SOME ASPECTS OF CHOLINE METABOLISM IN MAN

by

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Thesis

Submitted to the Faculty of

Graduate Studies and Research

in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

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Royal Victoria Hospital,

Montreal, Quebec, Canada.

September, 1949.

ACKNOWLEDGMENTS

Appreciation is expressed to Dr. J. S. L. Browne through whose kindness facilities for the experimental work were provided in the laboratories of the University Clinic of the Royal Victoria Hospital, and to Dr. Martin Hoffman for assistance and guidance through the project.

The technical assistance of Miss M. Desbarats, Miss M. Robinson and Mr. J. Penfield is gratefully acknowledged. The help and co-operation of numerous doctors and nurses on the Metabolism Ward and technicians in the Biochemistry and Endocrine Assay laboratories greatly facilitated the metabolic studies.

Miss Frances Mitchell prepared the drawings and charts for photography.

The receipt of a grant-in-aid from Frank Horner, Ltd., Montreal, for the purchase of equipment is gratefully acknowledged.

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THE LIPOTROPIC FACTORS

HISTORICAL REVIEW OF THE DEVELOPMENT OF THE CONCEPT OF LIPOTROPISM

Shortly after the production of experimental diabetes by von Mering and Minkowski in 1889 it was noted that the pancreatectomized dog manifested not only impairment of carbohydrate metabolism but evidence of altered fat metabolism as well with a tendency to deposit fat in the liver.

Sandmeyer in 1895 demonstrated the beneficial effect of feeding raw pancreas in the amelioration of this complication and postulated that the presence of certain digestive enzymes of the pancreas was essential for the prolonged survival of dogs after pancreatectomy.

In 1921 Banting and Best sacrificed dogs which had been kept alive for 10 weeks with insulin and remarked on the fatty changes in the livers. Long term studies from this laboratory were reported in 1924 by Allan, Bowie, Macleod and Robinson who found that fatty livers developed in pancreatectomized dogs maintained with insulin after periods ranging from one to seven months. Having considered and then discounted a possible toxic effect from the tricresol preservative in the insulin, they concluded: 'In some way or other the continued absence of the pancreas leads to a pathological condition of the liver cells and as a result fat accumulates in them.' They restated the suggestion made by Sandmeyer 30 years earlier that pancreatectomy deprives the animal of some enzyme required for the mobilization of fat. They began feeding their dogs supplements of raw pancreas and noted a disappearance of the manifestations of increased liver fat.

Almost simultaneously Fisher in Chicago reported that the survival of pancreatectomized dogs appeared to be related to the degree of regeneration of the pancreatic stump. However, he concluded, 'The marked degeneration of the

liver and the extreme arterial sclerosis developing in dogs on long insulin management indicates either diabetic processes not controlled by insulin, chronic toxic action of insulin itself, or such toxic action of other substances in the insulin mixture.'

In 1931 Hershey and Soskin demonstrated that the fatty liver of pancreatectomized dogs could be prevented as effectively by feeding crude egg yolk lecithin as by feeding minced pancreas.

Research on the nature of these fat-mobilizing substances was facilitated by the discovery that fatty livers could be produced in rats by dietary means. In 1932 Best, Hershey and Huntsman showed that the fatty liver which develops in these animals on a lean meat and sugar diet could be prevented or alleviated by the use of the choline moeity of the lecithin molecule in amounts as small as 1.0 mg. per rat per day. Choline was later shown to be equally effective in the prevention and treatment of the fatty livers of pancreatectomized dogs maintained with insulin.

Best and Huntsman (1932) demonstrated the effectiveness of naturally-occurring choline in retarding or preventing the development of fatty livers in experimental animals and speculated that choline might be a dietary essential for the rat. Its lipotropic effect was subsequently demonstrated in various other species in protection against fatty livers of dietary and toxic origin.

In 1935 Channon and Wilkinson noted that the ability of a diet to induce fatty livers was inversely proportional to its protein content provided that the choline intake was maintained at a constant suboptimum level. This suggested that certain amino acids might be converted into choline or betaine by methylation in the tissues. Simultaneously this effect of dietary protein was being studied in Toronto by Best, Mawson, McHenry and Ridout (1936) who found that 1.0 gm. of

protein exerted about the same effect as 5 - 6 mg. of choline.

Tucker and Eckstein (1937) simplified the problem somewhat when they demonstrated that the lipotropic effect of proteins was largely inherent in their methionine content but the interrelations between choline and methionine remained puzzling until du Vigneaud developed the concept of transmethylation.

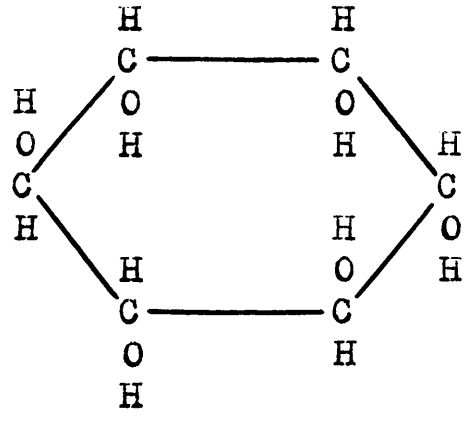
In 1936 Best, Grant and Kidout introduced the term 'lipotropic' to describe the effect of choline and related substances in the prevention and amelioration of fatty livers in animals. At this time it was generally considered that choline accounted for the anti-fatty liver activity of the pancreas. However in 1936 van Prohaska, Dragstedt and Harms reported the extraction of 'lipocaic' a so-called fat-metabolizing hormone occurring in pancreatic extracts and showing activity in amounts so small as to preclude the likelihood that its effect was due to contained choline.

Almost simultaneously Chaikoff and Kaplan reported that they had discovered an 'anti-fatty liver factor' in the external secretion of the pancreas. The nature of 'lipocaic' still remains in doubt but the active ingredients of Chaikoff's extract appear from recent work to be protein-splitting enzymes.

In 1941 Gavin and McHenry reported that inositol exerted a lipotropic effect under certain conditions. Since inositol is probably one of the less important lipotropic factors it will be considered briefly and finally in the following section.

INOSITOL

Inositol is a hexahydroxycyclohexane comparable to the cyclic form of glucose. It is a water-soluble, sweet-tasting crystalline compound sufficiently stable to resist strong acid and alkali treatment. The biologically active form is one of the optically inactive forms called meso-inositol which has a molecular weight of 216 and the following stereochemical structure:-



Inositol was first isolated from meat by Scherer in 1850 and has long been considered by chemists to be a substance intermediate between carbohydrates and aromatic compounds, because on one hand it has the same empirical formulae, taste and solubility as glucose and on the other hand is readily oxidized to a typical aromatic compound, tetrahydroxyquinone. Fisher (1945) has summarized his work demonstrating the interconvertibility of inositol and glucose, proving that glucose can be cyclized to from inositol, and conversely, that cleavage of the inositol ring gives rise to d-glucose.

Inositol is found widely distributed in plants and animals in an average concentration of 0.05 - 0.5%. Beef heart, the richest source, contains 1.6% inositol. Of many naturally-occurring forms only meso-inositol possesses nutritional activity.

This isomer occurs naturally in at least four forms:

Free inositol	Meso-inositol	Plants and animals.
Phytin	The calcium and magnesium salt of inositol hexa-phosphate.	Cereal grains, seeds, erythrocytes of chickens and turtles.
Lipositol	A phospholipid complex containing inositol mono-phosphate linked to galactose and combined with ethanol-amine, tartaric acid, oleic acid and various saturated fatty acids.	Phospholipids in plant and animal tissues, in tubercle bacillus, soy beans, cephalin of the brain and spinal cord.
Combined Inositol	A water-soluble, non-dialyzable complex.	Liver, heart and most other animal tissues.

Biological Functions of Inositol:

Earlier studies on this substance were concerned with its possible glucogenic function, and considerable evidence suggests that inositol is a reserve form of carbohydrate in certain species such as the shark and particularly in certain organs such as heart muscle (Winter,1944). By means of isotope tracers Stetten and Stetten (1946) showed that about 7% of inositol fed to phlorizinized rats was converted to glucose.

Vitamin-like activity was not attributed to inositol until 1928 when 'bios I' was identified as inactive inositol by Eastcott. It was shown to be a dietary essential for mice by Wooley in 1940, the deficiency sysndrome being characterized by alopecia. In rats, the 'spectacled eye' syndrome, so called because of peri-orbital alopecia and exudation, was attributed to inositol deficiency by Pavcek and Brown (1941) and was prevented and cured by the feeding of this vitamin.

In 1941 Gavin and McHenry reported the lipotropic action of inositol in the prevention of the 'biotin fatty liver' of rats and in this respect it was said to resemble 'lipocaic' since it prevented the accumulation of both cholesterol and triglycerides.

The possibility that inositol was the active principle of 'lipocaic' was explored but evidence adduced from work on dogs indicated 'lipocaic' was more active than an equal amount of inositol. Furthermore, inositol exerts strictly a lipotropic effect on pancreatectomized dogs, and unlike 'lipocaic' fails to influence the survival time, the increased insulin tolerance or the loss of body fat -- features considered by Dragstedt to be 'lipocaic deficiency' manifestations. (Owens, Allen, Stringer and Dragstedt, 1941; Rubin and Ralli, 1941). In therapeutic trials in man Abels, Kupel, Pack and Rhoads (1943) demonstrated an apparent lipotropic effect of 'lipocaic' in the prevention of fatty livers in patients with carcinoma of the gastro-intestinal tract. An amount of inositol equivalent to that occurring in a corresponding dose of 'lipocaic' proved equally efficacicus according to their observations.

Engel (1942b) induced fatty livers in rats by manipulations of the proportions of thiamine, riboflavin, pyridoxine, pantothenic acid, corn oil and choline in the diets. He found a maximum lipotropic effect from choline at a level of 10 mg. per rat per day but fatty livers were not completely cured unless a supplement of 3 mg. of inositol was provided. It was his opinion that pyridoxine was also required for the full effect of choline.

The possibility that inositol could exert its lipotropic effect only in the presence of certain fatty acids was explored by Beveridge in 1944. Unexpectedly he found that while the addition of corn oil enhanced the lipotropic action of choline, presumably by supplying fatty acids for phospholipid synthesis, it obliterated the lipotropic effect of inositol.

Handler (1946) confirmed the lipotropic effect of inositol in the so-called 'biotin fatty-liver' and in a type of 'fat fatty liver' of rats. However, if the experiment were carried on more than 24 days there was such a decline in

growth rate as a result of the dietary restrictions that the demand for lipotropic factors fell and the supply of inositol from synthesis by the intestinal flora became sufficient for the animals' requirements. Under these conditions choline alone sufficed to prevent fatty livers. However, if the synthesis of inositol by intestinal bacteria was inhibited by succinylsulphathiozole, dietary supplements of inositol again became necessary. Handler confirmed Engel's observations on the inhibition of inositol by unsaturated fatty acids, but failed to find evidence to support the contention of McHenry that inositol exerts selective activity for cholesterol esters rather than triglycerides. Forbes (1943) and Beveridge and Lucas (1945) likewise failed to note any such effect.

McHenry's group have continued in their efforts to devise dietary conditions which will produce 'cholesterol' fatty livers sensitive to inositol but resistant to choline. MacFarland and McHenry (1945) modified earlier opinions regarding the insensitivity of the 'biotin fatty liver' to choline and point out that the type of experimental fatty liver that resists choline and is sensitive to 'lipocaic' and inositol is that induced by feeding a beef liver fraction to rats on high carbohydrate, fat-free diet. Confusion arose from the fact that this type had previously been designated as the 'biotin fatty liver' whereas the true biotin fatty liver requires both choline and inositol for its prevention. These authors found that the addition of thiamine alone to the basal diets produced fatty livers responsive to choline (confirmed by Handler,1946), while the addition of other B vitamins such as in crude beef liver fraction, liver fraction eluate or biotin and folic acid resulted in fatty livers resistent to choline. McHenry admits, as Handler suggested, that the effect of dietary manipulations on the lipotropic action of inositol may be partly mediated through the altered nutritional status of the animals. However, he maintains that there is also a specific factor in the vitamin B supplements.

The mechanism of the lipotropic effect of inositol has been the subject of speculation. Attributing to it an action parallel to that of choline, McHenry and Patterson (1944) postulated that inositol was incorporated into certain phospholipids. Inositol-containing phospholipids have been isolated from bacteria and plants (Anderson et al, 1930,1938) and from animal tissues (Folch and Wooley,1942), but there is no evidence that inositol-containing phospholipids are implicated in conditions of altered metabolism in either animals or man.

