CCCXXXIII. THE EFFECT OF INCOMPLETE DIETS ON THE CONCENTRATION OF ASCORBIC ACID IN THE ORGANS OF THE RAT.

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WITH AN APPENDIX ON THE IDENTITY OF ASCORBIC ACID
AND THE REDUCING AGENT OF RAT'S GUT.

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I. EXPERIMENTAL RESULTS.

THE circumstance that certain animals, of which the rat is a typical example, synthesise ascorbic acid in the course of their metabolism raises questions of some scientific interest concerning the nature of its precursors and the seat or seats of its formation in the body. Experience with synthetic diets indicates that its precursors are derived from the main foodstuffs rather than from any minor constituent of the diet, and its constitution suggests of course that these would arise more directly from carbohydrate and perhaps also, less directly, from protein. As a preliminary to any attempt to determine the nature of possible intermediate products it seemed worth while to seek further information concerning its primary origin in the diet. Following Ray [1934], who studied its origin in plant seedlings, Guha and Ghosh [1934] carried out experiments *in vitro* which led them to believe that, as in the seedling, mannose is a preferential precursor for its synthesis in various tissues of the rat (see Section II). Neither mannose nor possible precursors of that sugar were present in the diets of which the effects were studied in the present research.

The paper describes experiments meant to determine the effect of varying the main constituents of the diet upon the concentrations of the vitamin in the liver and small intestine respectively. That it is normally present in the latter is now well known [Hopkins, 1934; Harde and Wolff, 1934; Zilva, 1935; *et al.*]. The estimations were made by the now familiar method of titration with 2:6-dichlorophenolindophenol in acid solution. Details of the method as employed, together with some comments upon certain apparent limitations in its application to animal tissues, will be found in the succeeding section. The animals chiefly used were male albino rats of weights not less than 150 g.

The results of the experiments show that certain fundamental modifications in the food supply, such for instance as the total omission of carbohydrate, or feeding on carbohydrate alone, are almost immediately followed by readjustments in the concentration, and especially in the relative concentrations, of the vitamin in the two organs studied. So rapid are these adjustments that significant changes are observed after the animals have received certain experimental diets for a few days only. By confining their administration for the most part to such brief periods less direct and therefore less significant results, due to the possible effects of abnormal one-sided diets in depressing the processes of general metabolism, have been avoided.

That the concentration of a vitamin in any organ would prove to display some degree of individual differences was to be expected. Even in circumstances apparently uniform variations may well be correlated with other differences in the metabolic condition of animals which are not obvious on inspection. The figures obtained in the following experiments display a scatter which is in some cases fairly wide; but, in general, with respect to the absolute concentrations in the two organs rather than to the relation between these concentrations. It is the latter which is of chief significance to conclusions based upon the results.

It was desirable, in any case, that the numerical data obtained should be submitted to statistical analysis. This has been most kindly carried out by Mr John Wishart of the Cambridge School of Agriculture. The following are the essentials of his report given nearly in his own words.

The most accurate comparisons can be made from the data which concern the ascorbic acid present in the liver and gut of the same animal, for in this comparison other possible sources of disturbance such as age, weight, genetic constitution *etc.* of the rats are standardised. The analysis deals first therefore with consideration of the significance which is attached to the average differences found between the liver and gut concentrations.

The method used for testing the significance of the results was that of "Student" and is expounded by Fisher [1925; 1935]. The assemblies of the organs examined may be treated as "populations" and the problem is to decide for or against an initial assumption (the "null hypothesis" of Fisher) that with respect to the attribute under study the two populations are identical.

If each gut-liver difference is denoted by x , and there are p such differences, the ratio (t) of the mean difference to its estimated standard error is calculated from the formula

$$t = \bar{x} \div \sqrt{\frac{\sum (x - \bar{x})^2}{p(p-1)}},$$

where $\bar{x} = \Sigma(x)/p$, and Σ denotes in both cases summation over p values. By consulting Fisher's table of t , with n (number of degrees of freedom) equal to $p-1$, we are enabled to approximate to the probability (P) that a difference equal to or greater than $\bar{x}$ should occur in a random sample from a population in which the true difference is zero. Conventionally we may reject this "null hypothesis" concerning the population when P is less than 0.01. This is expressed by saying that the difference is significant.

The data assembled in the tables which follow, in so far as they are assumed to prove a significant difference between the liver and intestine, are found on analysis fully to justify that assumption. In every such case the probability P (as above defined) is less than 0.0001. Other references to the results of the analysis appear later.

Effects due to a brief withdrawal of food.

Before placing the rats upon the experimental diets it was at first thought desirable to withdraw food for a short period in order to eliminate the influence of previous feeding. When however the effects of this abstinence itself were tested, an unexpected result came to light. When animals were kept without food for 48 hours and compared with others on a normal diet, a sharp rise in the concentration of the vitamin in the intestine was found to result from the fast, whilst the hepatic concentration was by then scarcely affected. It was impossible on this account to obtain on such lines basal values for the normal relation between the concentrations in these organs. The values found for rats taken from stock and receiving a mixed diet of oats, wheat grains and bread and milk were

therefore employed as "normals" from which to measure the amounts of change induced by the experimental diets. In such stock animals variation is considerable, but the average values obtained proved to be fully adequate for the purpose of comparison.

In Table I will be found a comparison of the values obtained from animals on the stock diet and those from others which had been deprived of food for 2 days (though never longer). The latter of course were supplied with water.

Table I.

