XX. THE INFLUENCE OF DEFICIENT NUTRITION ON THE PRODUCTION OF AGGLUTININS, COMPLEMENT AND AMBOCEPTOR.

By SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute.

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At least two diseases, scurvy and beri-beri, can definitely be traced to a prolonged consumption of a faulty diet. Both experimental and clinical data fully support this view which is now accepted by the majority of scientific workers and clinicians. Other diseases designated as "deficiency diseases," like pellagra, rickets, sprue, etc., are also attributed by some to a dietary deficiency, but the scientific evidence available at present is not sufficient to warrant any definite conclusion as regards the etiology of these diseases.

As is well known scurvy and beri-beri are caused by the absence from the diet of two principles of unknown chemical constitution. By depriving the diet of certain animals of these principles pathological conditions closely resembling scurvy and beri-beri can be induced. Lately Osborne and Mendel [1912, 1] and McCollum and Davis [1913] have demonstrated the necessity of a third accessory food factor provisionally named "the fat-soluble *A* factor" for the growth and existence of the rat. This has been confirmed by other workers. The effect of this deficiency on the human subject is still uncertain, but experimental evidence is gradually accumulating which promises to reveal it.

Besides the above principles of unknown chemical constitution it has been demonstrated experimentally on various kinds of animals that the removal of some chemically known compounds and elements from the diet restricts growth in the young and makes the food inadequate for sustenance in general. These products are of inorganic and organic nature.

The indispensability of the inorganic constituents of the diet was disclosed by the early researches in nutrition. The deficiency of certain elements such as calcium or phosphorus makes itself manifest very soon in growing animals. The presence of other inorganic elements in the food also exercises a very important physiological function.

The organic portion of the diet is limited by the character of the proteins as regards their amino-acid content. Willcocks and Hopkins [1906] and

Osborne and Mendel [1912, 1, 2, 3 and 1913] have shown that certain amino-acids are indispensable for existence and cannot be synthesised by the animal organism. Consequently these must be supplied in sufficiency through the medium of the consumed food in order to ensure the well-being of the animal.

When, therefore, any of the accessory food factors or the indispensable organic or inorganic constituents are eliminated from the diet, the animals cease to grow and eventually die. In the case of deficiencies in the antineuritic and antiscorbutic factors, as already pointed out, the animals succumb to clinically recognised diseases. The pathology of the other deficiencies is not so well defined. In all cases, however, where the diet is deprived partially or completely of an essential constituent the animals under experiment soon manifest the deficiency by a retarded rate or absolute cessation of growth, by restricted propagation and by a general unhealthy appearance, so that if any vital principle of the food is withheld for a prolonged period organic changes ensue which are very marked. But besides the radical changes produced by deficient nutrition already recorded it is quite possible that decided modifications may take place in the body tissues and fluids which are not discernible macroscopically or microscopically, but which may nevertheless restrict physiologically the functions of the organism. In this connection arises the interesting problem whether the resistance to disease of the animal is in any way influenced by deficient nutrition.

There is a general belief that underfed individuals are more susceptible to infection than well fed persons and that when the former contract a disease they show less resistance and are more prone to succumb to it. But terms like "malnutrition" or "underfed" are vague expressions and lack scientific definition. There is also no definite scientific evidence even of a general character to support this belief in spite of its probability. If, however, there is a connection between imperfect nutrition and susceptibility to disease the subject becomes one of the utmost theoretical and practical importance.

Even with our present knowledge of the various foodstuffs as regards their chemical constitution and content of the accessory food factors it becomes evident on reflection that the food of some people living under modern conditions may not contain an evenly distributed and adequate amount of all the vital factors which are necessary for normal existence. It is true that, excluding abnormal conditions like war or famine, the probability of a prolonged *total* deficiency of one or more essential principles from the diet is remote, but a prolonged partial deficiency or a rigorous deficiency lasting a short period is quite possible. The effect of such an irregularly balanced diet need not necessarily manifest itself by such drastic constitutional changes as those produced by the absolute deprivation of any essential principle for a long period, but it is only reasonable to suppose that certain systemic modifications may take place. This conjecture finds some support in the observations made by Zilva and Wells [1919] who found decided histological changes in the teeth of guinea-pigs kept on a scorbutic diet at a stage when the animal

was growing normally and was apparently in normal health. One may similarly imagine that the body cells and fluids may be altered at an early stage by deficient nutrition. It becomes necessary therefore to ascertain in the first place whether the immunity of an animal is affected by a rigorous and prolonged dietetic deficiency.

Immunity is a complicated biological phenomenon and our present knowledge of the subject is a collection of certain facts supposed to be connected with it, which are, however, insufficient to explain the actual mechanism involved. Immunity, therefore, is not very amenable to quantitative estimation. That it is a variable function is evident from empirical observations. Certain phenomena, however, like phagocytosis, complement fixation, agglutination, etc., connected with immunity can be estimated quantitatively for comparative purposes, and it is therefore possible to investigate the influence of diet on such phenomena.

So far there seems to be no record of any experimental work on this subject. It has been asserted by some observers that vegetarian races are less resistant to disease than races which eat meat. Even if that assertion be correct other differential conditions under which these races live would prevent one from ascribing the lower resistance of vegetarian races to their diet. Also the resistance of carnivorous animals towards certain diseases to which herbivorous animals are subject and *vice versa* cannot be entirely explained by the differential diet owing to the number of other factors concerned.

In studying the influence of diet on immunity one has to deal with a multitude of phenomena which offer a great variety of problems to be settled. As already mentioned a diet may be deficient by lacking one of the vital chemically known or chemically unknown principles, but the deficiency may also be of a multiple character. The conceivable changes in the factors connected with immunity which may be produced in the animal organism by any deficient diet are also numerous. The problem, therefore, is very complex and its investigation difficult.