To confuse the matter more thoroughly Ralli and Rubin (1942) have shown that inositol is the anti-lipotropic or lipogenic agent in dried liver or aqueous extracts of liver which has been repeatedly found since 1931 (Bollman and Mann) to induce fatty livers in animals.

In summary then, inositol appears to be lipotropic only under certain rigidly controlled conditions in certain species and in any case is active only when fat is absent from the diet. These limitations seem to relegate this substance to a relatively unimportant role as a lipotropic agent in man.

LIPOTROPIC SUBSTANCES OF THE PANCREAS

Shortly after the discovery of insulin it was noted by several workers that this did not constitute complete replacement therapy for depancreatized dogs since they developed fatty livers and other deficiency manifestations unless a dietary supplement of raw pancreas was fed.

Lipocaic:

In 1934 Dragstedt states that the interest of his group in Chicago was directed to the external secretion of the pancreas by the theory of Boldyreff (1934) that 'removal from the animal or human body of the external secretion of the pancreas leads to diabetes, hence the direct conclusion to treat diabetes by the introduction of pancreatic juice.' Repeated observations in many laboratories that pancreatic fistula does not lead to glycosuria or diabetes might be considered to have settled this question were it not for the fact that the usual technique for producing pancreatic fistula in dogs does not deprive the animal of all pancreatic juice due to the presence of accesscry small ducts.

Dragstedt, Montgomery and Ellis (1930) had devised a method of isolating a pouch of duodenum into which the ducts drained and from which pancreatic juice was continuously withdrawn by suction. By the use of such a fistula, Dragstedt, Von Prohaska and Harms, (1936,1936a) found that removal of pancreatic juice from dogs did not produce diabetes or lead to fatty livers and conversely feeding the external secretion to another group of depancreatized dogs did not relieve the diabetes or the tendency to develop fatty livers.

They concluded therefore that the substance in raw pancreas which was lipotropic was not found in pancreatic juice and hence must be an internal secretion.

They proceeded to fractionate extracts of pancreas and described the preparation of a fat-free alcoholic extract of beef pancreas which was said to contain a new 'fat-metabolizing hormone of the pancreas, 'lipocaic".

This substance was said to be effective in amounts so small as to preclude the possibility that its action was due to the contained lecithin or choline. Further evidence offered in support of this point was the finding that the feeding of other lecithin-rich viscera such as brain was not effective. The development of anorexia, apathy, weight loss, decreased glucose excretion and fatty livers in depancreatized dogs was attributed to 'lipocaic deficiency' and treatment with lipocaic was said to effect a reversal of these complications.

Since Dragstedt described 'lipocaic', the nature of this substance has been the subject of lively controversy and much experimental effort. It is perhaps desirable then to present coincidently with Dragstedt's evidence the opposing views of Chaikoff and others.

Dragstedt's method of collecting pancreatic juice in a duodenal pouch has been criticized by Entenman, Chaikoff and Montgomery (1944) as permitting reabsorption of some of the external secretion. Dragstedt's criterion of effectiveness, i.e. histological examination of liver biopsies, has been widely criticized particularly by Chaikoff and Kaplan (1937) who took the trouble to prove that the lipid content of liver tissue varies widely from lobe to lobe, and even in adjacent areas of the same lobe. Exactly the opposite finding was later reported by Dragstedt, Vermeulen, Donovan and Geer (1939) who found only slight difference in fat concentration from lobe to lobe and good correlation between comparative chemical and histological determinations.

The fact that feeding lecithin-rich brain did not prevent fatty livers proves nothing, since brain is also rich in cholesterol which is well known to promote fatty livers. (Okey,1934). The same objection holds for the failure of

raw liver to prevent 'lipocaic deficiency' since McHenry and Gavin (1940) showed that feeding liver extracts produces fatty livers regardless of the cholesterol content.

Frame (1942) criticizes the interpretation of the data from which Dragstedt initially concluded that choline was not the active principle of 'lipocaic'. Entenman, Montgomery and Chaikoff (1944) conclude that 'lipocaic' is a 'poor source of the anti-fatty liver factor of the pancreas' since an amount equivalent to 100 gm. of pancreas per day per dog does not prevent the development of fatty livers.

In the dietary fatty liver of rats Shapiro and Wertheimer (1937) and Channon, Loach and Tristam (1938) found that the activity of pancreatic extract could not be accounted for by the contained choline, while Aylward and Holt (1937), MacKay and Barnes (1938) and Best and Ridout (1938) found no effect from 'lipocaic' in rats which could not be predicted from the choline content. In short, a decision on the lipotropic action of pancreatic extracts in dietary fatty livers must await further investigation.

In 1939 Dragstedt, Vermeulen, Goodpasture, Donovan and Geer review the literature relative to fatty livers and diabetes and cite 2 cases of hepatomegaly in diabetic children which regressed on treatment with lipocaic and returned on cessation of therapy (Grayzel and Radwin,1938). However, Marble, White, Bogan and Smith (1938) review 60 cases of hepatomegaly in 1,077 children in Joslin's clinic and conclude that fatty liver in diabetics is indicative of poor control and is amenable to insulin therapy alone. It would seem likely that the fatty liver of human diabetics is analagous to the fatty liver of insulin deficiency in dogs which occurs rapidly and acutely after pancreatectomy, is associated with ketonemia and is not amenable to lipocaic therapy. (Dragstedt, Allen, Julian and Singer, 1941).

In 1940 Dragstedt made the interesting observation that dogs maintained on insulin for 8 months during which time fatty livers were repeatedly allowed to develop, showed marked arteriosclerotic lesions. In this review he reiterates his belief that 'lipocaic' is a hormone. McHenry and Patterson (1942) point out that the long latent period before symptoms develop is more typical of a dietary lack rather than a hormonal deficiency.

Noting the similarity between 'lipocaic deficiency' signs and those produced by injection of anterior pituitary hormone (Burn and Ling,1930) led to experiments by Julian, Clark, Van Prohaska, Vermeulen and Dragstedt (1942) showing that 'lipocaic' protected guinea pigs from the fatty livers that follow injections of 'anterior pituitary ketogenic hormone'. They theorize that this hormone controls the body fat to liver fat transfer (but only in part since Houssay dogs develop fatty livers) while 'lipocaic' controls the liver fat to body fat transfer.

Anti-fatty liver Fraction of Pancreas (Chaikoff):

Somewhat before the report on 'lipocaic' by Dragstedt, Chaikoff's group in California began publishing a series of papers (Chaikoff and Kaplan,1935) and (Kaplan and Chaikoff, 1937) describing similar studies on a lipotropic principle of pancreas which occurred in the external secretion. It was composed of a heat-stable fraction (later shown to be choline by Entenman and Chaikoff in 1944) and a heat-labile fraction which had the effect of raising the lowered blood lipids and decreasing the elevated liver lipids of depancreatized dogs maintained on insulin. 'Lipocaic' failed to prevent this fall of blood lipids after pancreat-ectomy, (Entenman, Montgomery and Chaikoff,1944). One gram per day of extract 'AR' (a dried, defatted fraction derived from 5.5 gm. of fresh gland) was found to prevent the development of fatty livers although it contained only 13 mg. of choline.

That the active principle occurred in the external secretion was inferred from the findings of Berg and Zueker (1931), Ralli, Rubin and Present (1938) and Montgomery, Entenman and Chaikoff (1939) who reported that pancreatic duct ligation in dogs produced fatty liver in dogs indistinquishable from that following pancreatectomy. Chaikoff's group found that it required 12 - 24 weeks to develop which is considerable longer than Dragstedt observed his dogs. Coincidently they noted a fall in blood lipids and at autopsy pancreatic atrophy was evident.

This fall in blood lipids is corroborated by Dragstedt, Donovan, Clark, Goodpasture and Vermeulen (1939). After pancreatectomy, the serum lipids rise for the first week, then fall to half the normal values as fatty livers and 'other signs of lipocaic deficiency' develop. Oral 'lipocaic' was stated to correct this hypolipemia in less than a week and maintain the blood fat at normal levels. Unfortunately phospholipids were not determined on this series.

Entenman, Chaikoff and Montgomery (1940) found that raw pancreas prevented both the fall in blood lipids and the fatty livers while 2.0 gm. of choline per day would prevent the fatty livers but did not prevent the drop in blood lipids.

Montgomery, Entenman and Chaikoff (1939) and Entenman, Chaikoff, Montgomery and Laurence (1941) then fed pancreatic juice and noted that 400 ml. per day restored to normal or prevented the fall of blood lipids. Montgomery et al (1940; 1941) showed that this treatment prevented the increase in liver fat in both the duct-ligated and depancreatized dog. In criticism of this work of Chaikoff's group it should be noted that between pancreatectomy and commencement of treatment with pancreatic juice the dogs were fed 125 gm. of raw pancreas per day until a vigorous appetite appeared (Dragstedt considers loss of appetite a manifestation of 'lipocaic deficiency'). In this period of from 3 - 8 weeks it is possible that the animals built up a store of the active principle.

Chaikoff's group have continued systematic studies to elucidate the nature of the anti-fatty liver factor. It appeared to be associated with choline metabolism as reflected in the rise in serum phospholipids, most of which are of the choline-containing type and contain about 95% of the total serum choline. (Taurog, Entenman and Chaikoff, 1944). They stress the role of phospholipids as vehicles for the transport of choline as well as fat. Their purified fraction '27C' was found to be effective at a level of 60 mg. per day while at least 350 mg. of choline is required for the same effect. (Chaikoff, Entenman and Montgomery, 1945). Later, they showed that in the depancreatized or duct-ligated dog, free methionine or hydrolyzed casein prevented the development of fatty livers while unhydrolyzed casein or lean meat containing an equivalent amount of methionine did not.

Since presumably there is no interference with the synthesis of choline from methionine in the depancreatized dog, it appeared that the anti-fatty liver factor was concerned with the liberation of methionine from proteins in the intestine, and hence was probably enzymatic in nature. (Chaikoff, Entenman and Montgomery, 1945). It is interesting that this concept was held by Sandmeyer in 1895 and Allen, Bowie, Macleod and Robinson in 1924.

In 1946 Best reported on the work of Palmer in his laboratory on the nature of the enzymes in Chaikoff's anti-fatty liver fraction. Palmer did isolate and identify trypsinogen and chymotrypsin 'in fair yield' but whether either is identical with the active principle remains to be established.

Thus it is seen that the results of Kelli's group and Chaikoff's group on one hand and Dragstedt's group on the other are diametrically opposed. If one accepts Chaikoff's rather convincing evidence for the existence of the factor in the external secretion this can best be reconciled with Dragstedt's findings by considering the possibility that the active

principle is the same in both cases. If one further admits the difficulty in collecting all pancreatic juice before some is reabsorbed, and realizes that different types of fatty livers develop depending on the species, age and diet of the animal, the chief source of contention is limited to the mechanism by which the active principle leaves the pancreas.

It can be said in summary that consideration of the available information suggests that choline is the final common substance through which betaine, methionine, proteins and pancreatic extracts exert their lipotropic effect.

METHIONINE AND OTHER METHYL DONORS

METHIONINE

Methionine was discovered in meat infusions by Mueller in 1921 and later studies by this author (1921a, 1922, 1923 and 1924) indicated that it contained a stable sulphur and nitrogen only in amino form. Barger and Coyne (1928) deduced the formula and synthesized methionine.

Formula -- Methionine is chemically designated as α-amino γ-methylthiol n-butyric acid and has the following formula:

$$CH_3S.CH_2CH_2\underset{\displaystyle NH_2}{\underset{|}{CH}} - COOH$$

Properties -- dl-methionine occurs as white, crystelline platelets or as a powder having a faint odour. Like other amino acids it has no well-defined melting point but undergoes decompensation and liquefaction between 270 - 280°C. Its solubility in gms. per 100 mls. of water is 1.82 at 0°; 5.4 at 25°; and 17.6 at 100°. It is soluble in dilute acids and alkalis, very slightly soluble in alcohol and practically insoluble in ether.

Methionine was established as one of the 10 amino acids essential for the growth of rats (Rose et al, 1936) and of dogs (Rose and Rice, 1939). Unlike some of the essential amino acids, both optical isomers of methionine are utilized. (Schmidt, 1944).

Metabolism -- Methionine occurs fairly widely in animal and vegetable proteins. The percentage composition of some common sources is: Casein 3.4; lactalbumin 2.6; egg albumin 4.6; wheat gliadin 2.1. ('Proteins and Amino Acids', 1944). After being freed by the action of proteolytic enzymes on ingested proteins, methionine is absorbed into the blood where

it is found as 'free' methionine in an average concentration of about 0.85 mg.%. (Silver, 1948). The studies of Horowitz (1947) using Neurospora mutants suggest that methionine can be synthesized from cystine via cystathionine and homocystine, and the findings of Teas, Horowitz and Fling (1948) indicate that in Neurospora homoserine can act as a precursor of methionine the reaction progressing via threonine, cysthathionine and homocystine. Evidence for these syntheses in mammals is not available but the synthesis of methionine by methylation of homocystine was indicated in rat growth experiments by du Vigneaud, Chandler, Cohn and Brown (1940), and by deuterium labelling. (du Vigneaud, Chandler and Meyer, 1941).

