AN EXPERIMENTAL STUDY OF SOME FACTORS INFLUENCING LIVER NECROSIS, AND THEIR RELATION TO CIRRHOSIS.

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by

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PREFACE.

This thesis describes experimental work carried out between 1937 and the outbreak of war in September, 1939. The author realises that much of it will appear incomplete and inconclusive, but he feels that it is better to report now the results that have been obtained, in view of the fact that under present circumstances there will be no o portunity of continuing the work for some considerable time.

R. M. C.

June, 1941.

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INTRODUCTION.

Chapter 1

The importance of liver necrosis in the pathogenesis of cirrhosis.

I first became interested in cirrhosis of the liver in 1937. A brief review of the literature at that time led me to form two general ideas which suggested the experimental work described in this thesis.

In the first place, it was evident that there had been what I will term an integration of the whole field of liver pathology. The older writers classified liver diseases fairly rigidly according to the postmortem appearance of the organ - there were acute yellow atrophy, subacute atrophy and the various types of cirrhosis. Both clinically and pathologically these conditions were quite distinct from each other. Modern pathologists, on the other hand, tend more and more to include all forms of diffuse damage to liver tissue under the term "hepatitis". According to this view, acute atrophy and cirrhosis are simply the two extremes of a series of inflammatory processes of varying degrees of acuteness. Thus Muir (1936) states "multiple nodular hyperplasia...... is of importance, as it represents, in a somewhat gross form and irregularly distributed, the lesions which occur more gradually and more generally in cases of cirrhosis. In fact, acute and subacute yellow atrophy, nodular hyperplasia and cirrhosis form a series of changes differing in extent and rapidity rather than in nature; and intermediate stages are met with." MacCallum (1937), Hurst (1937), Boyd (1938) and McNee (1939) all support this conception. It is a stimulating hypothesis which accords well with general pathological principles, and explains many ill-defined conditions in which the damage to the liver is less intense than in acute yellow atrophy, or as it should perhaps be termed, "acute fulminating hepatitis".

The second general conception follows as a corollary of the first, and concerns

the actual pathogenesis of cirrhosis. If we regard cirrhosis as "chronic hepatitis", then we clearly infer that the primary lesion is one of liver cells, the overgrowth of fibrous tissue being a secondary effect. The consensus of opinion appears to support this view. MacCallum (1937) states: "Although many conflicting views have been held, it seems clear enough that the injurious agent effects the destruction of the liver cells in the first instance, and that the scarring and the hyperplasia of the epithelial remnants are reparatory processes." Muir (1936), on the other hand, considers that damage of liver cells and overgrowth of fibrous tissue proceed concurrently, both being the result of some toxic agency. Eppen (1932) and Weiss (1935) lay stress on primary damage of liver cells, and McNee (1939) believes that "damage and destruction of liver cells is frequently repeated at short intervals in the production of cirrhosis." The evidence from animal experiments also points to liver cell damage as the important factor in producing cirrhosis. Moon (1934), in his comprehensive review of experimental cirrhosis, states that any substance, if it is to cause cirrhosis when given repeatedly, must produce necrosis as its acute effect. The essential process which initiates cirrhosis is repeated necrosis and repair. Cameron and Karunaratne (1936), using carbon tetrachloride, showed that doses large enough to produce necrosis could be administered indefinitely to rats without producing cirrhosis as long as the timing of the doses allowed for complete recovery after each dose. With more frequent dosage, cirrhosis resulted. From all these observations one is led to the conclusion that the extent and duration of necrosis are important and possibly determining factors in the development of cirrhosis. It therefore seemed to the author that a study of some of the factors which influence liver necrosis might be of value in relation to the etiology of human cirrhosis.

PART I.

The Relationship of Vitamin Deficiency to Liver Disease.

Chapter 2

Experimental: The Effect of Calcium Deficiency on Liver Necrosis.

There is a considerable body of evidence of the relation between calcium metabolism and carbon tetrachloride intoxication, (Minot, 1926-7; Lamson et al, 1928; Minot, 1927; Minot and Cutler, 1928; Cutler, 1932; Cantarow et al, 1938). Briefly, these authors found that the mortality in dogs after giving carbon tetrachloride by mouth could be greatly reduced by administering calcium chloride intravenously; they also showed that previous feeding on a low-calcium diet increased the mortality and the acute nervous symptoms. Various suggestions were made as to the cause of this, the most popular being that after carbon tetrachloride there is an acute hypocalcaemia, due to the combination of some of the serum calcium with the bilirubin, which is present in excess of normal; administration of calcium prevents death and relieves the convulsive symptoms by raising the serum calcium to normal. The point in which I was interested, however - the influence of calcium lack on the liver necrosis - was only mentioned in three papers. Minot and Cutler (1928) and Cutler (1932) could find no difference in the amount of liver necrosis between control dogs and those on a low-calcium diet; but Cantarow et al. (1938), using cats, reported definitely less histological damage in calcium-treated animals. It was therefore decided to repeat these experiments, using chloroform - a toxic agent very similar to carbon tetrachloride in its effects on the liver.

Methods.

White mice were used in these experiments. They were fed the following diets for periods from 14 - 28 days before being given chloroform. The diets were those used by Shelling (1932) for producing calcium deficiency in mice.

Diet A. (Adequate).		Diet B. (Calcium-deficient).	
White Wheat Flour	700 g.	White Wheat Flour	400 g.
Milk Powder (B.D.H.)	300	Wheat Starch (B.D.H.)	325
Marmite	50	Ashless Casein (Glaxo X.19)	100
		Wheat Gluten (B.D.H.)	50
		Butter	50
		Olive Oil	40
		NaCl	20
		KCl	15

The chloroform was administered by subcutaneous injection, mixed with an equal volume of liquid paraffin. It was found in preliminary experiments that 0.05 c.c. of chloroform by this route would produce in 24 hours a lesion involving about the central one-third of each lobule, and this dose was used throughout. The animals were killed at 24 or 48 hours after chloroform, and the livers fixed for histological examination. In some cases calcium deficiency was assessed by X ray examination; in others by chemical estimation of calcium in the carcase.

Results.

It seems unnecessary to describe the results obtained in detail in these early experiments. The numbers of animals used were not large, and the results obtained with rats in later experiments are of much greater significance.

Experiment 1.

Mice on diets A and B for 10 days. At this point the calcium-deficient group were beginning to lose weight. Both groups were given 0.05 c.c. $CHCl_3$ subcutaneously and killed after 24 and 48 hours. On comparing the livers histologically, it was found, rather surprisingly, that those of the calcium-deficient group were less extensively

damaged than the controls. This was exactly the opposite of what one had expected. In spite of this, X ray examination showed no osteoporosis and chemically there was no significant difference in the calcium content of the carcases; so that it seemed likely that the difference in liver necrosis could not be due to any change in calcium metabolism. In searching for some other difference between the two diets, it was realised that the low-calcium diet contained little or no vitamin B, and another experiment was therefore carried out, in which the white flour was replaced by whole-wheat flour, a rich source of the B complex.

Experiment 2.

Diets A and B - whole wheat flour replacing white flour. Mice on diets 21 - 29 days before injection of 0.05 c.c. $CHCl_3$. Killed 24 and 48 hours later. Carcases X rayed. Livers compared histologically.

In this experiment the calcium-deficient group did not lose weight, which suggested that in Experiment 1 the loss of weight had been due to vitamin B deficiency.

The skiagrams showed no osteoporosis.

There was no difference in the extent of liver necrosis in the two groups.

Experiment 3.

A repeat of Experiment 1, but the animals were kept on the diets 28 days before injection. By this time the calcium-deficient group had lost quite a lot of weight and were becoming bald and unsteady in their gait - symptoms suggesting a vitamin deficiency. 0.05 c.c. $CHCl_3$ injected. Killed 24 hours later. On comparing the liver damage, the calcium-deficient group were again found to have less necrosis than the controls.

Discussion.

The actual composition of the diets used was not determined chemically, but

the following figures have been estimated by using various food-tables:-

	With White Flour		With Whole-Wheat Flour	
	Diet A	Diet B	Diet A	Diet B
Protein	13.97	19.07	16.43	20.55
Fat	8.05	8.89	8.19	9.01
Carbohydrate	60.40	62.70	58.00	61.26
Ca	0.30	0.01	0.31	0.02
P.	0.37	0.13	0.47	0.19
Fe	0.07	< 0.001	0.07	0.001
Vitamin B	++	?absent	++	+
" A	+	?	+	?
" D	+	?	+	?

(All figures are percentages by weight).

It will be seen that the main effect of using whole-wheat instead of white flour is to abolish the deficiency of vitamin B in Diet B. The other differences between Diets A and B - in protein, Ca and Fe - are still present when whole-wheat flour is used. It therefore appears likely that the differences found in liver necrosis are explained by the varying content of the vitamin B complex, thus:-

Diet		Vitamin B	Growth	Liver Necrosis
White Flour	A	Present	Normal	+
	B	? Absent	Defective	0
Whole-wheat Flour	A	Present	Normal	+
	B	Present	Normal	+

From the point of view of calcium deficiency, these experiments were unsuccessful; no evidence of deficiency, as judged by X ray or chemical estimation, was

obtained. Possibly the animals were not kept long enough on the diets. While, therefore, the original object of the experiments was not attained, an unexpected finding of great interest resulted from them; namely, that a vitamin B-deficient animal is apparently protected in some way from the liver necrosis produced by chloroform. It was decided to pursue the study of this phenomenon with greater care in an animal more suitable for dietetic experiments - the rat.

Chapter 3

Experimental: The Effect of Vitamin B Deficiency on Liver Necrosis.

Experimental Methods.

Young, male, albino rats were used in nearly all these experiments. At the start of the experiment their average weight was 70 - 80 g., and they were weighed twice a week after being put on the various diets until one group began to show either definite loss of weight or other deficiency symptoms. This usually took 3 - 4 weeks. They were then injected subcutaneously with a mixture of equal parts of chloroform and liquid paraffin. The latter merely acts as a convenient vehicle for the chloroform, and has no effects of its own. A standard dose of 0.1 c.c. chloroform was used in most of the experiments, so that B-deficient animals received the same total dose of toxic agent as controls in spite of their smaller body weight. In some experiments the dose was adjusted to the weights of individual rats, but the same actual doses were used for the two groups. The rats were killed 24 hours after injection by breaking their necks, and portions of liver were fixed in 10% formol alcohol. Paraffin sections were cut at 5μ and stained with Ehrlich's acid haematoxylin and eosin and by Van Gieson's method. In some experiments fat was estimated histologically by fixing liver in formol saline and staining frozen sections with haematoxylin and Scharlach R.; in others the liver was weighed before and after removing a portion for histological examination, and the remainder of the liver was then immersed in hot KOH for the estimation of fat by the method of Leathes and Raper (1925). *

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* I am indebted to Dr. C.H. Gray of the Biochemical Department, King's College Hospital, for all such fat estimations.

Method of Assessing Liver Damage.

The effect of chloroform on the rat's liver has been fully described by many authors, and it seems necessary to give only a brief account here. . Within 24 hours of a moderate dose, one finds that the central part of each lobule has undergone a necrotic change characterised by loss of cellular outline and nuclear pyknosis; at the outer margin of the necrotic area there is usually a ring of swollen cells in a state of hydropic degeneration, and outside this ring again is an area of intense fatty degeneration. The periphery of the lobule is usually unaffected unless the dose has been very large. The cellular damage is usually fully declared by 24 or 36 hours, and reparatory processes are evident as early as 3 days. Within 7 days the necrotic cells have been removed and replaced by young cells produced by proliferation of the undamaged epithelium at the periphery of the lobule.

It is clear that the maximum damage falls on the centre of the lobule and that the various changes as one travels towards the periphery represent lessening degrees of cell damage. This is further borne out by the fact that a smaller dose of chloroform will produce merely hydropic change in the central ring of cells, while with a larger dose practically the whole lobule may be necrotic. There are, of course, two factors to be considered in assessing the extent of damage - (a) the area involved, and (b) the degree of cellular change, but these are closely associated. For example, it is unusual to find only hydropic change when more than the central one-third of the lobule is involved. It is therefore possible when looking at a section to form a fairly accurate estimate of the total degree of damage to the liver, and I have found that the best method is to select certain arbitrary grades of necrosis and to classify each liver on this scale. Other methods, such as tracing the outlines of the various areas and expressing them as a percentage, do not yield any more reliable results.

The four standards adopted are shown in the plate and are as follows:-

Grade 1. Not more than the central 2 - 3 rings of cells show hydropic change. There is no necrosis.

Grade 2. Not more than the central one-third of the lobule is hydropic. There

may be early necrosis of the central ring of cells.

Grade 3. Not more than the central one-third is necrotic. Surrounding this area is a hydropic ring of varying depth.

Grade 4, Includes all livers showing more than the central one-third necrotic.

Grade 0. Indicates that there is no visible change from normal.

By allotting each liver to one of these grades and then taking the mean of the values, one can form a fairly accurate estimate of the amount of liver damage in any group of animals. It is, however, most important to determine the statistical significance of any differences found in view of the wide variation between individual animals. It should perhaps be added that preliminary work showed the damage to be practically uniform throughout any one liver, so that it was not really necessary to examine more than one area; usually, however, two blocks were made from each liver.

Experiment 1. June, 1938.

It was decided first to confirm in rats the effect found in mice. Male albino rats weighing about 60 g. were put on the diets A and B used in the previous experiments; white flour was used so that diet B was deficient in the B complex. Three groups of rats were used:-

Group I. 18 rats on Diet A.

Group II. 13 rats on Diet B.

Group III. 12 rats on Diet B + an alcoholic extract of Marmite.

The third group would presumably be adequately supplied with vitamin B1 by the addition of the alcoholic extract of Marmite; no synthetic vitamin B1 was available at the time. The extract was prepared as follows:-

300 g. of Marmite were triturated with water to make a volume of 350 c.c. To this 350 c.c. of absolute alcohol was added. The mixture was allowed to stand 24 hours, filtered and the filtrate evaporated in vacuo at 50°C. till all the alcohol had disappeared and the volume was about 300 c.c. Distilled water was added to bring the volume accurately to 300 c.c. Then 1 c.c. of this extract $\equiv$ 1 g. Marmite. The

extract was added to Diet B in the proportion of 50 c.c. to 1 kilo, so that if a rat eats 10 g. of diet per day it is receiving the equivalent of 0.5 g. of Marmite - an adequate amount of vitamin B1.

