X. RESEARCHES ON THE FAT-SOLUBLE ACCESSORY SUBSTANCE. I. OBSERVATIONS UPON ITS NATURE AND PROPERTIES¹.

BY JACK CECIL DRUMMOND.

*From the Biochemical Laboratory of the Research Institute,
Cancer Hospital, London.*

(Received February 24th, 1919.)

OTHER than numerous observations upon the distribution of fat-soluble *A* in foodstuffs curiously little attention has been devoted to that important substance. The present study was begun with the object of ascertaining its chemical nature.

Few definite statements regarding the properties of fat-soluble *A* are to be found in the literature. By many it has been considered to be a relatively thermostable substance, a view which has led to suggestions being advanced that it was a more or less clearly defined chemical unit such as a lipoid. McCollum and Davis [1914, 1] reported that the factor was present in an ether extract of boiled eggs, whilst Osborne and Mendel [1915] found the growth promoting properties of butter fat unaffected by treatment with steam for over two hours.

More recently, however, the thermostability of the accessory factor has been questioned by Steenbock, Boutwell and Kent [1918], who have demonstrated that exposure of butter fat to a temperature of 100° C. for four hours is sufficient to destroy most, if not all, of its growth stimulating powers.

Regarding the chemical nature of fat-soluble *A* practically no information has been derived from a study of the literature. McCollum and Davis [1914, 2] hydrolysed butter fat in a non-aqueous medium at room temperature and, without attempting any separation of the products of hydrolysis, believed they had obtained evidence that fat-soluble *A* had survived the process.

A very considerable amount of work has been carried out in this laboratory aiming at the isolation and identification of fat-soluble *A*. Much of the earlier work, however, was based on an assumption that the factor is relatively stable to temperatures below 120°.

This assumption was founded partly on the results obtained by previous workers which have already been referred to, and partly on the observation

¹ The author wishes to express his indebtedness to the Medical Research Committee for a grant which defrayed part of the expenses of this investigation.

that many oils, in the preparation of which drastic processes employing high temperatures have been used, were found to be rich sources of fat-soluble *A*. Examples of oils of this type are many fish oils [Drummond, 1918].

Few of the early experiments will be described because they have scarcely any interest in the light of the results yielded by the examination of the stability of the accessory factor *A*.

The thermostability of this factor was not doubted until an enquiry into the effects of hydrogenation upon the factor present in oils was undertaken in collaboration with Professor W. D. Halliburton, at the request of the Oil and Fats Committee of the Food Investigation Board.

This investigation yielded results which demonstrated beyond any doubt that fat-soluble *A*, in the form in which it occurs in natural animal fats, is much less stable to high temperatures than has been previously assumed. Such an observation necessitated a complete revision of all the previous experiments to isolate and identify the factor, in many of which high temperatures had been employed.

EXPERIMENTAL METHOD.

At an early stage it became apparent that some more or less approximately standardised method of testing substances for the presence of fat-soluble *A* was necessary, if any results of a comparable nature were to be obtained. The elaboration of such a method presented very great difficulties, as may well be imagined by those who have had experience in this field of research.

Although the method to be described is far from perfect, it has nevertheless yielded somewhat more useful results than would have been obtained by direct feeding tests of the usual type. The method is at present being used as a routine in this laboratory and should be useful for comparative studies, when improved by certain modifications which are now under consideration.

The animals used in trials of this nature are selected from our own carefully bred stock, and are, therefore, of a higher standard than those which form the average stock supplied by outside breeders.

The beneficial results of using none but home-bred stock are quickly apparent in this type of research and should be borne in mind by anyone who attempts to undertake it. Young healthy rats selected from the main stock and weighing about 50 g. are fed upon an artificial ration constituted as given below:

Purified caseinogen	...	...	...	...	20 parts
„ starch	...	...	...	...	50 „
Salt mixture	...	...	...	...	5 „
Yeast extract (source of water-soluble <i>B</i>)	...	...	...	...	5 „
Butter fat (source of fat-soluble <i>A</i>)	...	...	...	...	15 „
Filtered orange juice (source of water-soluble <i>C</i>)	...	...	...	...	5 „

The last constituent is added because it has been recently shown that rats show a more satisfactory growth and development when their ration contains

the antiscorbutic factor in addition to the other requisites. [Harden and Zilva, 1918; Drummond, 1919.]

