VII. THE GROWTH OF RATS UPON ARTIFICIAL DIETS CONTAINING LACTOSE.

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It has been known for some time that many natural food-stuffs contain substances which, although in many cases present in very small quantities, play an exceedingly important rôle in the processes of growth and nutrition.

A large amount of literature has been published during recent years which deals with these substances in one way or another. Particularly has interest been centred upon those substances of this class which if absent or deficient from the diet may induce a definitely pathological condition.

We are, however, in this paper concerned with another group of these so-called accessory substances, namely, those which are apparently indispensable to the diet of a growing animal if satisfactory growth is to occur.

Milk has been shown to be peculiarly rich in its content of growth-promoting accessory substances. Hopkins [1912] showed that a very small ration of milk added to a diet consisting of purified caseinogen, starch, lard and salts—a diet that by itself was inadequate to supply all the factors necessary for the growth of young rats—at once rendered it sufficient for this purpose. Stepp [1909, 1911, 1913] had previously shown that dietaries which had been extracted with alcohol were rendered incapable of sustaining the growth processes of young mice, but when the extracted substances were completely replaced normal growth was at once possible.

Stepp demonstrated that the replacement of many of the individual components of the alcoholic extracts, such as purified natural fats and lipoids, was not followed by any improvement in the animal's condition.

He therefore assumed that the factors responsible were in all probability some unknown members of the class of lipoid substances.

Feeding experiments were accordingly made by several workers, in which attempts were made to rear young growing rats to maturity upon fat-free diets.

Osborne and Mendel [1912] carried out an extensive series of experiments of this nature, using diets similar in composition to those utilised by them in their earlier researches [1911], but which had been subjected to extraction with ether. With such diets they found that it was possible to obtain satisfactory growth in young rats, over a period of time equal to that during which the rat normally shows growth, and concluded that growth is possible upon a fat-free diet.

Since their diets had not been extracted with hot alcohol, they naturally could not claim that they were lipoid-free, and they were even forced to admit that they had not been able to obtain growth in young mice when fed upon diets similar to those employed by Stepp. In spite of these facts, however, the views of the latter author were subjected to criticism.

That the active substance or substances may in some cases be removed from diets by treatment with fat solvents was shown when McCollum and Davis [1913] announced that an ether extract of butter added to a diet, inadequate in accessory substances, at once rendered the diet satisfactory. Osborne and Mendel [1913, 1, 2] also demonstrated that unsalted butter and butter fat possessed the same power.

It was then soon found that many naturally occurring oils and fats have this property associated with them, while, on the other hand, many other representatives of this class are devoid of any such activity.

McCollum and Davis, in the communication referred to above, also attempted to feed young rats upon a fat-free diet, composed of caseinogen, lactose, agar, dextrin and a suitable salt mixture. They too recorded results which apparently demonstrated that growth for a certain period of time was possible upon such a diet, but that, invariably, sooner or later, the animals fed upon it declined and died. This decline and death was not the result if the diet contained the ether extract of butter or egg yolk, for then the animals grew normally in every way.

While much work was being done upon the presence of the accessory substances occurring in company with many of the fats and oils, the possibility of other fractions of a normal dietary also containing other unknown substances possessing equally important properties was almost neglected.

Funk [1914, 1] and Funk and Macallum [1914] have, however, subjected Osborne and Mendel to criticism for the extensive use which the latter authors make of "protein-free milk." In this criticism it was pointed out that it was unsatisfactory to introduce into diets such an unknown factor as this complex mixture represents. McCollum and Davis [1915, 1] have also criticised the use of "protein-free milk," and have pointed out that the very differences, recorded by Osborne and Mendel themselves, in the nutritive value of natural "protein-free milk," as compared with an artificial product made from lactose and salts, were sufficient to render evident the existence of substances of an unknown nature, other than those usually carried by certain fats.

In this respect an observation made by Stepp is of interest. He favoured the belief that the substances present in milk, which play the part of accessory substances, are present not in the fat but rather in the skimmed milk fraction.

That there was any serious objection to the use of lactose in diets employed in feeding experiments has, however, not been considered fully until recently, when Hopkins and Neville [1913] repeated some of the earlier work of Osborne and Mendel. The former authors found that when purification of the various ingredients of an artificial dietary was carried to a further degree than usual, the animals declined at a much more rapid rate than when fed upon a similar diet which was less pure. In the preparation of the purified diets, they purified the caseinogen and starch by extraction with alcohol, and several times reprecipitated the lactose from aqueous solution by alcohol. Their results demonstrated clearly the importance of purifying the lactose employed in dietaries. Funk [1914, 1] also speaks of commercial lactose containing traces of nitrogen and therefore considers it is an unreliable ingredient to include in diets which are to be considered as "pure."