The growth curves of these animals are shown in Chart 1. They prove that while Diet B is grossly deficient in growth factors as compared with Diet A, the addition of the alcoholic extract of Marmite to Diet B has only partially compensated for the deficiency. Diet B is probably deficient, therefore, in other growth factors besides vitamin B1, which are not soluble in alcohol.

Symptoms:

Group I (diet A) appeared normal. Group II (diet B) looked ill and emaciated, and showed the "high" gait characteristic of vitamin B1 deficiency. They could also be sent into convulsions by holding them up by their tails and spinning them. In addition, their fur was very ragged and some had partial baldness. Group III showed some baldness and ragged fur, but had none of the special signs of B1 deficiency.

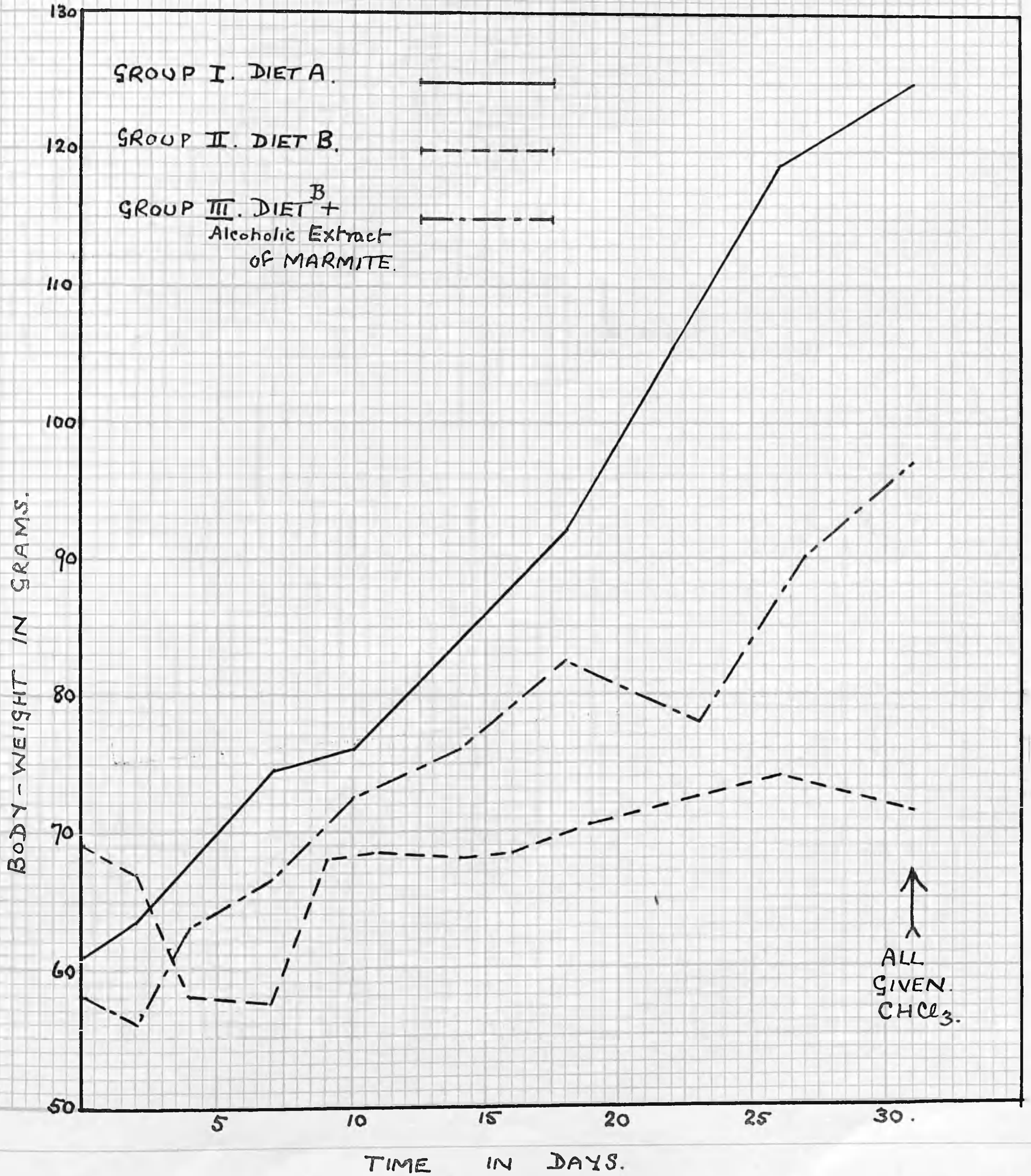
Results.

All the rats were given 0.1 c.c. chloroform, subcutaneously on the 31st day, and were killed 24 hours later. The liver damage was estimated as described above, with the following results:-

Group.	Diet.	No. of animals.	Degrees of Necrosis					Mean damage.
			0	1	2	3	4	
I	A	18	1	0	5	10	2	2.7
II	B	13	3	5	5	0	0	1.2
III	B + Alc. extract of Marmite.	12	4	5	2	1	0	1.0

There is a significant difference between Groups I and II ($t = 4.7$), but not between

CHART 1.



Groups II and III ($t = 0.6$). We conclude, therefore, that in rats, as in mice, a deficiency of some part of the vitamin B complex appears to protect the liver against the toxic effect of chloroform. The fact that the addition of vitamin B1, in the form of alcoholic extract of Marmite, to the B1-deficient diet did not lessen this protective action suggests that the factor concerned is not vitamin B1, but some other member of the B complex which is not soluble in alcohol. The growth curves showed that diet B was deficient in another growth factor as well as B1, and it is possible, although not proved, that this other growth factor is identical with the one responsible for the difference in liver damage.

Experiment 2. July, 1938.

It was now decided to test larger numbers of B1-deficient rats. They were obtained from another laboratory where they had been used in the assay of foodstuffs for vitamin B1 * and were all severely B1-deficient when given chloroform. The diet on which they had been maintained for varying periods was as follows:-

Diet B.O.		
	White Cane Sugar	60
	Light White Casein	20
	Arachis Oil	8
	Salt Mixture	5
	Autoclaved Yeast	15
	Cod Liver Oil	1 drop/rat/day.

The yeast was autoclaved at 2 atmospheres pressure for 6 hours in trays, not more than one inch deep.

Control rats were obtained from the same source and had been on the following diet for a long period:-

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* Supplied through the kindness of Dr. M.D. Wright of the Research Laboratories, Vitamins Ltd.

Diet C.7.

Skimmed milk	10
Bemax	15
Meat meal	5
Grass	5
Linseed meal	2
Yeast (fresh)	3
Salts	$\frac{1}{2}$
Yellow maize meal	$54\frac{1}{2}$
Arachis oil	5
Cod liver oil	2

These rats were a hooded black and white strain, and of both sexes. They were of the same age as those used in Experiment 1 at the time of injection. Preliminary experiments had shown them to be rather more resistant to chloroform than albino rats, and so they were all given a larger dose of chloroform subcutaneously - 0.2 c.c. mixed with an equal quantity of liquid paraffin. They were killed 24 hours later. In some cases, fat was estimated chemically in the livers.

Results.

Liver Damage.

Group.	Diet.	No. of animals.	Degrees of Necrosis					Mean damage.
			0	1	2	3	4	
I	C.7 (adequate)	28	2	5	8	11	2	2.2.
II	B.O. (B-deficient)	26	8	13	5	0	0	0.9.

There is a significant difference between the two means ($t = 5.2$).

Fat Content of Livers (% fat/liver wt.)

Group I (Adequate).	Group II (B-deficient).	
4.7	3.3	7.8
4.5	6.6	6.8
6.7	4.0	6.5
6.9	5.8	6.7
8.0	7.4	4.3
5.5	4.4	3.1
5.4	3.1	6.2
6.2	2.2	7.5
6.2	5.5	2.7
5.1	7.0	9.5
	4.2	2.6
Mean 5.9%	Mean 5.3%	

There is no significant difference between these means.

Discussion.

Here again, the livers of the B-deficient group showed less damage than the controls; they did not, however, show any difference in fat content, so that whatever the cause of this effect, it does not appear to act by increasing the degree of fatty change after chloroform.

It is not possible to reach any precise conclusion from this experiment as to the factor responsible for this effect. The approximate composition of the two diets is as follows:-

	Diet C.7 (Adequate)	Diet B.0 (Deficient)
Protein	19.6	20
Carbohydrate	?	60
Fat	9.9	8
Vitamins A and D	+	+
Vitamin B1 (thermolabile)	2.5 I.U./g.	NIL
Vitamin B2 (thermostable)	+	+
Another thermolabile factor	+	-

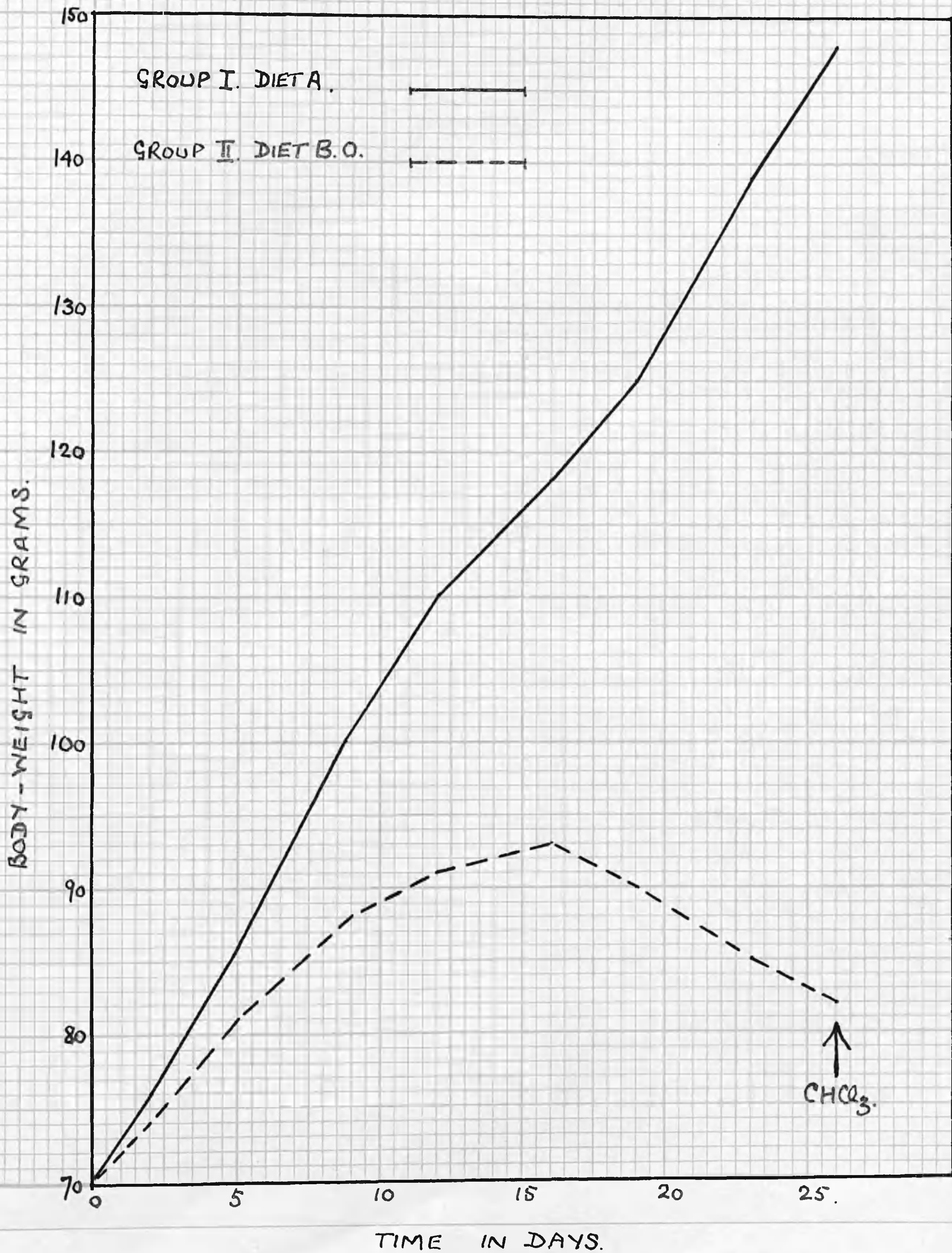
Diet B.O. was originally designed to produce a pure B1 deficiency, but unfortunately it is also deficient in some other thermolabile component. Dr. Wright tells me that she cannot maintain rats on it in health over periods of months with continued doses of of pure B1, whereas with doses of foods she can. I shall give confirmatory evidence of this later. All that one can conclude, therefore, is that the absence of the thermolabile part of the B complex in some way protects the liver against chloroform, but does not affect the amount of fat in the liver.

Experiment 3. October 1938.

The next step was obviously to compare two groups of rats on diets which differed only in the presence of the thermolabile part of the B complex. The two diets were the B.O. diet used above and the same diet in which autoclaved yeast was replaced by fresh, dried yeast - called diet A.

The rats were albino males, and the growth curves are shown in Chart 2. The group on diet B.O. developed all the changes described above - baldness and raggedness of fur, loss of weight, high gait, and a tendency to go into convulsions. On the 26th day four rats from each group were killed for liver fat estimation, and the remainder were injected with 0.1 c.c. chloroform subcutaneously and killed 24 hours later.

CHART. 2.



Results.

Rats Killed Before $CHCl_3$.

Group.	Rat No.	Body wt. g.	Liver wt. g.	Liver glycogen	Liver fat g.	Liver fat (% of liver wt)
I Diet A.	1	159	5	+	0.18	3.6
	2	105	4	-	0.15	3.8
	3	188	8	-	0.11	1.4
	4	191	8	-	0.11	1.4
Means =		161	6.3		0.14	2.6
II Diet B.O.	1	78	4	-	0.11	2.8
	2	80	3	+	0.10	3.3
	3	74	3	+	0.10	3.3
	4	107	4	-	0.15	3.8
Means =		85	3.5		0.12	3.3

There is no significant difference between the two groups of values, either for actual fat ($t = 0.9$) or for liver fat as percentage of liver weight ($t = 1.0$).