Stock diet			48 hours without food		
Weight of rat g.	Ascorbic acid mg./g.		Weight of rat g.	Ascorbic acid mg./g.	
	Liver	Gut		Liver	Gut
200	0.28	0.25	—	0.31	0.46
270	0.20	0.25	—	0.26	0.45
100	0.20	0.25	180	0.20	0.25
—	0.26	0.22	—	0.23	0.41
190	0.29	0.29	—	0.30	0.37
175	0.26	0.23	175	0.36	0.37
—	0.23	0.23	168	0.23	0.30
—	0.22	0.26	150	0.31	0.41
195	0.29	0.34	205	0.25	0.38
160	0.30	0.32	150	0.31	0.41
135	0.23	0.30	155	0.22	0.33
—	0.26	0.24	—	0.27	0.42
185	0.38	0.36	201	0.25	0.43
—	0.30	0.20	160	0.29	0.43
160	0.27	0.25	—	0.17	0.24
163	0.25	0.22	—	0.31	0.43
—	0.26	0.27	162	0.20	0.31
205	0.27	0.26	—	0.27	0.38
			191	0.30	0.42
Average	0.26	0.26	Average	0.27	0.40
Median	0.26	0.25	Median	0.27	0.41

It will be seen that in animals on a complete diet the average concentration in liver and gut is the same, 0.26 mg./g. Analysis of the figures as a whole confirms the fact that there is no difference of significance between them. Equality, or approximate equality, in these two organs seems indeed to be characteristic of normal nutrition. This tends to be the case in spite of individual variations in the absolute concentrations. After the brief abstinence on the other hand in which the average for the liver is scarcely affected, that for the gut rises to 0.40 mg./g.

Inspection of Table I will show that in the case of animals on the normal diet a significant departure from equality in the organs is seen in two only out of eighteen. On the other hand after abstinence the intestinal concentration is significantly higher than that in the liver in all save two out of nineteen. This noteworthy effect of fasting will receive further reference. Its occurrence illustrates the rapidity with which the concentration of ascorbic acid in an organ may be affected by a change in nutritional conditions.

Effects of incomplete diets.

The diets administered consisted respectively of carbohydrate alone (starch and cane sugar); fat alone (filtered butter and lard); protein ("light white casein") *plus* fat; protein *plus* carbohydrate; fat *plus* carbohydrate. A normal

salt mixture was added to each, but because of the short duration of the experiment no vitamin preparations were supplied. During the periods of administration the carbohydrate diet, and those containing protein and fat and protein and carbohydrate respectively, were well eaten, a normal daily calorie value being consumed. The consumption of the carbohydrate and fat mixture was somewhat less but adequate. The fat was always well eaten at first, though an occasional rat ate sparingly after the third or fourth day. The consumption of a pure protein diet was unsatisfactory, and estimations on this were discontinued.

Although abstinence from food involves the intestinal effect just discussed, experiments in which the diets were given for 3 days after the 2 days' fast proved nevertheless to be informative and were continued. In the tables the results of these appear side by side with data from other experiments in which the diets were fed for 5 days without a previous fast. A few results obtained when the administration was longer will be discussed.

It will be seen from what follows that were the changes found in the hepatic concentration considered alone there would be some grounds for the assumption that the vitamin is made in the liver from precursors derived from carbohydrate. When however the behaviour of the intestine is also considered it becomes clear that this assumption does not by itself cover all the facts.

In Table II will be found the concentrations found in the organs of rats fed upon a mixture of three parts starch and one part cane sugar. The results of 3 days' administration after the 48 hours' fast were identical with those following on 5 to 7 days of the diet without previous fasting. Whilst the concentration in the gut remained normal that in the liver was on the average some 30 % higher. This is not a large variation but the results are consistent, and analysis shows that they are significant. Individual rats showed little departure from the average, especially among the group fed without a fast, and all but two animals out of the nineteen fed show the increase in the liver and a concentration there greater than that in the gut.

Table II. *Carbohydrate diet.*

3 days on diet after 2 days' fast			5 to 7 days on diet without previous fast		
Weight of rat g.	Ascorbic acid mg./g.		Weight of rat g.	Ascorbic acid mg./g.	
	Liver	Gut		Liver	Gut
240	0.43	0.29	170	0.34	0.22
200	0.38	0.28	180	0.34	0.26
—	0.39	0.27	190	0.31	0.23
150	0.35	0.24	140	0.35	0.28
165	0.35	0.26	190	0.27	0.23
155	0.36	0.24	240	0.32	0.22
190	0.34	0.22	150	0.33	0.22
—	0.31	0.23	190	0.31	0.28
—	0.23	0.20	215	0.31	0.25
			215	0.31	0.23
Average	0.35	0.25	Average	0.32	0.24
Median	0.35	0.24	Median	0.32	0.23

Quite different were the results when carbohydrate-free diets were given. In Table III are figures from rats fed on a mixture of two parts protein and one part fat. When this diet was eaten after the 2 days' fast, the average hepatic