McCollum and Davis [1915, 1] have remarked that they appreciated the possible objection to the use of insufficiently pure lactose in diets. The samples of this sugar used by them, in their series of studies upon growth on fat-free foods, contained amounts of nitrogen varying from 0.02–0.034 %. In view of this fact they wisely refrained from any discussion as to the necessity of "vitamines," or hormones in the diets, until they had made an investigation of this point.

Sweet, Corson-White and Saxon [1915], in a research upon the influence of diet upon tumour growth in the rat, used diets similar to those employed by Osborne and Mendel, and they had occasion, from a consideration of their results, to believe that lactose does in some cases carry with it impurities

which may act as accessory substances to promote growth. They even explain some results obtained previously by van Alstyne and Beebe [1914], working upon the same subject, upon these grounds. The latter workers had fed rats upon two similar purified diets, one of which contained lactose, while the other did not. Investigations were made upon the effects of these two diets upon the growth of tumour grafts inoculated into the rats. From the results it was concluded that the carbohydrate of the diet, i.e. lactose, had had a beneficial effect upon the growth of the inoculated tumours in the rats. Sweet, Corson-White and Saxon now suggest that this result may be more correctly attributed to the presence of a growth-promoting accessory substance in the insufficiently pure lactose used in the diets.

In a recent paper Woglom [1915] has investigated the effect of lactose in diets upon the growth of tumours in rats and mice. The lactose was, however, added to their normal diet of bread and vegetables. From his results he concludes that lactose does not increase the receptivity of mice and rats for the transplantable carcinomata of their species and he proceeds to criticise the previous work of van Alstyne and Beebe. It must be remembered, however, that the latter authors added their lactose to a carbohydrate-free diet of caseinogen and lard, an important fact which Woglom has apparently passed over. The explanation of the results of van Alstyne and Beebe which has been given by Sweet, Corson-White and Saxon is, in all probability, the correct one. Any improvement in tumour growth in animals fed upon dietaries containing lactose is due, not to the lactose itself, but rather to the growth-promoting accessory substance which it so often carries, when insufficiently purified.

This work of Woglom therefore loses much of its value from the fact that he added the lactose to a diet already fully sufficient for normal growth. Consequently any action of the accessory substance, carried by the lactose he used, would fail to be apparent since both host and tumour-graft were already able to derive all the substances necessary for growth from the basal diet, without any addition of lactose.

Since the early spring of 1915, I have been making a large number of feeding experiments upon rats, with the object of obtaining sufficient experience in the technique to permit me to make an extensive examination of the effect of diet upon tumour growth. It was during these preliminary feeding trials that I was struck by the apparent effect of the purity of the lactose used upon the growth of the animal. Accordingly I made a few experiments to

ascertain the cause of this effect, and the results I have obtained are set forth in the present communication.

While the results were being compiled for publication, however, three papers appeared by McCollum and Davis [1915, 2, 3, 4], in which these indefatigable workers also record results consequent on the use of impure lactose in dietaries. They there put forward sufficient evidence to show that lactose, unless carefully purified, often does contain an accessory substance which helps to promote growth.

The identical conclusion to which the writer of the present paper had arrived, as a result of independent experiments, will now serve as a useful corroboration of the work of McCollum and Davis.

In view of the observations of Stepp, Hopkins, Funk and earlier workers, it has seemed a difficult matter to explain satisfactorily how Osborne and Mendel, and also McCollum and Davis, had been able to obtain a certain degree of growth in young rats fed upon fat-free diets composed of "purified" food-stuffs.

Osborne and Mendel have always been careful to utilise, throughout their extensive researches, proteins of a very high degree of purity, and yet they have gone to the other extreme in continuing to use in their diets "protein-free milk."

Reference has already been made to the criticisms which have been directed against the use of this complex substance, as a source of lactose and salts.

To determine whether any substance was contained in "protein-free milk" which would exert a growth promoting influence, two batches of young rats were taken and were fed upon the following diets.