In dealing with immunity one has to consider natural immunity and acquired immunity. The former is the inherent capacity of the animal to resist infection and the latter the immunity resulting from accidental or artificial infection previously sustained. The experimental study of natural immunity would have to be carried out by injecting standardised doses of a suitable lethal organism into animals fed on the various diets to be investigated and recording the relative mortality and time taken by the animals to succumb. In order to draw definite conclusions such experiments would require a great number of animals in each set. The evident drawback of such a plan of investigation is the amount of labour necessary for the preparation of purified diets for the great number of animals a systematic inquiry of such a nature would require. The task becomes almost impossible for a single experimenter to carry out. In investigating acquired immunity one is again confronted with the difficulty of choosing from the great number of factors

which undergo a change during artificial or accidental immunisation those most suitable for study.

In this investigation the study of the influence of deficient diets on the amboceptor and agglutinin formation and the complement content has been undertaken. Owing to the complexity of the subject the experiments carried out can only be considered of a more or less preliminary character. The results obtained although of an unexpected nature are nevertheless of general interest and suggest the necessity of further investigation.

The influence of the following dietetic deficiencies on the production of the above-mentioned antibodies was studied.

(a) The deficiency of each of the following elements Ca, Fe, K, Cl, P, and Na in the inorganic content of the diet.

(b) The deficiency of certain amino-acids.

(c) The deficiency of the antiscorbutic, antineuritic, and the fat-soluble A accessory factors.

The influence of a quantitatively restricted diet on the complement content was also investigated.

EXPERIMENTAL.

Rats were chosen for the investigation of the action of deficient proteins, restricted mineral salts and accessory food factors on the production of amboceptor and agglutinins. These animals are very suitable for the purpose as they readily consume the experimental diet. For the study of the influence of quantitatively restricted diet on the complement content of the blood and the effect of a scorbutically restricted diet on the production of amboceptor and agglutinins guinea-pigs were employed, because these animals are well adapted for complement production and are very susceptible to scurvy. In both these instances their ordinary basal diet of oats and bran was so adjusted that the requisite deficiencies were effected without making their food unpalatable. The rats employed were mostly of the albino variety and were specially selected either from stock bred in this laboratory or from healthy stock bought outside. The animals were kept separately in wooden boxes with perforated zinc tops. The bottoms of the boxes, except in some experiments to be mentioned later, were covered with a thin layer of sawdust. The diets were delivered daily in the form of weighed balls of suitable consistency. These were weighed back the following morning to ascertain the amount eaten. The drinking water which was kept in glass dishes was changed twice a day. All the animals were closely watched during the experiment, especially for scabies, and were weighed on the average about twice a week.

The guinea-pigs too were chosen from healthy stock, great care being taken to ascertain that they had not previously been used for any inoculation experiments. These animals were kept separately in wire cages and received daily measured quantities of their respective foods in clean glass dishes. The food remnants were measured back the following day.

The rats' rations were made up by thoroughly mixing all the constituents of the diet by means of a mechanical mortar. The mixtures were brought up to a convenient consistency by the addition of water and olive oil. The diets were made up twice weekly and stored in a refrigerating room until they were wanted so that no ration was older than 3 or 4 days.

The basal ingredients of the rats' food consisted of caseinogen, except in a few cases in which that protein was not compatible with the object of the experiment, starch and a mixture of mineral salts.

The caseinogen was obtained from a commercial firm. It was pale yellow and of light texture. For experiments on the accessory food factors it was previously purified. The purification consisted of shaking the caseinogen twice with ether and once with 95 % alcohol for several hours and filtering and drying. By testing the purified caseinogen on rats it was found to be free from the antineuritic and the fat-soluble *A* accessory factors.

Pure wheaten starch was employed. It was not found necessary to purify this ingredient as it was free from both the antineuritic and the fat-soluble *A* accessory factors.

The salt mixture was made up of pure reagents in the same proportion as that used by McCollum and his colleagues, and was constituted as follows:

NaCl	...	...	1.73 g.	CaH ₄ (PO ₄) ₂ ·2H ₂ O	...	...	5.4
MgSO ₄ (anhydr.)	...	...	2.66	Calcium lactate	...	...	13.0
NaH ₂ (PO ₄)·H ₂ O	...	...	3.47	Ferric citrate	...	...	1.18
K ₂ HPO ₄	...	...	9.54				

The fat-soluble *A* factor was supplied in the form of purified butter fat. Pure butter was melted at 40° and centrifuged. The supernatant layer of fat was then carefully syphoned off leaving behind the caseinogen, water and impurities.

Autolysed yeast was added to the diet to supply the antineuritic. Ordinary top fermentation brewer's yeast was pressed out in order to remove the adhering wort. It was then introduced into flasks plugged with cotton wool and placed in the hot room at 37° until it had liquefied. The autolysed mixture was then filtered through a Buchner funnel by means of the suction pump. The resulting filtrate was brown and fairly transparent, and was rich in the antineuritic factor. As the autolysed yeast solution contains a good deal of the inorganic constituents of the yeast it was necessary to treat it further when used for the experiments in which the inorganic constituents of the diet were limited. This treatment will be described later.

The antiscorbutic factor was introduced into the diet in the form of lemon juice treated according to the method described by Harden and Zilva [1918]. This solution has the advantage of containing almost all the antiscorbutic content of the lemon juice associated with little extraneous matter and can be used in very concentrated form if necessary.