Table III. *Protein and fat diet.*

3 days on diet after 2 days' fast			5 days on diet without previous fast		
Weight of rat g.	Ascorbic acid mg./g.		Weight of rat g.	Ascorbic acid mg./g.	
	Liver	Gut		Liver	Gut
—	0.16	0.40	180	0.26	0.40
180	0.21	0.35	215	0.25	0.37
—	0.14	0.44	170	0.24	0.38
170	0.23	0.48	215	0.25	0.41
160	0.17	0.32	185	0.25	0.34
160	0.16	0.32	175	0.26	0.44
—	0.20	0.27	190	0.30	0.39
180	0.19	0.41	180	0.25	0.38
—	0.17	0.35	—	0.24	0.37
160	0.23	0.34	—	0.26	0.41
160	0.21	0.46	Average	0.25	0.39
190	0.21	0.31			
170	0.21	0.31			
195	0.20	0.38			
195	0.23	0.33			
Average	0.19	0.37			
Median	0.20	0.35			

concentration on the third day, instead of rising as on the carbohydrate diet, was 0.19 mg./g. only, a figure significantly below the normal. At the same time the intestinal concentration rose sharply. Still confining attention to the liver it should be noted that in rats fed upon the diet for 5 days the concentration in the organ had become normal. That this rise after a preliminary fall is a genuine phenomenon is suggested by the consistency of the data and by the fact that like the earlier fall it is also seen in the case of the other carbohydrate-free diet employed, that containing fat alone (*v. infra*). It seems legitimate to correlate this rise with that adjustment of the liver to a carbohydrate-free diet which is indicated by the early disappearance of the ketosis first induced by such diets. Wigglesworth [1924] showed that in rats on a diet of fat this adjustment occurred about the fifth day. Gemmill and Holmes [1935], moreover, have shown that the glycogen content of the liver of rats fed on butter falls practically to zero on the first day, but gradually rises till on the fourth and fifth days it reaches a level of about 1%. It is justifiable to expect that if ascorbic acid is made from carbohydrate in the liver, the velocity of its formation will increase with increase in the glycogen present up to some normal limit. This limit may be of the low order of the 1% found by Gemmill and Holmes at the fourth or fifth day of a diet of fat. A few estimations of glycogen were made on the livers of rats employed in the experiments just described. Those of two rats which had received the protein *plus* fat diet for 3 days after the 2 days' fast contained 0.31 and 0.43% glycogen respectively (ascorbic acid 0.20 mg./g.); whilst the livers of two others after 5 days on the same diet without the fast contained 1.84 and 1.98% (ascorbic acid 0.26). Within higher ranges of hepatic glycogen, such as were found on diets containing carbohydrate, there was no correlation between it and the vitamin concentrations (see next section). On a carbohydrate-free diet, however, this normal hepatic concentration of the vitamin was not long maintained. Table IIIA gives data for rats kept for 12 days on the protein and fat diet which was consumed in almost normal amounts during that period. By then the average hepatic concentration is seen to have fallen again to the low figure

Table IIIA. *Protein and fat diet, 12 days.*

Weight of rat g.	Ascorbic acid, mg./g.	
	Liver	Gut
150	0.18	0.28
—	0.20	0.32
210	0.20	0.28
—	0.14	0.25
—	0.13	0.23
230	0.20	0.35
200	0.19	0.30
Average	0.18	0.29

of 0.18 mg./g. At the end of this period the intestinal concentration was also lower, but it remained somewhat above normal and much above that in the liver.

Table IV. *Fat diet.*

3 days on diet after 2 days' fast			5 to 7 days without fast		
Weight of rat g.	Ascorbic acid mg./g.		Weight of rat g.	Ascorbic acid mg./g.	
	Liver	Gut		Liver	Gut
150	0.20	0.36	240	0.28	0.40
—	0.21	0.36	145	0.28	0.35
—	0.20	0.32	190	0.31	0.41
210	0.13	0.21	220	0.30	0.28
195	0.17	0.28	180	0.23	0.37
—	0.17	0.27	190	0.26	0.31
165	0.18	0.27	190	0.27	0.37
—	0.16	0.34	200	0.24	0.40
Average	0.17	0.30	Average	0.27	0.36

In Table IV are the data for rats on a diet of fat alone. Protein and fat together form, as might be expected, a more satisfactory diet for testing the effect of lack of carbohydrate than one of fat only; but the results of feeding the latter, though fewer animals were employed, confirmed in essentials the most notable effects of the former. The food was less regularly consumed than when the fat was associated either with protein or carbohydrate. It was always eaten freely during 3 days after a fast, but on the fifth day without the preliminary fast an occasional rat was found to have its stomach and intestine empty. Such cases were rejected. It will be seen from Table IV that the group of eight animals fed on fat after the brief fast displayed that low concentration in the liver which characterised the protein *plus* fat diet, namely an average of 0.17 mg./g. only. Again, as on the protein and fat diet, the group of eight fed for 5 days without the fast displayed the secondary rise discussed above.

The results so far considered, showing that a diet of carbohydrate alone induces a relatively high concentration of the vitamin in the liver, whilst the immediate effect of diets lacking carbohydrate is a significant fall, suggest at least what is *a priori* likely, namely, that carbohydrates are the source of its precursors, and further that the liver, alone or in common with other organs, is a seat of its formation.

The behaviour however of the intestinal concentration when these diets are consumed requires full consideration. During the periods when the carbohydrate diet was consumed it remained essentially at the level proper to a mixed dietary and was therefore below the hepatic concentration. In marked contrast

the diet of protein and fat induced a rise in the gut comparable with that which followed the 2 days' abstention. The animals which had consumed this diet for 3 days after the fast showed an intestinal concentration of 0.37 mg./g. This might perhaps have been attributed to a survival from the influence of the fast, but for the fact that animals which had taken the diet for 5 days without a previous fast displayed a similarly high concentration in the gut—0.39 mg./g. (Table III). All the observations have shown, moreover, that the adjustments of vitamin concentrations to the diet are almost immediate. The carbohydrate diet for instance by the third day of its administration after a fast had completely removed the effect of the latter on the gut (Table II).