<i>Diet I.</i>				<i>Diet II.</i>			
Caseinogen	...	...	18.0	Caseinogen	...	...	18.0
Wheaten starch	...	...	30.0	Wheaten starch	...	...	30.0
Lard	...	...	22.0	Lard	...	...	22.0
"Protein-free milk"	...	...	30.0	Extracted "protein-free milk"	...	...	30.0

All the ingredients of the diet other than the "protein-free milk" were carefully purified. The caseinogen was precipitated from machine-skimmed milk by acidification. It was then reprecipitated twice from dilute solution in sodium carbonate by the addition of acid, washed, pressed and dried.

The dried caseinogen was extracted with two changes of alcohol for six hours at 60°, and finally subjected to ether extraction for six hours.

McCollum and Davis [1915, 4] have now shown that caseinogen should be purified very carefully, owing to the fact that changes may occur in the protein molecule, if it is subjected to purification by strong measures, which may greatly lessen its nutritive value.

I have not found that the purification, to which I subjected the caseinogen used throughout these experiments, has rendered it unsuitable for the rats to any marked degree. The only two occasions on which I suspected that the caseinogen was unsatisfactory for the nutritive demands of the animals were when it had been extracted with hot alcohol whilst in a slightly moist condition. In these cases every rat upon the diets containing this caseinogen died within a short space of time. I have not yet investigated the matter carefully, but the result may be attributable to the caseinogen having undergone changes of an unknown nature.

Chart I. Curve showing normal growth of young rats upon diet:

Milk powder	...	...	...	60
Lard	...	...	...	24
Starch	...	...	...	16

The wheat starch was also submitted to alcohol and ether extraction, while the lard was the purest obtainable.

The extracted "protein-free milk" was prepared by drying the product, obtained by following out the directions of Osborne and Mendel [1911], in an oven at 100° for six hours. The dried mass was then ground to a fine powder and extracted with alcohol at 60° C. and finally with ether.

To test whether the "protein-free milk" had been affected by the six hours' exposure to 100°, a third series of rats was placed upon a diet similar to Diet I, but which differed in containing the heated product.

Reference to Charts II, III, IV will show typical growth curves of rats fed upon these three diets, as compared with normal growth upon a milk powder diet, Chart I.

Chart II. Growth of young rats upon diet of:

Alcohol extracted caseinogen	...	...	...	18
Alcohol extracted starch	...	...	...	30
"Protein-free milk"	...	...	...	30
Lard	...	...	...	22

Growth was obtained upon the diet containing "protein-free milk" and also to about the same degree upon that containing the heated product. The growth curves in Chart II are in every respect similar to those published by Osborne and Mendel, using similar diets. Approximately normal growth

Chart III. Failure to grow of rats fed upon diet:

Alcohol extracted caseinogen	...	...	...	...	18.0
Alcohol extracted starch	...	...	...	...	30.0
Heated and alcohol extracted "protein-free milk"	...	...	...	...	30.0
Lard	...	...	...	...	22.0

occurs for periods of from sixty to eighty days, and is followed by a cessation of growth and a rapid decline. This cessation and rapid decline may be prevented, and the animal may continue to grow normally, if at the critical point one of the fats containing accessory substances is added to the diet.

The rats fed upon the diet containing heated and extracted "protein-free

milk," however, showed no signs of growth whatever (Chart III). Slow decline characterised the period during which they were fed upon the diet, and this decline usually terminated in death after some forty days or so.

Chart IV. Growth upon diet:

Extracted caseinogen	...	...	...	...	18.0
Extracted starch	...	...	...	...	30.0
Lard	...	...	...	...	22.0
Heated "protein-free milk"	...	...	...	...	30.0

Obviously, some substances had been removed from the "protein-free milk," by the alcohol extraction, that were indispensable.

Similar results, I find, are recorded by MacArthur and Luckett [1915] who, investigating the indispensability of lipins in the diet, also subjected their "protein-free milk" to a similar extraction in order to remove from it all traces of this class of compound.

Chart V. Slow growth, and subsequent failure of rats fed upon diet:

Alcohol extracted caseinogen	...	...	...	...	18.0
Alcohol extracted dextrin	...	...	...	...	56.0
Alcohol extracted agar	...	...	...	...	2.3
Salt mixture B	...	...	...	...	3.7
Lactose	...	...	...	...	20.0

Experiments of a similar nature were then made, using a fat-free diet which has been used extensively by McCollum and Davis [1915, 1].

Diet IV.