Whilst nourished upon this ration the young rats should show a strictly normal rate of growth and maintain good health. Any which fail to do so in this preliminary period are regarded as unfit for experimental purposes and are rejected. The remainder, having given evidence of a normal power to grow, are removed from the complete ration when they have attained an average body weight of 70 to 80 g. and are given a dietary similar to the above but in which the butter fat has been replaced by an equivalent amount of a fat known to be deficient in fat-soluble *A*.

For this purpose considerable use has been made of hardened linseed oil, which contains no detectable traces of the accessory substance *A*. Occasionally when a very important experiment was undertaken this fat was further purified by one recrystallisation from alcohol.

Fed upon this diet deficient in fat-soluble *A* the experimental animals should quickly cease growing. Occasionally a very vigorous individual will continue to grow for some weeks after the deficiency has been introduced and such animals should not be employed in experiments of a comparative nature. The majority of the young animals show greatly retarded growth after a week upon the deficient dietary. When it is definitely established that growth is inhibited by the deficiency of fat-soluble *A*, the linseed oil is either wholly or partially replaced by the substance to be tested. The behaviour of the animal is then closely watched during a period of from four to six weeks. An absence of fat-soluble *A* in the added fraction is indicated by no growth on the part of the animal, followed by a decline in health accompanied by the characteristic eye-condition which has somewhat loosely been termed a xerophthalmia.

On the other hand, if the animal shows growth, the rate of this may be taken to be roughly proportional to the amount of fat-soluble *A* which has been supplied by the added fraction. Finally, in cases where little or no growth has been observed during the period when the unknown substance was tested, the rats are placed upon the original complete dietary in order to determine whether they still possess the power to grow when all their nutritive requirements are supplied.

It will naturally be realised that it is of great importance to determine the food intake throughout experiments of this type, although there is no need for other than approximate values, unless the trial is one of a crucial nature. The importance of working along some such roughly standardised lines as these is quickly impressed upon one during the course of an investigation of this nature, for it has been ascertained that the requirements of animals for the fat-soluble accessory substance become smaller as maturity is approached, so that the age of the experimental animals and their food intake must be taken into careful consideration.

EFFECT OF TEMPERATURE ON FAT-SOLUBLE *A*.

As has already been stated this study arose from an enquiry into the effect of hydrogenation on the fat-soluble factor present in oils and fats. Very large quantities of oils are now hardened by the catalytic method of reduction with gaseous hydrogen, the products obtained being utilised widely for the manufacture of edible fats, such as butter and lard substitutes. [Halliburton, Noël Paton, Drummond and others, 1919.] The ever-increasing consumption of preparations of this type might conceivably lead in time to a grave disturbance of the health of the people, should it be found that they are seriously deficient in the fat-soluble factor.

Hardened vegetable oils are known to be useless as sources of fat-soluble *A* [McCollum, Simmonds and Pitz, 1916, 1; Halliburton and Drummond, 1917], but no information was available regarding the influence of the process upon animal fats. As a convenient product for examination whale oil was chosen. This oil contains fat-soluble *A* [Drummond, 1918], is readily procurable in large batches of uniform composition, is representative of a type which is extensively used for hardening, and does not require to be separated from other substances before use, as is necessary when butter is used.

The specimen of whale oil employed in most of this investigation was a mixed batch of uncertain origin. Its quality was of the commercial grade 0-1, and it was a clear pale yellow oil containing a small amount of crystalline "stearin."

For this oil I am indebted to Mr D. H. Simpson, Manager of Ardol, Ltd, Selby, Yorkshire, to whom I must also express my appreciation of his kindness in preparing several specimens of treated oils.

The main supply of oil was tested and found to be a satisfactory source of fat-soluble *A* (Table I, experiment 6). 20 % of this oil was apparently as efficient as 8 % of butter fat (Table I, experiments 1-5).

Mr Simpson kindly arranged for a batch of this oil to be hardened in the plant at Selby. This was effected by exposure of the oil to gaseous hydrogen at a temperature of 250° for four hours in the presence of a metallic catalyst. The pressure in the hardening chamber is usually about atmospheric.

