirmation with the biological assay. The stability of vitamin A in alcohol when aerated at its boiling point or treated with hydrogen peroxide at room temperature, and its stability when aerated in 20 % alcoholic potash at 98° were chosen for repetition. Stability of the vitamin under these conditions was demonstrated by preparing solutions in coconut oil of untreated and of treated concentrates, and comparing the vitamin A contents of these solutions by feeding tests. The solutions were prepared as follows:

Fig. 6. Biological confirmation of results obtained colorimetrically. *N*. Negative controls. *A*. Animals receiving untreated vitamin A concentrate. Daily dose per rat, 3 drops solution A, containing 0.05 mg. concentrate. *B*. Animals receiving vitamin A concentrate which had been aerated in boiling ethyl alcohol for 24 hours. Daily dose per rat, 3 drops solution B, containing 0.05 mg. concentrate.

Solution A. Solution of untreated concentrate, prepared by dissolving the vitamin A concentrate, 1 mg. to each gram of coconut oil.

Solution B. Solution of vitamin A concentrate aerated in alcohol. 190 mg. of the concentrate were dissolved in 19 cc. alcohol and aerated at the boiling point for 24 hours. The alcohol was evaporated under reduced pressure and the residue dissolved in 190 g. of coconut oil.

Solution C. Solution of vitamin A concentrate aerated in alcoholic potash. 80 mg. of concentrate were dissolved in 10 cc. of 20 % alcoholic potash and aerated at 98° for 24 hours. The solution was diluted with water and the vitamin extracted and dissolved in 80 g. coconut oil.

Solution D. Solution of vitamin A concentrate treated with hydrogen peroxide. 40 mg. of concentrate were dissolved in 10 cc. alcohol and 1 cc. of aqueous hydrogen peroxide (30 % H_2O_2 by weight) was added. The mixture was shaken and allowed to stand 24 hours at room temperature, after which the vitamin was extracted (see above) and dissolved in 40 g. of coconut oil.

Previous observation with the untreated concentrate had shown that if 3 drops of solution A were given daily to a rat deprived of vitamin A, sub-maximal growth was maintained. A group of matched albino rats was placed

Fig. 7. Biological confirmation of results obtained colorimetrically. *C.* Animals receiving vitamin A concentrate which had been aerated in alcoholic potash at 98° for 24 hours. Each rat received 3 drops solution C daily (containing 0.05 mg. concentrate). *D.* Animals receiving vitamin A concentrate which had been treated with hydrogen peroxide. Daily dose per rat, 3 drops solution D, containing 0.05 mg. concentrate. *E.* Animals receiving vitamin A concentrate which had been aerated in ethyl stearate at 98° for 4 hours. Daily dose per rat, 5 drops of solution E, containing 0.27 mg. concentrate.

on a vitamin A-free diet made up of 694 g. rice starch, 200 g. Glaxo "vitamin A-free casein," 50 g. salts, 1.1 cc. radiostol and 50 g. marmite. After depletion of its vitamin A reserves each animal was dosed daily with 3 drops of one or other of the test solutions. Four animals received untreated concentrate (solution A), four received concentrate aerated in alcohol (solution B), four received concentrate aerated in alcoholic potash (solution C), two received concentrate treated with hydrogen peroxide (solution D) and three others received no dose. The growth curves are given in Figs. 6 and 7 and it will

be seen that all four of the solutions were equally efficient in promoting growth. Within the limits of error of this test (which will be large owing to the small number of animals used) the solutions contain equal amounts of vitamin A, indicating the stability of the vitamin when exposed to oxygen or hydrogen peroxide under the conditions described, and confirming quantitatively the results obtained by colorimetric estimation.

2. Oxidation of vitamin A by aeration in ethyl stearate.

An observation was made to confirm the rapid oxidation of vitamin A when aerated in certain solvents; ethyl stearate was chosen as the solvent. A test solution of vitamin concentrate which had been aerated in ethyl stearate was prepared.

Solution E. 70 mg. of the concentrate were dissolved in 7 g. ethyl stearate and aerated at 98° for 4 hours. The solution was saponified and the residue dissolved in 21 g. coconut oil. This solution was given in a daily dose of 5 drops to each of four rats taken from the same group as those used in testing the other four solutions. The growth curves are reproduced in Fig. 7, and it will be seen that the dose of 5 drops was insufficient to support growth or even maintain the weight of the rats. As 5 drops of solution E contains 50/9 as much concentrate as 3 drops of solution A (untreated concentrate) it follows that the concentrate after aeration in ethyl stearate contained much less than 18 % of its initial content of vitamin A. The colorimetric test showed that aeration for the same period under the same conditions reduced the vitamin content of an ethyl stearate solution to 3 % of the initial value. In this experiment therefore the biological test affords a qualitative confirmation of the results obtained by the colorimetric method of estimation.

DISCUSSION.