Caseinogen	...	...	...	18.0
Dextrin	...	...	...	56.0
Lactose	...	...	...	20.0
Agar	...	...	...	2.3
Salt mixture	...	...	...	3.7

The salt mixture employed was one used with satisfactory results by these authors, and is composed of:

NaCl	...	...	...	0.173 g.
MgSO ₄ (anhyd.)	...	...	...	0.266 „
NaH ₂ PO ₄ H ₂ O	...	...	...	0.347 „
K ₂ HPO ₄	...	...	...	0.954 „
CaH ₄ (PO ₄) ₂ 2H ₂ O	...	...	...	0.540 „
Calcium lactate	...	...	...	1.300 „
Ferric lactate	...	...	...	0.118 „

Iodine was supplied in the drinking water (distilled) once weekly.

Upon this diet it was found that the stronger of the rats grew at a satisfactory rate for a period of about 50 days (Chart V). They did not reach as high a maximum weight as did the series fed upon Diet I, i.e. a diet in which the lactose was in a cruder form,—and having grown as indicated, they soon rapidly declined and died. Again in this case, the decline and death could be prevented by the prompt addition to the diet of a fat-soluble accessory substance.

As all the ingredients of this diet, other than the lactose, had been subjected to careful purification, the results obtained showed that it would be advisable to investigate the effect of purifying the lactose.

The lactose used throughout these experiments was bought from reliable English firms as “pure,” but notwithstanding this it usually gave, on analysis, evidence of the presence of nitrogen. The average figure obtained from analyses made on several samples of the sugar gave the percentage content of nitrogen as 0.024 %.

Samples of lactose as supplied under the description of “pure” and “purest” vary largely in their nitrogen content. I have had samples which were practically nitrogen free, and I can now attribute some of my failures to obtain growth upon such diets as Diet IV to this cause. The lactose was purified as Hopkins and Neville have described, namely, by precipitation from aqueous solution by means of alcohol.

A ration similar to Diet IV was then made up, containing the once recrystallised lactose instead of the cruder product.

The curves, showing the slight growth of rats fed upon this Diet V (Chart VI), demonstrate fully that the one purification of the lactose by recrystallisation had removed the majority of the growth promoting factor.

Charts VI and VII.

Chart VI. Slight growth, followed by rapid decline, upon diet:

Alcohol extracted caseinogen ...	...	...	18.0
Alcohol extracted starch ...	...	...	56.0
Alcohol extracted agar ...	...	...	2.3
Salt mixture B ...	...	...	3.7
Recrystallised lactose ...	...	...	20.0

Chart VII. Practically no growth upon diet:

Alcohol extracted caseinogen ...	...	...	18.0
Alcohol extracted starch ...	...	...	46.0
Alcohol extracted agar ...	...	...	2.3
Salt mixture B ...	...	...	3.7
Recrystallised lactose ...	...	...	20.0
Lard ...	...	...	10.0

A point of interest may be noted here. Using a diet similar to V, but containing some 10 % of lard, I was unable to obtain, in a large series of rats, more than one or two cases showing any growth whatever. The majority of the animals in this group slowly declined from the outset (Chart VII).

I cannot say, as yet, whether this result was purely accidental, or whether it had its origin in the inclusion of the lard in the diet. The possibility of the latter view being correct is not altogether out of the question, as parallel cases do exist. For instance, it is known that the quantity of carbohydrate in a diet plays a very important part in governing the utilisation of the

accessory substance which prevents beri-beri [Funk, 1914, 2; Braddon and Cooper, 1914].

Another diet was then tried which contained a much larger percentage of the unpurified lactose.

Diet VI.

Caseinogen	...	...	...	18.0
Lactose	...	...	...	56.0
Dextrin	...	...	...	20.0
Agar	...	...	...	2.3
Salt mixture	...	...	...	3.7

It was expected, from the greater percentage of lactose in this diet, that growth would be still more successfully promoted, and experimentally this expectation was realised (Chart VIII).

Chart VIII. Growth and early failure of young rats upon diet:

Extracted caseinogen	...	...	...	18.0
Extracted lactose	...	...	...	56.0
Extracted dextrin	...	...	...	20.0
Extracted agar	...	...	...	2.3
Salt mixture B	...	...	...	3.7

The animals survived, as a whole, longer, grew to a slightly greater maximum body-weight, and the subsequent decline did not occur at such an early date as in the cases of the rats fed upon a similar diet, which, however, contained only 20 % of lactose.