In studying the production of the antibodies the animals were fed on the restricted diet for several weeks, after which time they were immunised by

three injections of increased doses of organisms with an interval of 7 days between each injection. The animals were bled out a week after the last injection. The blood was then allowed to clot and centrifuged and the supernatant serum employed for the estimation of the agglutinin and amboceptor.

The immunisation was carried out with the typhoid organism "Simpson." Young 24-hour cultures were emulsified for the purpose and the emulsion brought up to a dilution of 1000 million per cc. It was then killed by heating it at 60° for $\frac{1}{2}$ hour. The same batch of emulsion which was kept in a refrigerating room was used for each group of experiments. The animals were weighed on the day of the injection and their doses were calculated in proportion to their weight. All the doses were brought up in volume with saline to 1 cc., and were injected subcutaneously in the abdominal region.

In estimating the agglutinins a "Simpson" typhoid emulsion containing 2000 million organisms per cc. was used. The different dilutions of the sera and the emulsion were kept at a temperature of 37° for 1 hour and read off 15 minutes after and again the following day.

The amboceptor was estimated by means of the complement fixation. For that purpose $\frac{1}{2}$ cc. of each dilution of the serum plus $\frac{1}{2}$ cc. of the "Simpson" emulsion of the same strength as that used for the agglutination plus $\frac{1}{2}$ cc. of complement were kept for one hour at 37°, after which time to each tube was added $\frac{1}{2}$ cc. of washed sheep's blood corpuscles and $\frac{1}{2}$ cc. of haemolytic amboceptor. The tubes were then kept for another hour at 37° and removed to the refrigerating room until the following morning, when the readings were taken. With each set two control tubes containing 1 in 25 serum without emulsion and a double quantity of emulsion, namely 1 cc., without serum were also set up. On each occasion a stock antityphoid serum was titred out for agglutinins and complement fixing bodies to demonstrate the activity of the reagents employed.

For the complement fresh guinea-pig serum diluted $\frac{1}{10}$ with saline was used. The haemolytic amboceptor was titrated the day of the estimation and double the registered strength necessary for haemolysis was employed.

Before proceeding with the experiments it was necessary to show that by injecting normal guinea-pigs and rats with the "Simpson" typhoid emulsion the production of agglutinins and amboceptor could be effected. It was also necessary to ascertain the number of injections required for the production of suitable quantities of agglutinins and amboceptor normally so that a restricted production of these antibodies could be demonstrated by comparison. For that purpose the following experiment was carried out.

Rat No. 55, weighing 54 g., received an injection of 1 cc. containing 40 million "Simpson" typhoid organisms, and was bled out a week after the injection.

Rat No. 56, weighing 46 g., received an injection of 1 cc. containing 40 million organisms and a week after another cc. containing 80 million organisms and was bled out a week after the second injection.

Guinea-pig No. 313, weighing 402 g., received an injection of 1 cc. containing 250 million organisms, and was bled out a week after the injection.

Guinea-pig No. 314, weighing 383 g., received an injection of 1 cc. containing 250 million organisms and another injection of 1 cc. a week after containing 500 million organisms. A week after the second injection this animal was bled out. Table I gives the agglutination and amboceptor titres of the above animals.

Table I.

In this and all the other tables + indicates agglutination or lysis.

<i>Agglutination.</i>								
	1/25	1/50	1/100	1/200	1/400	1/800	1/1600	1/3200
Rat 55 (one injection)	+++	+++	+++	++	+	-	-	-
Rat 56 (two injections)	+++	+++	+++	+++	+++	++	++	-
Guinea-pig No. 313 (one injection)	+++	+++	+++	++	+	+	-	-
Guinea-pig No. 314 (two injections)	+++	+++	+++	+++	+++	++	+	-
Stock antityphoid serum	+++	+++	+++	+++	+++	+++	+++	++
<i>Amboceptor.</i>								
	1/25	1/50	1/100	1/200	1/400	1/800	1/1600	
Rat 55 (one injection)	+++	+++	+++	+++	+++	+++	+++	
Rat 56 (two injections)	-	+	+++	+++	+++	+++	+++	
Guinea-pig No. 313 (one injection)	+++	+++	+++	+++	+++	+++	+++	
Guinea-pig No. 314 (two injections)	-	-	+	+++	+++	+++	+++	
Stock antityphoid	-	-	-	-	-	+	++	

It is seen from the above table that both in the normal guinea-pig and rat a very marked production of agglutinins was achieved with two injections. The amboceptor production did not respond so well but a decided change in the titre was effected with two injections. It was, therefore, decided to submit the experimental animals in this investigation to three injections with an interval of one week. The strength of the doses was to be calculated according to the body weight and was to be as follows: first injection 1 million organisms per g. body weight, second injection 2 million organisms per g. body weight, and third injection 4 million organisms per g. body weight. Lower dilutions of the serums, namely $\frac{1}{5}$ and $\frac{1}{10}$, were also to be titrated for the amboceptors.

As all the deficiency diets investigated could not conveniently be tested together, the experiments were divided into various sets. Several animals were put on each diet and their blood was pooled in order to obviate the error of idiosyncrasy. To make conditions as nearly as possible equal the weights, sex and colour of the animals of each set were kept the same. Each

set had a group of rats on a control diet which was theoretically complete and the daily ration consisted of the following ingredients:

Basal mixture	10.6 g.
Purified butter	1.7 „
Autolysed yeast	5 cc.
Olive oil	0.25 „
Antiscorbutic equivalent to lemon juice	5 „

The basal mixture was made up of 75 % of starch, 20 % of caseinogen and 5 % of salts.