The fate of methionine after absorption is not yet well elucidated. After intravenous injection of 1.5 gm. of methionine Kinsell et al (1948) found a rapid rise in blood level from a fasting 0.75 mg. to over 3.0 mg.% at 15 minutes and a subsequent gradual fall to 1.0 mg.% at 3 hours. Only 1/4 of the injected dose remained in the blood a few minutes after injection. They found that urinary excretion of the l-form was negligible but that excretion of d-methionine was roughly proportional to the blood level.

In general, methionine is utilized (a) as the intact molecule for the synthesis of body protein (b) as a source of dietary sulphur and (c) as a methyl donor. These functions of methionine and the interrelationships of this substance with cystine and choline will be elaborated upon in a later section.

The Lipotropic Action of Proteins -- Not long after the inception of studies on dietary fatty livers in rats, Best and Huntsman (1934) noted that replacing 20% of the carbohydrate fraction of the diet with casein protected the animals against the development of increased liver fat.

This was confirmed by Channon and Wilkinson (1935) who found that adult rats on a 40% fat diet did not develop fatty livers if 20 - 30% of the calories were supplied by casein. It was later shown that casein also had a lipotropic effect on the dietary fatty liver of rats produced by 20% fat - 2% cholesterol feeding, i.e. the so-called 'cholesterol fatty liver'.

In 1936 Best, Grant and Kidout found that casein exerted its maximal lipotropic effect at 30% of the diet. They also demonstrated that egg white protein and powdered beef muscle had similar activity but that gelatin was inactive lipotropically. However, Channon et al (1938a) demonstrated lipotropic action from zein, gelatin, gliadin, edestin, albumin and whole muscle protein in that order of potency.

These findings suggested that the lipotropic effect of proteins resided in one or more of the amino acid components. Beeston and Channon (1936) tested leucine, glutamic acid, aspartic acid, serine, glycine and phenylalanine but found no lipotropic activity. Singel and Eckstein (1941) reported a similar study which indicated that djenkolic acid, valine, leucine, and isoleucine were inert. Alanine, proline, hydroxyproline and arginine were likewise without lipotropic effect.

'Antilipotropic' Substances -- Several years before the concept of lipotropism was formulated, Curtis et al (1927) reported that a high cystine intake in the rat resulted in hemorrhage and necrosis in the kidneys and fatty changes and necrosis of the liver. In the course of testing various amino acids for their lipotropic activity, Beeston and Channon (1936) rediscovered this effect, noting that the addition of cystine to a low-protein, choline-free diet augmented the development of excess liver fat. Beeston, Channon and Platt (1937) showed a similar

'antilipotropic' effect of cystine in 'cholesterol fatty liver' and in 1938 Channon, Loach and Tristam showed that homocystine was 'anti-lipotropic', a finding confirmed by Singal and Eckstein (1939) who also reported a similar action of cysteine.

Reports of the 'antilipotropic' effects of cystine led Tucker and Eckstein (1937) to look for comparable properties of methionine, an amino acid whose metabolic functions had been related to those of cystine. (Jackson and Block, 1931,1932: du Vigneaud and Dyer, 1937). Unexpectedly, the opposite effect was demonstrated, and a reduction of liver fat resulted from adding methionine to the diet to the extent of 0.5%. This lipotropic effect of methionine was confirmed by Channon, Manifold and Platt (1938) and Best and Ridout (1940) who found that as little as 0.125% methionine in the diet of rats prevented fatty livers and that this effect could be nullified by the simultaneous addition of 0.1% of cystine.

The net lipotropic activity of the various proteins was now explained by Eckstein, Tucker et al (1938, 1940) as representing the balance between the constituent amounts of lipotropic methionine and 'antilipotropic' cystine and to no other factors. However, Best and Ridout (1940) showed that increasing the methionine content of diets to more than 0.5% did not increase the lipotropic effect and Channon, Manifold and Platt (1940) likewise concluded that apparently methionine was prevented from exerting its full lipotropic effect in the absence of some other factor.

Treadwell, Grcothius and Eckstein (1942) reported that dietary methionine in the free state was lipotropically more active than casein containing an equivalent amount, presumably because the latter was largely utilized for protein synthesis as attested by the increased growth of

the casein-fed rats. This finding actually bolsters the opposing argument of Mulford and Griffith (1942) that the augmented growth of the animals increases the demand for lipotropic factors and thus leads to a relative deficiency. They point out that cystine, by providing needed dietary sulphur, permits more rapid growth and thereby increases the demand for lipotropic factors rather than exerting any specific 'antilipotropic' action. As a matter of fact, Miller, Rose and Whipple (1940) reported that in dogs cystine as well as methionine protected against liver damage from chloroform. Drill and Loomis (1946) showed that a protective effect from methionine against carbon tetrachloride liver damage in dogs could only be demonstrated if the animals were protein depleted.

Beveridge, Lucas and O'Grady (1944) in attempting to reconcile these conflicting views, generalize in the opinion that the lipotropic action of dietary methionine is markedly influenced by the abundance or lack of other essential amino acids, and contrary to previous reports, state that free methionine and protein methionine have the same lipotropic potency, all other things being equal.

The nutritional role of methionine is a triple one: it acts as a 'building stone' of tissues either per se or as a precursor of cystine; it is a source of dietary sulphur; it is a source of labile methyl groups for the formation of choline and the methylation of other compounds. According to Best (1948) it is only through the methylation of choline precursors that methionine exerts its lipotropic effect.

A fourth action of methionine has been postulated: Draft, Sebrell and Lillie (1942), in a study of the hemorrhagic and necrotic lesions of rats produced by dietary means (Gyorgy and Goldblatt, 1941) found that

apparently cirrhosis, and hemorrhage and necrosis, were separate and distinct entities. They attributed to cystine and methionine a specific and direct action in prevention of the hemorrhage and necrosis whereas methionine must act through choline in the prevention of dietary fatty livers and fatty cirrhosis. Himsworth and Glynn (1944) corroborated this finding and working independently in Germany with low sulphur diets Hock and Fink (1943) made similar observations. However this hypothesis does not go unchallenged since other groups including Best's have failed to induce necrotic lesions with identical diets.

McKittrick (1947) differentiated in a quantitative way between 'essential' and 'replaceable' methionine and almost simultaneously Jukes (1947) came to the conclusion that there was no sharp preferential affinity for methionine possessed by reactions relative to protein synthesis as opposed to reactions concerned with the transformation of methionine to produce choline. Although there is no general agreement on some aspects of this subject it is evident that nutritional adequacy particularly as regards amino acids is important in assessing the effect of lipotropic substances.

Therapeutic Trials of Methionine in Human Disease -- Beattie and Marshall (1944, 1944a, 1944b) indicated equivocal results from the use of methionine in treatment of post-arsenical liver damage, while one case of carbon tetrachloride poisoning reported by Beattie and associates (1944d) was apparently favourably influenced by methionine. Eddy reported a few cases of carbon tetrachloride poisoning (1944) and trinitrotoluene poisoning (1944a) treated with a high protein diet and 4 - 6 gms. of methionine per day, but in the absence of controls it is difficult to ascribe the beneficial results to the methionine itself. In a series of cases of infectious hepatitis, Wilson, Pollock and Harris (1945) found no demons-

trable effect from the use of methionine on the course or severity of the disease. As a result of a survey of the literature, The Council on Pharmacy and Chemistry of the American Medical Association (1947) felt constrained to refuse recognition of the claims made for the clinical usefulness of methionine. However in April of the following year there was evidence of a softening in the editorial policy regarding methionine and in October the decision of the Council was reversed and methionine was accepted 'for its lipotropic activities'.

BETAINE

The discovery of lipotropic activity for glycine-betaine by Best and Huntsman (1932) and its confirmation, (Chandler and du Vigneaud, 1935; Channon and Smith, 1936; Welch and Welch, 1938) led to the investigation of at least 10 other betaines of which only alanine-betaine (Welch and Welch, 1938) and cystine-betaine (Singal and Eckstein, 1941) showed any activity. That betaine acts simply as a methyl donor in this respect was later proven by tracer experiments. (Stetten, 1941a, 1941b). The effectiveness of betaine is only one-third that of choline suggesting that only one methyl group is available for trans-methylations. This indicates that not all methyl groups in biologically important compounds are available for transmethylation, a point that is emphasized by the absence of lipotropic effect from creatine (methyl guanido acetic acid) or sarcosine (methylaminoacetic acid).

Although the methyl group of sarcosine can be removed resulting in the formation of glycine (Gordon and Jackson, 1935; Abbott and Lewis, 1939) the methyl group is apparently destroyed by oxidation and is not available for transmethylations, as indicated by the lack of methyl-donor effects, i.e., in vivo formation of methionine from homocystine (du Vigneaud, Chandler and Moyer, 1939) and prevention of hemorrhagic kidneys in young rats (Griffith and Evans, 1942). Thus a distinction is noted between 'demethylation' and 'transmethylation'.

METHYL PURINES

The weak lipotropic activity of caffeine, theobromine and theophylline presumably results from donation of methyl groups in the course of their metabolic degradation. (Heppel, Porterfield and Peake, 1947).

TRANSMETHYLATION REACTIONS

The interrelationships between choline, methionine and allied substances was the source of much controversy and confusion until du Vigneaud and collaborators elucidated the concept of transmethylation reactions. In 1932 Butz and du Vigneaud prepared homocystine by heating methionine with sulphuric acid and showed that it was capable of serving in lieu of cystine in promoting growth in methionine-deficient animals. Many studies were carried out between 1932 and 1939 on homocystine and in the latter year du Vigneaud, Ayer and Kies reported that homocystine or cystine could not support growth unless certain sources of B vitamins were available. That choline was the active component of these B complex mixtures was shown by du Vigneaud, Chandler, Meyer and Keppel (1939), thereby suggesting that homocystine was converted to methionine by transfer of a methyl group supplied by choline, a hypothesis later proven by deuterium labelling. (du Vigneaud, Chandler and Meyer, 1941).

The same group demonstrated transfer of methyl groups from methionine to choline and creatine and postulated that the body was incapable of generating methyl groups which must therefore be supplied in the diet in a biologically labile form such as occurs in methionine, betaine and choline. However in a later communication the possibility of limited synthesis of labile methyl groups by intestinal bacteria in the white rat is recognized. (du Vigneaud, Simmonds, Chandler and Cohn, 1945).

CHOLINE

Synonyms -- Sincaline, Bilineurine, Amanitine, Gossypine, Vidine, Luridine, Fagine, trimethylaminoethanol, trimethylhydroxyethyl ammonium hydroxide.

Historical Note -- Choline was discovered by Strecker in 1849 in hog's bile, not a particularly rich source of the material. In 1862 he named the substance, described a technique for its purification and deduced the formula correctly except for one molecule of water.

In 1852 Von Babo and Hirschbrunn isolated choline from sinapin, a mustard seed alkaloid and in 1865 Liebreich isolated a base from hydrolyzed brain which he called 'neurine' but which was, in reality, choline. Wurtz in 1867 synthesized choline by two different methods: by warming trimethylamine with ethylene chlorhydrin producing choline chloride; and by treating concentrated aqueous trimethylamine with ethylene oxide at room temperature yielding the free base.

The acetic acid ester of choline first prepared by Baeyer in 1867 remained only of chemical interest until the discovery, by Hunt and Taveau in 1902, of its great physiological activity. In 1914 both Ewins and Dale discovered acetylcholine in extracts of ergot and in 1929 Dale presented in the Croonian lectures the accumulated evidence for the existence of acetylcholine in animals and its role in the transmission of impulses in parasympathetic and other nerves.

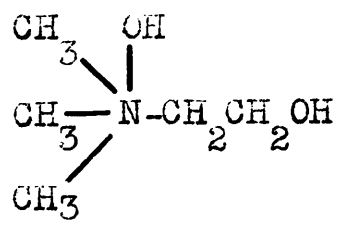
The role of choline-containing phospholipids as intermediaries in fat metabolism was postulated as early as 1891 by Loew (cited by Bloor, 1943), who believed that lecithin was a soluble transportable form of the fats. This concept has been explored and extended by a number of workers in more modern times.