A sample of the hardened product was forwarded to me and was a clean white fat resembling mutton fat both in taste and smell. It has entirely lost all trace of the fishy smell possessed by the original oil. The hardening process had, however, also destroyed all power to act as a source of fat-soluble *A* (Table I, experiment 7). Young rats fed upon a diet containing 20 % of this fat as a sole possible source of fat-soluble *A* failed to grow and sooner or later developed the characteristic infection of the external eye.

The question then arose as to whether destruction of the active principle had been caused by the reduction of an unsaturated body or by the elevated temperature employed in the process.

Table I.

No. of experiment	Substance tested for fat-soluble A, and percentage in diet				Average weights in g. of three rats every seven days						Remarks
					0	7	14	21	28	35	
1	2 % butter fat	...	...	...	90	96	106	109	116	119	Comparative tests with butter fat
2	4 % "	...	...	...	92	102	111	115	118	121	
3	6 % "	...	...	...	100	111	122	131	140	141	
4	8 % "	...	...	...	85	100	119	134	136	149	
5	10 % "	...	...	...	85	99	119	137	151	156	
6	Stock whale oil 20 %	...	...	...	85	108	124	131	141	147	Fat-soluble A present
7	20 % hydrogenated whale oil	...	...	...	90	93	105	90	—	—	A completely destroyed
8	20 % whale oil heated 250° for 4 hours	...	...	...	101	108	112	118	109	—	A destroyed
9	" " 100° "	...	...	...	98	102	103	115	106	—	"
10	" " 75° "	...	...	...	92	96	99	97	—	—	"
11	10 % butter heated 100° for 1 hour	...	...	...	75	77	80	78	—	—	"
12	" " 100° for 2 hours	...	...	...	95	100	106	109	107	100	"
13	" " 100° for 4 "	...	...	...	101	112	112	108	—	—	"
14	" " 75° for 4 "	...	...	...	76	87	101	111	116	117	Some destruction
15	" " 50° for 4 "	...	...	...	81	94	106	119	130	132	Slight "
16	10 % butter fat heated 37° for 21 days (rancid fat)	...	...	...	80	95	113	131	132	135	V. sl. destruction
17	20 % whale oil exposed to air at 37° for 18 days	...	...	...	72	78	76	75	—	—	Destruction of A
18	20 % whale oil exposed to air at room temperature for 18 days	...	...	...	70	86	92	100	104	112	Some destruction
19	20 % whale oil heated to 37° in absence of air for 14 days	...	...	...	72	85	90	96	—	—	" "
20	20 % whale oil kept at room temperature in contact with O	...	...	...	76	94	116	133	140	—	No destruction
21	Aqueous extract representing 20 % whale oil	...	...	...	91	108	114	109	106	—	A probably not present
22	20 % water-extracted whale oil	...	...	...	80	97	104	116	122	132	No destruction
23	Dilute acid extract representing 20 % whale oil	...	...	...	75	83	95	83	—	—	A not present
24	20 % acid-extracted whale oil	...	...	...	80	97	110	122	129	130	Very slight loss
25	Alcohol extract representing 20 % whale oil	...	...	...	73	85	96	106	109	—	A probably present
26	20 % alcohol-extracted whale oil	...	...	...	79	99	110	121	120	126	Very slight loss
27	Unsaponifiable matter representing 15 % butter fat	...	...	...	96	102	100	92	—	—	A absent
28	15 % fatty acids from butter fat	...	...	...	88	86	97	92	—	—	"
29	Unsaponifiable matter representing 25 % whale oil	...	...	...	85	101	109	106	94	—	"
30	20 % whale oil fatty acids	...	...	...	72	78	82	76	—	—	"
31	Unsaponifiable matter + fatty acids from 25 % whale oil	...	...	...	80	80	92	106	92	—	"
32	Glycerol water representing 25 % whale oil	...	...	...	91	90	72	63	—	—	"
33	Unsaponifiable matter representing 25 % whale oil. Special preparation	...	...	...	80	89	86	90	—	—	"
34	20 % fatty acids from whale oil. Ditto	...	...	...	75	80	89	92	86	—	"
35	Glycerol water representing 20 % whale oil	...	...	...	81	88	90	82	—	—	"
36	Unsaponifiable matter + fatty acids from 20 % whale oil	...	...	...	78	93	105	91	—	—	A present?
37	Unsaponifiable matter + fatty acids + glycerol water from 20 % whale oil	...	...	...	80	87	93	89	—	—	A absent
38	1 % cholesterol	...	...	...	90	88	85	80	—	—	A absent
39	1 % lecithin (Merck)	...	...	...	98	99	100	82	—	—	"
40	0.5 % sphingomyelin fraction	...	...	...	70	68	72	66	—	—	"
41	0.5 % phrenosin	...	...	...	95	93	90	86	—	—	"
42	Crude carrotene fraction approx. 0.003 % pigment	...	...	...	108	114	123	135	—	—	Slight traces of A present
43	0.003 % crystalline carrotene	...	...	...	111	114	113	104	—	—	A absent