The above diet contains all the known accessory factors. For the sake of convenience, the fat-soluble *A*, the antineuritic and the antiscorbutic factors will be referred to in this communication as *A*, *B* and *C* respectively and the theoretically complete control diet as the *ABC* diet.

Fig. 1.

The first thing to ascertain was how rats fed on an *ABC* diet respond to injections in agglutinin and amboceptor formation in comparison with rats fed on an ordinary mixed diet. Two groups of rats each containing 3 animals were employed in this experiment. One group received an *ABC* diet, the other was fed on a mixed diet consisting of cabbage, boiled potatoes, scraps of bone and meat, biscuit meal, bread, carrots and boiled rice. After having subsisted for 6 weeks on the above diets the animals were immunised and bled out 9 weeks after the commencement of the experiment. The group receiving the *ABC* diet yielded 8 cc., the other group 9 cc. of blood. As will be seen from Fig. 1, which represents the growth curves of the two groups, the animals in both groups gained well in weight and the final weights were about the same in both cases. The increase in weight of the mixed diet group, however, was more erratic than that of the *ABC* group, which was most probably due to the lack of well-balanced uniformity in the diet of the former group, the

animals of which were left to choose their food from the offered mixture by instinct.

Table II gives the agglutination and amboceptor titres of the two groups. In both groups there was a very marked production of agglutinins and amboceptor, especially of the former, so that any differential production of these antibodies could not be missed. There is, however, no marked differentiation to be observed in the production of either of the antibodies. There is a slight difference in the amboceptor titres of the two groups, but it is of an order that may be neglected.

Table II.

	<i>Agglutination.</i>			<i>Amboceptor.</i>							
	1/800	1/1600	1/3200	1/10	1/25	1/50	1/100	1/200	1/400	1/800	1/1600
<i>ABC</i> purified diet	+++	+	-	-	-	+	++	+++	+++	+++	+++
Mixed diet	+++	+	-	-	+	++	+++	+++	+++	+++	+++
Stock anti-typhoid serum	+++	+++	+++	-	-	-	-	-	-	+	++

Having ascertained that rats fed on an *ABC* diet respond to injections in the formation of agglutinins and amboceptor under the conditions described equally well as rats fed on a good mixed diet, it was possible to employ this diet as a control in the study of the various deficient diets.

It was previously mentioned that it has been definitely established that the inorganic constituents of the food are indispensable for existence. Lately Osborne and Mendel [1918] made a detailed study of the influence of various inorganic elements in nutrition and found that calcium and phosphorus were the only elements which when singly reduced to very low limits had a retarding effect on the growth of rats. In the present investigation the study of the influence of a deficiency of iron, calcium, potassium, phosphorus, chlorine and sodium on the production of the immune bodies was attempted. For this purpose in devising the diets precautions had to be taken in order to reduce the presence of the respective elements to a minimum. This was accomplished in all the cases with the exception of the sodium and chlorine, in which owing to an oversight the amounts of those elements consumed were rather higher than was intended. In all the groups wheaten starch was used as it was found to be fairly free from the inorganic constituents under investigation. Caseinogen was employed for the protein portion of the diet with the exception of the group which received a phosphorus-free diet, in which case egg-white was used instead. The egg-white was obtained from a commercial firm and did not contain any phosphorus.

The autolysed yeast could not be used in the ordinary way to supply the antineuritic factor as it contained a good proportion of the inorganic constituents of the yeast. It was, therefore, treated in a manner which reduced its salt content to a minimum. The procedure was as follows. Five parts by volume of autolysed yeast were mixed with 95 parts of absolute alcohol and

well stirred. A viscous precipitate containing mostly proteins and their degradation products and salts was formed. This was filtered off yielding a clear yellow-coloured filtrate. The alcoholic filtrate was then evaporated to dryness *in vacuo* at 40° and the residue obtained dissolved in a volume of distilled water equivalent to the original juice. This solution was tested on a polyneuritic pigeon and was found to be active. 3 cc. of this active solution was given in the daily ration and was sufficient to keep a control group of rats in good health. The treated lemon juice which was used to supply the anti-scorbutic factor in the diet contained so little inorganic matter that it could be neglected. The purified butter with the exception of the sodium chloride adhering to it contained little inorganic matter. Unfortunately the sodium chloride supplied by this source, although very low, was overlooked and, as has already been mentioned, made the diets intended to be deficient in these elements contain rather significant traces of sodium and chlorine. The inorganic constituents of the various diets were made up as follows:

Table III.

	Diet low in Iron	Diet low in Calcium	Diet low in Potassium
	g.	g.	g.
NaCl	4.6	4.6	4.6
MgSO ₄ (anhydrous)	7.4	7.4	7.4
NaH ₂ PO ₄	9.4	9.4	9.4
K ₂ HPO ₄	25.8	25.8	—
CaH ₂ (PO ₄) ₂	14.6	—	14.6
Ca lactate	35.3	—	35.3
Ferric citrate	—	3.2	3.2

All the above were made up with starch to 100 g.

Diet low in Sodium.

KCl	6.0	CaH ₂ (PO ₄) ₂	14.6
MgSO ₄ (anhydrous)	7.4	Ca lactate	35.3
K ₂ HPO ₄	25.8	Ferric citrate	3.2

Made up to 100 g. with starch.

Diet low in Chlorine.

MgSO ₄ (anhydrous)	7.4	CaH ₂ (PO ₄) ₂	14.6
NaH ₂ PO ₄	9.4	Ca lactate	35.3
K ₂ HPO ₄	25.8	Ferric citrate	3.2

Made up to 100 g. with starch.

Diet low in Phosphorus.