Before injection, therefore, there was no difference in the amount of liver fat in the two groups of rats. Glycogen was estimated roughly by the iodine test; there is no gross difference between the two groups.

Rats Killed 24 hours after $CHCl_3$

Group.	Rat No.	Body wt. g.	Liver wt. g.	Liver glycogen	Liver fat g.	Liver fat (% of liver wt.)	Grade of damage (histological)
I Diet A.	5	104	6	-	0.15	2.5	2
	6	153	8	++	0.19	2.4	3
	7	130	5	+	0.15	3.0	3
	8	139	7	-	0.20	2.9	2
	9	135	7	-	0.16	2.3	2
	10	125	6	-	0.14	2.3	4
	11	134	7	?	?	?	1
	12	128	5	trace	0.11	2.2	3
	13	144	6	+	0.11	1.8	3
	Means =	132	6.3		0.15	2.4	2.6
II Diet B.O	5	87	4	-	0.15	3.8	1
	6	88	3	+	0.18	6.0	1
	7	92	4	+	0.18	4.5	1
	8	88	4	trace	0.15	3.8	1
	9	52	2	+	0.18	9.0	4
	10	91	?	?	?	?	1
	11	75	4	-	0.15	3.8	2
	12	79	4	trace	0.14	3.5	1
	13	68	3	?	?	?	1
	Means =	80	3.5		0.16	4.9	1.4

There is a significant difference between the amount of histological damage in the two groups ($t = 2.7$) and between the fat/liver weight percentages ($t = 3.5$).

Here again, we have the B-deficient group showing less liver damage than the adequately fed controls. But since the only difference between the two diets is that fresh yeast in diet A is replaced by autoclaved yeast in diet B.O., we can say quite definitely that the factor we are concerned with is present in yeast and is destroyed by autoclaving. Another point which emerges from this experiment is that the difference in necrosis does not depend on the level of liver fat before or after injection. The two groups killed before chloroform showed no significant difference in the percentage of fat in the liver tissue, or in the total quantity of liver fat. After chloroform, there was a significantly higher percentage of liver fat in the B-deficient group than in the

controls, although the total quantity of fat was the same. The higher liver fat percentage in the B-deficient animals might have been expected to lead to a more severe degree of necrosis, whereas the opposite result was obtained. It seems clear, then, that the apparent resistance of the liver in a B-deficient animal cannot be explained on the basis of altered fat content. This point is of some importance, since McHenry (1937) has shown that vitamin B1 increases the liver fat of rats which are fed on a diet low in ~~chlorine~~ choline. This subject is discussed more fully later.

The method used for estimating glycogen is only semiquantative, but it can be seen that there is no gross difference between the two groups.

Experiments 4, 5 and 6. January - April 1939.

The results of these three experiments can conveniently be considered together, since they were all designed to answer the same question - whether the "protective" action of the B-deficient diet could be explained on the basis of food-intake. It did, of course, seem unlikely that the low food-intake of B-deficient animals could protect the liver - indeed it has been repeatedly shown that starvation renders the liver more vulnerable to poisons; but it was felt that the question of food-intake had to be ruled out as a possible cause of the phenomenon.

Three groups of male albino rats were used:-

Group I - Diet A.

Group II - Diet B.O.

Group III - Diet B.O. + 15 γ vitamin B1/rat/day (= Diet B.X.)

The vitamin B1 solution was made up from a stock solution of the pure solid in N/100 HCl containing 3 mg/c.c. This stock solution keeps for two months in the refrigerator. For use, it was diluted 1 : 20 with a mixture of equal parts of glycerine and N/50 HCl. The resulting solution contains 150 γ of B1 per c.c., and 0.1 c.c. (= 15 γ) was given by mouth to each rat daily, using a calibrated capillary pipette. The glycerine ensures that the solution is readily swallowed, and the rats soon became quite eager for their daily ration.

The animals were kept in individual cages. The daily amounts eaten by group II (B-deficient) rats were recorded by weighing the feeding dishes before and after filling each day. Each rat in group II had an opposite number in groups I and III who received the next day what the group II rat had eaten the day before. Since B-deficient animals always eat less than normal animals, one could be sure that both groups had the same food-intake; the rats receiving vitamin B1 all showed extreme hunger towards the end of the experiment, and usually finished their daily ration within a few minutes of its being put into the cage.

The growth curves are shown in Chart 3. (Incidentally, it is interesting to note that all three groups have roughly the same type of curve. This suggests that the failure to grow of B-deficient animals can be entirely accounted for by the diminished food-intake). After 26 days on the diets, all the rats were injected with 0.1 c.c. of chloroform subcutaneously and killed 24 hours later. The B-deficient group (II) showed the usual symptoms of B-deficiency; the other two groups, although rather wasted from partial starvation, had healthy coats and no nervous symptoms.

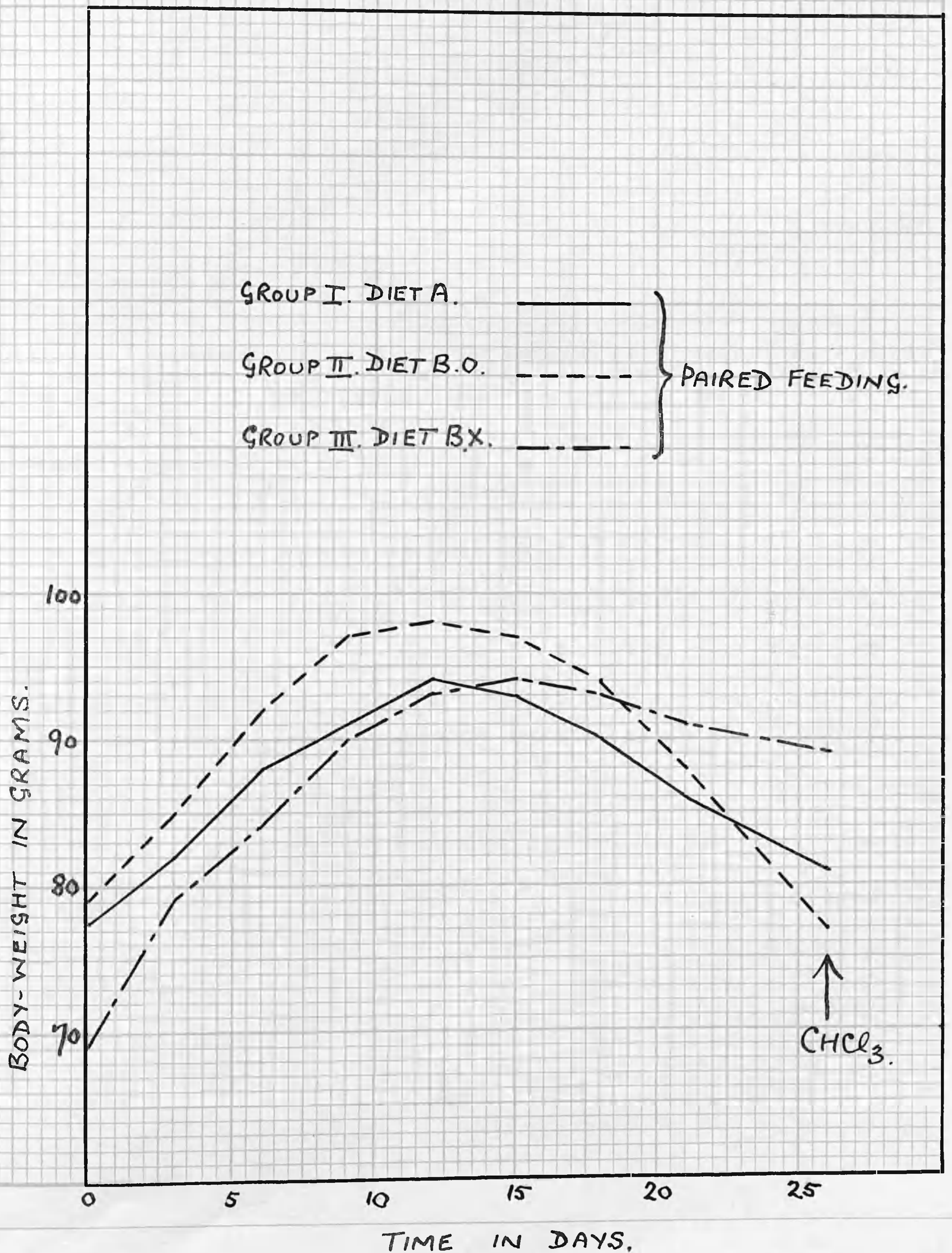
Results.

Liver Damage.

Group.	Diet.	No. of animals.	Degrees of Necrosis					Mean damage.
			0	1	2	3	4	
I	A (Adequate)	6	0	0	2	3	1	2.8
II	B.O. (B-deficient)	14	0	6	7	1	0	1.6
III	B.X (B.O. + Vitamin B1)	13	4	3	4	2	0	1.3

There is a significant difference between groups I and II ($t = 3.7$), but not between groups II and III ($t = 0.9$).

CHART. 3.



Liver Fat Estimations.

Group.	Rat No.	Body wt. g.	Liver wt. g.	Liver glycogen.	Liver fat g.	Liver fat (% liver wt.)	Histological grade.
I. Diet A.	1	92	4	trace	0.15	3.8	4
	2	97	3	-	0.29	9.7	2
	3	80	3	+	0.14	4.7	3
	4	85	3	+	0.12	4.0	3
	5	88	5	+	0.16	3.2	2
Means =		88	3.6		0.17	5.1	2.8
II. Diet B.O.	1	92	3	trace	0.15	5.0	1
	2	86	3	+	0.16	5.3	2
	3	70	3	trace	0.14	4.7	1
	4	66	3	-	0.15	5.0	1
	5	68	4	-	0.15	3.8	1
Means =		76	3.2		0.15	4.8	1.2

Discussion.

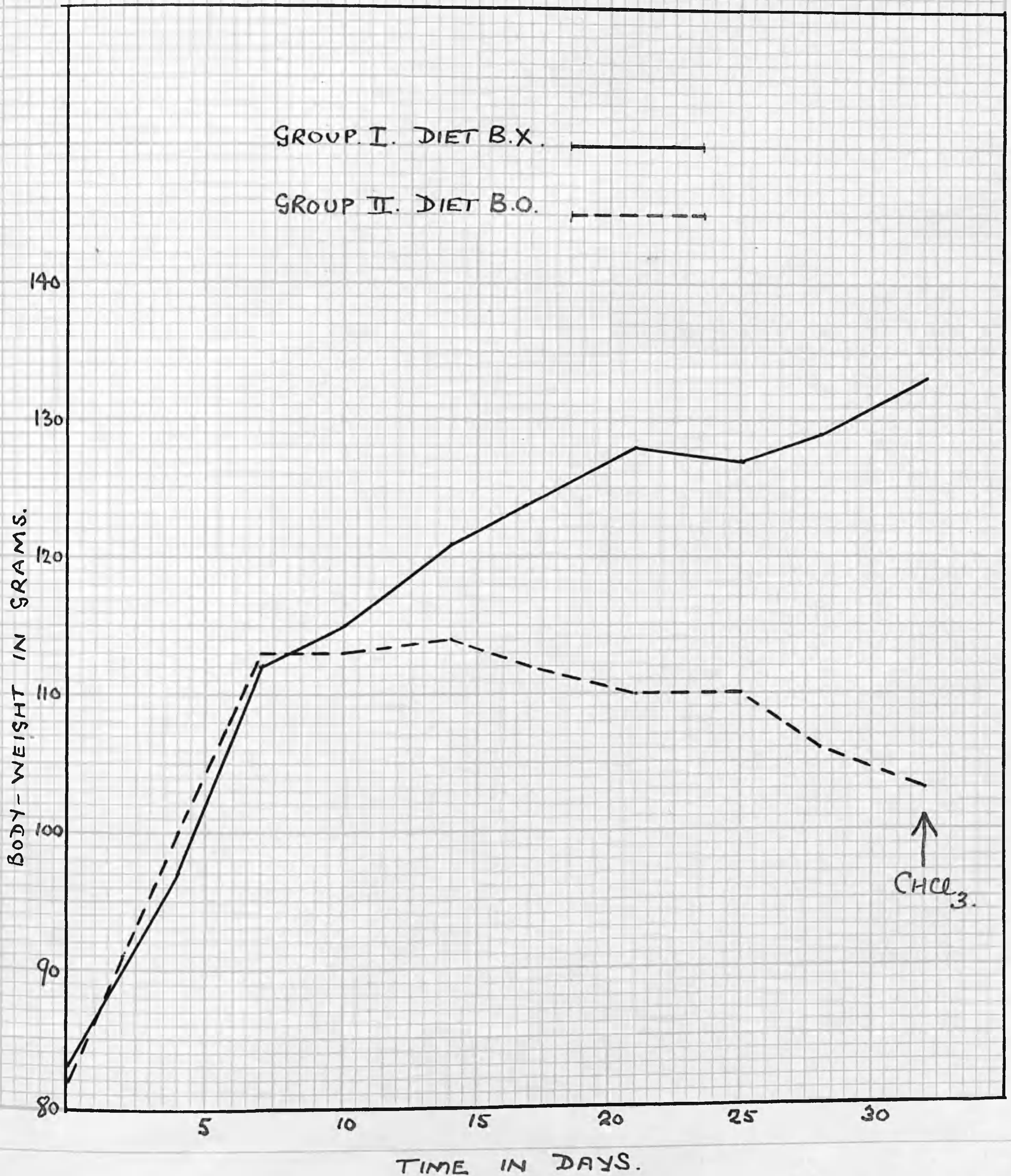
In this experiment we again find the B-deficient animals suffering less liver necrosis, in spite of the identical food intake of the two groups. Further, the addition of vitamin B1 to the B-deficient diet has not abolished the protective effect, and it must therefore be some other thermolabile substance present in yeast which is concerned. This conclusion was already suggested in Experiment 1, and receives further proof below. The results of fat estimations are similar to those obtained previously; the lesser degree of necrosis is not associated with any lower level of liver fat.