McCollum and Davis have just pointed out [1915, 3] that, if the lactose in a diet similar to this is entirely replaced by dextrin, no growth whatever is obtained, whilst Lander [1915] found no growth in young rats fed upon purified diets containing cane-sugar instead of lactose.

In another series of feeding experiments, lactose was used which had been twice recrystallised in the manner already indicated. No growth at all resulted, and the rats rapidly declined and died [Chart IX].

Chart IX. Rats fed upon diet:

Caseinogen	...	...	...	...	18.0
Dextrin	...	...	...	...	56.0
Agar	...	...	...	...	2.3
Salt mixture	...	...	...	...	3.7
Twice recrystallised lactose	...	...	...	...	20.0

A preliminary attempt was made to concentrate the active substance to some extent in the following manner.

The mother-liquors from a series of recrystallisations of lactose were concentrated down to dryness at 40° under diminished pressure. The resulting impure lactose was then recrystallised once from aqueous solution by means of alcohol. The mother-liquor from this recrystallisation was evaporated as before to dryness, and the product obtained was utilised in a diet, as the source of lactose, to the extent of 20 %. The effects of this concentration of the accessory substance upon the growth were not as marked as were expected. Chart X illustrates the type of growth curve of rats fed upon this ration. The maximum body-weight attained was slightly greater than that reached upon Diet IV, but no postponement of the rapid decline was observable. A further attempt is being made in this direction.

One cannot help being much impressed by the apparent need of the growing animal organism for more than one type of accessory substance. The one factor that has been studied in this paper is itself useless to make good the inadequacy of a pure diet so that complete growth can result. This can only occur when the diet also contains one of the fat-soluble accessory substances.

McCollum and Davis, in the recent papers referred to, have shown that many food-stuffs carry a water-soluble accessory factor that is of quite as great importance as those usually associated with many fats.

Thus wheat-embryo, egg-yolk and other substances are shown to contain water-soluble substances which may now be classified as accessory substances. Oseki [1914] had already shown that the accessory substance present in rye

is soluble in water, while Funk and Macallum [1914] have suggested the likelihood that two classes of accessory substances—called by Funk “vitamines”—exist, one occurring in water-containing food-stuffs, such as fruit.

Chart X. Growth upon diet:

Caseinogen	...	...	...	...	...	18.0
Dextrin	...	...	...	...	...	56.0
Agar	...	...	...	...	...	2.3
Salt mixture B	...	...	...	...	...	3.7
Lactose + concentrated impurities	...	...	...	...	...	20.0

whilst the other is generally found in the company of fats such as butter and cod-liver oil.

CONCLUSIONS.

It is apparent, from a consideration of the results set forth in the present communication, that lactose may contain effective amounts of a growth promoting factor, soluble in alcohol and stable on exposure to 100° for six hours.

These results are in entire agreement with those recently published by McCollum and Davis, and both sets of conclusions show that the suspicion with which other workers, to whom reference has been made, have regarded the inclusion of insufficiently pure lactose in experimental diets was well grounded.

It is now established conclusively that the views, held for some time past by Stepp, Hopkins, Funk and other workers, are correct, and that growth is impossible in the young animal when the diet supplied to it is deficient in certain indispensable accessory substances.

Our knowledge of the action of these unknown factors is so slight, that we shall be forced to reconsider many of the conclusions which have been drawn from the results of feeding experiments, involving the use of diets which we now know were not pure.

Much extremely valuable work has been done in this field, but the fact that many of the diets employed contained lactose, in an impure form, will make one await with interest the results which will be obtained when these experiments are repeated with diets containing a purer carbohydrate.

In this light, we may now regard many of the failures to obtain growth in young rats upon artificial food rations, which have been recorded by numerous workers, as evidence that they were truly dealing with diets of purified substances, and not as being due to faulty technique as has been suggested by some workers, whose own methods are now open to criticism.

SUMMARY.

1. Lactose, prepared from milk, may contain traces of an impurity which acts as a growth-promoting accessory substance.
2. This substance is soluble in water and alcohol, and is not destroyed by exposure to 100° for six hours.
3. Before complete growth can occur in a young animal, the diet, besides being adequate as regards its proteins, carbohydrates, fats and salts, must contain both fat-soluble and water-soluble accessory substances.
4. No growth is possible in the animals fed upon a purified diet which is entirely deficient in these accessory substances.

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