To test this point a sample of the oil was subjected at Selby to the conditions of hardening without the presence of hydrogen. This sample, after exposure to 250° for four hours, was inactive as a source of fat-soluble *A* (Table I, experiment 8).

As this result indicated that heat was the destructive agent the effect of lower temperatures was investigated. In view of the previous findings of the American workers it was somewhat surprising to find that exposure of the oil to temperatures of 75° or 100° for four hours was sufficient to render it valueless as a source of the accessory dietary unit *A* (Table I, experiments 9 and 10). Just about this stage of the investigation the paper by Steenbock, Boutwell and Kent [1918] reached this country, and in view of the results described therein it was decided to carry out some experiments on butter fat.

Their observations upon the destruction of butter fat at 100° were confirmed by the results of these experiments. Exposure of butter fat to 100° for periods of from one to four hours destroys its growth promoting power entirely, so far as can be determined by experiments on young rats (Table I; experiments 11-13).

The effect of lower temperatures was also investigated and it was ascertained that the nutritive value of butter fat may be appreciably lowered by four hours' exposure to temperatures ranging from 50°-75° (Table I, experiments 14-15).

Interest was immediately focussed upon the probable nature of the destruction, for obviously fat-soluble *A* is a substance of a much more unstable nature than had been previously believed.

The chemical changes which occur in oils and fats on heating at relatively low temperatures are as yet imperfectly understood. They are grossly represented by alterations in the acid value and the iodine value, which indicate changes of a hydrolytic and oxidative nature. It was therefore possible that inactivation of fat-soluble *A* was a result of oxidation or of increase in the proportion of free acid present.

Both these changes occur when fats undergo the alteration known as rancidity. A sample of butter was permitted to become markedly rancid by exposure to a temperature of 37° in contact with air for a period of slightly over three weeks. The acid value of this sample showed that rancidity was well advanced. Tested upon rats, this sample appeared to have lost very slightly its value as a source of fat-soluble *A* (Table I, experiment 16). This result at first led to the adoption of the view that oxidation was a factor in destruction but the following experiments with whale oil soon dispelled that idea.

Samples of whale oil were allowed to become oxidised by contact with air when spread out in thin layers in porcelain dishes. One sample was kept at room temperature whilst the other was maintained at 37° in an incubator. Both samples absorbed oxygen as shown by a drop in the iodine values (Table II) and the films of oxidised oil which formed on the surface were

frequently broken so as to expose the lower layers. Oxidation under these conditions was permitted to go on for eighteen days.

The feeding tests upon the animal gave surprising results. The sample oxidised at 37° was quite inactive as regards fat-soluble *A*, whilst the sample which had been kept at room temperature had lost but little of its activity (Table I, experiments 17 and 18). The iodine value of the two samples showed that oxidation had been more extensive at 37°, so that it was necessary to test whether this was responsible for the result.

A bottle was completely filled with oil, closely stoppered and sealed, after which it was maintained at 37° for fourteen days. This oil had undergone no changes of an oxidative nature as a result of this treatment as was shown by the iodine value (Table II). It had, however, very appreciably decreased in efficiency as regards fat-soluble *A* (Table I, experiment 19). A further sample of oil, given opportunity for oxidation at room temperature by prolonged shaking in the presence of gaseous oxygen, when tested was found to have lost little if any of the accompanying accessory substance (Table I, experiment 20). Apparently these experiments prove that oxidation plays no primarily important part in the destruction of fat-soluble *A* in animal oils.

