THE RELATIVE EFFECTS OF PROTEIN, CHOLINE, AND METHIONINE IN THE TREATMENT OF EXPERIMENTAL DIETARY CIRRHOSIS IN THE RAT*

By IRVIN C. PLOUGH,‡ M.D., ARTHUR J. PATEK, JR., M.D., AND MARGARET BEVANS, M.D.§

(From the Research Service, First (Columbia University) Division, Goldwater Memorial Hospital, and the Department of Medicine, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, May 22, 1952)

It is established that rats fed diets low in protein and deficient in lipotropic substances develop fatty changes and cirrhosis of the liver. Although many studies have been made relative to the production and prevention of dietary cirrhosis in the rat, few reports have dealt with the treatment of this disease. The fact that choline prevents this form of cirrhosis suggests that it may be of value in the treatment. However, this may not be the case, since a substance which prevents hepatic injury need not necessarily promote repair and regeneration of the injured liver. Moreover, multiple factors may be involved in the reparative process.

Lowry, Daft, Sebrell, Ashburn, and Lillie in 1941 (1) reported results of short term treatment in a small series of rats with nutritional cirrhosis. The addition of choline to a basal diet containing 4 per cent leached casein, or the change to a diet containing 50 per cent casein brought about the disappearance of liver fat and regeneration of liver cells, but the excess of connective tissue seemed to remain unchanged. In a later study (2) the periods of treatment were extended up to 1 year following a diagnostic liver biopsy. The rats surviving the operation were divided into two groups. One group received the 4 per cent casein diet with added choline and a second group received a 30 per cent casein diet. Findings were similar to those of the earlier experiment in which there were disappearance of fat and regeneration of liver cells, but no apparent alteration of connective tissue. The addition of choline

* This work was supported in part by grants from the Public Health Research Institute of the City of New York, Inc., and the Albert and Mary Lasker Foundation Gift for the Study of Animal and Human Arteriosclerosis.

‡ Major, Medical Corps, United States Army. Present Address: Army Medical Service Graduate School, Army Medical Center, Washington, D. C.

§ Fellow of the Public Health Research Institute of the City of New York, Inc., Research Fellow, Research Service, First (Columbia University) Divison, Goldwater Memorial Hospital.

to the basal diet seemed more efficacious than the change to a 30 per cent casein diet.

Sellers, Lucas, and Best in 1948 (3) tested various therapeutic regimens in rats with cirrhosis due to carbon tetrachloride poisoning. Animals placed on a control "hypolipotropic" diet experienced high mortality rates and their livers were very fatty. Those animals receiving choline, methionine, or casein showed striking changes with loss of hepatic fat, regeneration of cells, and disappearance of fibrous tissue. Although the observations are of great interest, the experimental conditions are not analogous to the present study and so the findings are not suitable for comparison.

The report of György and Goldblatt (4) throws some doubt on the therapeutic value of choline and methionine in experimental nutritional cirrhosis. Rats were placed on a low protein, cirrhosis-producing diet for about 21 weeks and were then treated for a corresponding period with various dietary factors. Tests were made of high and low protein diets, of choline, methionine, liver extract, and thiouracil singly and in combination. Because the procedure was attended by a high mortality rate, liver biopsy was performed only in a limited number of animals. The authors concluded that lipotropic substances were effective in moderate degrees of cirrhosis, causing reduction in fat, regeneration of liver cells, and resorption of connective tissue. However, in severe cirrhosis the therapeutic value of lipotropic substances was disappointing. The best results were obtained by a combination of casein and liver extract.

The problem was reexamined because of these inconclusive and divergent results. In the experiments to be described, a diet deficient in protein and lipotropic substances was employed to produce cirrhosis of the liver in rats. An attempt was made to evaluate the effects of dietary protein and of lipotropic factors separately and together in reversing the disease process. These studies suggest that high protein feeding produces reparative effects greater than those attributable to choline and methionine.

Methods[1]

Albino rats of the Sherman strain ranging from 6 to 8 weeks in age and weighing from 40 to 130 gm. were employed. The average weight was 90 gm. The animals were housed in separate cages. They were weighed at weekly intervals. Between weaning and the beginning of the experiment the animals were fed a commercial dog "chow" diet. The experimental diet was patterned after that devised by Daft, Sebrell, and Lillie (5) to produce cirrhosis. This diet, which was low in protein, choline, and methionine, was fed for a period of 16 to 19 weeks.