It is now necessary to consider the results which follow when carbohydrate is fed together with either protein or fat. In Table V are the data for a diet of protein together with a starch-cane sugar mixture. Most of the animals received one part of the former to two of the latter, but it may be remarked that in these binary mixtures even extreme variations in the ratio of the constituents made no difference in the results. This is not surprising. The total amounts of the vitamin produced in the body are small, and if either constituent of the food functions as an efficient precursor very little of it need be present in the mixture.

Table V. *Protein and carbohydrate diet.*

3 days after fast			5 days without fast		
Weight of rat g.	Ascorbic acid mg./g.		Weight of rat g.	Ascorbic acid mg./g.	
	Liver	Gut		Liver	Gut
165	0.19	0.18	120	0.29	0.31
170	0.15	0.25	155	0.20	0.23
150	0.22	0.28	120	0.20	0.26
150	0.21	0.34	145	0.27	0.35
230	0.29	0.35	150	0.25	0.30
160	0.27	0.34	155	0.23	0.28
190	0.29	0.28	240	0.22	0.20
170	0.26	0.34	300	0.26	0.27
170	0.19	0.30	230	0.21	0.24
150	0.23	0.26	280	0.25	0.30
Average	0.23	0.29	Average	0.24	0.27
Median	0.22–0.23	0.29	Median	0.24	0.27–0.28

It will be seen from Table V that the diet of protein and carbohydrate induced that approximation to equality in the hepatic and intestinal concentrations which is characteristic of the influence of a complete dietary. This is not so marked in the rats fed for 3 days after the fast, and the data from this group of animals displayed a somewhat wide scatter. Nevertheless the average concentrations come much nearer together than with any of the experimental diets previously discussed. In the ten animals fed for 5 days without previous fasting the average, derived from consistent individual results, was for the liver 0.24 mg./g. and for the intestine 0.27 mg./g. It will be remembered that the average value on a complete diet was 0.26 mg./g. for both.

This same approximation to equality in the concentrations, yielding in each organ an average value of the same order as that found in a normal mixed dietary, was also displayed by rats on a diet of fat and carbohydrate (Table VI). As with protein and fat there was irregularity in the figures when the mixture was taken for 3 days only after a fast, whilst for some reason in the case of the fat and carbohydrate the average figures (from four rats only) though equal were

low. 5 days' feeding, however, undisturbed by the preliminary fast, induced the approximate equality in question at a level close to the normal. Out of sixteen rats placed for this period on carbohydrate in association with either protein or fat only three showed any important departure from this behaviour. Although the approach to equality in the concentrations when the two diets last mentioned were consumed is clear, analysis shows that the rather higher figures for the gut are still significant. It would seem that the intestine adjusts to this binary diet somewhat more readily than the liver.

Table VI. *Fat and carbohydrate diet.*

3 days on diet after 2 days' fast			5 days without fast		
Weight of rat g.	Ascorbic acid mg./g.		Weight of rat g.	Ascorbic acid mg./g.	
	Liver	Gut		Liver	Gut
170	0.21	0.13	170	0.23	0.26
220	0.18	0.19	150	0.19	0.20
190	0.18	0.16	200	0.23	0.23
190	0.19	0.20	210	0.26	0.36
			220	0.19	0.25
Average	0.19	0.17	175	0.19	0.22
			160	0.21	0.27
			225	0.27	0.30
			200	0.26	0.29
			200	0.25	0.28
			170	0.21	0.21
			—	0.30	0.31
			Average	0.23	0.26
			Median	0.23	0.26-0.27

Certain of the experimental results may be here assembled. The following are the average values after 5 days' consumption of the diets, without the preliminary fast. It must not be forgotten that in the case of the carbohydrate-free diets the normal figures given for the liver were preceded by the earlier fall in the concentration.

With carbohydrate.

	Liver mg./g.	Intestine mg./g.
Carbohydrate alone	0.32	0.24
Protein and carbohydrate	0.24	0.27
Fat and carbohydrate	0.23	0.26
Normal mixed diet	0.26	0.26

Without carbohydrate.

Protein and fat	0.25	0.39
Fat alone	0.28	0.36

It should be emphasised that in these experiments only in animals on the diet of carbohydrate alone was the hepatic concentration ever above normal, or above that of the intestine; and only when carbohydrate was entirely absent did the (more marked) rise in the intestine occur. It would appear that carbohydrate induces approximate equality of concentration in the two organs by inhibiting the rise in the gut which occurs in its absence, while the presence of protein and, apparently, also of fat, inhibits the (less marked) rise in the liver, which the uncontrolled influence of a carbohydrate diet induces.

If the assumption be made that the vitamin is produced in both organs it would appear that when at any time carbohydrate is not available in the diet for production in the liver, synthesis in the gut, presumably from other precursors, proceeds with increased velocity. Such a happening would be rare in the life of the animal, but the capacity of the gut to react in this way would seem to possess physiological interest. It would seem that there must be a mechanism capable of securing some mutual adjustment in the activities of the two organs. The possibilities are further discussed in a later section.

A few animals were kept for longer periods (10 to 12 days) upon each of the diets. It may be said that in general as in the case of the experiment of Table IIIA, the difference in the relative concentrations in liver and gut earlier induced by a diet was still found in each case, but the absolute concentration was in both organs lower.