NaCl	4.6	CaCO ₃	6.3
MgSO ₄ (anhydrous)	7.4	Na ₂ CO ₃	4.2
K ₂ CO ₃	20.0	Ferric citrate	3.2
Ca lactate	35.3		

Made up to 100 g. with starch.

The basal mixtures for these experiments consisted of 80 % of starch, 20 % of protein and 5 % of the respective salt mixtures. The diets were made up in the same proportions as the *ABC* diet with the exception that only 3 cc. of the treated autolysed yeast was given.

Samples of the diets were analysed for the various elements with the following results:

Diet low in iron			Absent
„	„	calcium ...	0.014 %
„	„	potassium ...	Undeterminable traces
„	„	sodium ...	0.014 % calculated from Cl
„	„	chlorine ...	0.021 %
„	„	phosphorus	0.05 %

All the rats in this experiment received distilled water to drink and were housed in boxes containing perforated zinc floors. It was not advisable to use sawdust in this instance as some of it might have been consumed with the food and thus formed a source for inorganic constituents.

For convenience these experiments were divided into two sets. In the first set the deficiency of iron, calcium and potassium was investigated. In the second set the deficiency of sodium, chlorine and phosphorus was studied. Each set had a control group of animals. Each group of the first set consisted of five rats. These animals were kept 85 days on the experimental diets. The group on diet low in Ca yielded 16 cc., in K 20.5 cc., in Fe 21.5 cc. and the control 19 cc. of blood. Fig. 2 shows that the animals receiving the diets deficient in Fe and K grew almost as well as the control group of rats, although the K group showed a slight decline towards the end of the experiment. On the other hand, the group deficient in Ca showed a decided restriction in their development. By examining Table IV it will be seen, however, that the agglutinins and amboceptor titres are almost identical in all the groups.

The second set of animals had 3 rats in each group. It will be seen from Fig. 3, that although the groups deficient in sodium and chlorine developed as well as the control in this set, the group receiving the diet low in phosphorus not only failed to show any growth but actually declined. In order to obviate the loss of the animals in this group the experiment was terminated after 54 days. The following quantities of blood were obtained. The sodium group yielded 9 cc., the chlorine 10 cc., the phosphorus 9 cc. and the control 9 cc. of blood. It is to be noticed that the phosphorus group although the lightest in weight yielded as much blood as the other groups. By examining Table V it is seen that no differentiation in the agglutinins and amboceptor formation in the groups which received the diets deficient in Na and Cl could be observed. The animals which were fed on the diet low in phosphorus showed a decidedly lower titre in the agglutinins and amboceptor. In fact hardly any amboceptor formation took place in this group. As will be seen from the other experiments which follow, this was the only group that showed a lower amboceptor titre.

In arranging the above diets it was impossible to eliminate from the basal

salt mixture any one element without at the same time altering quantitatively the content of some of the other constituents. This alteration was not of an important character as care was taken not to establish a second deficiency, and in view of the results obtained it can be ignored.

Table IV.

	<i>Agglutination.</i>					<i>Amboceptor.</i>				
	1/800	1/1600	1/3200	1/6400	1/25	1/50	1/100	1/200	1/400	1/800
Control diet	+++	+++	+++	+	-	++	+++	+++	+++	+++
Ca-free diet	+++	+++	+++	+++	-	+	+++	+++	+++	+++
K-free diet	+++	++	-	-	-	++	+++	+++	+++	+++
Fe-free diet	+++	+++	++	-	-	+++	+++	+++	+++	+++
Stock antityphoid serum	+++	+++	+++	+++	-	-	-	-	++	+++

Table V.

<i>Agglutination.</i>									
	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800		
Control diet	+++	+++	+++	++	++	++	+		
Diet low in phosphorus	+++	++	+	trace	-	-	-		
Diet low in sodium ...	+++	+++	+++	+++	++	+	-		
Diet low in chlorine ...	+++	+++	+++	+++	++	+	-		
Stock antityphoid ...	+++	+++	+++	+++	++	++	+		
serum									

<i>Amboceptor.</i>									
	1/5	1/10	1/25	1/50	1/100	1/200	1/400	1/800	1/1600
Control diet	+	++	++	+++	trace +++	+++	+++	+++	+++
Diet low in phosphorus	trace +++	trace +++	+++	+++	+++	+++	+++	+++	+++
Diet low in sodium	-	-	+	+	+++	+++	+++	+++	+++
Diet low in chlorine	-	-	+	trace +++	+++	+++	+++	+++	+++
Stock antityphoid serum	-	-	-	-	-	+	++	++	+++

On examining the composition of the diet low in phosphorus it is seen that two other factors were also altered. The salt mixture was slightly more alkaline owing to the higher content of the carbonates of sodium and potassium which replaced the phosphates of those salts, and the caseinogen was replaced by egg-white, which from observations made in control experiments does not seem to be as good a source of protein as caseinogen. As the result obtained with this group is interesting the subject will have to be studied further before definite conclusions can be arrived at.

The next thing to be investigated was the influence of diets deficient in certain amino-acids on the production of the antibodies in question. Two sets

of experiments were devoted to this subject. In the first set a low intake of caseinogen was studied, in the second the action of gliadin as the sole source of protein in the diet.

Osborne and Mendel [1915] were the first to demonstrate that if caseinogen formed 18 % of a diet it was adequate to ensure normal growth in rats; if, however, the content of that protein in the diet was reduced to 9 % the rate of growth of the animals was greatly impaired. The explanation was advanced that cystine was the limiting factor, caseinogen having a low cystine content. When it forms as much as 18 % of the diet the amount of cystine supplied through its medium is sufficient to satisfy the requirements of the animals, when, however, it forms only 9 % of the diet the amount of cystine consumed becomes deficient and manifests itself by the restricted growth of the animals. This explanation was confirmed by the fact that the addition of cystine to a low caseinogen diet raised its nutritive value to the same level as that of lactalbumin.