[1] In a preliminary study, 55 rats were employed, with a sex ratio of 1 male to 1.4 females. After being fed a 4 per cent casein diet for a period of 16 weeks a representative sample of 8 rats was sacrificed and the remaining animals placed in two dietary groups, namely 30 per cent casein and 4 per cent casein with supplementary choline. Results were consistent with findings in this report. However, it became apparent that sampling was not a dependable technique in a series of this size, and that the male was more susceptible to cirrhosis than the female, as others have observed (7). Therefore in the present series animals of the same sex were employed and sampling was abandoned for biopsy of the liver, permitting appraisal of the disease process before dietary treatment was begun.

Liver biopsies were then performed, and the rats were separated into groups comparable according to the severity of the lesions in the liver. These groups were placed on several test diets for periods of 15 to 19 weeks. The animals were then sacrificed, and comparison was made of the livers before and after dietary therapy. The composition of the experimental diets employed is shown in Table I. The Hubbell-Mendel salt mix (6) was employed. Daily vitamin supplements were fed as follows: thiamine chloride 100 μg.; riboflavin 50 μg.; pyridoxine 20 μg.; calcium pantothenate 20 μg.; and niacin 1 mg. Once weekly each rat was fed percomorph liver oil with added tocopherol to provide 2000 I.U. vitamin A, 400 I.U. vitamin D, and 3 mg. alpha-tocopherol per week.

TABLE I

Composition of Experimental Diets

Low protein		High protein	
	per cent		*per cent*
Vitamin-free casein	4.0	Vitamin-free casein	30.0
L-cystine	0.5	L-cystine	0.5
Wesson oil	5.0	Wesson oil	5.0
Salt mixture	3.0	Salt mixture	3.0
Corn-starch	87.5	Corn-starch	61.5

TABLE II

Plan of Experiment

Group No.	Foreperiod (16–19 wks.)	Treatment period (15–19 wks.)
1	4 per cent casein diet	Biopsy 4 per cent casein diet + choline + methionine
2	" " " " "	" 30 per cent casein diet + choline
3	" " " " "	" " " " " " "
4	" " " " " (control)	" 4 " " " " "
5	30 " " " " + choline (control)	" 30 " " " " + choline

Choline or methionine or both were added to some of the diets. When employed, choline chloride was mixed in dry form with the diet in a proportion of 0.5 per cent. Thus, the average amount consumed per rat per day, 8 gm., contained 40 mg. In the low protein diets containing methionine, the DL form was added at a level of 0.8 per cent. This level was estimated to be equivalent to the methionine content of the 30 per cent casein diet. The plan of the experiment is indicated in Table II. Actually, two separate series were studied, but, since similar methods were employed and similar results were obtained, the data are combined in this report.

Liver biopsies were performed as follows: Under ether anesthesia a longitudinal incision was made in the epigastrium just to the left of the midline. A wedge-shaped piece, about 0.5 cm. square, was cut from the presenting lobe of the liver. The defect was filled with a small piece of dry gelfoam and the wound was closed with interrupted silk sutures.

After the period of dietary treatment the rats were sacrificed by means of intraperitoneal injections of nembutal. At autopsy the livers were weighed, and specimens were taken from

the liver, spleen, kidney, heart, and lungs for histologic examination. In animals which were subjected to biopsy and which subsequently died or were sacrificed, sections of the liver were taken both from the area of the biopsy scar and at a distance from this site.

Duplicate specimens of tissue were fixed in Zenker's solution and in 10 per cent formaldehyde. Routine paraffin sections, cut at 5 μ, were stained with hematoxylin and eosin. Trichrome stains were employed for the better definition of connective tissue. Acid-fast stains were used for the demonstration of ceroid pigment, and frozen sections stained with oil red O and hematoxylin for more accurate definition of fat.

The degrees of gross cirrhosis and fat in the liver in both biopsy and autopsy specimens were graded on an arbitrary scale of 1 to 4 plus by two observers. Microscopic sections of the liver were also evaluated in this manner and, in addition, necrosis, cellular infiltration, bile duct proliferation, and the amount of ceroid pigment were similarly estimated.