This result confirms those obtained in the experiments described by Steenbock, Boutwell and Kent [1918] who found that butter fat was as rapidly inactivated by shaking for twelve hours at 40–60° with carbonated water as when atmospheric oxygen was present.

Table II.

Sample of oil						Value as source of fat-soluble <i>A</i>	Acid value	Iodine value
Original whale oil	...	...	...	...	...	+++	4.9	118.5
Whale oil heated 100° for 4 hours	...	...	...	...	...	—	9.5	116.2
“ “ 75° “	...	...	...	...	...	—	9.2	116.0
Whale oil exposed in thin layer to air at 37° for 18 days	...	...	...	...	...	—	6.5	97.7
Whale oil maintained at 37° in absence of oxygen for 2 weeks	...	...	...	...	...	+	4.95	118.7
Whale oil exposed in thin layer to air at ordinary temperature for 18 days	...	...	...	...	...	++	3.55	112.0
Whale oil shaken in contact with gaseous oxygen for 2 weeks	...	...	...	...	...	+++	4.82	114.0

It remains, however, to reconcile these findings with that described in an earlier part of this paper, where butter was found to have lost but little of its fat-soluble *A* after exposure to 37° in contact with air for over three weeks.

So far the only explanation of the different behaviour of the two oils is that butter fat is a much richer source of fat-soluble *A*. It is, therefore, possible for destruction to have occurred, and yet sufficient to have been left untouched to supply the requirements of the rats when their diet contained 10 % of the preparation. As a matter of fact the animals on which the rancid butter fat was tested did not show quite as satisfactory a rate of growth as those which received the untreated fat.

As all the experiments up to this point had been carried out on what may be termed a secondary source of fat-soluble *A*—that is an animal fat containing the accessory substance derived from plant sources—it appeared desirable for tests to be made of the thermostability of the factor in its primary environment.

Accordingly, a series of experiments was begun upon the factor as present in green leaves and seed embryos.

Neither of these investigations has yielded definite results and they are as yet incomplete. The experiments with cabbage gave evidence that drying may reduce the efficiency of leaves as a source of fat-soluble *A*, but no definite opinion can yet be given. The experiments with wheat embryo were a complete failure. Whenever this foodstuff was present to any considerable proportion in their diets the animals soon showed disturbances of health which quite upset the purpose of the experiments.

This point has not been investigated further as it leads too far away from the main enquiry. It is, however, interesting to note that McCollum, Simmonds and Pitz [1916, 2] have described the presence in wheat embryo of a substance distinctly toxic for rats.

A recent paper by Osborne and Mendel [1919] contains information that spinach and alfalfa are rich sources of fat-soluble *A*, even after the leaves have been dried at 50–60° in the air, but they give no comparative tests with the fresh vegetables.

Delf [1918], and Delf and Skelton [1918], report observations which lead them to believe that high temperature or the drying of cabbage leaves may effect a destruction of the fat-soluble accessory.

ATTEMPTS TO FRACTIONATE FATS CONTAINING FAT-SOLUBLE *A*.

Extraction of oils with water.

It was stated by McCollum, Simmonds and Steenbock [1917] that shaking melted butter fat with repeated changes of water removed the fat-soluble accessory substance from the fat. Many attempts to repeat this apparently simple method of separation were made in this laboratory. Butter fat, whale oil and cod liver oil were all treated as described but in no case was an active aqueous extract obtained. In the case of butter fat confirmation of their observation that the fat becomes inactivated by this treatment was obtained, but whale oil and cod liver oil appeared to lose little of their activity during the process (Table I, experiments 21 and 22).

One of the authors responsible for the original statement has now amplified it somewhat [Steenbock, Boutwell and Kent, 1918]. These investigators report that neither the washed butter fat nor the aqueous extract contains fat-soluble *A*. The factor had been destroyed during the process and their further experiments indicate that this is due to the temperature used during the extraction.

Extraction of whale oil with dilute acid.

At one time there was a suggestion that butter fat contained substances containing nitrogen and phosphorus which might be removed by extraction with dilute acids. Funk and Macallum [1914] were under the impression that a substance of the accessory factor type might be removed by this process.