The experiments described above establish definitely that the solvent plays a cardinal part in determining the rate of oxidation of dissolved vitamin A by atmospheric oxygen. This is well illustrated by the data showing the percentage of the vitamin which is oxidised in one hour under similar conditions

Table I. *The percentage of dissolved vitamin A which is destroyed by aeration for one hour at 98°.*

Solvent	% oxidised	Solvent	% oxidised
Ethyl alcohol*	0	Ethyl laurate	67
<i>n</i> -Butyl alcohol	0	Ethyl stearate	87
<i>n</i> -Amyl alcohol	22	Ethyl oleate	81
Cetyl alcohol	0	<i>n</i> -Amyl acetate	23
Acetic acid	89	Triacetin	0
Caproic acid	54	Tributylin	31
Lauric acid	93	Triolein	36
Stearic acid	94	Coconut oil	29
Oleic acid	92	Arachis oil	60
Ethyl acetate†	0	20 % alc. KOH	0

* At 78°.

† At 66°.

in different solvents. No correlation between the rate of oxidation of vitamin A and the molecular constitution of the solvent is apparent although it is perhaps significant that the rate is great in all the acids tried, while it is negligible in some alcohols and esters and also in strong alcoholic potash. Similarly no correlation between the rate of oxidation and the solubility of oxygen in the solvent can be discerned.

The work of Huston, Lightbody and Ball [1928] and of Mattill [1931] suggests that the phenomena of autoxidation may play a part in determining the stability of the fat-soluble vitamins, but at the present time the theory of autoxidation cannot be applied to the elucidation of the detailed results reported above. Until observations can be made upon the oxidation of pure vitamin A dissolved in rigorously purified solvents, this must inevitably remain true, as the effects of traces of impurities cannot be predicted. Any substance present in traces might exert a pro-oxidant or an anti-oxidant effect upon the oxidation of the vitamin A; moreover a single substance is able to act as pro-oxidant under some conditions and anti-oxidant under others [*cf.* Moureu and Dufraisse, 1926].

The results (obtained with the ordinary grade compounds easily available in the laboratory) bear directly upon questions concerning the concentration and preservation of vitamin A preparations. For instance, it is evidently superfluous to take special precautions to prevent the oxidation of vitamin A during saponification processes, although this has been considered necessary [Drummond *et al.*, 1925; Smith and Hazley, 1930]. The effect of ethyl alcohol in protecting vitamin A from oxidation has already been applied successfully in the treatment of concentrates. Although they deteriorate rapidly in air, if dissolved in ethyl alcohol they appear to escape oxidation completely for days or weeks, without special precautions, and only lose their potency slowly over a period of months. Moreover, some observations on the colour change which occurs in alcoholic solutions of vitamin A when kept for several months suggest the possibility that the destruction of the vitamin in this solvent may not be due to oxidation, but to isomerisation. Further work upon this question is now in progress.

SUMMARY.

Vitamin A is rapidly oxidised by air when dissolved in some solvents, and slowly or not at all in others. It is particularly stable in ethyl alcohol, alcoholic potash and ethyl acetate. In ethyl alcohol it is also stable toward hydrogen peroxide.

The solvent (or impurities associated with the vitamin or solvent) appears to play a leading part in determining the stability of vitamin A.

I wish to express my indebtedness to Dr L. J. Harris and Dr T. Moore for much help and advice; also to the Medical Research Council for the tenure of a part time grant during the progress of this work.

REFERENCES.

- Blix and Löwenhielm (1928). *Biochem. J.* **22**, 1313.
Cady and Luck (1930). *J. Biol. Chem.* **86**, 743.
Dann (1931). *J. Soc. Chem. Ind.* **50**, 832.
Drummond, Channon and Coward (1925). *Biochem. J.* **19**, 1047.
— and Coward (1920). *Biochem. J.* **14**, 734.
Dunn (1924). *J. Biol. Chem.* **61**, 129.
Hopkins (1920). *Biochem. J.* **14**, 725.
Huston, Lightbody and Ball (1928). *J. Biol. Chem.* **79**, 507.
Marcus (1931). *J. Biol. Chem.* **90**, 507.
Mattill (1931). *J. Biol. Chem.* **90**, 141.
McCollum and Davis (1915). *J. Biol. Chem.* **23**, 247.
Moore (1930). *Biochem. J.* **24**, 699.
Moureu and Dufraisie (1926). *Chem. Rev.* **3**, 113.
Olcovich and Mattill (1931). *J. Biol. Chem.* **91**, 105.
Osborne and Mendel (1916). *J. Biol. Chem.* **24**, 37.
Rosenheim (1927). *Biochem. J.* **21**, 386.
Scheunert (1931). *Biochem. Z.* **236**, 39.
Smith and Hazley (1930). *Biochem. J.* **24**, 1942.
Steenbock, Boutwell and Kent (1918). *J. Biol. Chem.* **35**, 517.
Takahashi, Nakamiya, Kawakami and Kitasato (1925). *Scientific Papers Inst. Phys. and Chem. Research, Tokyo*, **3**, 81.
Wokes and Willmott (1927). *Biochem. J.* **21**, 419.