EXPERIMENTAL

Fig. 1 illustrates the average growth curves in one series of rats employed in this study. During the foreperiod the rats on the 30 per cent control diet grew rapidly, whereas those on the 4 per cent casein diet grew slowly. In 2 groups of animals there was an abrupt acceleration of growth when their diets were changed from the 4 per cent to the 30 per cent casein,—with or without supplements of choline. The addition of choline and methionine to the 4 per cent casein diet provided no apparent stimulus to growth. Those animals on the 4 per cent casein diet appeared stunted, feeble, and irritable, whereas those on the 30 per cent casein diet were sleek and vigorous. These differences in growth were not correlated with corresponding differences in food intake. Animals on both low and high casein diets consumed approximately the same amount of food (in grams), the average being 8 gm. per day. The differences in growth appeared to be related to qualitative differences in the diet and to differences in utilization of the diets.

In the course of this experiment certain of the rats, particularly those fed the 30 per cent casein diet, developed a dermatitis. This was characterized by scaling, swollen tails and feet, loss of hair, and ecchymoses near the eyes and ears. The lesions resembled the description of deficiency of unsaturated fatty acid (8, 9). According to the manufacturer, Wesson oil contains 43 per cent linoleic acid. Therefore 8 gm. of any of the diets contained approximately 150 mg. This is five times the stated daily requirement of the rat for linoleic acid (10). In spite of doubt as to the etiology of the dermatitis a small supplement (15 to 30 mg. daily) of linoleic acid was fed all animals in the final 8 weeks of the experiment. Although there seemed to be partial recovery, two rats of the high protein control group died during the 16th week of the treatment period. These rats had severe renal lesions, similar to those described as occurring in deficiency of unsaturated fatty acid.

Mortality was high among those rats fed the 4 per cent casein diet during the foreperiod. In the early stage of this period death was due chiefly to hemorrhagic cortical degeneration of the kidneys, presumably from choline deficiency. Those

deaths occurring later in the foreperiod were due chiefly to hydronephrosis secondary to renal calculi. Only 10 animals died from cirrhosis. 9 of 79 rats sub-

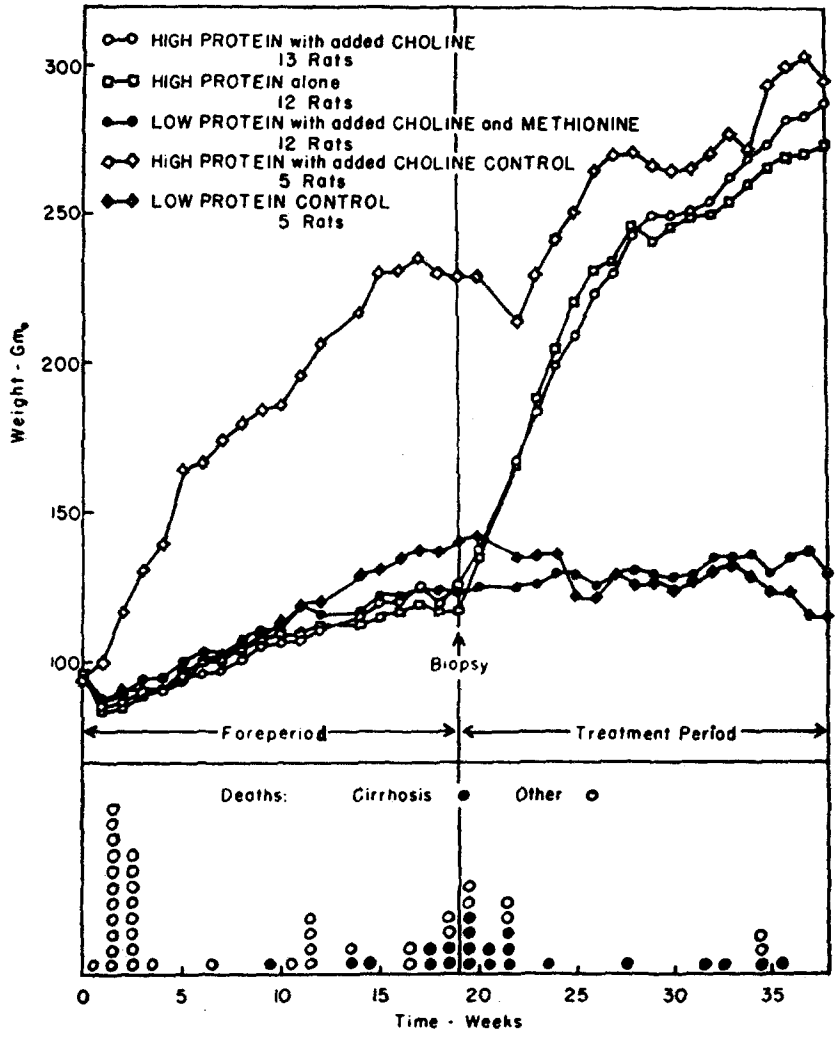

FIG. 1

jected to biopsy of the liver died within a week of this procedure, a mortality rate of 11.4 per cent. The fatalities usually occurred in animals with very severe cirrhosis. During the treatment phase of the experiment there was a sharp reduction in death rate in all groups, once the animals had survived a critical period of about 2 weeks. This reduction in death rate applied to the control

animals as well as those receiving therapeutic diets. During the period of treatment 18 rats died of various causes. There were more deaths in the group fed 4