The role of choline as a lipotropic factor was established by Best and Huntsman in 1932 and a variety of other biological functions have been described since that time.

Properties, Formula, etc. -- Choline usually occurs as a syrupy mass due to its extreme hugrospocity but it can be obtained with difficulty in crystalline form. The crystals are colourless, odourless and have a bitter taste.

Choline is a strong base which liberates ammonia from its salts and precipitates as hydroxides the salts of heavy metals. Its solutions dissolve fibrin and prevent the coagulation of proteins.

Choline has a molecular weight of 121.13 and the following structural formula:



Solubility -- Choline is soluble in water, formaldehyde, methyl and ethyl alcohol. It is insoluble in dry ether, petroleum ether, benzene, toluene, carbon bisulphide or carbon tetrachloride. However, moist ether dissolves traces of choline and ether containing phospholipids may dissolve significant amounts. (Klein and Linser, 1932).

Stability -- On heating crystalline choline breaks down to trimethylamine, glycol and small amounts of dimethylaminoethanol without melting or distilling. Pure dry choline decomposes even at 40°C. under reduced pressure.

Dilute, aqueous solutions acted upon by bacteria may give rise to a poisonous base 'neurine' (vinyl trimethyl ammonium hydroxide). Wurtz (1868) and Nothnagel (1894), found that aqueous solutions up to 4% were stable at

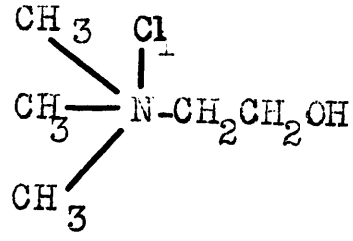
boiling temperatures and that the chloride was more stable to heat than the free base. Alkaline solutions are stable to prolonged boiling. On the other hand, evaporation of solutions of choline to dryness on a water is accompanied by losses which are roughly proportional to the concentration; if the concentration is 0.3 mg. per ml. the loss is less than 10%, but if the concentration is 10 micrograms per ml. the loss on evaporation is almost 100%. (Roman, 1930). Although losses are less on evaporation in vacuo, they are still considerable.

Pharmocological, choline has a muscarine effect (stimulation of parasympathetic nerve endings) and a nicotine effect (stimulation and paralysis of autonomic ganglia). It produces a fall in blood pressure, slows the heart by vagal stimulation, stimulates gastric and intestinal peristalsis, increases lacrimal, salivary and other secretions. (Merck Index, 1940).

Salts of Choline and Their Properties -- The chloride, nitrate, sulphate, carbonate, acetate, oxalate and picrate are freely soluble in water and alcohol.

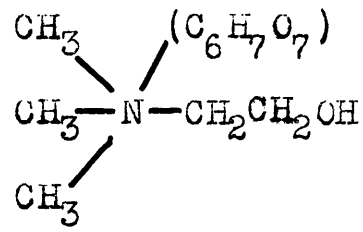
The periodide, phosphotungstate, phosphomolybdate, reineckate and double salts with gold chloride, platinum chloride and mercuric chloride are insoluble in water and this property has been exploited for the isolation of choline by several methods which will be mentioned later. The properties of these various salts are listed by Lucas and Best, 1943.

Choline chloride, one of the more useful salts, has a molecular weight of 139.57 and the following formula:



It is a colorless, deliquescent, crystalline compound with a fish-like odour and a bitter taste. It is very soluble in water, ethyl and methyl alcohol and formaldehyde. It is less soluble (but more so than free choline) in acetone, chloroform and carbon tetrachloride. It is insoluble in ether, petroleum ether, benzene, toluene and carbon disulphide. The aqueous solution is practically neutral.

Choline dihydrogen citrate is a white crystalline salt with a sour taste. It has a molecular weight of 295.29 and the following chemical formula:



It is soluble in water and alcohol and has the advantage of being less deliquescent and its solutions more pleasant tasting than the chloride.

Occurrence of Choline -- Choline is widely distributed in nature, both in plants and animals where it is usually found incorporated in the lecithin molecule. This wide distribution has made it difficult to select choline-free diets for animals, and some of the conflicting experimental results are attributable to choline being inadvertently supplied in the ration.

Choline Content of Common Foodstuffs, in mg. per Kg.

	Fletcher, Best & Solandt, 1935	Engel, 1942	McIntyre, et al, 1944	Borglin, 1947
Beef	760	-	-	440, 755
Pork	440	-	1200	480, 540
Lamb	-	1300	770	770
Veal	-	-	1100	-
Chicken	-	-	-	840, 730
Beef pancreas	2300	-	-	-
Beef liver	2700	6300	5100	-
Pork liver	-	-	-	1700
Fish	-	870	-	590 - 830
Cod liver	-	-	-	1700
Milk	-	147	-	45 - 159
Canadian cheese	190	480	-	--
Butter	130	50	-	0 - 10
Margarine	-	50	-	0
Eggs (whole)	-	-	-	3500-11700
Eggs (yolk)	-	17130	-	17500
Eggs (white)	20	0	-	7 - 17
Sugar	0	-	-	0
Wheat flour	1400	520	-	225 - 550
Rye flour	-	940	-	220 - 250
Barley flour	-	880	-	-
Potatoes	0	2380	-	33 - 108
Vegetables	-	-	-	80 - 230
Fruits	-	-	-	45 - 130
Beer, stout	-	-	-	90 - 225
Brandy	-	-	-	0
Brewer's yeast	2400	4300	-	3600
Corn oil	0	-	-	-

Requirements of Choline -- The daily requirement in the dog to permit normal growth and to prevent fatty liver is 10 - 100 mg./Kg. or 0.1 - 0.3% of the diet. (Fouts, 1943). The rat requires 120 - 200 mg. /Kg. or 0.1 - 0.2% of the diet for the prevention of fatty liver and hemorrhagic kidneys. (Engel, 1942; Treadwell, 1945). In the chick and turkey choline at a level of 0.15 - 0.3% of the diet prevents perosis and permits normal growth. (Jukes, 1940).

Assuming that choline requirements of man are of a similar order, (i.e., 0.1 - 0.3% of the diet), an adult would require 1.5 - 3.0 gms. per day. Other

estimates are lower. Borglin (1947) considered 300 - 500 mg. per day to be 'a suitable dietary supply' since it represented the choline content of the average Swedish hospital diet. From the interrelationships between choline, methionine and related substances it is apparent that the amount of choline required will be altered by the quantities of other dietary factors available. Sellers, Lucas and Best (1948) estimate the average Canadian diet to contain 300 - 900 mg. of choline plus 2.0 - 4.0 gm. of methionine from which a further 150 - 400 mg. of choline may be derived.

Toxicity of Choline -- In addition to effects of choline on the hematopoeitic system of the dog as discussed on page 33 there are reports of hemorrhagic lesions in the central nervous system after doses of choline, prostigmine and carbamyl choline. (Davis and Fletcher, 1946).

In chicks, addition of choline chloride at levels of 1%, 2% and 4% of the diet decreased growth rate by 12.0%, 13.8% and 23.8% respectively with marked reduction in body fat but no other pathological lesions. (Melass, 1946)

In the rat similar dosage depresses the growth rate (Hodge, 1945) and the $L.D._{50}$ when given by stomach tube is from 3.4 - 6.1 gm./Kg. depending on the concentration. (Neuman and Hodge, 1945).

In mice, using 2% choline chloride intraperitoneally the $L.D._{50}$ is 320 mg./Kg. (Hodge and Goldstein, 1942).

If these figures can be applied to man, mild toxicity might be expected from 15 - 70 gm. per day and the $L.D._{50}$ would be between 200 and 400 gm. per day by mouth.

NON-LIPOTROPIC FUNCTIONS OF CHOLINE

(Strictly applied this designation includes all biological effects except those concerned with promoting mobilization of lipids from the liver.)

Relations to the Kidney -- In 1939 Griffith and Wade noted the appearance of hemorrhagic lesions of the kidneys and decreased renal function within a few days after weanling rats were placed on low choline diets. Engel and Salmon (1941) showed that with further restriction of methionine intake similar lesions developes in adrenals, lungs, myocardium and eyes as well. These changes were prevented by feeding 1 - 2 mg. of choline per day. Other vitamin B factors were without effect (Griffith and Wade, 1939) and cystine aggravated the lesions in the kidneys, (Griffith,1941) but only when the animals were on a low sulphur intake. (Griffith and Mulford, 1941a). This suggests that the cystine exerts its effect by permitting faster growth and thereby increasing the demand for the raw materials for protein synthesis. The renal hemorrhages are apparently due to deficiency of some factor required for tissue synthesis at a critical period of development since only young growing animals are susceptible.

Gyorgy and Goldblatt (1940), Christensen (1940), Hartroft (1948) and others have described the renal lesions. In the acute phase there is engorgment and mottling of the cortex due to interstitial hemorrhages. The tubular epithelium becomes necrotic in both distal and proximal convoluted tubules and hyaline casts appear in the lumen. If the animal survives there is recession of the congestion, regeneration of epithelium, interstitial fibrosis and some calcification, and shrinkage and scarring of glomeruli. Later there is a reduction in the number of functioning

glomeruli with distension of surviving nephrons and varying degrees of vascular damage from cellular proliferation of the media and adventitia to intimal hyalinization and fibrinoid necrosis. In such cases Hartroft and Best (1949) were able to demonstrate progressive renal failure and hypertension in rats that had undergone only a few days of choline restriction in early life. These animals finally succumbed to cardio-vascular-renal disease produced for the first time by dietary deficiency alone.

Relations to Experimental Neoplasms -- Between 1935 and 1937 several workers in Japan (cited by White and Edwards,1942) described the production of hepatic tumours in rats by the feeding of p-dimethylaminoazobenzene (butter yellow). The rats are most susceptible on a diet of polished rice and carrot and varying degrees of protection are conferred by supplements of riboflavin, casein, liver, wheat (Kensler et al,1941) or choline (Gyorgy, Poling and Goldblatt, 1941).

Choline-deficient rats which survived 8 months or longer were noted to be unduly liable to neoplastic tumours. 30% developed adenocarcinoma of the liver, 10% scattered hemangioendotheliomata and 6% retroperitoneal sarcoma. (Lillie, Daft and Sebrell, 1941).

Relations to Avian Nutrition -- Perosis of fowl, or 'slipped tendon disease', is characterized by shortening and thickening of the tarsus and tibia of young birds. It was shown in 1936 to be due to manganese deficiency but Jukes (1939) found that choline was also necessary, and proved to be protective for both chickens and turkeys. (Jukes 1941a, 1941b). Since birds cannot synthesize choline, methionine cannot substitute for it in this situation.

Hyperplasia of the Forestomach of Rats -- On a white flour diet rats develop a deficiency syndrome characterized by abnormal epithelial pro-

liferation in the forestomach. This is prevented by protein diets or by rice polishings. In 1940 Sharpless showed that choline was the essential principle in the rice polishings.

Relation to the Hemopoietic System -- Davis (1939) noted that choline depressed the experimental polycethemia of dogs produced by cobalt feeding. The same effect occurred with 10 gm. of soybean lecithin (3% choline) per day (Davis, 1943) or with injections of acetylcholine, 3 mg. b.i.d. (Davis, 1946). In normal dogs a hyperchromic anemia developed with a drop in erythrocyte count beginning about 5 days after the start of the feedings and reaching a maximum decline of 15 - 25% after 12 - 25 days. Since the effect was abolished by atropine it was explained as being due to the muscarine effect of choline which produced vasodilatation of the bone marrow sinusoids, increasing oxygenation and depressing hematopoesis. Other vasodilator drugs such as nitrites and aminophylline were found to have similar effects. (Davis, 1944a). Treatment with stomach preparations, liver or folic acid produced reticulocytosis and a remission of the anemia, (Davis 1944b, 1946) coincidently with a marked increase in the serum cholinesterase content. Davis suggests that relapse in pernicious anemia is related to the increased activity of acetylcholine which produces dilatation of marrow sinusoids, increases oxygen tension thereby depresses hemopoiesis. In corroboration of this work are the findings of Meyer et al (1948) who noted particularly the low level of erythrocyte cholinesterase in pernicious anemia and its rise coincidently with the reticulocyte response.

The findings of Davis regarding the production of anemia in dogs are not confirmed by Best and Clarkson (1947) using oral choline or by Kunkel, Krop and Wescol (1948) using parenteral acetylcholine. In the rat, Bucciero

and Ortin (1948) not only failed to prevent cobalt-induced polycythemia with choline but found that choline at levels from 2.0 - 6.0 gm./Kg. of diet induced polycythemia to the same extent as cobalt itself.