Experiments 7 and 8.

Two further experiments were now carried out to confirm the fact that vitamin B1 is not the factor concerned. Groups of male albino rats were put on diet B.O. (B-deficient) and on diet B.X. (= diet B.O. + 15 γ vitamin B1 per rat per day). The vitamin B1 was administered daily to each rat with a pipette, as described above.

The growth curves are shown in Chart 4. After 32 days the B-deficient group had the usual nervous symptoms; both groups showed some baldness, which suggests a

CHART 4.



deficiency of some other factor in both diets. Another point is that the group receiving vitamin B1 did not grow as fast as rats on a diet containing fresh yeast, e.g. group I in Experiment 3 (Chart 2). It has already been pointed out that diet B.O. is deficient in some other thermolabile factor in addition to vitamin B1.

All the rats were given 0.1 c.c. chloroform subcutaneously on the 32nd day and killed 24 hours later.

Results.

Liver Damage.

Group.	Diet.	No. of animals.	Degrees of Necrosis					Mean damage.
			0	1	2	3	4	
I	B.X (= B.O. + vitamin B1)	21	5	6	6	4	0	1.4
II	B.O. (B-deficient)	12	4	2	6	0	0	1.2

There is no significant difference between the mean damage in the two groups ($t = 0.5$).

It is clear from these results that vitamin B1 deficiency cannot be responsible for the relative immunity to chloroform necrosis of the livers of rats on diet B.O.

Experiment 9.

At this point it was realised that the diets B.O., A. and B.X. were probably all deficient in choline. Using the choline estimations made by Fletcher et al. (1935), we obtain:-

Diet B.O.	g.%	Choline Content mg.
Sugar	60	NIL
Casein	20	o.7
Autoclaved yeast	15	37.5
Salt mixture	5	NIL
Arachis oil	8	NIL
	100 g.	38.2 mg.

If a rat ingests 10 g. of this diet daily, it will therefore receive only 3.8 mg. of choline. This quantity is probably not large enough to maintain the liver fat at a normal level, although it is large enough to exert some lipotropic effect (Best and Channon, 1935; Channon et al. 1938). Diets A and B.X. will have the same choline content (choline is heat stable, and pure vitamin B1 is choline-free).

In this experiment, therefore, each of the three diets was supplemented by choline. Choline chloride was mixed with the food each day in the proportion of 15 mg. per rat. Three groups of rats were kept on the diets for 28 days:-

Group I - Diet A
Group II - Diet B.O.
Group III - Diet B.X.
} + 15 mg. choline chloride/rat/day.

The growth curves are shown in Chart 5. The addition of choline has had no effect on growth (cf. Chart 2 for same diets without additional choline). It is also obvious that diet B.O. is deficient in another growth factor as well as vitamin B1, since growth on diet B.X. is not so good as on diet A. This has been already discussed. All the rats were injected on the 28th day and killed 24 hours later. A dose of 0.65 c.c. $CHCl_3$ per kilo body weight was given to group I (diet A), and the same actual quantities to groups II and III, giving the largest dose to the heaviest rat, and so on. Thus the dose was adjusted to the weight of each rat, but all three groups received the same actual quantity of chloroform.

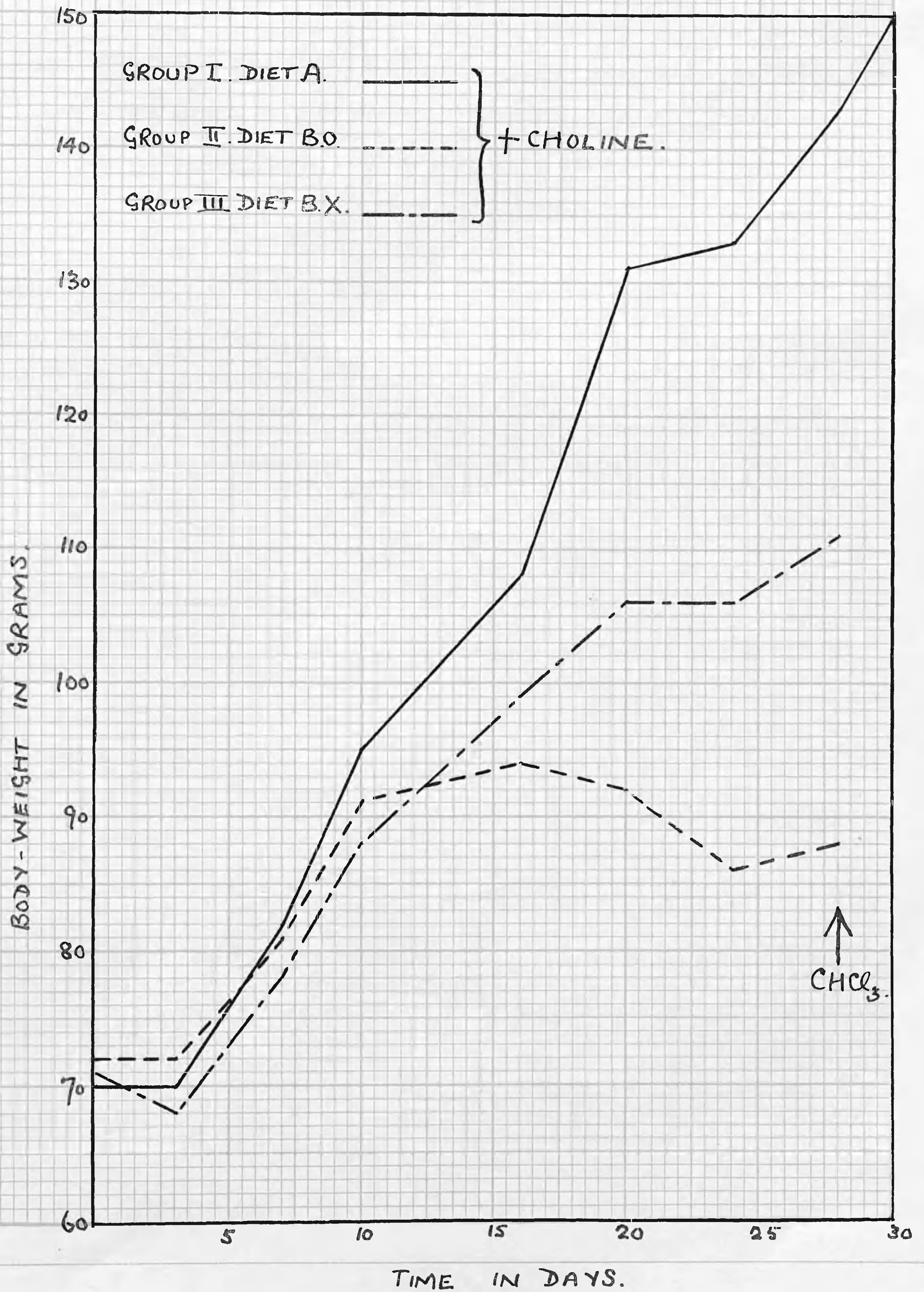
Results.

Liver Damage.

Group.	Diet.	No. of animals.	Degrees of Necrosis					Mean damage.
			0	1	2	3	4	
I	A (Adequate)	7	5	2	0	0	0	0.3
II	B.O. (B-deficient)	7	2	0	3	1	1	1.9
III	B.X. (B.O. + vitamin B1)	8	1	1	3	2	1	2.1

CHART 5.

EXPERIMENT 9.



There is a significant difference between the mean damage in groups I and II ($t = 2.7$), but not between groups II and III ($t = 0.3$).

Here we have exactly the opposite result from that obtained in all the previous experiments; the addition of 15 mg. of choline daily to each rat's diet has reversed the effect, so that now the rats on diet A (adequate) suffer less liver damage than those on diet B.O. The addition of vitamin B1 to diet B.O. has made no difference to the necrosis, as in previous experiments. Unfortunately, it was not possible to make any liver fat estimations on these animals, so that we cannot say whether the additional choline produced any lowering of liver fat before chloroform was given; but the liver fat values for animals before chloroform obtained in Experiment 3 (means 2.6 and 3.3%) are well within what is regarded as "normal" for rat liver by other workers, so that one doubts whether the reversal of effect on necrosis in this experiment could be explained on the basis of fat content.

General discussion of all these experiments.

It seems advisable at this point to summarise the experiments done and the conclusions to be drawn from them.

Expt.	Diets.	Thermo-labile Vit. B.	Thermo-stable Vit. B.	Growth.	Mean Liver Damage.	Conclusions.
1.	"Normal" (Made up with White Flour)	+	+	++	2.7	1. Deficiency of "B complex" may 'protect' liver. 2. Factor concerned is not present in alcoholic extract of Marmite.
	"Low Ca" (Made up with White Flour)	–	–	0	1.2	
	"Low Ca" + alc. extract Marmite (Made up with White Flour)	+	?+	+	1.0	
2.	Bemax C.7 (adequate)	+	+	++	2.2	Factor is probably a thermolabile part of B complex.
	B.O. (B1-deficient)	–	+	0	0.9	
3.	A. (= B.O. with fresh instead of autoclaved yeast).	+	+	++	2.6	Factor is present in fresh yeast and is destroyed by autoclaving.
	B.O.	–	+	0	1.4	
4, 5 & 6.	A. (Paired feeding)	+	+	0	2.8	1. Effect not due to differences in food-intake. 2. Factor is not vitamin B1.
	B.O. (Paired feeding)	–	+	0	1.6	
	B.X. (= B.O. + vitamin B1) (Paired feeding)	(B1 present)	+	0	1.3	
7 & 8	B.X.	(B1 present)	+	+	1.4	Factor is not vitamin B1.
	B.O.	–	+	0	1.2	
9.	A. (+ 15 mg. choline/rat/day)	+	+	++	0.3	1. Addition of choline reverses effect. 2. Vitamin B1 still has no effect.
	B.O. (+ 15 mg. choline/rat/day)	–	+	0	1.9	
	B.X. (+ 15 mg. choline/rat/day)	(B1 present)	+	+	2.1	

Note:- It is not possible to compare the amount of liver damage between different experiments, since the conditions varied, e.g. the dose of $CHCl_3$ was not constant.

In the first place, let us consider the results of Experiments 1 - 8. These have provided evidence that liver necrosis after chloroform is diminished by the absence of a factor which is:-

1. Absent in white flour but present in Marmite. } Expt. 1.
2. Not present in alcoholic extract of Marmite. } Expt. 1.
3. Not present in Bemax Diet B.O. - Expt. 2.
4. Present in yeast but destroyed by autoclaving. - Expt. 3.
5. Not vitamin B1. - Expts. 4, 5, 6, 7 and 8.

The only thermolabile factors recognised in the B complex are vitamin B1 and factor W (Elvehjem et al. 1936). The latter is a thermolabile factor present in liver extract which is essential for normal growth of rats. It seems quite possible that this factor is the one concerned in the present experiments, particularly since defective growth was always present in the groups of animals whose livers were protected. Further experimental work is obviously necessary to isolate the factor concerned and to establish whether it is identical with any of the recognised factors. In the meantime, for convenience it will be referred to as factor N.

There is another possible explanation of the different degrees of liver necrosis, which deserves consideration - that the 'protection' on diet B.O. is due to the presence of some substance produced in yeast during the autoclaving process, and not to the absence of any vitamin. This appears a possibility until one recalls that in Experiment 1 (and in the earlier experiments on mice), the B-deficient diet contained no autoclaved yeast. There is, therefore, fairly good evidence that the effect is due to the absence of a thermolabile member of the B complex, possibly factor W.

Turning now to experiment 9, where 15 mg. of choline was added to the diet of each rat daily, we find a most surprising "reversal" of effect - here the livers of the B-deficient group are more extensively damaged. This is the result that one might originally have expected for several reasons:

1. The same quantity of chloroform was given to both B-deficient and control animals. Since the B-deficient group are lighter in weight, they are receiving a larger dose of chloroform per kilogram.
2. Starvation is known to render the liver more vulnerable. B-deficient animals have a lower food-intake than controls.
3. Deficiency of various members of the B complex has been shown to have a depressing effect on oxidation mechanisms in the liver - B1 (Goldschmidt and Lewin, 1937), B 2 (Hastings et al., 1939; Dontcheff, 1939), and B6 (Muus et al., 1937). Since chloroform also interferes with oxidation mechanisms, one would have expected deficiency of vitamin B to accentuate the liver damage.

These reasons are perhaps rather vague, general conceptions; but they lead one to regard the result of experiment 9 as much more "normal" than the opposite effect found in experiments 1 - 8, where the B-deficient group suffered less damage than the controls. The determining factor therefore appears to be the balance between choline and an unidentified member of the B complex in the diet - factor N, which is possibly identical with Elvehjem's factor W.

Choline Deficient	B1 adequate,	Factor N present,	Liver Damage +
	B1 deficient,	Factor N absent,	Liver Damage -
	B1 adequate,	Factor N absent,	Liver Damage -
Choline Adequate	B1 adequate,	Factor N present,	Liver Damage -
	B1 deficient,	Factor N absent,	Liver Damage +
	B1 adequate,	Factor N absent,	Liver Damage +

In other words, if choline is deficient in the diet, it is better from the point of view of the liver that factor N should also be deficient; if choline is adequate, factor N should also be adequate.

Another point which requires emphasis is that the quantities of choline "reversing" the effect are not large. In experiments 1 - 8, the calculated amount of choline in the diet was 3.8 mg. per rat per day. The liver fat content of the two groups of rats killed before chloroform in experiment 3 (means 2.6 and 3.3%) are certainly not above normal for rat liver; it is unfortunate that liver fats could not be estimated in experiment 9 after the addition of choline to the diets. In any case, it is probable that the effect of choline on necrosis is independent of any lipotropic action. The liver fat values obtained in experiments 2, 3, 4, 5 and 6 showed that the difference in necrosis could be dissociated from any effect on the fat content of the liver. Nor does it seem possible to explain the findings as due to altered glycogen content; the estimation of glycogen, although only semi-quantitative, showed no constant differences between the different groups of rats. The mode of action of the dietary balance of choline/factor N is therefore quite obscure.

Conclusions:

1. The dietary blance of choline and a thermolabile factor in the B complex has an influence on the degree of liver necrosis after subcutaneous injection of chloroform in rats.