Whale oil was extracted with three changes of 5 % hydrochloric acid for several hours at room temperature by repeated shaking. The acid extract after neutralisation with dilute sodium hydroxide solution was evaporated *in vacuo* at 30° to half its original volume. This fraction was totally inactive as regards the fat-soluble factor whilst the extracted oil, after being freed from traces of acid by extraction with water, was practically as active as before treatment (Table I, experiments 23 and 24).

Extraction with alcohol.

As there are definite grounds for believing that fat-soluble *A* is soluble under certain conditions in alcohol [Osborne and Mendel, 1915], a sample of whale oil was repeatedly shaken with several changes of cold absolute alcohol at room temperature.

The alcoholic extracts after removal of the alcohol *in vacuo* at 32° were found to possess a slight activity, but a larger proportion of the fat-soluble factor remained behind in the extracted oil (Table I, experiments 25 and 26).

A few attempts to modify the experiments carried out by Osborne and Mendel [1915] and thereby obtain an alcoholic extract of butter fat containing a high concentration of fat-soluble *A* failed. Destruction of the active principle apparently occurred owing to the exposure to the temperature of boiling alcohol, whilst the extracts when prepared appeared to lose their activity somewhat readily on storage. This confirms the later observations of Osborne and Mendel [1916].

ATTEMPTS TO SEPARATE FAT-SOLUBLE *A* FROM THE PRODUCTS OF THE
HYDROLYSIS OF FATS.

Prior to the appreciation of the thermolability of fat-soluble *A* much work along these lines was carried out. Butter fat, cod liver oil, whale oil and liver tissue, all of which are rich in fat-soluble *A*, were hydrolysed by hot alkalies and various fractions were prepared from the saponified products. No trace of any activity was found in any of these products.

As soon as the temperature destruction was known it was obvious that any further attempts along these lines must employ less drastic methods of hydrolysis.

McCollum and Davis [1914, 2] have already advanced evidence that the accessory factor accompanying butter fat can resist the conditions necessary for the hydrolysis of fats in a non-aqueous medium at ordinary temperature.

The first experiments along these lines were made with butter fat.

This fat was saponified by the method of Henriques [1895], a process that completely hydrolyses the ordinary type of fats in 24 hours at room temperature. After removal of the solvents *in vacuo* at 35°, the soaps were dissolved in distilled water at 35° and the unsaponifiable matter removed by extraction with ether. The fatty acids were then liberated by a very slight excess of mineral acid and removed by ether extraction. Both fractions were well washed with water and freed from ether by evaporation under reduced pressure at 37°. Animal feeding tests showed no activity on the part of either fraction (Table I, experiments 27 and 28). A similar experiment was made with the products isolated from cold saponified whale oil and similar results were obtained (Table I, experiments 29 and 30).

The possibility of fat-soluble *A* being a mixture of two or more substances suggested itself and tests were made with the united unsaponifiable and fatty acid fractions. No activity was observed (Table I, experiment 31). The glycerol fraction after the removal of ether and neutralisation was also inactive (Table I, experiment 32).

Although the possibility of oxidation during the processes being the cause of the destruction was not considered probable, in the light of the results detailed in the earlier work on whale oil, nevertheless a more careful experiment was carried out.

A sample of whale oil was hydrolysed by Henriques' method, and throughout all subsequent operations involved in the separation of the various fractions, great care was taken to exclude air. Distillations and operations of that kind were conducted in an atmosphere of carbon dioxide, while the fractions when prepared were stored in highly evacuated and darkened desiccators.

Particular care was taken in the selection of the animals employed in the testing of these particular fractions. No evidence of the presence of fat-soluble *A* in the unsaponifiable matter fraction was obtained (Table I, experiment 33).

Two of these animals whose diet contained 20 % of the fatty acids derived from whale oil showed practically no growth during a period of observation of five weeks and at the end of that time exhibited the external eye trouble. They speedily recovered health and resumed growing when given the original whale oil ration (Table I, experiment 34).

The third animal grew during the whole five weeks although the rate was sub-normal throughout. It did not at any time exhibit sore eyes. This animal is not included in the average.