Determinations on the organs of the guinea-pig were made, but they were few and call for brief reference only. In guinea-pigs normally fed the concentration of ascorbic acid was found to be much lower than in rats, alike in liver and gut, but, as in the latter, approximately equal in both. Four animals yielding closely concordant results gave for the liver an average of 0.13 mg./g., and for the intestine 0.15. It is noteworthy that a 48 hours' fast produced no rise in the latter; rather an appreciable fall. Four animals after fasting showed for the liver an average of 0.13 mg./g., and for the gut one of 0.11. It has been shown by others that the concentration falls in the liver and adrenals during the development of scurvy in the guinea-pig. The intestines of four animals showing marked, but not extreme, symptoms between the fifteenth and twentieth days of a scorbutic diet displayed very low values: 0.02, 0.04, 0.02 and 0.03 mg./g. In these the liver values were 0.07, 0.07, 0.08 and 0.11 respectively.

II. EXPERIMENTAL DETAILS.

The excellent micro-method of titration with Tillman's reagent described by Birch *et al.* [1933] was closely followed. The acid tissue extracts, of known volume, were run from a standard micro-burette into a measured quantity of the dye; usually 0.05 ml. The solutions of the dye used (approximately 0.01 *M*) were standardised at frequent intervals by titration against pure ascorbic acid. Bezssonoff *et al.* [1934] have suggested that the reduction of the dye is a process with a large dilution factor. This is certainly not true within the range of concentrations employed in the experiments here described. Within such a range a solution of ascorbic acid of given strength may be diluted twice or even thrice and then again titrated without any appreciable effects upon the calculated result. Nevertheless the final volume of each extract employed in the experiments was so adjusted to the weight of tissue taken that the titration figures all fell within a relatively small range; in most cases between 0.3 and 0.7 ml. of extract for 0.05 ml. of the reagent.

Several authors have reported general agreement between the results of the titration method and those of biological tests, and it has now become common to accept the former without question. This however might seem unjustifiable in the case of material such as the tissues of gut which have not been tested biologically.

It proved impossible to administer intestinal tissues or aqueous extracts made from them to guinea-pigs for adequate periods. Attempts to do so have given indications of protection, but the animals have nearly always died prematurely and adequate biological proof that the gut contained ascorbic acid in concentration corresponding with the titration results was not obtained.

On the other hand it seems that evidence on the following lines is in itself sufficient to show that when applied to intestinal extracts the figures obtained by titration represent ascorbic acid and that substance alone.

In acid solutions the reduction of Tillmann's reagent by ascorbic acid proceeds with a velocity which is out of all proportion greater than reduction by any other substance (cysteine, glutathione, phenols *etc.*) known to react with the dye. It is easy on the other hand to show by relatively simple means that if the amount of the vitamin in a given intestinal extract be assumed to correspond with the titration figure, and if a solution of pure ascorbic acid be prepared of equivalent strength and similar p_H , the extract and the solution will reduce a given amount of dye with a velocity which to all appearances is the same. To give precision to such evidence Dr G. A. Millikan kindly undertook to determine quantitatively the rate of reduction by previously titrated extracts, comparing it with that of reduction by pure ascorbic acid and cysteine solutions of equivalent strength. He has provided an account of his experiments which should be of interest to other workers (see Appendix, p. 2817).

Having regard to very great differences in the velocity of reduction by ascorbic acid and cysteine respectively, and to the circumstance that, roughly observed, the reduction of the dye by *e.g.* phenolic substances, while slow, differs in velocity from one case to another, the identity of the velocity constants for pure ascorbic acid and the reductant from the intestine seems strong evidence in favour of the titration method.

While this applies to the gut and, it would seem, almost certainly to most other organs, it is well to note that there may be exceptions. The kidney, for instance, to judge at least from experience gained in the course of this research, is an organ which offers difficulties. In titrating acid extracts of the gut and liver (when the organs have been removed from the body without undue delay) there is, as in the case of solutions of pure ascorbic acid, no difficulty in deciding upon the end-point, and different observers readily agree when determining it. Moreover, it is significant that in the case of extracts from these organs the actual rate of titration may be widely varied without affecting the final result. It is otherwise with extracts from the kidney. If in the case of this organ an extract is first titrated quickly and then more slowly, it is found that the results are by no means the same. If indeed (the extract being in the burette) a pause is made between each successive addition so that the titration occupies a minute or longer, decoloration of the dye may occur when no more than, say, half of the extract required in the quick titration has been added. Slowly reducing substances in exceptional amounts are usually if not always present in kidney extracts. It is known to histologists and others that the kidney is exceptional in the rapidity with which it undergoes change after removal from the body. Catheptic activity for example is exceptionally great in this organ. Whether autolytic products account for the difficulty in titration is uncertain, though likely. It occurs, however, when there has been no more than ordinary delay in the treatment of the organ after its removal. It is sure that, even in the case of the liver or intestine, long standing even at room temperature before extraction makes titration more difficult in the above sense. Needless to say this occurs more rapidly on incubation of the organs. Guha and Ghosh [1934] have described experiments to show that various organs can produce ascorbic acid from mannose but not from glucose when incubated with these *in vitro*. The titration values rise considerably however when tissues are incubated alone. It would seem therefore that, in order to make the evidence on these lines for a real increase of the vitamin of any value control samples must in any case be incubated side by

side with the experimental samples while the final titrations must be fast and very uniform in rate. It is probable that the above authors are aware of this difficulty and have allowed for it. The circumstance that they report negative results with some sugars seems proof of this.