2. The thermolabile factor is not vitamin B1. It is present in yeast, insoluble in alcohol, and destroyed by autoclaving. Factor W has these properties, and the two may therefore be identical. The liver necrosis factor is termed "factor N" for convenience.

3. Vitamin B1 deficiency has no effect on liver necrosis after chloroform.

4. Deficiency of factor N with adequate choline, or the converse, will increase liver damage as compared with animals in which both factors are adequate, or both are deficient.

5. These effects cannot be explained on the basis of alterations in liver fat, or in liver glycogen.

Chapter 4

Experimental: The Effect of Vitamin B-Deficiency on Experimental Cirrhosis.

The results obtained in Chapter 3 at once suggested that it would be of great interest to know whether the diets used there would have any effect on the development of an experimental cirrhosis. For this purpose carbon tetrachloride was used as a toxic agent, since there were available full details of its mode of action and histological effects in the paper by Cameron and Karunaratne (1936). Other groups of animals were given alcohol in an attempt to produce cirrhosis by the combined effects of alcohol and vitamin B deficiency. At the time these experiments were begun, no one had succeeded in producing a convincing cirrhosis in animals by the use of alcohol alone. Since then, however, Connor and Chiakoff (1938) have produced definite cirrhosis in dogs by giving alcohol along with a high fat diet.

Methods.

Male albino rats were used, which weighed about 70 g. at the beginning of the experiment. The two diets were diet A and diet B.O., already described in Chapter 3. The only difference between them is that the autoclaved yeast of diet B.O. is replaced by fresh yeast in diet A, so that diet B.O. is deficient in the thermolabile part of the B complex - vitamin B1 as well as "factor N". Both diets probably contain suboptimal amounts of choline. The aim was to maintain rats for a long period on diet B.O. by giving them a day or two on diet A when necessary. The rats were divided into 6 groups:-

Group I. 12 rats on Diet A + Alcohol twice weekly.

Group II. 12 rats on Diet B.O. + Alcohol in doses equal to those given to group I.

Group III. 12 rats on Diet A + Carbon Tetrachloride twice weekly.

Group IV. 12 rats on Diet B.O. + Carbon Tetrachloride in doses equal to those given to group III.

Group V. 4 control rats on Diet A.

Group VI. 4 control rats on Diet B.O.

Alcohol: was given by stomach tube. At first 1 c.c. of 25% alcohol was as much as the rats could stand, but the dose was gradually worked up as their weight and toleration increased, till they were having 3 c.c. of 50% alcohol by the end of the experiment. The animals were usually very quiet for the rest of the day after alcohol but showed few symptoms of drunkenness beyond this. There were some deaths during the experiment from over-dosage with alcohol.

Carbon Tetrachloride: was injected subcutaneously in doses of 0.1 c.c. twice a week. The area of injection was changed on each occasion, but even so some of the rats had to be killed because of ulcers developing at the site of injections.

The experiment was originally intended to last at least six months, but the outbreak of hostilities in September 1939 made it necessary to kill the animals; some groups had been going for nearly five months but others only about three and a half months.

Results.

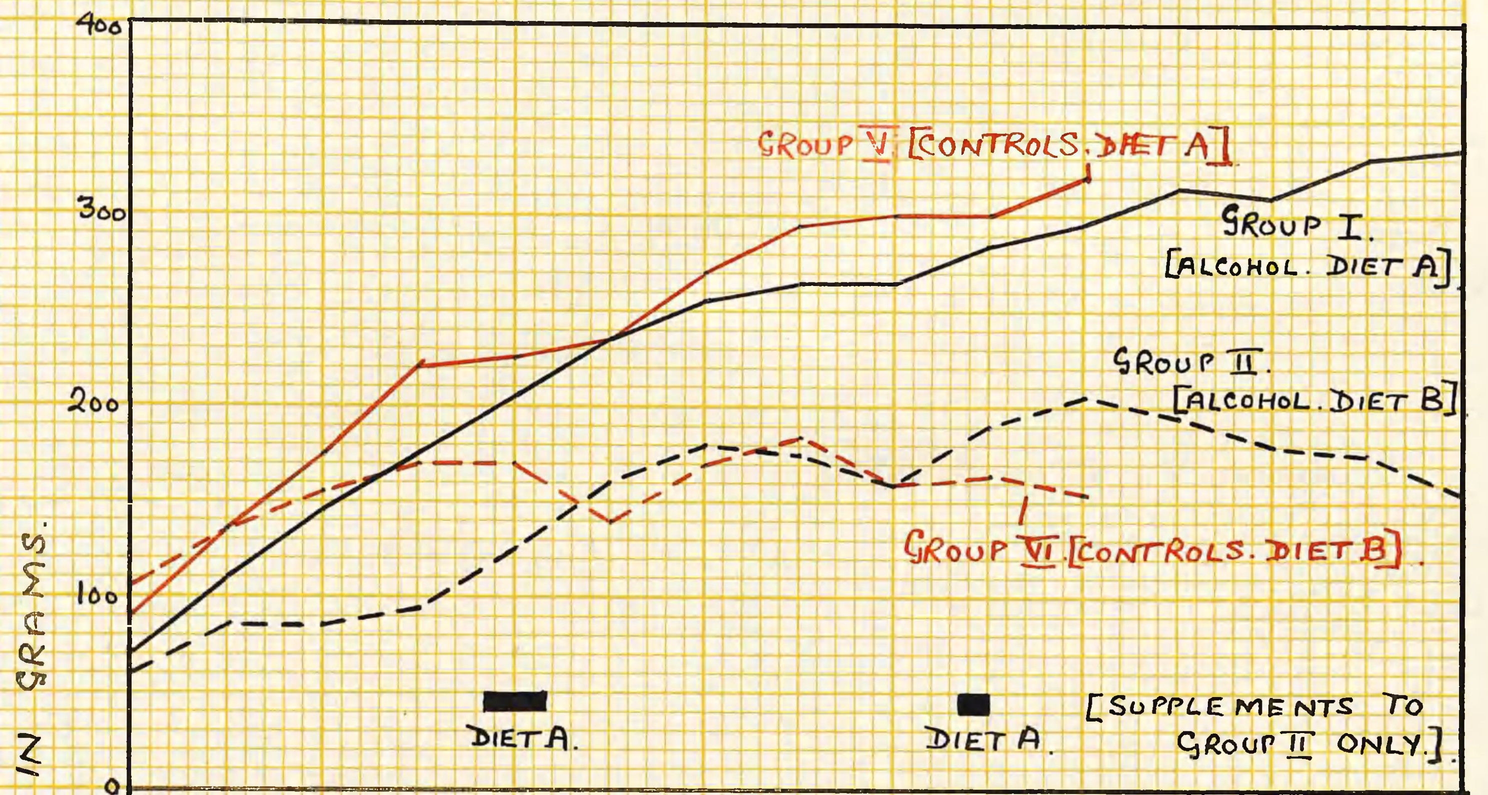
Growth.

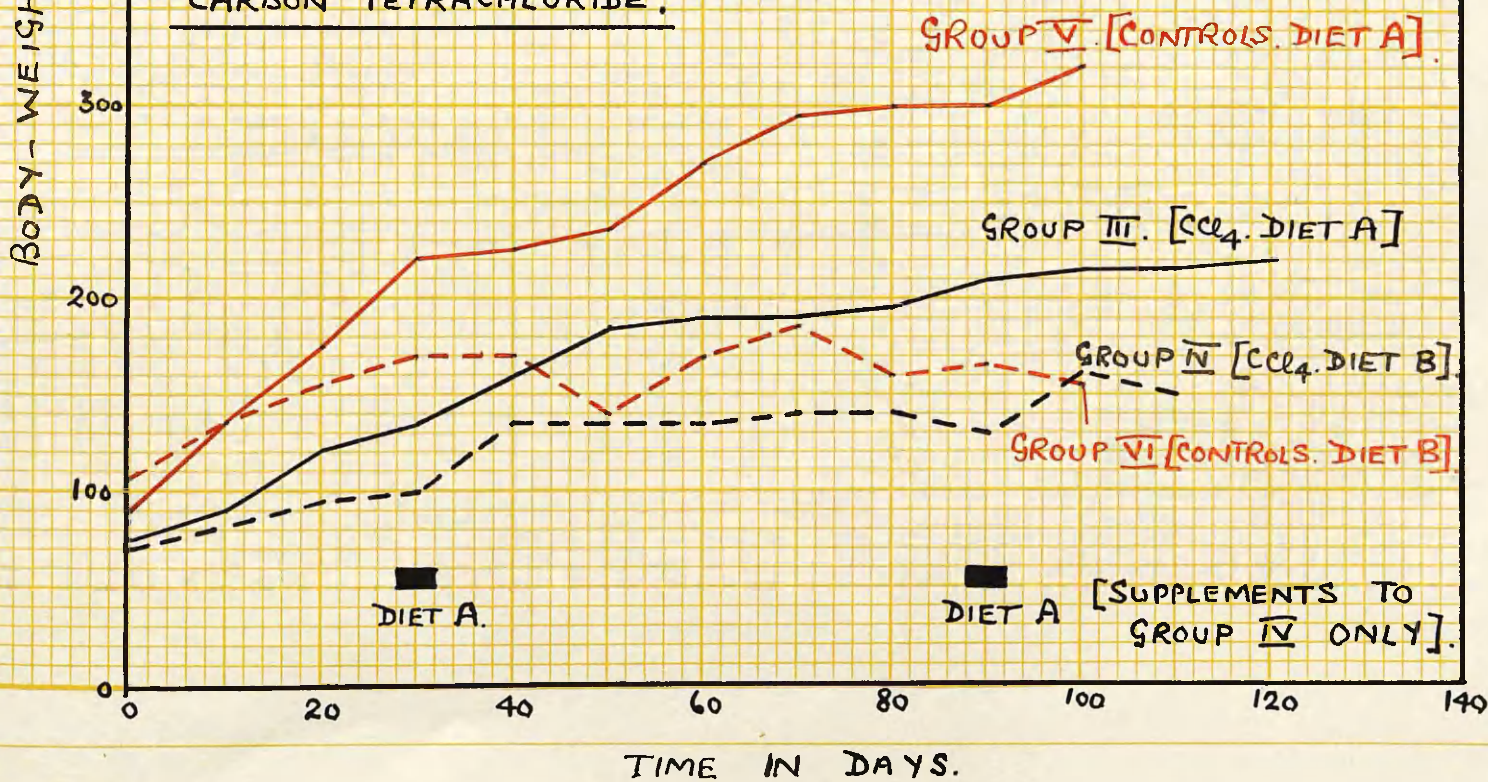
Chart 6 gives the growth curves. The most striking fact is that carbon tetrachloride has depressed growth on both diets, while alcohol has had no effect on growth. Since one of the great functions of the liver is to deal with the products of digestion and pass them on to the tissues in a form suitable for repair and growth, we might consider this as indirect evidence that carbon tetrachloride has depressed liver function, while alcohol has had no effect.

CHART. 6.

"CIRRHOSIS" EXPERIMENT.

[CONTROLS IN RED].





Histological Changes in Livers.

Controls - Groups V and VI.

No abnormality was found in any of the livers in these groups. Prolonged subsistence on diets A and B.O. does not, therefore, by itself have any effect on the liver.

Alcohol: Groups I and II.

Group I: Diet A.

65th day: (18 doses of alcohol)

1. Normal.
2. Normal.
3. Normal.
4. Marked fatty infiltration at centres of lobules.

84th day: (24 doses of alcohol)

5. Marked central fatty infiltration.
6. Similar.

133rd day: (38 doses of alcohol)

7. }
8. } All normal.
9. }

Group II: Diet B.O.

56th day: (16 doses of alcohol)

1. Marked central fatty infiltration.
2. Normal.
3. Normal.

87th day: (24 doses of alcohol)

4. Normal.
5. Normal.

126th day: (36 doses of alcohol)

6. }
7. } Both showed a very slight increase of perilobular connective tissue, as compared with controls (groups V and VI).

The results of alcohol administration are clearly negative. It is most unfortunate that the animals had to be killed so soon, since the last two of group II showed what may very well have been the first stage of a true alcoholic cirrhosis. No conclusions as to the effects of diet can be drawn.

Carbon Tetrachloride: Groups III and IV.

Well-marked cirrhosis had developed in most of the livers from rats killed after 100 days. 10 from group III (diet A) and 8 from group IV (diet B.O.) were killed on the 119th and 112th day respectively, and are therefore comparable. The extent of cirrhosis was estimated by tracing on paper the outlines of fibrous tissue from sections stained by van Gieson's method with the aid of a projecting microscope. The fibrous tissue areas were then cut out and weighed accurately, the weights of the remaining pieces of paper also being determined. It was thus possible to express the fibrous tissue as a percentage of the whole microscopic field. Several fields from each liver were estimated in this way, and the average figure determined, with the following results:-

Percentage of Fibrous Tissue in Cirrhotic Livers.

Rat.	Group III (diet A)	Group IV (diet B.O.)
1	24	9
2	18	27
3	6	9
4	3	37
5	13	15
6	8	14
7	18	23
8	31	5
9	12	Mean = 17%
10	8	
	Mean = 14%	

There is no significant difference between these means ($t = 0.65$).

The different diets have had no effect on the incidence or extent of cirrhosis produced by carbon tetrachloride. This is a disappointing result, in view of the definite difference observed in necrosis after chloroform on these two diets. The degree of vitamin deficiency in this experiment was, of course, much less acute than in the experiments described in Chapter 3, and this may explain the negative result. It would be interesting to repeat the experiment with the addition of choline to each diet, since this was shown to reverse the dietary effect on necrosis.

Conclusions:

1. The administration of alcohol twice a week to rats for a period of 5 months produced only a certain degree of fatty infiltration of the liver. Two rats kept on Diet B.O. (deficient in thermolabile vitamin B) showed a slight increase of perilobular connective tissue after 31 doses of alcohol.

2. Deficiency of thermolabile vitamin B had no effect on the incidence or extent of cirrhosis produced by carbon tetrachloride.

Chapter 5

A Review of the Literature on the Relation between Diet and Liver Disease.