Fig. 4

In order to study the deficiency of cystine, diets of low caseinogen content were used in this investigation. Three groups of rats receiving 20 %, 12 % and 8 % respectively, were used. With the exception of the varied quantities of the caseinogen and starch the diets were in other respects similar to the *ABC* diets. Six animals were started in each group, but only two survived in the group receiving a diet with 8 % of caseinogen. Fig. 4 gives the weight curves of the three groups. It is seen that the group which received the 8 %

caseinogen diet did not grow at all. The group fed on the 12 % caseinogen diet grew but did not do as well as those on the 20 % diet. When killed 23 cc. of blood was collected from the group of six rats fed on the 20 % caseinogen diet, 15 cc. from the group of six rats which received 12 % diet, and $4\frac{1}{2}$ cc. from the group of two rats which survived on the 8 % diet. Table VI shows that no marked difference can be observed either in the agglutinins or in the amboceptor formation of the three groups in spite of the restricted growth which the groups receiving 12 % and 8 % of caseinogen manifested.

Table VI.

	<i>Agglutination.</i>					<i>Amboceptor.</i>		
	1/800	1/1600	1/3200	1/10	1/25	1/50	1/100	1/200
Diet containing 20 % caseinogen	+++	+++	++	-	+	+++	+++	+++
Diet containing 12 % caseinogen	+++	+++	-	-	+	+++	+++	+++
Diet containing 8 % caseinogen	+++	++	-	-	++	+++	+++	+++
Stock antityphoid serum	+++	+++	+++	-	-	+	++	+++

According to Osborne and Mendel [1914, 1916], gliadin as the sole protein in a diet suffices to maintain rats, but is insufficient to promote growth in young animals unless it is supplemented by lysine, an amino-acid which this protein does not contain. The influence of gliadin was studied in this investigation on a set of rats containing one group receiving an *ABC* diet with caseinogen as the protein and another group having the caseinogen replaced by gliadin.

The gliadin was prepared from wheat flour by the method described by Osborne [1910]. The product was precipitated twice with water and once with absolute alcohol and was thus carefully purified. The basal mixtures in these experiments were made up as follows:

Control diet.

Starch 77 %, Caseinogen 18 %, Salts 5 %.

Gliadin diet.

Starch 77 %, Gliadin 18 %, Salts 5 %.

The above basal mixtures were mixed with the necessary ingredients in proportion already described to form an *ABC* diet.

The weight curves of the two groups (Fig. 5) show that rats which were fed on the gliadin diet failed to grow, thus confirming the observations of Osborne and Mendel. Six animals were started in each group, but only four survived in the gliadin group and consequently the blood of four animals in each of the above groups was pooled for examination. These rats were immunised after 38 days instead of 6 weeks as it was feared that more animals

in the gliadin group might die if the experiment were prolonged. The animals in the gliadin group yielded 9 cc. of blood, the four animals in the control group yielded 16 cc. of blood. Table VII shows that the gliadin diet made no difference to the agglutinin and amboceptor production, although the animals in this group showed no growth owing to the restriction in the diet. The view that the nutritive restriction of gliadin is due to the lack of lysine is not altogether supported by the observations of McCollum, Simmonds and Pitz [1917] and Geiling [1917]. The former found that when maize kernel, which is poor in lysine, is supplemented by gelatin, which is very rich in that amino-acid, the nutritive value of the maize is not enhanced. The latter is led to believe by his investigation that lysine is not indispensable for existence of the mouse. Whatever the deficiency of gliadin may be due to it does not inhibit the production of agglutinins and amboceptor.

Fig. 5

Table VII.

	Agglutination.				Amboceptor.				
	1/800	1/1600	1/3200	1/6400	1/100	1/200	1/400	1/800	1/1600
Control diet containing 18 % caseinogen	+++	+	-	-	-	+	+++	+++	+++
Diet containing 18 % gliadin	+++	++	-	-	-	+++	+++	+++	+++
Stock antityphoid serum	+++	+++	++	-	-	-	+	++	+++

The following sets of experiments were devoted to the investigation of the influence of the lack of the accessory factors. Observations were carried out on animals subsisting on diets deficient in the antineuritic, fat-soluble *A*, and antiscorbutic factors. McCollum and Davies have revealed by their researches that rats lacking the antineuritic factor in their diet fail to grow and die after about 6 weeks, often manifesting decided neuritic lesions. This was confirmed by Drummond [1917] and by Harden and Zilva [1918, 2]. If these animals are deprived of the fat-soluble *A* factor they may show a certain amount of growth during the initial stage of the experiment, but eventually also cease to develop and die. In most of these cases the animals display a condition of the eyes resembling xerophthalmia. Although rats do not actually manifest scorbutic lesions when kept on a scorbutic diet there seems according to Harden and Zilva [1918, 2] and Drummond [1919] to be evidence that they do not thrive as well on such diets as they do when the antiscorbutic factor is supplied. The standard *ABC* diet used in these experiments contained all the three factors. The *AB* diet was deprived of the treated lemon juice, the *BC* diet of the butter fat, and the *AC* diet of the autolysed yeast. They were made up as follows:

AB diet daily ration.

Basal mixture	...	...	10 g.	Autolysed yeast	...	...	5 c.c.
Purified butter	...	...	1.85 g.	Olive oil	...	...	2.5 c.c.

BC diet daily ration.