In the greater number of the experiments male albino rats were used, of weight varying from about 150 to 200 g. Quite young animals (50 g. or under) tend to show higher absolute values for the vitamin concentrations than those more mature, so that in testing the effect of a given dietary they are not strictly comparable with the latter. It was thought that females might show greater variation than males in the normal amount of vitamin so, perhaps without sufficient reason, they were infrequently used.

The animals were killed instantaneously by stunning, and the throat was immediately severed to allow free drainage of blood. The organs were removed as quickly as possible and ground up under trichloroacetic acid in the presence of a little sand. The final volume was in each case made proportionate to the weight of tissue taken and the extracts as titrated contained approximately 2.5 % trichloroacetic acid.

It is perhaps desirable to describe here the preliminary treatment of the gut before extraction. It was uniform in all experiments. The small intestine was cut at its pyloric and caecal ends and removed intact; the mesentery being peeled off during the gradual removal of the gut from the abdomen. Its contents were washed out by a stream of water at 37°, and the residual water removed by "milking" the gut with moistened fingers. It was then gently squeezed between filter-papers. To make sure that such treatment did not leave a variable amount of adherent moisture a portion of the intestine was in a large number of cases reserved for the determination of water content. The variations were small; the departures from the average of 78.2 % total solid matter being such as would affect the figures obtained for ascorbic acid by at most a unit in the second decimal place. In the case of the liver the whole organ or a suitable proportion of it was weighed and ground up at once under trichloroacetic acid. The metabolic condition of the liver—its variable content of water, glycogen or fat—must of course indirectly affect to some degree the observed concentration of the ascorbic acid. It seems clear, however, that variations in these were not of sufficient magnitude or, alternatively, not of the right kind, to diminish the significance of the variations displayed by the vitamin concentration.

In a proportion of cases a sufficient amount of the liver was reserved for estimations of the glycogen by the method of Good, Kramer and Somogyi, and in some others the total nitrogen was estimated by Kjeldahl. In the case of the groups compared when one was fed carbohydrate only and the other protein and fat (Tables II and III) the livers of two rats from the former group contained 5.64 and 3.67 % glycogen respectively, whilst those of two from the latter group contained only 0.31 and 0.43 %. This difference would of course somewhat disturb a comparison of the true cytoplasmic concentrations of ascorbic acid, but it is clear that any attempt to correct for this would somewhat increase and not diminish that difference between the groups in question which has been claimed as significant. The differences in glycogen content of livers from sample animals from groups otherwise fed and compared were much less than this. The total nitrogen of the livers of animals fed on carbohydrate alone showed an average, based on twelve cases, of 2.84 % from which individual departures were remarkably small. The average in the livers of those fed protein and fat was 3.30, again with small departures from this mean. If the total nitrogen be taken as a measure of the cytoplasmic contents of the livers, this again shows that the difference between

the true concentrations of ascorbic acid in the two groups in question (the significant point) is somewhat greater than the figures indicate. The nitrogen figures found for animals on diets of protein or fat together with carbohydrate have differed but little from those of animals on complete synthetic dietaries.

III. DISCUSSION.

The experimental results reported and briefly discussed in the first section show that when rats are placed for short periods on certain dietaries (incomplete in the sense that one or more of the three main organic foodstuffs was lacking) the concentration of ascorbic acid in the two organs specially studied, namely the liver and the small intestine, may vary with the nature of such diets. The absolute concentrations may rise or fall with a change in the nature of the diet, and the relation between the concentration in one organ and that in the other may come to show wide differences. Any such adjustment to a change in the diet is always rapid.

The essential experimental results can be summarised in the following statements. The numerical data upon which the statements depend have been shown by statistical analysis to possess the significance here claimed for them (see p. 2804).

1. During the consumption of a normal mixed diet the average concentration of ascorbic acid was found to be the same in both organs, namely 0.26 mg./g.

2. A fast of only 48 hours was found to produce a rise in the intestinal concentration which reached an average value of 0.40 mg./g. The concentration in the liver was by then scarcely affected (Table I).

3. On a diet containing carbohydrate alone, whether administered for 3 days after a fast of 2 days, or for 5 days without the previous fast, the hepatic concentration rose to an average of 0.33 mg./g., whilst that in the gut remained normal (Table II). The increase in the liver, though not large, is outside the limits of experimental error and is statistically significant.

4. When on the other hand a diet containing protein and fat without carbohydrate was consumed for 3 days after a fast, the concentration in the liver fell to the low average value of 0.19 mg./g., while the intestinal concentration rose to 0.37, this high value being also displayed after 5 days of the diet without the fast (Table III). On a diet of fat alone the results were essentially the same (Table IV).

The immediate fall in the liver induced by carbohydrate-free diets was, it is true, followed by a return to normal on the fifth day of feeding. It is almost sure, however, that this is correlated with the known circumstance that the livers of rats adjust to a carbohydrate-free diet on about the fifth day of its consumption. The hepatic glycogen which in rats fed upon fat alone at first falls to near zero, rises by then to concentrations round 1% [Gemmill and Holmes, 1935], and this is associated with the disappearance on or about the same day of the ketosis first produced by a fat diet [Wigglesworth, 1924]. If therefore the precursors of ascorbic acid in the liver arise from carbohydrate it is clear that this becomes more available for the purpose at about the fifth day of a diet of fat or of protein and fat.