Cartwright and Wintrobe (1945) demonstrated that in the low doses of 10 mg. per Kg. per day choline chloride does not produce anemia in humans, but Loewy et al (1942) found that a single dose of 400 mg. plus 1/4 pound of butter produced a significant drop in erythrocyte count followed by reticulocytosis in 3 - 5 days. This anemia was considered analagous to the hemolytic anemia produced in dogs from the hemolytic action of 'fatty acids and soaps' rapidly absorbed from the intestine under the influence of choline. (Davis and Gross 1944, 1945; Davis 1947).

Choline has been used in the treatment of liver-resistant macrocytic anemia associated with fatty infiltration of the liver with reported success. Its role is that of a lipotropic agent removing hepatic fat and normallizing liver function. (Moosnick, Schleicher and Peterson, 1945; Watson and Castle, 1945; Davis and Brown, 1947).

In 1948 Jiminez-Diaz et al in Spain and Engel in America reported the development of anemia in rats on low-choline, low-protein diets which is cured by choline supplements. This raises the question as to whether the anemia of malnutrition in man might have a similar genesis.

Relation to Acetylcholine -- Biosynthesis of acetylcholine from choline, acetate and adenosine triphosphate is catalyzed by choline acetylase. The reaction requires anaerobic conditions and the presence of potassium and a co-enzyme as yet unidentified but found in yeast and animal tissues. Increased yield results from the addition of α-tocopherol. (Nachmanson and John, 1945; Nachmanson and Berman, 1946). The possibility that increase or decrease in intake of choline might affect the rate of formation

of acetylcholine has been the subject of speculation and some investigation by Solandt and Best (1939). They found some evidence that choline depletion results in decreased vagus effect in rats.

This interesting lead was pursued by Abdon and Borglin (1946) who found that in choline-deficient animals the heart did not respond to vagus stimulation but did respond 10 - 15 minutes after choline was injected. This lag period suggests that synthesis of 'acetylcholine precursor' is relatively slow compared to the rate of formation of acetylcholine from the precursor, which may be choline itself or a choline complex. (see also section on 'Relation of Choline to the Heart').

Relation to Intestinal Absorption -- Frazer (1946) showed in rats that choline increased the rate of absorption of fats into the intestinal cells. He also maintains that choline is essential for the transport of fat through the inner cell membrane of the intestinal mucosa. Using radioactive phosphorus, Artom and Cornatzer (1946) showed that feeding of choline increased the rate of accumulation of phospholipids in the intestinal mucosa.

Choline increased the rate of absorption of vitamin A provided the animal was on a low-fat diet. (Bentley and Morgan, 1946).

Choline as a Growth Factor -- Choline was reported to be a growth essential for the rat by Best, Huntsman, McHenry and Ridout in 1935 and this finding was confirmed by further experiments of McHenry (1935), Griffith and Wade (1939), Sure (1940b) and others. It was subsequently found to be essential for the growth of chicks and turkeys by Jukes (1940, 1940b, 1941a), Jukes and Welch (1942) and Almquist and Grau (1944). McKittrick (1947) showed that with regard to growth of poultry both choline and methionine could be separated into an 'essential' portion for tissue construction and a

'replaceable' portion for methylation processes. For optimum growth the 'essential' requirement was found to be 0.5% methionine and 0.1% choline. The growth-promoting effect of choline can be differentiated from the anti-perosis action in fowl and from the anti-hemorrhagic kidney and anti-fatty liver action in rats.

Moyer and du Vigneaud (1942) have summarized in tabular form the lipotropic, anti-perotic, anti-hemorrhagic kidney and growth-promoting effects of various compounds. A high degree of structural specificity is noted for methyl donors as tested for by growth-promoting effect. Of 35 compounds studied only 5 were active: choline, phosphoryl-choline, dimethylethylhydroxyethyl ammonium chloride, lecithin and betaine.

Although choline has come to be regarded widely as a growth essential for most animals a recent report by Treadwell (1948) suggests that it is effective only as a result of its methionine-sparing action. He found that in rats a maximum growth-promoting effect was realized at a level of 200 mg. per rat per day.

Relation to Cardiac Function -- In 1946 Abdon and Borglin reported experiments to support their hypothesis that an acetylcholine precursor of unknown identity is essential for the metabolism of heart muscle. This is suggested by the finding that the weakening beat of the isolated perfused heart is restored after addition of choline, but the effect is not realized for 10 - 15 minutes which presumably represents the time required for sufficient synthesis to restore the depleted 'acetylcholine precursor'. Acetylcholine is rapidly derived from precursor and almost immediately broken down by tissue cholinesterase. Depletion occurs in the perfused heart because the freed choline is washed away in the perfusate.

Abdon postulates that liberation of acetylcholine from precursor

and synthesis of precursor from choline takes place in the absence of functioning cholinergic nerves and hence 'such metabolism belongs to the normal metabolism of heart muscle'.

Choline-deficient rats show bradycardia and decrease in muscle choline content and isolated hearts of choline-deficient rats show decreased ability to contract. It suggests that an adequate supply of acetylcholine precursor is essential not only for vagal transmission but for muscular functions of the heart as well. (Abdon and Borglin).

Further evidence of this metabolic role of choline in the myocardium might be adduced from the finding of Gremels (1936, 1937) that the low oxygen consumption of the Starling heart was increased to near normal by addition of acetylcholine, choline or choline-containing compounds such as crude lecithin.

Choline and Arteriosclerosis -- Page (1945) has reviewed the relationships of altered fat metabolism to the development of arteriosclerosis. He discusses the various theories of production of atheromatous lesions and the relation of diet to blood lipid levels. A high fat diet plus lecithin leads to a high blood cholesterol in dogs. (Corwin, 1938). Even with a fat intake less than 3%, addition of phospholipid to the diet produces a sustained post-absorptive lipemia. (Flock, Corwin and Bollman, 1938). In humans, Steiner and Domanski (1941) showed that feeding 100 gm. per day of egg yolk powder (lecithin 14 gm.; cholesterol 8 gm.) resulted in rises of from 40 - 218 mg.% in serum cholesterol after 6 - 10 weeks. Rabinovitch (1935) presented statistical evidence to show that in diabetics the degree of lipemia was influenced by diet. However in the diabetes of depancreatized dogs as discussed previously the lipemia is dependent on the insulin control and the presence of adequate lipotropes

rather than on diet. (Dragstedt, 1945).

There is evidence to indicate that hyperlipemia predisposes to the development of arteriosclerosis: Gibbs et al (1933) noted increase in the free cholesterol of diabetics with advanced arteriosclerosis; Rabinovitch (1935) noted a similar relationship; White (1934) calculated that diabetics with hyperlipemia were 15 times as likely to develop severe arteriosclerosis; in rabbits (Leary, 1934) and chickens (Dauber and Katz, 1943) increased blood cholesterol predisposed to arteriosclerosis.

However it is well known that arteriosclerosis can develop without hyperlipemia (Lande and Sperry, 1936; Page et al, 1936) and it is likely that at least two factors are important in arteriosclerosis in humans -- increased blood lipids and local factors in the vessel walls.

In view of its lipotropic action and its effect on blood lipid levels in animals it was logical to test the use of choline in experimental arteriosclerosis.

In preliminary reports Steiner (1933, 1938a) suggested that choline exhibited a temporary action in inhibiting development of cholesterol arteriosclerosis in rabbits and might even aid resorption of the lesions. This was not confirmed by Himsworth (1938) or Baumann and Rusch (1938) but they used proportionately less choline. However, Andrews and Broun (1940) showed a protective effect from choline in rabbits and Kesten and Silbowitz (1942) found choline or soya lecithin effective.

Hermann (1946) found that choline 0.5 gm. per day given to old hens considerably decreased the free and ester cholesterol of the blood, aorta, heart muscle and liver.

Steiner (1948) has recently reported a well-controlled study using cholesterol-fed rabbits one group of which received supplements of 0.5 to

1.0 gm. of choline per day. Although there was no change in blood cholesterol from choline feeding this group were free of arteriosclerotic lesions after 40 - 80 days while of the control group 10 out of 11 showed aortic atheroma. It is suggested that its mode of action is similar to that by which it exerts its liver lipotropic effect. Almost identical results are reported by Morrison et al (1949).

Thus the bulk of evidence favours the impression that experimental arteriosclerosis is ameliorated by the exhibition of choline presumably acting by virtue of its lipotropic properties.

Choline and the Organs of Reproduction -- Sure (1940) reported that choline is essential for lactation in the rat and for the growth and health of the suckling young. Similarly choline has been shown to improve reproduction and lactation in mice. (White and Cercedo, 1947).

Morphological and functional changes in genital organs of choline-deficient rats have been recently described by Peet and Sampson (1948). They found atrophy of uterus and ovaries and loss of contractibility of the uterus both spontaneously and after stimulation.

Deficient egg production has been noted in choline-deficient chickens. (Abbott and deMasters, 1940).

The Influence of Choline on Protein Metabolism -- Rats maintained on a low-protein intake for long periods show less depletion of protein if they are given a small supplement of choline. (Jiminez-Diaz and Vivanco, 1948).

Miscellaneous Effects of Choline -- Bronze hair pigmentation has been noted in rats on high choline intake. (Higgins, Joneson and Mann, 1945).

Choline is said to be essential for the formation of histiocytic cells. (Chrevemont, 1945).

Choline is essential for the growth of Type III pneumococcus (Badger, 1944) and Neurospora mutants. (as detailed subsequently).

THE LIPOTROPIC ACTION OF CHOLINE IN RELATION TO LIVER FAT

Historical Review -- The fatty livers of depancreatized dogs had long been known to be prevented by feeding raw pancreas. (Sandmeyer, 1895). When fatty livers developed in spite of good insulin control (Allen et al, 1924; Fisher, 1924) fresh stimulus was given to a search for the principle in pancreas which prevented liver fat accumulation. Hershey (1930) tried lecithin, prompted by the reasoning that the fatty livers resulted, not from an absence of digestive enzymes in the intestine, but rather from a disturbance of fat metabolism in the liver, since he noted that one of Fisher's animals died in hypoglycemic convulsions although no insulin had been given for days. Since phospholipids had long been attributed a role in fat transport, and had been specifically implicated in the transport of fat from the liver (Leathes and Raper, 1925), Hershey decided to feed lecithin in the hope of supplying an essential factor for fat metabolism and transport. Subsequent findings have proven the soundness of his reasoning.

The Toronto group using rats with dietary fatty livers as experimental animals, first proved the effectiveness of purified lecithin (Huntsman, Hershey and Best, 1932a) and then tested the various components of the phospholipid molecule. Best and Huntsman (1932) found that choline was the active lipotropic principle of lecithin and that it was effective in amounts as small as 1.0 mg. per rat per day. Extending the work to other species, Best, Huntsman and Solandt (1932) prevented dietary fatty liver of mice by choline and Best, Ferguson and Hershey (1932) demonstrated its effectiveness in prevention and cure of the fatty livers of depancreatized dogs.

Definition of the effectiveness of choline led to the reorientation

and expansion of research on this problem chiefly by Best's group in Toronto and later by Channon in Liverpool. Low choline diets were devised (Best and Channon, 1935) which, combined with cholesterol supplements, produced a type of fatty liver high in cholesterol ester content and relatively resistant to choline therapy. In fact, the principle effect of choline in 'cholesterol fatty livers' and in the 'fat fatty livers' of high fat diet appeared to be on the glyceride fraction. (Best, Channon and Ridout, 1934; Channon and Wilkinson, 1935; Best and Ridout, 1933 and 1936). That neither high cholesterol nor high fat intake were the sole determining factors in these diets was shown by Best and Huntsman (1935) who produced fatty livers in rats by high carbohydrate diets free of choline. In phosphorus, chloroform and carbon tetrachloride poisoning in rats the development of fatty livers was not prevented by choline but the removal of the fat was accelerated. (Best, McLean and Ridout, 1935; Barrett, Best and Ridout, 1938; Barrett, Best, McLean and Ridout, 1939).

<u>The Mechanism</u> of Action of Choline as a Lipotropic Principle -- Among the first to speculate on this subject were Best, Channon and Ridout (1934) who found little to substantiate the phospholipid fat transport theory of Leathes and favoured a process by which choline 'accelerates either directly or indirectly the oxidation of fatty acids and cholesterol by the liver itself'. At that time action of choline through liver phospholipids was discredited because investigators could detect no significant change in phospholipid content of the livers of choline-deficient rats. (Best, Channon and Ridout, 1934; Channon, Platt, Loach and Smith, 1937; Loizides, 1938). Best's group specifically measured the choline-containing phospholipids but found no change in short term studies lasting 21 - 26

days. In experiments lasting 2 - 3 months, no change was found in choline content of liver, kidneys or brain (Jacobi, Baumann and Meek, 1941) or of the whole animal (Jacobi and Baumann, 1942). Brante (1943) confirmed these findings.