Basal mixture	...	...	12 g.	Antiscorbutic, equivalent to			
Autolysed yeast	...	...	5 c.c.	lemon juice...	...	...	5 c.c.
				Olive oil	...	...	0.75 c.c.

AC diet daily ration.

Basal mixture	...	...	10 g.	Centrifuged butter fat	...	1.8 g.
Antiscorbutic, equivalent to				Olive oil	...	1.6 g.
lemon juice	...	...	5 c.c.			

The basal mixtures in all the above diets were of the same composition as the control *ABC* diet already described.

As these experiments were intended to last 9 weeks the majority of the animals subsisting on the *BC* and *AC* diets would by that time have succumbed to the deficiency and thus frustrated the object of the experiment. It was, therefore, found necessary to add at times small quantities of butter and autolysed yeast to the respective groups in order to prolong the existence of the animals, but the quantities of these addenda had to be of an order insufficient to promote normal growth. It will be seen from Fig. 6 that the *ABC* group grew best, the *AB* group did almost as well, while the *BC* group and more so the *AC* group show restricted development. Both these groups exhibited the characteristic appearance of animals existing on restricted diets. Five rats were used in each group of this experiment. By examining Table VIII it is seen that there was no change in the amboceptor production, but the

agglutinin titres showed a great variation, none of the groups receiving the deficient diets producing as much as the control group. Taking this as an indication that the absence of the accessory factors from the diets influences the agglutinin production in the animals it became desirable to repeat the experiment. A set of animals, therefore, similar to the above were again submitted to the same treatment as the first, but as will be seen from Table IX no differentiation in the agglutinin titres was obtained the second time. This would suggest that the impaired agglutinin formation could not be ascribed to the consumption of the deficient diets. Another experiment about to be described gives further support to this view.

Fig. 6

Table VIII.

	<i>Agglutination.</i>						<i>Amboceptor.</i>							
	1/100	1/200	1/400	1/800	1/1600	1/3200	1/10	1/25	1/50	1/100	1/200	1/400	1/800	1/1600
<i>ABC</i> purified diet	+++	+++	+++	+++	+	-	-	++	+++	+++	+++	+++	+++	+++
<i>AB</i> purified diet	+++	+++	+	-	-	-	-	-	++	+++	+++	+++	+++	+++
<i>BC</i> purified diet	+++	+++	+	-	-	-	-	-	+	++	+++	+++	+++	+++
<i>AC</i> purified diet	+++	+	-	-	-	-	-	+++	+++	+++	+++	+++	+++	+++
Stock antityphoid serum	+++	+++	+++	+++	+++	+++	-	-	-	-	-	-	-	-

Table IX.

	<i>Agglutination.</i>					<i>Amboceptor.</i>				
	1/3200	1/6400	1/10	1/25	1/50	1/100	1/200	1/400	1/800	1/1600
<i>ABC</i> purified diet	+++	-	-	-	++	+++	+++	+++	+++	+++
<i>AB</i> purified diet	+++	+	-	-	++	+++	+++	+++	+++	+++
<i>BC</i> purified diet	+++	-	-	+	+++	+++	+++	+++	+++	+++
<i>AC</i> purified diet	+++	++	-	-	+	+++	+++	+++	+++	+++
Stock anti-typhoid serum	+++	+++	-	-	-	-	+	+	++	+++

It has already been mentioned that rats kept on a scorbutic diet do not show scorbutic lesions. It was, therefore, of interest to study the influence of the antiscorbutic deficiency on the production of the antibodies in guinea-pigs, which are very susceptible to scurvy. These animals, if kept on a scorbutic diet, manifest signs of scurvy after about 9 days and succumb to the disease about a month after. After about 15 days these animals usually cease to grow, and eventually decline in weight. It is possible by administration of small doses of antiscorbutics just to maintain the weights of the guinea-pigs and keep the animals alive in this way for months. Animals in such a condition are decidedly scorbutic, as can be proved by chloroforming them and holding a post mortem examination. By taking advantage of this fact it was possible to investigate the subject on guinea-pigs. Three groups of guinea-pigs were employed in this experiment. One group received a basal diet of oats and bran and 40 cc. of autoclaved milk and occasional doses of orange juice given *per os* at intervals sufficient to maintain the weight of the animals constant. The second group received a quantitatively restricted mixed diet containing plenty of antiscorbutics. The food was given in quantities sufficient to maintain the weights of the animals, but insufficient to permit growth. It consisted of a daily ration of 5 g. of cabbage, 10-20 g. of bran and 30 cc. of fresh milk containing 10 cc. of treated lemon juice. The third group received oats, bran, milk, cabbage and roots *ad lib*. All the animals were kept 73 days on their diets. The animals received 250 million organisms in the first, 500 million in the second, and 1000 million in the third injection per 400 g. of body weight. Fig. 7 shows the weight curves of the three groups. Four animals were bled out in each group and the unrestricted mixed diet group yielded 48 cc., the restricted mixed diet group 31 cc. and the scorbutic group 26 cc. All the animals of the scorbutic group showed well-marked signs of scurvy, whereas all the animals of the other groups were entirely free from any trace of scorbutic lesions. The agglutination and amboceptor titres, which are represented in Table X, do not show any differentiation in the three groups. These results go further to support the view that the impaired agglutinin production observed

in the first set of rats fed on the diets deficient in the accessory factors was not due to the diet.

It is well known that guinea-pigs form a good source of complement and it was of interest to ascertain whether, if these animals are restricted in their diet, the complement activity of their blood is impaired. The blood of the animals of the three groups described above was therefore tested for its complement content. Varying quantities of the serums were added to tubes containing a haemolytic amboceptor and washed blood corpuscles. As Table XI discloses, no variations in the titre could be observed, thus showing that the bloods of the three groups were of almost the same activity.