TABLE III

*Relation of Diets to Mortality**

Foreperiod (16–19 wks.)

No. of rats	Diet	Deaths during foreperiod
143	4 per cent casein	69
5	30 " " " + choline	0
Total.....148		Total, *per cent*.....48

No. of biopsies.....79
Deaths within 1 week of biopsy.....9
No. of rats available for treatment.....70

Treatment period (15–19 wks.)

Group No.	No. of rats	Diets	Deaths (2–9 wks.)	Deaths (10–19 wks.)
1	23	4 per cent casein + choline + methionine	5	5
2	23	30 per cent casein + choline	2	0
3	14	30 per cent casein	2	1
4 (control)	5	4 per cent casein	1	0
5 (control)	5	30 per cent casein + choline	0	2

* Groups 1, 2, 3, 4 were fed 4 per cent casein diet during foreperiod of 16 weeks. After being placed in comparable groups, according to histologic appearance of livers, they were fed various diets as listed. Group 5 was maintained on a diet containing 30 per cent casein plus choline throughout the foreperiod and treatment period. Two of this group died of renal disease.

Animals that had not died during the period of treatment were sacrificed at the end of 15 to 19 weeks.

per cent casein supplemented with choline and methionine than in those fed 30 per cent casein diets (*cf.* Table III).

Pathologic Changes

The pathology of the lesions of dietary cirrhosis produced by various protein deficient regimens has been accurately described by many investigators (4, 7,

11–13). Therefore only a cursory description of the changes pertinent to the conditions of this experiment will be included in this report.

In animals dying as early as 4 weeks after the institution of the cirrhosis-producing regimen the gross appearance of the liver was altered. Large, irregular areas of congestion appeared on the pale yellow surface and the consistency of the organ was flabby. Microscopically the sinusoids were congested and the parenchymal cells showed great variation in size largely due to the presence of fat droplets within the cytoplasm. This accumulation of lipid which began in the cells around the central vein accounted for the appearance of irregularity and disarrangement of the liver cords. Small foci of necrosis involving groups of 5 or 6 cells with minimal or no cellular reaction about them were present in these early lesions. Disintegrated cells and debris were frequently present in the lymphatic channels, as was hemorrhage about the central veins.

As the duration of the experiment lengthened the livers increased in size and were pale yellow and finely reticulated. Microscopically the huge lipodiastemata described by Hartroft (13) seemed to replace up to 90 per cent of the parenchyma. Concurrently with these changes connective tissue proliferation was evident about the hepatic vein areas and extended in strands along the sinusoids to the adjacent central zones. Within the connective tissue groups of disintegrating parenchymal cells, histiocytes, lymphocytes, and occasional polymorphonuclear cells were enmeshed. Bile duct proliferation was not prominent but ceroid pigment was observed in the connective tissue and later in macrophages. With the increase in fat content, connective tissue proliferation, focal necrosis, and distortion of the lobule occurred.

When the low protein, cirrhosis-producing diet was supplemented by choline and methionine, with or without additional protein, certain changes occurred fairly consistently though with varying degrees of rapidity. The most striking alteration was the rapid reduction of fat. Animals dying 1 or 2 weeks after biopsy showed appreciable reduction in visible and stainable fat. It disappeared first from the cells about the portal triads. Within the cells the lipid droplets became finely divided. Later a faint, homogeneous blue-green perinuclear halo remained in hematoxylin and eosin preparations. Comparable sections, appropriately stained, showed this material to be sudanophilic but not acid-fast. However, complete resolution of intracellular lipid in parenchymal cells occurred only in those animals on the high protein regimens.

It was difficult to evaluate the connective tissue changes which occurred after treatment. Before treatment the connective tissue appeared edematous; the fibrocytes were large and the collagenous fibers hazy. As the duration of the experiment lengthened with or without treatment, the fibers condensed and in some sections seemed hyalinized.