On the other hand, after only 10 days of choline-free diet, Stetten and Grail (1942) found the livers of young rats to contain only half as much choline as the controls. By addition of methyl-consuming guanidoacetic acid to the diet complete disappearance of liver lecithin was effected and extensive fatty infiltration ensued.

These apparently conflicting results can probably be reconciled in the light of the isotope studies of Perlman, Ruben and Chaikoff (1938) and Perlman and Chaikoff (1939) which indicate that phospholipid metabolism in the liver is very rapid and that it is accelerated by choline, suggesting that the rate of metabolism of phospholipids is the determining factor rather than the amount in the liver cells at any given time.

Logically it would appear that an increase in liver fat might result from one or a combination of the following processes:

(a) Increased intake of fat in the diet (as in experimental fatty livers of high fat feeding).

(b) Increased synthesis of fat from carbohydrate, a process accelerated by thiamine as proven by McHenry and Gavin, (1938, 1939) and Boxer and Stetten, (1944).

(c) Decreased degradation of fat. This probably has no relation to choline deficiency since Stetten and Grail (1943) showed that fatty acid catabolism is normal in choline-deficient rats.

(d) Increased transport of fat from depots to liver. This is probably the process by which anterior pituitary extracts (likely acting

through stimulation of 'S' hormones of the adrenal cortex) exert their effect. (Barrett, Best and Ridout, 1938; C.H. Li, Lecture, University of Montreal, April, 1949).

(e) Decreased transport of fat from liver to depots. This is apparently the process in which choline is most important. Stetten and Salcedo (1944) used isotope studies to show, that in choline-deficient rats, newly synthesized fatty acids do not readily escape from the liver to the depots.

There is considerable evidence that the action of choline in promoting transfer of liver fat to peripheral depots involves the intact molecule. Welch (1936) prepared arsenocholine and found that it possessed lipotropic properties similar to choline. In later studies Welch and Landau (1942) proved that arsenocholine was utilized in the synthesis of lecithin apparently as the intact molecule since it is inactive as a methyl donor in the rat (Moyer and du Vigneaud, 1942) and in the chick (Almquist and Jukes, 1942). The proven lipotropic activity of the arsenocholine molecule (Best and Huntsman, 1932; Platt, 1939) and its anti-hemorrhagic effects (Welch, 1941) suggest that choline too may exert its lipotropic and anti-hemorrhagic effects through reactions involving the intact molecule. This is further substantiated from a consideration of the following list of lipotropic compounds which act directly, and not by supplying groupings for the synthesis of choline. It appears that the general configuration of choline is required although methyl groups can be replaced by ethyl as in triethyl choline and the nitrogen replaced by other elements of suitable valency as in phosphorylcholine and arsenocholine.

Compounds Related to Choline Which Posses Lipotropic Activity --

Arsenocholine chloride Calcium phosphorylcholine chloride	Welch and Welch, 1938.
Homocholine	Channon, Platt and Smith, 1937.
Triethyl choline	McArthur, Lucas and Best, 1947.

The possibility that choline must be oxidized before it can exert its lipotropic activity is suggested by Jacobi and Baumann (1942). The physiological role of choline oxidase is not yet established (Bernheim and Bernheim, 1938) and it may be significant that the two organs most sensitive to choline deficiency, liver and kidney, contain the highest concentration of the oxidase. (Lucas and Best, 1943). In vitro fatty acids inhibit choline oxidase (Bernheim, 1940) and in the fatty liver of methionine-deficient rats choline oxidase activity is likewise considerably depressed. (Handler and Bernheim, 1942).

Summary -- Taken together, these findings suggest that the mechanism of the lipotropic effect of choline is stimulation of phospholipid interchange between the liver and other tissues, the liver being most active in the process as shown by tracer studies. However, the more intimate details of the process remain to be elucidated.

CHOLINE AND CIRRHOSIS

Intelligent application of therapy is, in general, conditioned by an understanding of the disease process under treatment. This is particularly true of metabolic disorders of the liver. Here the problem is complicated by the manifold character of liver disease and by the fact that most classifications are based on morphologic or symptomatic characteristics rather than on metabolic genesis and evolution. In the following section an attempt will be made to outline a systematic treatment of the subject from the metabolic point of view utilizing as a framework the classification proposed by Himsworth in 1947.

The Pathogenesis of Hepatic Cirrhosis

Introduction -- The hepatic fibrosis to which Laennec gave the term cirrhosis was at that time considered a primary inflammatory and sclerotic lesion of supporting tissues. Only toward the end of the nineteenth century was the importance of degeneration of the parenchymal cells recognized. At first parenchymal degeneration was regarded as a result of connective tissue sclerosis, then as a concomitant, and now as a cause. Cirrhosis is in no sense a specific condition but rather is to be regarded as a non-specific sequel to a variety of lesions which cause parenchymal cell dissolution.

Classification -- Hepatic fibrosis in animals and man can be divided into two anatomical types:

A. Post-necrotic scarring, which follows acute massive necrosis.

B. Diffuse hepatic fibrosis, which may occur:

1. As a sequel to biliary obstruction.

2. As a sequel to repeated attacks of zonal necrosis.

3. As a sequel to prolonged fatty infiltration.

POST-NECROTIC SCARRING

In 1935 Weichselbaum showed that rats maintained on low protein diets finally died with hemorrhagic hepatic lesions which were preventable by the use of the sulphur-containing amino acids. Later as papers appeared on the production of experimental cirrhosis there were occasional reports of the development of hepatic hemorrhages in rats given diets designed to produce fibrosis. Gyorgy and Goldblatt (1939) recognized these lesions as acute necrosis and subsequent speculation as to the etiology has varied from the view of Best's group that it is caused by an infection secondary to nutritional depletion, to the more widely held opinion of Himsworth that it is a specific form of nutritional hepatic injury. Like acute yellow atrophy in man it progesses to coarse scarring and nodular hyperplasia.

Relation to Diseases of Man -- In addition to the implied relation to acute yellow atrophy, Himsworth relates post-necrotic scarring to the 'tropical cirrhosis' which follows the 'toxic jaundice' prevalent in undernourished pcpulations particularly in the Far East. Aberration of the regenerative process in these cases is invoked to explain the high incidence of primary carcinoma of the liver found in the same populations.

Another interesting correlation is the association of post-necrotic scarring and nodular hyperplasia in humans with deToni-Fanconi syndrome which is characterized by chronic loss of amino acids in the urine. Loss of serine which is required for the synthesis of cystine from methionine may amount to 2.0 gm. per day (Dent, 1942) so that a cystine deficiency might explain this occurrence of acute hepatic necrosis as it does in cystine-deficient rats. (v.i.)

Etiological Relation to Cystine Deficiency -- To briefly state the results of a considerable amount of experimental work, the etiology of acute massive necrosis can be regarded as due to cystine deficiency with further sensitization resulting from a deficiency of tocopherols. (Glynn, Himsworth and Neuberger, 1945; Himsworth, 1947).

The inconstancy with which these lesions can be reproduced has been variously explained as reflecting genetic differences in rats, differences in the tocopherol content of dietary fats, differences in intestinal flora, or some unknown factors.

Cystine in Relation to Hepatic Injury -- In an effort to simplify a confusing subject the manifold and paradoxical effects of cystine in experimental liver injury will be summarized.

1. Cystine deficiency leads to acute hepatic necrosis. (v.s.)

2. Cystine accelerates growth on diets suboptimal with respect to methionine and may in this way increase the demand for lipotropes, i.e., exert a so-called 'anti-lipotropic' effect. (Mulford and Griffith, 1942).

3. Cystine accentuates the hyperplasia which occurs in fibrotic lesions of the liver. (Himsworth, 1947).

4. By a direct toxic action large doses of cystine produce hemorrhagic necrosis in the liver and moderate doses produce a diffuse necrosis not unlike that which follows carbon tetrachloride poisoning. (Curtis and Newburgh, 1927; Miller, Ross and Whipple, 1940; Daft, Sebrell and Lillie, 1941).

B. DIFFUSE HEPATIC FIBROSIS

1. As a sequel to biliary obstruction -- A discussion of clinical and experimental 'biliary cirrhosis' is beyond the scope of this presentation.

2. As a sequel to repeated attacks of zonal necrosis such as occur following prolonged administration of carbon tetrachloride. (Lamson and Wing, 1926; Moon, 1934; Sellers, Lucas and Best, 1948). Repeated and frequent injections are required to establish the picture of cirrhosis as though extended exposure to high concentrations of the products of cellular injury is required to stimulate the proliferation of fibrous tissue. Thereafter, up to a point, removal of the noxious agent is followed by resorption of the fibrous tissue, but there is a stage beyond which the process is irreversible (Cannon and Karunaratne, 1936), and indeed may be self-perpetuating, as suggested by the work of Cameron and Oakley (1934) who showed that transplants of normal liver provoked a fibrous reaction in surrounding tissues while boiled liver did not.

It is conceivable that a small proportion of clinical portal cirrhosis might follow repeated exposure to chemical hepatotoxins or infection with the virus of infectious hepatitis.

3. As a sequel to prolonged fatty infiltration -- A century ago Rokitansky (1849) wrote in reference to alcoholic cirrhosis, 'The latter (adipose deposit) may be the primary affection on which the granular disease is grafted in the shape of cirrhosis.' Early French clinicians of the nineteenth century regarded the enlarged fatty liver of 'steatosis' as preceding the later stage of 'cirrhosis' when the organ is shrunken and atrophic.

There is now considerable evidence to support this concept. Connor (1938) reviewed the literature relating to the association between fatty infiltration and subsequent fibrosis and described histological studies of the transition from fatty liver to cirrhosis as encountered in autopsy material. He found that accumulation of fat from whatever cause led to pressure anoxia and slow necrosis of cells followed by proliferation and then condensation of reticulum. As the fibrosis progressed the fat often disappeared, possibly reflecting terminal exhaustion of body fat in untreated cases or the effect of dietary lipotropes in treated cases.

This transition from fatty infiltration to cirrhosis has been repeatedly observed in animal experiments as well. In depancreatized dogs maintained with insulin, fibrosis supervened in 8 of 16 animals after fatty changes had been present from two to five years. (Chaikoff, Connor and Biskind, 1938).

In 4 of 16 dogs cirrhosis was superimposed on fatty livers induced by high fat feeding plus alcohol, (Connor and Chaikoff, 1938) of by fat feeding alone. (Chaikoff and Connor, 1940).

The concept that conditions which produce fatty liver in short term experiments will eventuate in cirrhosis if carried on long enough receives further support from the reports of Blumberg and McCollum, 1941; Webster, 1941; Daft, Sebrell and Lillie, 1941; and Gyorgy and Goldblatt, 1942.

In this regard the time relationship is of interest. In the depancreatized dogs of Chaikoff fatty livers developed in a few months but cirrhosis supervened only after years. Similarly in rats dietary fatty livers can be established in a few days while it requires 80 - 150 days

for cirrhotic changes to develop. (Sellers, 1948).

In man as well there are a number of clinical studies, some employing serial liver biopsies, which support the view that fatty infiltration is the usual precursor of portal cirrhosis. (Gillman and Gillman, 1945; Hoagland, 1946; Buck, 1948). Some reports emphasize the potential reversibility in animals and man of this pre-cirrhotic fatty infiltration and even of early cirrhotic fibrosis by the use of diets high in lipotropes and calories. (Russakoff and Blumberg, 1944; Barker, 1945; Beams, 1946; Sellers, Lucas and Best, 1948: and Buck, 1948).

Clinically then, the problem of portal cirrhosis largely resolves itself into prevention of increased liver fat, or its early detection when present, because the effectiveness of therapy is inversely related to the degree of fibrosis and this in turn is a function of the duration and degree of fatty infiltration.

To this end it might be profitable to examine the etiology of experimental fatty infiltration and then apply to human disease any analogies which become apparent.

Experimental Fatty Livers

Increased deposition of fat in the liver can be induced in a variety of ways:

(1) Dietary Fatty Livers --

(a) Ingestion of a low protein, low choline diet. (Best and Channon, 1935). This type is characterized by predominent deposition of triglycerides and responds to choline feeding.

(b) Ingestion of an extremely high fat diet. (McHenry and Patterson, 1944). Like (a) this produces a 'fat fatty liver' sensitive to choline.