It now seems advisable to review the literature of the experimental work which has been done on the influence of diet on liver disease, in order that the results described above may be viewed in their proper setting. The subject is a large one, and some sides of it are no more than touched on in this review.

The Effects of Proteins, Fats and Carbohydrates on Liver Disease.

A. Experimental Necrosis.

Before dealing with the effects of vitamin deficiency on the liver, which concerns us most closely, it will be convenient to survey briefly the effects which alterations in the three main dietary constituents may have upon liver necrosis. A lot of work has been done on this subject, and some of the results are summarised in the table:-

The Effects of the Three Main Food Constituents on Liver Necrosis.

Author.	Toxic Agent.	Carbohydrate	Protein	Fat
Opie & Alford 1915	$CHCl_3$	Protects		Aggravates
Davis & Whipple, 1919	"	Protects	Protects	
Simonds, 1919	"	Protects		
Moise & Smith, 1924	"	Protects	Protects	Aggravates
de Zalka, 1926	"	No effect		
Goldschmidt et al., 1939	"	Protects	Protects	Aggravates
Miller & Whipple, 1940	"		Protects	
Davis, 1924	CCl_4	Protects	Protects	Aggravates
Chandler & Chopra, 1926-7	"	No effect		
Cutler, 1932	"	No effect	No effect	No effect
Craven, 1931	Salvarsan	Aggravates	Protects	Protects
Schifrin, 1933	"	No effect	?Protects	No effect
Messinger & Hawkins, 1940	"	Protects	Protects	Aggravates
Opie & Alford, 1915	Phosphorus		Aggravates	
Simonds, 1919	"	Protects.		

It must be understood that these results refer only to the degree of liver damage inflicted, and not to any other criterion of toxicity such as mortality-rate or survival time. What is so surprising is the good agreement among all these workers in view of the many differences in experimental methods - species, toxic agent, type of diet, etc.

Carbohydrate. There is general agreement that a high-carbohydrate diet protects the liver. Until recently this was regarded as due to a high glycogen content of the liver cells, but Goldschmidt et al. (1939) found that the level of hepatic glycogen *per se* had no influence on the amount of damage; they consider that carbohydrate protects the liver by reducing the liver lipoid concentration, and regard the latter as the important factor. Craven's finding that carbohydrate increased liver lesions after salvarsan has been criticised by Messinger & Hawkins (1940) on the grounds that he killed his animals at the first appearance of jaundice - a widely varying interval.

Protein. The usual finding is that a high-protein diet has a protective action. Miller & Whipple (1940) demonstrated the protective action of protein most convincingly. They rendered dogs hypoproteinaemic by plasmapheresis or a low protein diet; such dogs suffered a maximum liver injury after chloroform, but even a single meal of protein given two days beforehand greatly reduced the liver damage. Goldschmidt et al. also consider that protein has a protective action and that the effect of starvation in aggravating liver damage is due to gradual depletion of the protein stores of the body.

Fat. In all the papers except one, fat was found either to aggravate or have no effect on liver damage. It is to be noted that most liver poisons are fat-soluble, and the idea that the injurious effect of a high fat diet is due to the increased solubility of the poison in the liver cell protoplasm is often referred to as Well's hypothesis. In studying the effects of vitamin deficiency, it is therefore important to consider what part alterations in liver fat may play. The important work of Connor and his associates on the relation of fatty change to cirrhosis is discussed below.

Starvation: has always been recognised as rendering the liver more vulnerable. Why it should do so is not certain. The older view was that reduction of liver glycogen reduced the resistance of liver cells, but more recent work regards protein-depletion as the important factor (Miller & Whipple; Goldschmidt et al.).

To sum up, then, carbohydrate and protein have a protective action against liver necrosis. Fat increases liver damage after most poisons, and starvation also renders the liver more vulnerable. It is important to bear these facts in mind when considering the effects of vitamin deficiency.

B. Experimental Cirrhosis.

There has been much less work on the effect of diet on experimental cirrhosis, but what there is suggests, as we would expect, that a high carbohydrate diet exerts a protective influence against cirrhosis, while fat has a deleterious effect. v. Glahn et al. (1938) fed rabbits, rats and ferrets on copper and lead arsenates. These substances produce focal necroses in the liver about 3 days after ingestion, and repeated feeding leads to a cirrhosis. By feeding rabbits a high carbohydrate diet they were able to reduce the incidence of cirrhosis from 91% to 9%. Bollmann (1940) administered carbon tetrachloride three times a week to rats which were maintained on various diets. He gives no details of the histological changes in the livers, but the survival times were as follows:-

(Controls	100%)
High CHO diet	156%
Protein	105%
Fat	87%

This suggests a protective action by carbohydrate and an aggravation by fat.

Connor's work on the relation of fatty change to cirrhosis is more fully discussed below, but it should be pointed out here that he has recently produced cirrhosis in dogs simply by feeding on a high fat diet (Chiakoff & Connor, 1940).

Four normal dogs were given a diet consisting of Lard, 10 g., and Lean Meat, 7 g. per kilo per day with supplements of salts and vitamins. They were fed by stomach-tube when they refused food. One died after 138 days and showed no cirrhosis, but the other three, dying at 246, 298 and 386 days, all showed diffuse fibrosis, gross distortion of liver architecture, bile-duct proliferation and excessive fatty change. Connor seems therefore to have proved his point that fatty change, if persisting long enough, will of itself lead to cirrhosis. The importance of dietary factors which lead to accumulation of fat in the liver is obvious.

Another effect of diet is its effect on the regenerative powers of liver cells. Machella et al. (1940) performed partial hepatectomy on rats and then measured the rate of regeneration. Animals which were forcibly given a fixed amount of food daily by stomach-tube regenerated the liver mass more quickly than another group which were allowed to eat freely; the food consumption of the latter group was smaller, and the inference is that a large food intake has a stimulating effect on regeneration. Brues et al. (1936), in a very accurate study of liver regeneration, found that starvation caused a slower increase of liver mass but had no effect on the increase of cell numbers. A high fat diet, on the other hand, inhibited the rate of cell number increase.

To sum up, the diet which will tend to prevent the development of a cirrhosis ought to provide:-

1. A high caloric intake, with adequate protein.
2. A large proportion of carbohydrate.
3. A minimal quantity of fat.

These conclusions will be of importance when we come to consider the therapeutic aspect.

The Effects of Vitamin Deficiency on the Liver.

It should be made clear in the first place that it is only as an etiological factor that vitamin deficiency is being considered here. The opposite sequence -

liver disease leading to vitamin deficiency, owing for example to the poor capacity of a cirrhotic liver to store vitamins - is an entirely different question. There are four main ways in which vitamin deficiency may affect the liver:-

1. By a specific action on liver cell metabolism.
2. By lowering the resistance of the mucous membrane of the alimentary canal to trauma, with consequent infection and exposure of the liver to toxins.
3. By influencing the storage of substances in the liver, e.g. fat.
4. By altering the relative regenerative powers of liver cells and interstitial cells so that fibroblastic proliferation is stimulated.

In considering the different vitamins these four points will be discussed as far as possible.

Fat-Soluble Vitamins.

1. Vitamin A.

The liver is the great storehouse of vitamin A in the body, and it is therefore not surprising that deficiency of vitamin A should accompany disease of the liver. Hori (1895) and Jeghers (1937) reported night-blindness and keratomalacia in liver disease; and more recently Patek and Haig (1939) and Wohl and Feldman (1940) have confirmed by more accurate methods that vitamin A deficiency is present in many cases of cirrhosis. It seems very likely, however, that this is an effect of the cirrhosis rather than its cause. Cirrhosis has not been described as a postmortem finding in patients with xerophthalmia. On the other hand, it is well known that the integrity of the alimentary tract is dependent on an adequate intake of this vitamin and that local infections result from a deficiency, (Cramer & Kingsbury, 1924; Mellanby, 1926). The liver is therefore exposed to various toxins in conditions of deficiency, and this might act as a secondary factor in producing cirrhosis.

On the experimental side there is little evidence that any very great changes occur in the liver when vitamin A is deficient. Moro (1922) reported that

the liver was normal. Jackson (1929) said that no effects of A deficiency on the liver had been described, and suggested that this was due to the large amount of the vitamin normally present in the liver. Ruddy (1939), however, found by the Warburg technique that there was a lowering of oxygen uptake in A-deficient livers. It does not seem likely that deficiency of vitamin A plays much part in liver disease, apart from its secondary action in favouring local infections of the alimentary tract.

2. Vitamin D.

The liver is said to be "somewhat fatty" in rickets by Price (1937), but neither Muir nor MacCallum mention the liver when describing rickets. I have been unable to find any papers on the effect of experimental deficiency on the liver. Jackson (1929) states that deficiency of vitamin D has "no special effect" on the liver.

Water-Soluble Vitamins.

1. Vitamin C.

Several authors have described fatty change in the livers of C-deficient guinea-pigs (Meyer & McCormick, 1928; Spellberg & Keeton, 1939), but the diets used may have been deficient in other factors such as choline, and the fatty change may not have been a specific effect of C-deficiency. Murakami (1939) found that liver function, as judged by the dye test, the santonin test and indol detoxication, was lowered in C-deficient guinea-pigs. In man, there are no characteristic liver changes in scurvy.

The effect of C-deficiency on wound healing is now well established. The administration of vitamin C to a deficient guinea-pig causes a fibroblast response in a healing wound. If, therefore, C-deficiency were present during the development of a cirrhosis one might expect it to have, if anything, a retarding influence on the growth of fibrous tissue. On the whole, vitamin C does not appear to be of much importance in relation to the liver.

2. Vitamin B.

None of the vitamins considered so far have had any very striking association with liver disease, but when we come to the B complex we find that there is a much larger body of evidence for some such relationship.

In the first place, let us consider the evidence for the relation between the complex as a whole and liver disease. In human conditions of gross B deficiency - beriberi and pellagra - there are no constant liver lesions. McCarrison and Norris (1924) state simply that the liver is "enlarged" in beriberi. Price describes nutmeg liver, which is merely a result of the congestive heart failure present in the wet form of the disease. In pellagra no special liver changes are described. All we can conclude from this is that the particular deficiency present in these diseases does not have any gross effect on the liver. In human beriberi the actual deficiency is still in dispute - Williams et al. (1940) were unable to produce the disease in human volunteers fed on a diet deficient only in vitamin B1. In pellagra the deficient factor is nicotinic acid.

On the experimental side, a paper by Heaton (1926) provides interesting evidence of some relation between vitamin B and liver cell metabolism. In tissue cultures of embryonic chick organs, he was able to show that liver cells were stimulated by a thermostable, water-soluble factor present in yeast. Fibroblasts, on the other hand, were inhibited by this factor, and for stimulation required a thermolabile factor present in embryonic tissue extract. Both factors are present in extracts of adult liver, but when the extract is concentrated the fibroblast-inhibiting factor predominates. The importance of these observations when considering the pathogenesis of cirrhosis is obvious. Yeast would therefore appear to have a protective effect on the liver, and further evidence of this is provided by other workers. v. Glahn & Flinn (1939) found that the addition of yeast to a diet of hay and oats protected rabbits against the cirrhosis produced by lead arsenate. Drill & Hays (1940) showed that yeast improved the liver function, as judged by dye excretion, of rabbits fed on thyroid. But the most important paper on this subject is

undoubtedly that by Rich & Hamilton (1940). These workers have produced in rabbits a cirrhosis closely resembling human cirrhosis by dietary means alone. The factor whose deficiency leads to cirrhosis is present in yeast, and is not protein, carbohydrate, fat, salts, or vitamins A, D, E, B1, B2, B6 or nicotinic acid. The addition of 5 g. of dried yeast per day to a rabbit's diet prevents cirrhosis. The authors suggest that the factor may be choline (see below).

We may also refer briefly to some Japanese work on the effects of feeding animals on diets consisting of rice. Ogata (1920), Murata (1923) and Yoshida (1924) all reported varying degrees of fatty change and fibrosis of the liver on rice diets. It is obvious, however, that many deficiencies are present in such diets and the real significance of the results is not clear. Another evidence of a relation between vitamin B and the liver is the fact that yeast feeding inhibits the production of carcinoma of the liver by butter yellow (Ando, 1938; Nakahara et al. 1939).

Before we consider the various factors included in the B complex in turn, it seems advisable to present a brief summary of the present views as to their nature and properties. Only well-recognised factors are included; many others have been described.

The Vitamin B Complex (Water-Soluble)

	Factor	Necessary for:	Deficiency produces:	Daily Human Requirement	Rich Sources.
"VITAMIN B1". Thermolabile.	Vitamin B1, (aneurin, thiamin)	All species	Beriberi (?).	1-2 mg.	Yeast Husks of grain etc.
	Factor W	Rats	Poor growth	?	Liver
"VITAMIN B2". Thermostable.	Riboflavin.	Man, & others	1. Dermatitis 2. Nervous changes	1-2 mg.	Yeast Liver Vegetables
	Nicotinic Acid	Man & others	Pellagra	10-20 mg.	Meat Yeast
	B.6	Rats Chicks ? Man	Dermatitis (rats)	1-2 mg. ?	Yeast Liver
	Pantothenic Acid	Chicks Rats ? Man	Poor growth (rats)	?	Cereals Yeast Liver
	Choline	All species?	Fatty liver	?	Yeast White Flour

(Choline is now included in the B complex by most authorities; its distribution in foodstuffs is very similar).

Vitamin B1. (Aneurin, Thiamin).

The evidence presented in Chapter 3 shows that vitamin B1 deficiency does not influence the liver necrosis produced by chloroform in the rat. Drummond et al. (1938) maintained rats for their whole lifetime on diets providing suboptimal amounts of vitamin B1, but could find no effect on the livers. Aneurin does not therefore have a direct effect on the resistance of liver cells. It is, of course, essential for the normal metabolism of these cells as for all cells in the body, and the liver may even have a special role in the metabolism of this vitamin. Ochoa & Peters (1938)

state: "the liver certainly participates in the metabolism of vitamin B1, which is rapidly taken up by the liver and synthesized to co-carboxylase". It is possible that liver disease might interfere with such synthesis and produce a degree of deficiency; but as we pointed out in the case of vitamin A, this is the opposite sequence from the one we are discussing. Golschmidt & Lewin (1937) reported that B1 deficiency led to low oxygen uptake of liver slices (Warburg technique). The figures they obtained for controls, however, are also much lower than usual, so that one hesitates to put much significance on their results. On the whole, there is little evidence of a specific effect of B1 deficiency on liver cell metabolism.