Without doubt the presence of fat influenced the estimation of the connective tissue seen in sections of the liver, since large amounts of it tended to obscure and perhaps to compress the strands of connective tissue. For this reason, where the amount of fat had decreased from 4+ to 0, the amount of connective tissue appeared to be slightly increased.

It seems reasonable to believe that when condensation of connective tissue occurred the strands shrank in width. This may have accounted for some of the improvement noted in the amount of connective tissue in the livers of rats

under all types of therapy. Therefore only extensive changes were considered significant. Sellers, Lucas, and Best (3) reported actual disappearance of connective tissue in treated animals with carbon tetrachloride cirrhosis. In the present study complete restoration to normal did not occur once an increase in connective tissue had been noted.

Small foci of necrosis persisted throughout the therapeutic period only in those animals on a low protein intake with or without added lipotropic factors.

TABLE IV

*Histologic Appearance of Liver as Determined for Specimens at Biopsy and Autopsy**

Group No.			Fatty infiltration		Connective tissue	
			Biopsy	Autopsy	Biopsy	Autopsy
1	4 per cent casein + choline + methionine (18 rats)	Absent	0	4	2	2
		Minimal	3	10	8	3
		Abundant	15	4	8	13
2	30 per cent casein + choline (21 rats)	Absent	2	10	1	7
		Minimal	8	11	13	7
		Abundant	11	0	7	7
3	30 per cent casein alone (12 rats)	Absent	0	7	1	1
		Minimal	3	4	6	6
		Abundant	9	1	5	5
4	4 per cent casein control (4 rats)	Absent	0	0	1	0
		Minimal	0	0	2	0
		Abundant	4	4	1	4
5	30 per cent casein control (5 rats)	Absent	5	5	5	5
		Minimal	0	0	0	0
		Abundant	0	0	0	0

* Only those specimens are compared in which animals received dietary treatment for 10 or more weeks.

Histologic changes in the livers of the experimental animals both before and after treatment are indicated in Table IV. The tabulation is restricted to fatty infiltration and connective tissue, since other changes were difficult to appraise in this qualitative fashion. At the time of biopsy the rats were placed in comparable groups according to the degree of hepatic cirrhosis. Since a fair trial of treatment was considered to be at least 10 weeks, those animals dying in less than 10 weeks were omitted from the computation in this table. This resulted in a certain degree of imbalance among the various groups. Nonetheless certain trends seem fairly evident. For example, a sharp reduction of liver fat

was observed in rats when their 4 per cent casein diet was supplemented with choline and methionine. A still greater lipotropic effect was seen in livers of rats whose diets were changed from 4 per cent to 30 per cent casein, with or without added choline. Fibrosis appeared to progress in rats on the 4 per cent casein diet despite the addition of choline and methionine. In contrast, those rats whose diets were changed from 4 to 30 per cent casein showed either moderate regression (group 2) or arrest (group 3) of this process. In this small group of animals it is doubtful whether the differences between groups 2 and 3 are significant, whereas the differences between group 1 and either group 2 or group 3 appear significant.

DISCUSSION

During the treatment phase of the experiment there was a sharp reduction in mortality, once a critical period of 2 weeks after biopsy was survived. This decline in death rate was seen not only in those animals receiving supplements of choline and methionine or those changed from a 4 per cent to a 30 per cent casein diet, but also in the control rats maintained on the 4 per cent casein diet. From these data it does not appear that the change of diet or that lipotropic factors alone were responsible. The animals that survived the foreperiod and the biopsy procedure were in a sense selected. It is possible that they were stronger and that they were destined constitutionally to survive longer.

The change from a 4 per cent to a 30 per cent casein diet was followed by partial or complete restoration of normal liver structure with respect to fat and liver cells. The fibrous tissue appeared to be less conspicuous. The factor in casein responsible for this is not evident. Casein contains approximately 1.5 to 3 gm. of methionine per 100 gm. However, the addition of methionine in a concentration of 0.8 gm. per 100 gm. to the 4 per cent casein diet was less effective than the 30 per cent casein diet containing approximately 0.4 to 0.9 gm. methionine per 100 gm. Likewise the addition of choline in a concentration of 0.5 gm. per 100 gm. to the 4 per cent casein diet was less effective than the 30 per cent casein diet. Thus casein seemed to exert a therapeutic effect not attributable to its content of methionine or its choline equivalent.