(c) Ingestion of excessive amounts of cholesterol has been reported by many investigators (since Chalatow, 1912) to lead to deposition of liver lipids characterized by a high concentration of cholesterol esters. (Okey, 1933). This cholesterol component is mobilized slowly and incompletely by ordinary doses of choline but large doses over a long period will mobilize both cholesterol and triglycerides. (Best and Ridout, 1933, 1935; Best, Channon and Ridout, 1934; Channon and Wilkinson, 1934). Lipocaic and inositol are said to mobilize cholesterol from the liver in cases in which choline is not completely effectual. (Gavin, Patterson and McHenry, 1943).

(d) Ingestion of certain watery extracts of meat and liver. As early as 1915 Opie and Alford showed that a lean meat diet aggravated the hepatic lesions of phosphorus poisoning in dogs. In 1931 Bollman and Mann showed that it was not the meat protein which was toxic but some substance present in watery and alcoholic extracts of meat and liver. Blatherwick and associates (1931, 1932, 1933) considered this effect was probably due to cholesterol while Gavin and McHenry (1941) suspected biotin. However, Ralli and Rubin (1942) were able to induce fatty livers in dogs by a lean meat diet and identified the active toxic substance in watery extracts as inositol, which paradoxically had been shown to be lipotropic with respect to the biotin and cholesterol fatty livers of rats. (Gavin and McHenry, 1941; Gavin, Patterson and McHenry, 1943).

(e) Ingestion of certain of the B complex vitamins. McHenry (1937) showed that addition of thiamine increased liver fat deposition at levels of fat intake from 0 - 58% and in high carbohydrate diets as well. It was postulated that thiamine promotes synthesis of fat from

carbohydrate and exerts its greatest effect in the presence of choline and with an intake of fat equal to 40% of the diet. (McHenry, 1937a). Conversely a deficiency of thiamine protects against development of fatty liver. Biotin is another B vitamin which promotes fatty livers of a type resistent to choline but affected by inositol or lipocaic. (Gavin and McHenry, 1941).

McHenry and Patterson (1944) have tabulated the response to lipotropes of several types of fatty livers induced by manipulation of diet and vitamin B intake:

	Choline	Lipocaic	Inositol
Rats: High fat diet, thiamine	++	O	−
High fat diet, all B vitamins	++	−	−
High fat diet, cholesterol	+	O	+
Fat-free diet, thiamine	++	−	O
Fat-free diet, thiamine and riboflavin	++	−	−
Fat-free diet, thiamine, riboflavin, pyridoxine & pantothenic acid	+	−	+
Fat-free diet, 4 above vitamins and biotin	O	++	++
Fat-free diet, B vitamins and cholesterol	+	+	+
Dogs, depancreatized	++	++	−

Handler and Bernheim (1943) are not in agreement with McHenry's group with respect to the effect of B vitamins in fatty livers and conclude that, 'the effect of deficiencies of members of the vitamin B complex in preventing the appearance of fatty livers due to choline deficiency is the result of an impairment of the overall metabolism of the rat rather than some specific defect in the metabolism of the liver.'

(2) Fatty Livers Induced in Dogs by Removal of the Pancreas --

This subject is treated fairly comprehensively in previous sections including Historical Review, Methionine and Pancreatic Lipotropes. In addition to the fatty liver due to insulin deficiency which develops rapidly after operation and responds to insulin control of the diabetes, there is the slowly-developing fatty liver due to deficiency of pancreatic lipotropes (q.v.) which are probably enzymes required to free methionine from ingested protein.

(3) Fatty Livers Resulting from Starvation --

During starvation fat is mobilized from body depots and accumulates in the liver. There is almost unanimous opinion that choline administered during the fast does not prevent the fatty livers in fasting rats, dogs, mice, rabbits and guinea pigs, but will hasten reabsorption during refeeding. (Barrett, Best and Ridout, 1938; Best and Ridout, 1938).

(4) Fatty Livers Produced by Toxic Substances --

Experimental fatty livers have been induced by a variety of toxic agents such as chloroform, carbon tetrachloride, tannic acid, ethyl formate, phosphorus and alcohol. The action of such poisons is said to be through interference with carbohydrate metabolism in liver cells (Quastel, 1932; Peters and VanSlyke, 1931; Goldschmidt et al, 1937) probably due to an inhibitory effect on cell respiration. (Himwich et al, 1933).

Alcohol has long been associated by clinicians and the laity alike with the genesis of certain disorders of the liver, notably cirrhosis. As early as 1820 James Johnson in England remarked, 'In this country,

among the internal exciting causes of hepatic inflammation, the ingurgitation of inebriating liquors and particularly ardent spirit has always been ranked foremost.' Yet in 1934 when Moon evaluated the literature up to that time he concluded that alcohol per se had not been proven to cause liver injury, although Lamson and Wing (1926) had demonstrated that it aggravated the lesions of carbon tetrachloride poisoning, and Bollman and Mann (1935) found that alcohol feeding increased the rapidity of development of liver lesions in dogs on high fat diets. Later, with the elucidation of dietary causes of fatty livers and cirrhosis and the recognition of various deficiency syndromes in alcoholics, the tendency was to attribute the liver damage of alcoholics to dietary deficiency alone. However since Cannon (1940) produced hepatic fat infiltration, necrosis and fibrosis in rabbits by adding alcohol to an adequate diet and Ashworth (1947) confirmed this finding in the rat, the conclusion of Beazell and Ivy (1940) and others that alcohol alone cannot produce experimental cirrhosis may have to be revised.

(5) Fatty Livers Induced by Hormones --

The effect of the 'ketogenic factor' of the anterior pituitary and the well-known amelioration of the diabetes of depancreatized dogs by hypophysectomy led Chaikoff et al (1936) to study the fat metabolism of these animals. It was found that removal of the pituitary did not appreciably alter the blood fat nor prevent the accumulation of fat in the liver , but 'ketogenic hormone' injections increased liver fat at the expense of body fat. (Best and Ridout, 1938; Best and Campbell, 1936, 1938). This was confirmed by MacKay and Barnes (1938) who found

that neither choline nor lipocaic would prevent this fatty liver but would hasten the removal of fat in the recovery phase. However, in 1943 Julian et al using guinea pigs reported from Dragstedt's laboratory that lipocaic was effective and postulated that 'ketogenic hormone' controlled the body fat to liver fat transfer while 'lipocaic' controlled the reverse process. The questionned existence of either of these 'hormones' together with the fact that Houssay dogs will develop fatty livers casts considerable doubt on this attractive hypothesis, however.

The importance of the adrenal in this regard is suggested by the fact that adrenalectomy protects against the fatty liver that follows pancreatectomy (Long and Lukens, 1936), partial pancreatectomy (MacKay and Carne, 1938; Berman, Sylvestre, Hay and Selye, 1947) or injection of crude anterior pituitary extracts. (MacKay and Barnes, 1937; Fry, 1936).

Ingle (1943) reviewed the relationship of the adrenal cortex to fat metabolism and concluded that cortical insufficiency is characterized by a slower-than-normal rate of fat absorption, decrease in fat transported to and deposited in the liver and a diminished response to ketogenic agents, these effects being mediated through changes in carbohydrate and electrolyte metabolism.

However adrenalectomy does not alter the fat-metabolizing effect of purified growth hormone as measured by ketogenesis (Fry, 1936; Bennett, Kreis, Li and Evans, 1948; Szego and White, 1949) so it would appear that the action of growth hormone in promoting transient deposition of fat in the liver is not mediated through the adrenal.

C.H. Li (1949) has reported that purified adrenocorticotrophic hormone induces a prolonged deposition of fat in the liver of rats presumably by virtue of metabolic changes consequent on increased activity

of the 'S' hormones. (Lecture, University of Montreal, April, 1949).

The inverse relation between the level of blood lipids and thyroid activity was studied in the dog by Chaikoff et al (1941) who found alterations particularly in cholesterol esters and fatty acids with less consistent changes in cholesterol and phospholipids. The blood lipid elevation following thyroidectomy is dependent on adequate nutrition and can be inhibited by fasting (Entenman, Chaikoff and Reichert, 1942a) or exaggerated by removing the hypophysis also and thereby presumably preventing stimulation of any accessory thyroid tissue. (Entenman, Chaikoff and Reichert, 1942b).

Chaikoff et al (1948) showed that thyroidectomized dogs developed fatty livers even though maintained on diets adequate with respect to protein and vitamins. This fatty liver showed little tendency to progress to fibrosis while in those dogs with both thyroid and hypophysis removed the fatty infiltration rapidly progressed to fibrosis. In the latter two types of fibrosis were recognized histologically and it was postulated that removal of the hypophysis permitted the development of a particular type of hepatic fibrosis not dependent on pre-existing fatty metamorphosis. They demonstrated that this was not associated with accidental operative trauma to the hypothalamus. Less complete studies on hepatic fibrosis in hypophysectomized dogs were reported by Graef, Negrin and Page (1944).

Hypophysectomy alone does not alter liver fat but thyroidectomy alone does, and the combined operation results in earlier and more severe fat deposition. Addition of free choline to the diet is protective and since the lean meat diets used were known to contain adequate choline precursors these findings suggest some hormonal control of the release,

absorption or utilization of such lipotropes as methionine by some obscure mechanism involving the thyroid.

In the rat, however, Handler noted just the opposite effect, i.e., that the hepatic lesions of choline deficiency were retarded by decreased secretion and aggravated by thyroid feeding. It is possible that those types of hepatic damage which are made worse by thyroid overactivity are those in which tissue anoxia is the critical factor.

GENERAL PRINCIPLES REGARDING LIPOTROPE THERAPY IN LIVER DISEASE

Post-necrotic Scarring -- There is no evidence that lipotropes have any value in acute yellow atrophy in man or in the post-necrotic scarring which follows in non-fatal cases. It is possible that supplementary cystine would be useful prophylactically in cases of de Toni-Fanconi syndrome characterized by loss of the cystine-precursor, serine. Diets adequate in lipotropes would likewise be of prophylactic value in lowering the incidence of 'tropical cirrhosis' which appears to result from a combination of nutritional and infective factors.

Diffuse Hepatic Fibrosis -- 'Portal cirrhosis' as encountered in man is usually the sequel of prolonged fatty infiltration. (v.s.). In animals there is some evidence that recovery is possible even after considerable fibrosis has supervened, but in man experience to date indicates that recovery is unlikely except in those cases with large fatty livers in which there is little or no fibrous tissue proliferation. (Wade et al, 1948; Buck, 1948). Other authors also noted a clear relationship between liver size and prognosis. (Fleming and Snell, 1942; Berne, 1946).

Since the degree of fibrosis (and resistance to therapy) is probably a function of the degree and duration of fatty infiltration the necessity of finding cases early is evident. This will require a higher index of suspicion than is currently prevalent since most cases now are diagnosed and futilely treated only after the appearance of late signs such as ascites, ankle edema, jaundice, varicosities, etc. The incidence of fatal 'portal cirrhosis' can be reduced when diagnostic tests are applied to individuals exposed to excessive alcohol and/or malnutrition in the way that diagnostic tests are applied to contacts exposed to tuberculosis.

Among the more sensitive liver function tests available for this purpose are the cholesterol ester-total cholesterol ratio, serum albumin level, cephalin-cholesterol flocculation, thymol turbidity and flocculation, and bromsulphalein retention. Needle biopsy of the liver provides the most sensitive and accurate means of diagnosing pre-cirrhotic fatty infiltration and its more frequent use may be required to convince the patient and the physician of the need for vigorous therapy.

It is evident from the foregoing that rigid exclusion of all hepatotoxic agents including alcohol, carbon tetrachloride, phosphorus, etc. is a prime requisite of therapy. It is probably desirable to restrict fat to the lowest palatable level in view of the well-recognized lipogenic activity of cholesterol.

Positive therapy involves the feeding of not only an adequate diet but a high calorie, high protein diet supplemented with vitamins and choline, (and probably methionine), keeping in mind the fact that cirrhotics suffer from depletion of both serum and tissue proteins so there will be keen competition for the amino acids on which liver regeneration depends.

Most cases of pre-cirrhosis in man are of the fatty type secondary to deficient intake of lipotropes, but for the sake of completeness, mention will be made of the fatty livers which complicate cystic disease of the pancreas and which are analagous to the fatty livers of depancreatized dogs. In these the deficiency is in the proteolytic enzymes of the pancreas required to free methionine from ingested protein so that treatment would consist of supplying a maintainence dose of free methionine. In addition there are the rare cases of cirrhosis which follow scattered zonal necrosis due to chemical toxins or the virus of infectious hepatitis, and in these the value of lipotrope therapy is in doubt.

THERAPEUTIC TRIALS OF CHOLINE IN DISEASES OF THE LIVER

The first methodical approach to the therapy of cirrhosis was marked by the regime of high protein and high vitamin intake proposed by Patek and Post in 1937. This therapy proved efficacious in many cases including some with marked ascites. Similar treatment and results was reported by Fleming and Snell (1942) although they noted that advanced cases showed no improvement in survival time as a result of therapy.