As a doubtful result was thus obtained in a very important experiment a further test was made in which two young rats whose growth had been stunted and in which eye disease had been produced by a deficiency of fat-soluble *A* were given the ration containing 20 % of the whale oil fatty acids. No resumption of growth has yet been observed during fourteen days and the eye condition is becoming progressively worse.

It is, therefore, concluded that there is no evidence which identifies fat-soluble *A* with any of the fatty acids occurring in whale oil, and the rat which was observed to grow on that fraction must have been one of those individuals which are frequently encountered and which exhibit a power to grow for long periods of time although receiving a diet seriously deficient in fat-soluble *A*.

When a diet containing both the unsaponifiable matter fraction and the fatty acid fraction was tested a slight initial growth was observed in some cases. This was not maintained, however, during the following weeks of observation (Table I, experiment 36). Likewise, no positive results were obtained when the glycerol fraction was tested (experiment 35) or when all three fractions were combined (experiment 37).

It must be remarked that in all these feeding tests the food consumption was determined, even if only approximately. In every case except where the dietaries contained the glycerol fractions derived from the hydrolysed oils the food consumption was satisfactory. This excludes any complications arising from a failure of the animals to partake of sufficient food to ensure an adequate intake of the substance being tested.

DISCUSSION.

Speculation as to the nature of "fat-soluble *A*" as it occurs in oils and fats is perhaps unwise until this investigation has advanced a few stages further.

It appears doubtful whether the substance known as fat-soluble *A*, in the form in which it occurs in animal oils, is a clearly defined chemical substance of comparatively simple constitution. Long before the discovery of the thermolability of the factor made such tests valueless, many attempts were made in this laboratory to identify fat-soluble *A* with certain known constituents of fats.

Unsuccessful attempts were made to replace the fat-soluble accessory factor in the diet by a number of the commoner fatty acids, glycerol, cholesterol, lecithin, sphingosin, phrenosin, kephalin and the lipochrome carotene (Table I, experiments 38-43).

The discovery of the destruction at comparatively low temperatures at once narrowed the field of possible identity very greatly.

Further, when it no longer appeared probable that oxidation was responsible for the destruction, it was possible to rule out many substances such as the unsaturated acids and the lecithins.

It is of interest to refer here to the question of the therapeutic value of cod liver oil. For many years this foodstuff has been widely employed in the treatment of diseases of malnutrition, and there have been numerous speculations as to why this particular oil should be of value in such cases.

Small traces of iodine and other substances are frequently found in cod liver oil, and by some these impurities were considered to be responsible for the high nutritive value of the oil. It has been shown, however, that this view

is incorrect, and that oils which are free from impurities containing phosphorus, iodine and sulphur may give as good results in the treatment of wasting diseases as those which are less pure [Williams, 1912, 1].

Osborne and Mendel [1914], after having discovered the beneficial influence of cod liver oil on growth, remarked that it is perhaps more than a coincidence that the oil has so long enjoyed a reputation for nutritive virtues which can scarcely be attributed to its fat content *per se*. The existence of an accessory substance in certain fats was at that time only just being suspected and it is interesting in the light of present knowledge to quote the final paragraph of their paper. "Perhaps experiences such as have been reported in this paper will pave the way for a clearer understanding of the physiological potency of natural products like butter, egg-yolk and cod liver oil, which have long enjoyed a popular, yet inexplicable, reputation for unique nutritive potency."

Williams [1912, 1, 2] has suggested that in the case of cod liver oil it is owing to the presence of peculiar unsaturated fatty acids. The work described in this paper does not tend to identify the fat-soluble *A* factor with any of the fatty acids present in the oils in which it is found, and the theory of Williams would appear to lack support.

From the results obtained during this study one is led to conclude that the chief agent in the inactivation of fat-soluble *A* is temperature. It must, however, be borne in mind that all these speculations refer solely to the factor as present in secondary sources, such as the animal oils. It is quite possible that the factor occurs in a different form in its primary environment in the plant tissues; in fact McCollum, Simmonds and Pitz [1916, 1, 2] have already intimated that the factor may be present in the embryos of seeds in combination with some cell constituents such as protein.