The present findings are at variance with other reports (2–4) which describe the arrest or decrease of connective tissue and restoration of normal liver structure when lipotropic substances were added to low casein diets. They tend to support the more recent findings of György and Goldblatt (4) who observed that mild or moderate degrees of cirrhosis responded favorably to lipotropic substances and high casein diets, whereas advanced cirrhosis responded poorly or not at all.

The differences among the findings of different workers are understandable in that conditions of the experiments have varied with respect to age, sex, and diets employed. Moreover, there may be multiple factors involved in the

reparative process. It is possible that the addition of choline or methionine to the basal low protein diet indeed causes transitory improvement, but that as the experiment is extended the need develops for other factors. Thus the longer interval of therapy may bring into play factors essential for growth and repair which are present in the 30 per cent casein diet but deficient in the 4 per cent casein diet with added choline and methionine.

In recent papers Popper, Koch-Weser and Szanto (14), Drill and McCormick (15), and György and Rose (16) have described experiments in which vitamin B_{12} exerted lipotropic and protective effects in the liver. The leached casein employed was reported to contain negligible amounts of vitamins, although there are no analyses of the vitamin B_{12} content. It seems unlikely that the lipotropic activity of vitamin B_{12} was a factor in the present study.

Ever since the demonstration that choline protects against experimental dietary cirrhosis, choline has been advocated for the treatment of human Laennec's cirrhosis. It has been suggested that a highly nutritious diet is effective largely because of its choline content. However, it has been difficult to demonstrate the therapeutic value of choline clinically. These experiments suggest that choline is of limited therapeutic value in cirrhosis produced by a low protein diet. A high level of dietary protein is of considerably greater benefit. If the experimental disease can be compared to the human disease, the addition of choline to a highly nutritious diet is of doubtful value.

SUMMARY AND CONCLUSIONS

Cirrhosis of the liver was produced in rats by feeding a diet low in protein (4 per cent casein) and deficient in lipotropic factors. The degree of liver cirrhosis was determined from specimens obtained at biopsy. Comparable groups of animals then were treated with diets containing 4 per cent casein and 30 per cent casein. The 4 per cent casein diets were supplemented with choline and methionine; the 30 per cent casein diets were fed with and without added choline.

On supplementing the low protein diet with choline and methionine the animals remained feeble, their growth remained stunted, and their livers showed signs of progressive cirrhosis. In contrast, animals fed the higher protein diet (with or without added choline) grew normally, and their livers showed signs indicating arrest and regression of the disease process.

These studies suggest that the feeding of high protein (30 per cent casein) diets to rats with nutritional cirrhosis produces reparative effects greater than those attributable to the supplements choline and methionine.

BIBLIOGRAPHY

1. Lowry, J. V., Daft, F. S., Sebrell, W. H., Jr., Ashburn, L. L., and Lillie, R. D., *Pub. Health Rep., U.S.P.H.S.*, 1941, **56**, 2216.

2. Lowry, J. V., Ashburn, L. L., and Sebrell, W. H., Jr., *Quart. J. Stud. Alcohol*, 1945, **6**, 271.
3. Sellers, E. A., Lucas, C. C., and Best, C. H., *Brit. Med. J.*, 1948, **1**, 1061.
4. György, P., and Goldblatt, H., *J. Exp. Med.*, 1949, **90**, 73.
5. Daft, F. S., Sebrell, W. H., Jr., and Lillie, R. D., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 228.
6. Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition*, 1937, **14**, 273.
7. György, P., and Goldblatt, H., *J. Exp. Med.*, 1949, **89**, 245.
8. Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, 1929, **82**, 345.
9. Borland, V. G., and Jackson, C. M., *Arch. Path.*, 1931, **11**, 687.
10. Martin, G. J., *J. Nutrition*, 1939, **17**, 127.
11. Ashburn, L. L., Endicott, K. M., Daft, F. S., and Lillie, R. D., *Am. J. Path.*, 1947, **23**, 159.
12. Glynn, L. E., Himsworth, H. P., and Lindan, O., *Brit. J. Exp. Path.*, 1948, **29**, 1.
13. Hartroft, W. S., and Ridout, J. H., *Am. J. Path.*, 1951, **27**, 1951.
14. Popper, H., Koch-Weser, D., and Szanto, P. B., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 688.
15. Drill, V. A., and McCormick, N. M., *Proc. Soc. Exp. Biol. and Med.*, 1949, **72**, 388.
16. György, P., and Rose, C. S., *Proc. Soc. Exp. Biol. and Med.*, 1950, **73**, 372.