There are, however, two ways in which vitamin B1 deficiency may indirectly affect the liver. Firstly, there is a condition of general atony of the alimentary canal which may possibly expose the liver to various toxic substances. Drummond et al. (1938) reported a higher incidence of ulcerative lesions of stomach and intestines in B1-deficient rats. Secondly, vitamin B1 is known to affect fat storage in the liver, and this is one of the most important factors governing the susceptibility of the liver to poisons. McHenry (1937) showed that this vitamin increased liver fat in rats, but only when the diet was low in choline, which has the opposite effect. When choline is present even in quite small quantities, its effect of reducing liver fat masks the opposite action of vitamin B1. I was unable to find any significant difference between the liver fats of B1-deficient and control rats (Chapter 3), but the diets probably contained quite enough choline (3.8 mg./rat/day) to mask the action of vitamin B1. It seems unlikely that human diets would ever be deficient enough in choline to allow vitamin B1 to exert its effect. Finally, an interesting effect described by Engel & Phillips (1938, 1939) may be mentioned. They found that the injection of a large dose of thiamin to B1-deficient rats led to an excessive production of free fat in the liver with consequent disruption of normal cell structure. Here is another example of disturbed balance of vitamin intake producing deleterious effects; we shall have more to say of this when considering treatment.

Factor W.

This factor is only mentioned here to recall the fact that it may be identical with the factor responsible for the effect described in Chapter 3, which I referred to as "factor N". There is no information in the literature about the effect of factor W on the liver.

Vitamin B2 (= Thermostable part of B complex).

The thermostable fraction of the B complex appears to have greater importance for the liver than any other vitamin. Evidence for its relation to liver cell metabolism is provided by the observation of Hastings et al. (1939) that deficiency in rats leads to fatty infiltration and lowered oxygen consumption of liver slices; and by the finding of Dontcheff (1939) that vitamin B2 antagonised the action of thyroxin in depressing the oxidation of alcohol by the liver. Rhoads & Miller (1938) found that B2 deficiency in dogs led to a failure of liver function, as judged by the bilirubin excretion test. Gyorgy & Goldblatt (1939) have recently produced liver damage in rats simply by deficiency of an unidentified member of the B2 fraction. The histological changes were very like those produced by carbon tetrachloride, but the necrosis was less uniformly central and there was more haemorrhage and less fatty change. The factor concerned is present in yeast and in Peter's eluate and it is not B1, riboflavin, or B6. It is possibly identical with the factor described by Rich & Hamilton (1940) as preventing cirrhosis in rabbits.

We shall now survey the individual members of the B2 fraction as far as they concern the liver.

1. Riboflavin.

Lillie & Sebrell (1933) described a "yellow liver" in dogs with black tongue - a condition due to deficiency of nicotinic acid; but Sebrell & Onstott (1938) have pointed out that the diet then used was also deficient in riboflavin, and were

able to produce such livers with pure riboflavin deficiency. The main change in the livers of these dogs was an intense degree of fatty change, but there were also scattered necrotic cells, more numerous at the centres of the lobules. Recently Sebrell has described a specific syndrome in humans called cheilosis, which is due to riboflavin deficiency, but no information is available as to whether it is associated with a fatty liver.

2. Nicotinic Acid.

I have been unable to find any information on the effect of deficiency on the liver; no characteristic changes are described in human pellagra.

3. Vitamin B6.

Muus et al. (1937) found that the livers of B6-deficient rats had a low oxygen uptake associated with fatty infiltration. Halliday (1938) found a higher liver fat content than normal in B6-deficient rats; by feeding choline he reduced this fat level but could not bring it back to normal. Further work will probably show that this vitamin is of great importance for the liver. Dr. T.F. Macrae (1941) tells me that he finds B6 deficiency particularly affects the liver.

4. Pantothenic Acid.

Phillips & Engel (1939) described "fatty livers and some hydropic change" in chicks deficient in pantothenic acid, but they give no histological details.

5. Choline.

It is not proposed to review here the rapidly growing literature on the various lipotropic factors which influence the level of liver fat. The whole subject has been fully reviewed recently by Best & Ridout (1939). What we are more interested in is whether choline deficiency will interfere with liver function, or render the liver more vulnerable. As regards liver function, there is a good deal of evidence that choline deficiency depresses it. There is a failure of gluconeogenesis in depancreatised dogs maintained on a low choline diet (Hershey & Soskin, 1931; Best et al., 1933). Dye excretion was lowered in rats fed on a choline-deficient diet (McLean et al., 1937). Welch et al. (1935) found that liver from choline-deficient

animals had a lowered oxygen uptake. The question now arises, whether the accumulation of fat or the lowering of function is the primary effect. A large excess of fat in the cell might easily interfere with normal metabolism; but on the other hand a depression of metabolism might easily result in the accumulation of fat. It seems clear that fatty infiltration does not necessarily interfere with the glycogenic function of the liver (Kaplan & Chiakoff, 1936). On the other hand, the production of cirrhosis in dogs by simple feeding on a high fat diet by Chiakoff & Connor (1940) suggests that prolonged accumulation of fat in the liver cell will ultimately lead to a failure of function. Further work is necessary to decide the question.

Secondly, as regards the effect of choline deficiency on liver changes produced by toxic agents, the evidence is contradictory. Best et al. (1934-5) and Mackay & Barnes (1941) reported that choline had no influence on the fatty change produced by phosphorus, but Laszt & Verzar (1936) claimed that choline had an inhibiting effect. In a careful paper Barrett et al. (1939) found that choline had no effect on the initial accumulation of fat in the liver after carbon tetrachloride, but that the removal of fat was accelerated by choline. No authors have reported any effect of choline on the extent of necrosis.

The results I obtained in Chapter 3 throw little light on these points, except to stress the importance of the balance between choline and the rest of the B complex as a factor influencing liver necrosis. The degree of choline deficiency in my experiments was not enough to cause any significant increase of liver fat, and yet it was enough (in association with deficiency of another factor) to influence the amount of liver necrosis produced by chloroform. This suggests that choline may directly affect liver cell metabolism apart from any lipotropic effect.

However choline may act, it seems probable that a deficiency will render the liver more liable to damage.

Summary of Chapter 5.

Dietary Constituent.	Relationship with Liver Disease.
Carbohydrate:	Protects liver (? by reducing liver fat).
Protein:	Protects liver. Necessary for regeneration.
Fat:	Renders liver more vulnerable. (In excess may produce cirrhosis - Connor).
Vitamin A:	Deficiency favours local infections of alimentary tract.
Vitamin D:	--------
Vitamin C:	Deficiency may retard fibrosis.
Vitamin B Complex:	Protects liver (as a whole).
Vitamin B1:	a. Deficiency favours ulceration of alimentary canal. b. Increases liver fat when choline is deficient.
Factor W:	Probably = factor N (Chapter 3).
Vitamin B2 (whole):	Necessary for normal liver cell metabolism.
Riboflavin:	Deficiency produces fatty change.
Nicotinic Acid:	--------
Vitamin B6:	Deficiency produces fatty change.
Pantothenic Acid:	Deficiency produces fatty change.
Choline:	Deficiency: a. Increases liver fat. b. May interfere directly with liver function. c. Decreases rate of fat removal after poisons.
Factors described by Rich & Hamilton; Gyorgy & Goldblatt (?= choline)	Absence leads to necrosis and cirrhosis.

The main conclusions from this survey of the literature are therefore that carbohydrate, protein and the Vitamin B complex have a special importance in maintaining the integrity of the liver. The importance of ensuring an adequate and balanced supply of all the factors in the B complex is also evident. The accumulation of fat in the liver will favour maximal liver damage.

Chapter 6

The Etiology of Human Cirrhosis.

In the previous chapter we have traversed rather a wide field in discussing the experimental relation between diet and liver disease. It now remains to apply the conclusions we reached there to the problem of human cirrhosis, as far as is possible in the present state of knowledge.

Until recently there was a serious inconsistency between experimental and clinical knowledge of the subject. Moon, reviewing the experimental side of the question in 1934, stated that to cause cirrhosis, any substance must cause necrosis as its acute effect; alcohol will not produce necrosis and therefore cannot of itself be the cause of cirrhosis. The clinicians were equally emphatic that one great factor associated with cirrhosis in man is chronic alcoholism. From the days of Fagge (1875) till today (Hall & Morgan, 1939), the fact that cirrhosis and alcoholism go hand in hand has been constantly demonstrated. This does not mean, of course, that all cases of cirrhosis occur in alcoholics. There are many well-authenticated cases in children where there is no question of alcohol playing a part. Some authors even suggest the abolition of the term "alcoholic cirrhosis" (Boles & Clark, 1936). But on the whole the impression persists that alcohol has a close association with cirrhosis.

The discovery that another disease associated with chronic alcoholism - peripheral neuritis - was in reality due to deficiency of vitamin B1 at once suggested that avitaminosis might provide the answer also to the problem of cirrhosis. Goodhart & Jolliffe (1938), in reporting the successful cure of alcoholic neuritis with vitamin B1, speculated along these lines, and suggested that the vitamin should be tried in the treatment of cirrhosis. It is no doubt as a consequence of these ideas that so much experimental work on the relation between vitamin deficiency and the liver has been carried out in the last two years. Of all this work, the papers

by Rich & Hamilton (1940) and by Gyorgy & Goldblatt (1939) are of first importance, since they were able to produce liver damage by dietary means alone; simple omission of a part of the B2 complex led to the appearance of liver lesions. They also suggested that the factor concerned might be choline, and this naturally leads us to consideration of Connor's important work on the relation of fatty change to cirrhosis.

As long ago as 1870, Ruge showed that alcohol produced a fatty liver in dogs and that carbohydrate feeding diminished the degree of such fatty change. Connor (1938), after a careful survey of the clinical and pathological findings in his cases of chronic alcoholism, came to the conclusion that there was no dividing line between those with fatty infiltration of the liver and typical cases of Laennec's cirrhosis. He suggested that the first effect of alcohol was to cause fatty change in the liver, which gradually gave place to an overgrowth of fibrous tissue; finally the fat might disappear leaving the typical shrunken liver of atrophic cirrhosis. He has subsequently confirmed this hypothesis to a great extent by animal experiments. Chiakoff et al. (1938) reported cirrhosis in depancreatised dogs maintained for long periods on insulin; here we can exclude alcohol as a direct factor and conclude that fatty change persisting for long enough will lead to cirrhosis. Connor & Chiakoff (1938) then reported the production of cirrhosis in dogs by giving alcohol in combination with a high fat diet, and finally (Chiakoff & Connor, 1940) by giving a high fat diet alone. We must therefore revise Moon's dictum that any agent to produce cirrhosis must produce necrosis as its acute effect. Connor has provided examples of cirrhosis following pure fatty change.

Let us now consider the ways in which alcohol may affect the human liver:-

1. By direct interference with oxidation mechanisms, leading to accumulation of fat in liver cells.
2. By causing vitamin deficiency:
   (a) by producing anorexia and low intake of protective vitamins and carbohydrates,
   (b) by providing calories with no corresponding vitamins,
   (c) by leading to gastritis with malabsorption of vitamins.

It has been shown in Chapter 5 that the most important vitamins affecting the liver are included in the B2 group and that deficiency of many of these, including choline, will lead to accumulation of fat in the liver cells. It is a matter for further research to find out which factors are actually deficient in cases of alcoholism, and with the newer methods of estimation this should not be long delayed.

In conclusion, the author would like to put forward the following as a possible etiology for cirrhosis:-

In many cases, the original cause is the ingestion of alcohol which leads to an accumulation of fat in the liver, partly by a direct effect on liver cell metabolism but probably also by producing a deficiency of various members of the B complex - notably choline. Fatty change may be produced in other ways, e.g. in diabetics maintained on insulin, but the essential is that it should persist for long periods. If such is the case, the fatty change will gradually give place to an overgrowth of fibrous tissue, which produces the classical signs and symptoms of cirrhosis. The fat may then disappear from the liver, leaving the shrunken atrophic organ so often met with at postmortem.

Other cases may have necrosis and not fatty change as the initial lesion. It is not impossible that a single dose of a toxic agent may lead to progressive failure in function of liver cells, with consequent overgrowth of interstitial tissue. For example, Boyland & Mawson (1938) found that bile-duct proliferation in the livers of mice was present as long as 170 days after a single intraperitoneal injection of a carcinogenic hydrocarbon, 3:4:5:6-dibenzcarbazole. Whatever the toxic agent producing the original lesion, vitamin deficiencies may again play a part in maintaining conditions in the liver which favour overgrowth of fibrous tissue, and at the same time inhibit liver cell regeneration.

Chapter 7

Therapeutic Suggestions.

The therapeutic indications arising from all these observations are few in number and quite definite in character. The regime on which a case of cirrhosis is put should provide:-

1. An adequate supply of carbohydrate and protein.
2. A minimum quantity of fat.
3. A good supply of all the vitamins, but particularly vitamin B complex.

The use of high carbohydrate diets in liver disease is well recognised and need not be stressed. Bach & Klemperer (1929), Althausen (1933) and McNee (1939) have described very adequately the wide field which carbohydrate therapy finds here. The dietary protein should be adequate in quantity and of first-class biological quality.

The use of vitamins in cirrhosis was first described by Patek (1937), who reported that 10 out of 13 cases were definitely improved by being put on a well-balanced diet supplemented by cod liver oil concentrate, orange juice, liver extract and crystalline vitamin B1. Unfortunately he had no controls on the diet without supplements, so that it is difficult to know how much of the improvement was due to the vitamins. This is a fallacy to be avoided in therapeutic experiments. The institution of an ordinary hospital regime with regular meals and a limited consumption of alcohol would probably improve most cases of cirrhosis. Another point to be remembered is that the administration of vitamin B1 often leads to an improvement of appetite, with the result that larger quantities of the other vitamins are ingested. Most cases of human cirrhosis have probably a multiple deficiency, and one should therefore examine carefully any claims that vitamin B1 has produced improvement. The experimental work described in this thesis suggests that vitamin B1 is of less

importance for the liver than various other members of the B complex. The successful use of lipocaic in the treatment of fatty liver was reported by Rosenberg (1938); lipocaic is a fat-free alcoholic extract of beef pancreas quite distinct from choline, and its efficacy in restoring the liver to normal was proved in this case by biopsy.