After much careful consideration of the experiments which have been described it is felt that there is justification for advancing the suggestion that the accessory growth promoting factor, provisionally termed "fat-soluble *A*," is not a clearly defined chemical substance but rather that it is a labile substance perhaps possessing characteristics resembling those of an enzyme.

Although this hypothesis is advanced with some hesitation it is the only one which has been found to fit in with the majority of the experimental results.

Attempts have been made in collaboration with Dr O. Rosenheim to determine whether the factor can pass through rubber membranes, but unfortunately the experiments have so far failed. At present it is believed that toxic substances are dissolved from the rubber dialysis membranes and pass into the solutions of the oil rendering it impossible to carry out successful feeding experiments with the final products. When a more satisfactory technique has been evolved for the purpose of this experiment it is hoped to gain information as to the class of substance to which fat-soluble *A* belongs.

Much further work will be necessary before a definite conclusion upon the important question of the identity of fat-soluble *A* can be arrived at, but in

the meantime it may be useful to test the possibility of it being an ill-defined and labile substance such as an enzyme.

It is surely legitimate to conceive the animal organism being as dependent upon the plant kingdom for a supply of an essential complex of that nature as it is for the supply of many indispensable substances of more clearly defined constitution.

I am indebted to Professor W. D. Halliburton for his interest and advice during the course of these experiments and to Dr O. Rosenheim for many valuable suggestions and for purified specimens of certain lipoids and carotene.

SUMMARY.

1. The fat-soluble accessory food factor *A* present in certain oils, as for example butter fat and whale oil, is readily destroyed by short exposure (one hour) to a temperature of 100°.

2. Destruction occurs, but is less rapid, when the exposure is to temperatures ranging from 50° to 100°.

3. Exposure of the oil to a temperature of 37° for several weeks may cause destruction of the accessory factor.

4. Destruction is apparently not a result of oxidation or hydrolysis.

5. Fat-soluble *A* is not extracted from oils by water or dilute acid.

6. Fat-soluble *A* is soluble in alcohol, and may be removed in small quantity from oils by cold extraction with that solvent.

7. Hydrolysis of oils in a non-aqueous medium at room temperature causes disappearance of fat-soluble *A*, as the factor is not found present in any of the fractions derived from the original oil.

8. So far, there is no evidence which suggests that fat-soluble *A* is not a single substance.

9. Fat-soluble *A* has not been identified with any of the recognised components of fats, such as glycerol, saturated or unsaturated fatty acids, cholesterol, lecithin, phosphatides or the lipochromes.

10. In view of the low temperatures at which destruction may occur it is suggested that fat-soluble *A* may be a labile substance of ill-defined constitution.

REFERENCES.

- Delf (1918). *Biochem. J.* **12**, 416.
Delf and Skelton (1918). *Biochem. J.* **12**, 448.
Drummond (1918). *J. Physiol.* **52**, 95.
—— (1919). *Biochem. J.* **13**, 77.
Funk and Macallum (1914). *Zeitsch. physiol. Chem.* **92**, 13.
Halliburton and Drummond (1917). *J. Physiol.* **51**, 235.
Halliburton, Noël Paton, Drummond, and others (1919). *J. Physiol.* **52**, 328.
Harden and Zilva (1918). *Biochem. J.* **12**, 408.
Henriques (1895). *Zeitsch. angew. Chem.* **7**, 721.
McCollum and Davis (1914, 1). *Proc. Soc. Exp. Biol. Med.* **11**, 101.
—— (1914, 2). *J. Biol. Chem.* **19**, 245.
McCollum, Simmonds and Pitz (1916, 1). *Amer. J. Physiol.* **41**, 361.
—— (1916, 2). *J. Biol. Chem.* **25**, 105.
McCollum, Simmonds and Steenbock (1917). *J. Biol. Chem.* **29**, xxvi.
Osborne and Mendel (1914). *J. Biol. Chem.* **17**, 401.
—— (1915). *J. Biol. Chem.* **20**, 379.
—— (1916). *J. Biol. Chem.* **24**, 37.
—— (1919). *J. Biol. Chem.* **37**, 187.
Steenbock, Boutwell and Kent (1918). *J. Biol. Chem.* **36**, 577.
Williams (1912, 1). *Pharm. J.* **35**, 806.
—— (1912, 2). *Brit. Med. J.* Sept. 21.