5. Whilst the hepatic concentration was raised by feeding carbohydrate exclusively, and the intestinal concentration was raised when diets without carbohydrate were consumed, it was next found that a diet of protein and carbohydrate produces approximate equality of concentration in the two organs at a figure corresponding to that found in rats on a normal mixed diet (Table V). A diet of fat and carbohydrate produced the same situation (Table VI).

If the behaviour of the vitamin in the liver were considered alone, showing the rise above the normal concentration, and above that in the gut, after a diet containing only carbohydrate, and on the other hand a rapid fall on the two carbohydrate-free diets, there would be sufficient evidence to support the natural expectation that carbohydrates supply precursors for ascorbic acid, and, further, the suggestion that the liver, alone or in common with other organs, is the seat of the formation. The behaviour in the intestine however clearly raises other issues.

The intestinal concentration seems to be more flexible than the hepatic, and the notable rise in the former displayed by all the animals when placed on carbohydrate-free diets, is the most striking effect which the experiments under discussion have revealed. It seems fully justifiable to believe that the intestinal rise from 0.26 to 0.40 mg./g. found to follow a brief fast is itself due to a specific effect of failure in the carbohydrate supply, since it was entirely prevented by the consumption of a starch-sugar mixture alone, whereas a rise of a magnitude similar to that induced by the fast occurred rapidly on the diets which were carbohydrate-free. Data from nearly ninety rats deprived of carbohydrate were therefore recorded during the experiments, and the high intestinal concentration was displayed by every animal save four or five. The gut of no single rat out of nineteen on a carbohydrate diet displayed this behaviour, and among the same number of animals upon a normal diet yielding the average of 0.26 the gut of only one was found to depart so far from the average as to approximate to the high concentration in question.

The experimental result which has finally to be taken into consideration is the maintenance by diets of protein and carbohydrate, or fat and carbohydrate, of approximately equal concentrations in the two organs at a value which is that maintained by a normal mixed dietary.

In considering the results as a whole it seems justifiable to dismiss the suggestion that the organs studied represent seats of storage for the vitamin rather than seats of formation. It is very unlikely that changes in diet would promptly affect the relation between the concentration in the liver and that in the intestine if each organ was receiving its supply from another source.

If, as seems certain, ascorbic acid is produced in both of the organs studied, the results of the experiments almost compel the assumption that the intestine can call upon a source yielding primary precursors for synthesis which is less readily available for the liver. They suggest moreover that there must be some mechanism through which a mutual adjustment in the productive activities of these organs is established. With the diminution of production in the liver owing to a failure in the carbohydrate supply, the rate of synthesis in the intestine is—as it were vicariously—increased.

It may be useful at this point to recall the following results. On a diet of carbohydrate alone the average concentration in the gut was 0.24 mg./g., on carbohydrate with protein 0.27, and on carbohydrate with fat 0.26; that is to say, on all diets containing carbohydrate when consumed for 5 days the concentration was essentially the same, being that found on a normal mixed dietary and, incidentally, essentially also the same as that in the liver when protein or fat was fed with carbohydrate. It seems reasonable to suppose therefore that so long as carbohydrate is supplied in the food it is the preferential source of the vitamin precursors for both organs alike. On the other hand with a protein and fat diet the average intestinal concentration was 0.39 mg./g., with fat alone 0.36 and, after a fast, 0.40. Thus in all cases where no carbohydrate was supplied, the gut concentration was essentially the same at an average figure some 50 % higher than that so consistently found when carbohydrate was available in the food.

It would seem clear therefore that in the rat the cells of the intestinal epithelium—unlike the hepatic cells, or much more readily than they—can, when deprived of carbohydrate, turn to protein or fat as a primary source of precursors for the synthesis of ascorbic acid. Owing to the constitution of the vitamin it is even then likely that carbohydrate formation is an intermediate stage, and that therefore the primary source is most probably protein. It is true that the intestinal rise occurs on a diet of fat alone, no less than on one of protein and fat, and in the case of that temporary adjustment in the liver to a carbohydrate-free diet earlier discussed, it is likely, to judge from the experiments of Gemmill and Holmes, that an adequate amount of carbohydrate had in the case of that organ been derived from fat. Evidence that carbohydrate can be formed in the body from fat seems to be growing, but it does not suggest its formation in the intestine. It must in any case be a process of low velocity compared with that of the formation of glucose from certain amino-acids [*cf.* Csonka, 1915; Janney, 1915]. It is, on the other hand, not difficult to believe that failure in a carbohydrate supply involves some increase of protein breakdown in the intestinal epithelium, and that precursors of ascorbic acid may thus undergo a local endogenous increase. This seems at least a reasonable hypothesis on which to explain the well established behaviour of the gut concentration whenever there is failure in a carbohydrate supply. It is well to remember that the search for the precursors of a vitamin produced in the body differs from most studies of intermediary metabolism in that the amounts of materials necessarily involved are so small.

Complete absence of carbohydrate from its food would be a rare event in the life of the animal, but the capacity of the gut to increase its production in circumstances which lead to a decrease in the liver, and possibly in other organs, is a case of physiological adjustment not without interest. Whether other organs which may function in the production of ascorbic acid resemble the liver or the intestine in their reaction to dietetic factors was not adequately investigated in this research.

In the case of the guinea-pig, which is of course unable to produce ascorbic acid endogenously, a few experiments showed that any increased intestinal concentration due for instance to a brief fast was entirely absent. In scorbutic animals the concentration in the intestine was found to be very low (average 0.03 mg./g.).

SUMMARY.

In rats consuming a normal mixed dietary the average concentration of ascorbic acid in the liver and in the small intestine is the same.