It is clear that until we are able to determine the precise deficiency present in each case of cirrhosis, we can only ensure that all necessary factors are supplied in the diet in adequate amounts; and even if we could say exactly which factors were deficient, it would still be better to administer a preparation containing the entire B complex rather than a pure vitamin. If there is one fact obvious from the experiments I have described, it is that the balance between the various members of the B complex is just as important as deficiency of any one of them. With Elvehjem (1940), therefore, one feels quite strongly that the present tendency towards the use of highly concentrated or pure factors is wrong, and that what we should aim at in treatment is to administer all the factors known to be necessary in a more natural form, such as dried yeast. Deficiency of a single factor is unlikely to occur in man in view of the very similar distribution of the different factors in natural foods. Perhaps it will exercise a cautionary effect against the indiscriminate use of pure vitamins in large doses if we recall the histological changes produced in the livers of B1-deficient rats by the injection of vitamin B1 (Engel & Phillips 1938).

In conclusion, it is interesting to record that Askey (1939) has recently suggested that the balance between choline and vitamin B1 in the diet may be of importance in the pathogenesis of cirrhosis. He gives no evidence in support of his suggestion, however. I have shown above that the balance of choline and a thermolabile factor other than vitamin B1 (possibly factor W) exercises an important influence on the response of the rat's liver to chloroform, and my experiments also suggest that vitamin B1 has less importance for the liver than some other members of the B complex.

PART II.

The Relation Between Leucocytosis and Liver Damage.

Chapter 8

Experimental: The Protective Action of Xanthine and Allied Substances on the Liver.

Forbes et al. (1936) reported that a liver extract injected subcutaneously protected rats' livers against necrosis from chloroform and carbon tetrachloride. Further purification of this extract proved that the active principle was mono-sodium-2,6-dioxypurine, or sodium xanthine, (Neale, 1937). It was also shown that synthetic sodium xanthine had a similar protective action, and that other purines - sodium guanine, guanosine, hypoxanthine, and uric acid - exerted similar although less powerful protection (Neale & Winter, 1938). The mechanism of the protective action was discussed in the last of these papers, and it was even suggested that xanthine should be tested clinically in acute toxic conditions where liver damage was suspected. Barrett et al. (1938) confirmed the protective action of the liver extract prepared by Forbes, and found also that the healing of the liver lesion after carbon tetrachloride was accelerated by the injection of xanthine. Fitzhugh (1939) found that the protective action was hardly apparent at 24 hours but was definite at 48 hours when the xanthine was injected 24 hours before the toxic agent (carbon tetrachloride); but he could find no evidence of quicker regeneration of liver cells in xanthine-treated animals. Drinker (1939) is the only author who failed to obtain protection from xanthine, but he used a mixture of penta- and hexa-chlornaphthalenes as the toxic agent, and administered most of the xanthine by mouth; the other workers had all injected xanthine subcutaneously. The whole subject has been recently illuminated by the work of Ravdin et al. (1939). These authors first confirmed the protective action of sodium xanthine against chloroform necrosis, but were struck by the fact that xanthine, and all the other purines which have a protective action, are practically insoluble. When the animals were killed, the xanthine could be found still at the site of injection, and histologically there was always an

inflammatory reaction round it with leucocytic infiltration in animals which had been protected. They therefore tested other substances which would induce an inflammatory reaction and found that both sodium ricinoleate and a colloidal suspension of carbon were more effective than xanthine. It is clear, therefore, that the effect of xanthine is not specific and is simply due to its capacity for producing a local inflammation, with emigration of leucocytes. Ravdin suggested that the insoluble matter injected would liberate protein-split products from the surrounding tissues, and that these were responsible for reducing the liver damage. Forbes & Outhouse (1940) agreed that other insoluble substances had a protective effect.

Experimental Methods.

Albino rats weighing from 100-200 g. were used throughout. Xanthine and various other substances were injected subcutaneously at varying intervals before the toxic agent - chloroform, which was mixed with equal parts of liquid paraffin before injection and given in a dose of 1 c.c./Kg. The rats were usually killed 24 hours later. Portions of the liver and of the site of xanthine injection were taken for histological examination. In one experiment the amount of chloroform present in the mass of xanthine was estimated, since it was thought that simple adsorption of chloroform by the insoluble material injected might account for the liver protection. The method used was that devised by Cole (1926-7) - a quantitative modification of Fujawara's colour reaction. It was carried out as follows:-

The mass of xanthine and surrounding tissue was cut up into small pieces and then ground with sand in a mortar under water slightly acidified with HCl. The mortar was left to stand for an hour, when the supernatant fluid could be poured off and made up to a convenient volume with distilled water. Then in a narrow 10 c.c. test-tube were placed 2 c.c. of 20% caustic soda, 1 c.c. of pure pyridine, and 1 c.c. of the aqueous tissue extract. The loosely plugged tube was then immersed in boiling water for exactly one minute. A red pigment is produced in the ~~absence~~ presence of

chloroform, which settles at the top. This was taken off with a pipette and compared colorimetrically with a series of standards. These were prepared from a solution of basic fuchsin in 15% alcohol + 0.01% HCl, and were standardised against known amounts of chloroform. This method has an error of 5 - 10% and is applicable to chloroform concentrations of 100 - 0.1 mg. per cent.

Liver Damage: was assessed in exactly the same way as in Part I of this thesis. (See Plate for standards).

Experiment 1.

The experimental rats were injected with sodium xanthine. This was prepared from xanthine by the method of Neale & Winter (1938) and suspended in water in a concentration of 200 mg. per c.c. It was found necessary to use a wide-bore needle for the injections, since blocking occurred very easily. Five experimental rats were given 100 mg. of sodium xanthine subcutaneously on two successive days, while five controls received an equal volume of saline at the same times. On the third day all the rats were given 0.1 c.c. chloroform subcutaneously and were killed 48 hours later. Sections of liver were stained with haematoxylin and eosin, and for fat with Scharlach R. and haematoxylin, with the following results:-

No.	Xanthine-treated.		Controls.	
	Grade.	Fat.	Grade.	Fat.
1	0	0	2	+
2	2	+	2	+++
3	1	++	2	+
4	0	+	2	++
5	0	0	1	+++
Means =	0.6		1.8	

These results are not conclusive, but suggest that xanthine has lessened both the degree of necrosis and the amount of fatty change produced by chloroform.

Experiment 2.

In this experiment the protective effect of xanthine was compared with that of other insoluble substances. Preliminary work showed that as far as the stimulation of leucocyte infiltration goes, these substances could be arranged in the order:-

Indian Ink > Charcoal Suspension > Xanthine Suspension > Red Blood Cells > Carborundum

With carborundum there was very little invasion of the mass by leucocytes 24 hours after injection.

Various groups of rats were injected with these substances, as shown below. All the rats, including controls, received 1 c.c. chloroform per Kg. subcutaneously and were killed 24 hours later.

Degrees of Liver Damage 24 hours after 1 c.c. $CHCl_3$ /Kg.

Group.	"Protective Injection"	Interval between injection and $CHCl_3$	Local Leucocyte Reaction	No. of Rats	Mean Liver Damage
I. Controls	NIL	-	NIL	12	2.6
II. Carborundum	1 c.c. Carborundum Suspension	24 hrs.	±	6	2.1
III. R.B.C.	1 c.c. thick suspension of rat's R.B.C.	24 hrs.	+	6	1.5
IV. Xanthine	100 mg. Xanthine per 100g. body weight	40 hrs. 24 hrs.	++ +	6 6	1.3 1.7 } 1.5
V. Charcoal	1 c.c. thick watery suspens. charcoal	24 hrs.	++	6	1.1
VI. Indian Ink	1 c.c. Indian Ink	5 days 40 hrs. 24 hrs. NIL	+++ +++ +++ ++	6 6 6 6	0.3 1.1 0.3 1.0 } 0.7

There is a significant difference between the liver damage of groups I and IV ($t = 3.2$) and between groups I and VI ($t = 7.7$).

It can be seen from these results that charcoal and Indian Ink have a stronger protective action than xanthine, in the doses employed. Even when injected at the same time as chloroform, Indian Ink will exert quite a definite action. In all groups whose livers were protected, there was a leucocytic reaction at the site of injection of the insoluble material, and as can be seen from the table, there is fairly good correlation between the extent of this reaction and the degree of protection afforded. Thus carborundum, which stimulates hardly any inflammatory reaction, has very little protective action, while Indian Ink which spreads widely in the subcutaneous tissue, and is rapidly invaded by Leucocytes, has a very marked protective action.

The Quantity of Chloroform Absorbed by the Injected Mass.

One possible explanation of the protective action of these insoluble substances might be simple absorption of the toxic agent by the injected mass. The quantity of chloroform in the injected mass was estimated, as described above, in three groups of rats when they were killed 24 hours after chloroform. In each group the injection masses were pooled, and the total quantity of chloroform in the group determined.

Group.	Total $CHCl_3$ recovered.	Total $CHCl_3$ injected to group.
IV - Xanthine 40 hrs. before $CHCl_3$	$<$ .0001 c.c. (no colour)	2.18 c.c.
VI - Indian Ink 40 hrs. before $CHCl_3$	$<$ .0001 c.c. (no colour)	2.28 c.c.
VI - Indian Ink at same time as $CHCl_3$	$<$ .0001 c.c. (no colour)	3.18 c.c.

It is clear, therefore, that the protection of the liver is not due to the retention of chloroform by the material injected.

The Histological Reaction at the Site of Injection.

The type of reaction was not found to vary greatly with the different substances used. It consists in a typical inflammatory reaction of mild degree in the loose areolar tissue surrounding the inert material. There are the usual vascular dilatation and leuocyte emigration; but the emigration of large mononuclear phagocytes is perhaps earlier than in the average inflammatory process. Twenty-four hours after an injection of Indian Ink, most of the carbon mass has been invaded by phagocytes - polymorphonuclear leucocytes and large mononuclear cells in about equal numbers, with smaller numbers of lymphocytes. Phagocytosis was much less active after injections of carborundum and rat red cells, and the correlation of leucocytic response with liver protection has been mentioned above. It may be added that in none of the animals was there evidence of the presence of the injected material in the reticuloendothelial system generally - no pigment was found in the Kupffer cells in the liver or in the cells lining the splenic sinuses.

Discussion.

The results of these experiments are in complete agreement with the work of Ravdin et al. (1939). They suggest that the effect of xanthine and allied compounds when injected is quite non-specific, and is associated with their capacity of inducing a local inflammatory reaction. They do not act by absorbing the toxic agent.

The mode of action of such insoluble substances on the liver is quite obscure; they do not themselves gain access to the blood stream in the course of 24 hours. It is possible that they may liberate locally the products of protein breakdown, as suggested by Ravdin, but it seems doubtful whether the amounts produced would be able to protect the liver. In this connection it is interesting to note that Madden et al. (1940) could find no evidence of increased plasma protein production after the development of a turpentine abscess in hypoproteinaemic dogs; one would

imagine that the amount of tissue destruction produced by carbon will be much less than that produced by the injection of turpentine.

The essential factor must be in some way associated with the local inflammation, since when this is absent no protection is afforded. We must therefore look for some general effect of a local inflammation. Such an association was described by Blumenthal (1939), who found that the alterations in the differential white count of the peripheral blood following the transplantation of various tissues were closely parallel to the type of leucocyte response around the transplant. Here we have an example of a local inflammatory process producing a systemic effect. It seems likely that an injection of carbon will be associated with a general leucocytosis, and this is an obvious line for further research. It would be very interesting, for example, to know whether substances such as peptone and nucleic acid which produce a general leucocytosis exert any protective effect on the liver; and conversely whether liver damage could be increased by depressing leucopoiesis with benzol or X ray radiation of the marrow.

There is one other paper which suggests that there is some association between haemopoietic tissue and the resistance of the liver. Whipple (1912) found that pups were comparatively insusceptible to chloroform necrosis of the liver during the first week of life; searching for some explanation, he realised that this period coincided with the time when active haemopoietic tissue was present in the liver - in the form of "blood islands". He suggested that the presence of this tissue in some way protected the liver cells from injury. Blood islands begin to disappear from the liver during the second and third weeks of life and are absent after the fourth week; pups older than four weeks suffer the same liver damage from chloroform as adult animals. These facts are suggestive, and it would be interesting to explore the question further.

The Relation of these Observations to Cirrhosis.

Experimentally, Forbes (1939) was able to prevent carbon tetrachloride

cirrhosis in rats by giving an injection of sodium xanthine before each dose of carbon tetrachloride. This is the result that we should expect in view of the protective action of xanthine against necrosis.

Turning to the bearing these observations may have on the pathogenesis of human cirrhosis, there is one well-known fact which seems relevant. The role played by alcohol in the etiology of cirrhosis was discussed in Part I, and the general conclusion reached was that while not to be regarded as a direct cause of cirrhosis, it is in most human cases the predominant factor. Now, it is well known that alcoholics usually fail to exhibit the normal leucocytic reaction in the presence of an acute infection such as lobar pneumonia; this suggests that alcohol has some depressing action on the haemopoietic tissue or on the mechanism by which it is stimulated to combat an infection. Is it possible that leucocytes normally exert a protective action on liver cells and that alcohol lessens this action, and thus lays the liver cell open to every injury which the portal blood-stream has to offer?

Conclusions from Part II.

1. The following substances, when injected subcutaneously, were found to exert a protective action on the liver of the rat against chloroform necrosis:-

   Indian Ink > Charcoal > Xanthine > Red Blood Cells > Carborundum.

   They are arranged in order of efficacy.

2. There is an association between the degree of inflammatory reaction at the point of injection and the protection afforded to the liver.

3. The protective action is not due to retention of chloroform by the substance injected.

4. It is suggested that a general stimulation of haemopoietic tissue may result from such an injection and that leucocytes may protect liver cells.

5. The bearing of these observations on the pathogenesis of human cirrhosis is discussed.

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