Table X.

	<i>Agglutination</i>					<i>Amboceptor</i>								
	1/200	1/400	1/800	1/1600	1/3200	1/10	1/25	1/50	1/100	1/200	1/400	1/800	1/1600	
Scorbutic diet	+++	+	-	-	-	-	+	++	+++	+++	+++	+++	+++	
Restricted mixed diet	+++	++	-	-	-	-	+	++	+++	+++	+++	+++	+++	
Unrestricted mixed diet	+++	++	-	-	-	-	-	+	+++	+++	+++	+++	+++	
Stock antityphoid serum	+++	+++	+++	+++	+++	-	-	-	-	-	-	-	-

Fig. 7

Table XI.

Complement $\frac{1}{4}$			0.2 c.c.	0.1 c.c.	0.05 c.c.
Scorbutic diet	...	...	+++	++	-
Restricted mixed diet	...	...	+++	++	-
Unrestricted mixed diet	...	...	+++	+++	-

Table XII.

Complement $\frac{1}{4}$		0.2 c.c.	0.1 c.c.	0.05 c.c.	0.025 c.c.	0.01 c.c.
Restricted mixed diet	...	+++	+++	++	-	-
Unrestricted mixed diet	...	+++	+++	-	-	-

Another set of experiments on the subject was instituted with the object of ascertaining whether a longer period of dietetic restriction would influence the activity of the complement.

Fig. 8

Two groups of six guinea-pigs each, kept on the experimental diet for 202 days, were investigated. One group received a quantitatively restricted mixed diet and was of the same nature as the one described above. The other group received a mixed diet *ad lib*. Fig. 8 shows their weight curves which are similar in character as those of the previous experiment. At the end of the experiment these animals were bled out and their blood was pooled. The restricted mixed diet group yielded 67 cc. and the unrestricted mixed diet group 111 cc. of blood. As will be seen from Table XII the prolonged dietetic restriction did not influence the complement content of the blood of these animals.

CONCLUSION.

Of all the groups of animals tried on the various deficient diets only the group which received a diet deficient in phosphorus showed a decidedly poorer response to the inoculations in the production of amboceptor and agglutinins. One set of animals subsisting on diets deficient in the accessory food factors showed a low production of agglutinins only, but as this did not agree with the results obtained on guinea-pigs fed on a scorbutic diet this particular experiment was repeated. The results obtained on repetition failed to confirm the differential production of the agglutinins in rats fed on diets deficient in the accessory food factors. On the other hand, the majority of the groups of animals fed on deficient diets failed to show any difference in the production of both agglutinins and amboceptor. This becomes more remarkable when it is seen that some of the groups owing to the dietetic deficiency manifested very restricted growth or no growth at all and were altogether in a much poorer condition than the control animals. It is true that none of these experiments has been repeated, but we have several instances in this investigation where owing to various nutritional deficiencies the animals have displayed a very inferior condition to the control rats fed on a theoretically complete diet and yet have shown no differential production of the antibodies. The results of the groups fed on the 12 % and 8 % caseinogen diets, on the diet low in calcium and on the gliadin diet may be cited in this connection. It is, therefore, interesting that the group of animals fed on a diet low in phosphorus should be the only one showing a well marked differentiation in their agglutinins and amboceptor titres. This, of course, invites further investigation in order to establish the fact definitely. The results of the experiment can at present only be considered as an indication.

This investigation has only dealt with two antibodies produced under certain definite conditions. What actual bearing these antibodies have on immunity is not well established, but evidently they have some unexplained indirect connection with it, as the Widal's reaction seems to suggest in the case of agglutination. On the whole the subject is still very obscure. In the case of bacteriolysins it is known that as a result of infection or inoculation the corresponding bacteriolytic amboceptors are formed. This is demonstrated by bacteriolysis *in vitro* and *in vivo*; yet this bacteriolytic power of the blood of the organism does not remain, while the increased resistance to the disease is sometimes established for years. It is, therefore, plain that the actual capacity of the animal for antibody formation cannot be taken as a definite indication of the power to resist disease, although in conjunction with other observations it may give valuable information on the subject. A systematic study of the influence of nutrition on the production of immunity, although complex and laborious, is urgently called for and may not only prove to be of direct clinical value but may elucidate many obscure points in the complicated mechanism of immunity.

SUMMARY.

The influence of deficient diets on the production of agglutinins and amboceptor in rats was investigated.

The following deficiencies were studied.

1. Diets low in iron, calcium, potassium, sodium, chlorine and phosphorus.
2. Diets containing 12 % and 8 % of caseinogen as a source of protein.
3. Diets containing 18 % of gliadin as the sole source of protein.
4. Diets deficient in each of the three accessory food factors.

Although several of the deficiencies became manifest by the restricted growth and the poor condition of the animals, no differentiation in the titres of the agglutinins and amboceptor could be recorded, except in the group receiving the diet low in phosphorus. Guinea-pigs fed on an unrestricted mixed diet, quantitatively restricted mixed diet and a scorbutic diet showed no differentiation in the amboceptor and agglutinin titres, and in the complement activity of the blood.

Guinea-pigs kept on an unrestricted mixed diet and a quantitatively restricted diet for about 6 months showed no differentiation in the complement activity of the blood.

In conclusion, I wish to express my gratitude to Dr H. Schütze for the valuable help and advice he has given me in connection with the bacteriological work of this investigation. Dr Schütze has been good enough to check independently all the readings of the agglutinin and amboceptor titres.

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