When carbohydrate forms the sole organic constituent of the food consumed, the hepatic concentration immediately rises, becoming higher than that in the intestine.

On the other hand, when carbohydrates are absent from the food the concentration in the liver rapidly falls, while that in the intestine rises to a high value.

Possible explanations of these readjustments in the concentrations are discussed.

APPENDIX.

THE IDENTITY OF ASCORBIC ACID AND THE REDUCING AGENT OF RAT'S GUT.

BY GLENN ALLAN MILLIKAN.

The present experiments, undertaken at the suggestion of Sir F. G. Hopkins, were designed to provide a rather critical test for the identity of the reducing agent found in trichloroacetic acid extracts of rat's gut and ascorbic acid. They consisted in measuring comparatively the rate of reduction of Tillman's reagent (2:6-dichlorophenolindophenol) by these two substances, as well as by cysteine. Good agreement was found between the gut extract and ascorbic acid. Taken in conjunction with the widely divergent cysteine value, the result provides strong evidence for the suspected identity.

Since the reactions were too fast to follow by ordinary procedures, the continuous flow method introduced by Hartridge and Roughton [1923], and developed for non-haemoglobin reactions by Millikan, was used. The solutions were prepared by Hopkins, using the extraction method described by him in the accompanying paper. A solution of Tillman's reagent was made up to a concentration suitable for the kinetic measurements (usually 0.2 mg. per ml.), and the extract was standardised against it by titration, its concentration being adjusted until it was 20–40 % greater than that of the dye, the solution containing 2.5 % trichloroacetic acid. An exactly equivalent solution of ascorbic acid, also in 2.5 % trichloroacetic acid, was then prepared, its concentration being checked by titration against the same dye. The solutions were placed at once in the storage reservoirs of the apparatus, which was arranged so that one could quickly change from the gut extract to the ascorbic acid as the reducing agent (Fig. 1).

Fig. 1. Simplified diagram of the apparatus.

In measuring the velocity of reduction, the two reacting fluids were drawn into their respective syringes from the storage vessels, and were then driven by an electric motor at a known constant rate through the two converging tubes to

the mixing chamber, whence they issued as a single thoroughly-mixed stream down the observation tube. The extent of the reaction as measured by the decoloration was observed at different points along the tube, the colorimeter being a simple photo-electric one using colour filters and a rectifier type of cell [Millikan, 1933]. A 4 volt, 8 watt lamp was used as light source, the two filters being Wratten No. 29, red, and No. 56, green. The device is suitable for measuring reaction times from about 1/1000 sec. to 1/2 sec., and the concentrations of the reactants were so chosen that the rates fell into this range.

The results of a typical experiment are shown in Fig. 2. It is seen that over the very critical early portion of the curve—in fact up to 60 % completion of the reaction—the two solutions reduce with identical velocities. At the end of the

Fig. 2. Rate of reduction of Tillman's reagent.

reaction, the gut extract was sometimes a little slower in its action. It is not thought, however, that this final spread between the two curves is significant, because it is always small, and because it only shows itself near the end, when the reaction is proceeding slowly, and where one might expect it to be more susceptible to inhibiting effects of foreign substances in the gut extract. No attempt was made to purify the extract other than by filtering through paper, and slight effects (due perhaps to the slow adsorption of the dye on colloidal molecules in the extract solution) are almost to be expected. The striking fact, on the contrary, is that the two agree as well as they do, when it is considered that large changes in the rate are brought about by small alterations in the concentrations of the reactants and in the p_H of the solutions, as found from the control tests described below.

In contrast to these small differences, the rate of reduction of Tillman's reagent by cysteine is about 350 times as slow as that by ascorbic acid when measured under the same conditions. Thus, in the first one-tenth of a second, ascorbic acid has more than half completed reduction of the dye, while cysteine has only reduced about one-thousandth of it. A few orienting experiments of Hopkins indicate

that glutathione and materials precipitated from tissue extracts by mercuric acetate act even more slowly than does cysteine so that of these possible claimants ascorbic acid is the only one which is even in the running.

Controls. 1. *Decay with time.* Since the reducing power of solutions of ascorbic acid is known to fall off in the course of time, it was thought desirable to sandwich the gut extract readings between two sets of observations on the ascorbic acid (hollow circles and dots respectively in Fig. 2). These showed that there was no decay in the rate during this time.

2. *Effect of p_H .* The rate of the reaction is very sensitive to alterations in the p_H of the solution, becoming progressively slower as the alkalinity is increased, until at neutrality the rate is about 1/40 of that in the trichloroacetic solutions (which have a p_H of about 2). This explains the necessity for using the same solvent for the ascorbic acid and for the gut extract in the kinetic experiments.

The bimolecular order of the reaction.

A tenfold variation in the concentration of the ascorbic acid, and a twofold variation in that of the dye left the bimolecular velocity constant unaffected:

Conc. of ascorbic millimols/litre	Conc. of dye millimols/litre	Bimolecular velocity constant, K 1/millimols $\times$ sec.
0.073	0.057	30 ± 5
0.136	0.112	30 ± 5
0.71	0.125	34 ± 5

Temp. 20°. (An experiment at about 22° gave a value for K of 39 ± 2 , indicating a high temperature coefficient for the reaction, but the data are insufficient for its calculation.)

If the reaction were of either higher or lower order than the second, the value of K would have varied widely. The agreement obtained is therefore strong evidence that the reaction actually proceeds according to the expression:

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