Choline had been established as a lipotropic agent for almost 10 years before Broun and Meuther (1942) tried a small supplement of choline (1.0 gm. per day) in conjunction with high protein, low fat diet in the treatment of 4 cases of cirrhosis. They were favourably impressed but of course failed to prove whether or not choline contributed to the results. Yater (1943) in discussion of a paper, expressed disappointment with the effect of choline in 15 cases. On the other hand, Russakoff and Blumberg (1944) cite 3 cases of alcoholic cirrhosis which resisted therapy until choline was added in doses of 2 - 6 gm. per day. Others attributing beneficial effects to the use of choline include Barker (1945), Wade (1945) and Beems (1946).

Steigmann (1948) studied the mortality figures of 247 cases of cirrhosis divided into 3 groups with respect to therapy. Those receiving lipotrope supplements including choline, methionine and cystine showed a greater survival rate than those receiving ordinary diets or those on a Patek regime. The treated series is relatively smaller than the others and is not broken down with respect to the agent used so results are difficult to interpret. Furthermore their findings are at variance with those of Wade et al (1948) who failed to influence the life span of patients with cirrhosis by supplementing their diets with 1 - 8 gms.

of choline and 0.9 gm. of methionine per day. Buck (1948) likewise found that lipotropes were of no added value in patients who could eat an adequate diet.

THE NEED FOR OBJECTIVE CRITERIA IN ASSESSING THE THERAPEUTIC REQUIREMENTS AND EFFECTIVENESS OF LIPOTROPES

Even from a non-critical survey of the therapeutic trials just cited it is evident that objective and quantitative criteria are required, (a) to assess the degree and nature of lipotrope deficiency (if such exists) in human liver disease, particularly cirrhosis; and (b) to judge the therapeutic requirements and effectiveness of lipotrope therapy in correcting this, as yet hypothetical, deficiency.

Heretofore all reported series have utilized lipotropes only as supplements to diets already high in lipotropes or their precursors, and therefore are of doubtful value as indicators of therapeutic effectiveness. Furthermore it is unlikely that this point can be established by clinical methods for some time since this would involve risking the chances for recovery of the control group. In such an experiment the series would require to be large and carefully selected and both control and treated groups would have to be maintained for long periods on basal diets suboptimum with respect to lipotropes. Only then could differences in the rate of progress of the two groups be attributed to the lipotrope therapy used in the treated cases.

It is evident then that information of practical value is more likely to accrue from the application of some direct laboratory test as an aid to establishing the indications for and the efficacy of lipotrope therapy. To this end a method was sought for the quantitative estimation of choline in biological fluids.

PART II

METHODS FOR THE ASSAY OF CHOLINE

METHODS FOR THE ASSAY OF CHOLINE

REVIEW OF CHEMICAL METHODS

Periodide -- As early as 1885 Gries and Harrow utilized the insolubility of the periodide salt to isolate choline. In 1896 the Florence test for semen was developed which depended on the finding of typical brown crystals. Within a year a number of German workers reported positive Florence tests on a variety of human secretions other than semen, and on hydrolyzed egg yolks and juices of plants including vines. Within 5 years it was firmly established that Florence crystals were choline periodide. Rosenheim (1905) combined the periodide reaction on the microscopic slide with chloroplatinate precipitation in alcohol as a characteristic test for choline, and Booth (1935) restudied the procedure, noting that the limiting dilution of choline was 1:50,000. Kahane and Levy (1939) attempted to modify the method for assay by noting the greatest dilution of an unknown solution which would yield characteristic crystals.

Tri-iodide -- Stanek (1905) devised an assay utilizing choline tri-iodide but it was later reported by Stanek and others that betaine, proteins, peptones, purines, alkaloids and various other substances were also precipitated. Modifications of the Stanek method were described by Sharpe (1923), Roman (1930) and Maxim (1931). Roman's method was the first microchemical method and was stated to be capable of assaying as little as 5.0 micrograms. Erickson, Avrin, Teague and Williams (1940) added certain improvements to the tri-iodide method but the physical conditions still require careful control and the method is not very specific.

Reineckate -- In 1929 Paal used ammonium reineckate to precipitate the choline freed from hydrolyzed lecithin and Kapfhammer and Bischoll (1930)

nd Beattie (1936) modified the method to a colorimetric procedure tilizing the pink color developed by dissolving the precipitate in cetone. However, other bases besides choline are precipitated and alues are generally too high. Certain modifications including control of the physical conditions as developed by Strack and Schwaneberg (1936), increased the specificity. Shaw (1938) combined reineckate precipitation with iodemetric titration and Jacobi, Bauman and Meek (1941) adapted the method for photoelectric colour measurement.

Engel (1942) added further improvements using methanal for more complete extraction of lipidsand altering the technique so that alkaline hydrolysis of phospholipids was more complete.

Possibly the most sensitive and specific of the reineckate procedures is that of Marenzi and Cardini (1943) which is based on the colorimetric determination of the chromium content of the reineckate (Cazeneuve, 1900) by which as little as 15 micrograms can be determined with accuracy. However, even this degree of sensitivity is rather inadequate for a study of biological fluids.

Oxidation -- Lintzell and Fomin (1931) utilized the oxidation of choline to trimethylamine for devising an assay based on the titration of the volatile bases which distill from the reaction mixture. The method was modified later and conditions devised which increased its specificity but it is still not applicable in the presence of other quaternary ammonium bases.

In summary, the chemical micromethods are suitable for estimation of relatively large amounts of choline in choline-rich material such as phospholipids from which other quaternary ammonium bases are absent. They do not possess satisfactory sensitivity or specificity for the assay of

the small 'free choline' fraction in body fluids. The situation was appraised by Lucas and Best (1943) as follows: 'Failure to discover a precipitant or chromogenic compound with high specificity for choline leaves the chemical estimation a problem for the future.'

REVIEW OF BIOLOGICAL METHODS

Acetylcholine Bioassay -- First described by Hunt and Taveau in 1906 this method exploites the powerful physiological activity of acetylated choline which under certain conditions is from 1,000 to 100,000 times as powerful as choline. The method was developed in detail by Hunt (1914; 1915) using the isolated frog heart for bioassay; by Guggenheim and Loffler (1916) and Fuhner (1916) using the isolated guinea pig's intestine. The latter author noted in 1913 that eserine potentiated the effect of acetylcholine on the dorsal muscle of the leech. Minx (1932) and Chang and Gaddum (1933) in further studies of the eserinized leech muscle method found it to be both specific and sensitive. The initial problem is to separate the choline fraction which is then acetylated by the method of Abderhalden and Paffrath (1925) as used by Fletcher, Best and Solandt (1935) or by the method of Abdon and Ljungdahl:Osterg (1944) and then assayed by its inhibitory effect on the contraction of the dorsal muscle of the leech. This method has not been more widely applied because as Best (1943) remarks 'it possesses many dangerous pitfalls for the chemist and is only reliable in the hands of a competent physiologist. Alcohol, calcium, potassium, histamine, adenosine compounds, traces of lecithin, protein, cholesterol, etc. may cause profound disturbances in the assay.'

Rat Bioassay -- Engel (1942) described a crude bioassay using the protective effect of choline against the development of hemorrhagic kidneys in young rats on deficient diets.

Neurospora Microbiological Assay -- In view of the deficiencies of chemical methods and the difficulties of the acetylcholine method, there was considerable interest in the description by Horowitz and Beadle (1943) of a Neurospora microbiological method which claimed both sensitivity and specificity. Before describing the method of Horowitz and Beadle or its modification for blood and urine (Luecke and Pearson, 1944), it is perhaps desirable to outline the scope and nature of the role of Neurospora mutants in biochemistry. This is particularly true because of the newness of the subject and the increasing applications of these organisms to the study of biochemical problems.

APPLICATIONS OF NEUROSPORA TO BIOCHEMISTRY

Neurospora is the name given to a group of pink-colored, heat-resistant bread molds which have long been familiar pests around bakeries, particularly before the use of mold inhibitors. The first scientific study of Neurospora was made in the late nineteenth century by a Dutch botanist, Went, who obtained cultures from peanut meal and carried out studies of the growth requirements. On one occasion he noted remarkable growth stimulus from the use of a new batch of sugar in the medium. This was almost certainly the result of contamination with biotin, the only vitamin-like substance required by the organism. Although puzzled by this effect, Went failed to recognize it for what it was and vitamins went undiscovered for another generation.

In 1927 Dodge named the genus Neurospora (formerly Sitophila), and described three species -- sitophila, crassa and tetrasperma. He pointed out the possibilities inherent in these organisms for genetic studies and the contribution which they made to science during the next decade was in this direction.

LIFE CYCLE OF NEUROSPORA CRASSA

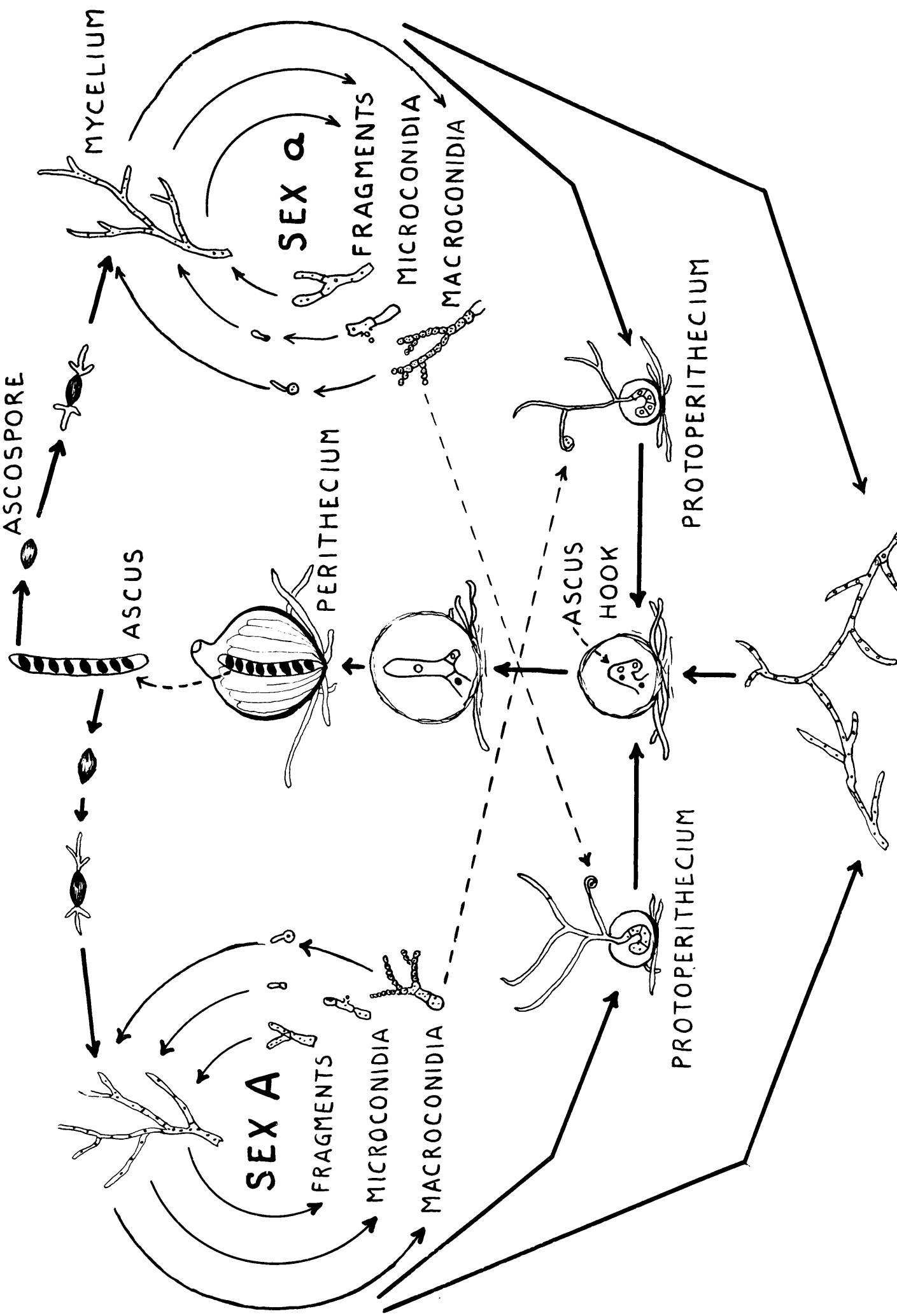


FIGURE I

(See descriptive text on the following page)

The Life Cycle of Neurospora (See Fig. I, page 67) -- The mycelium consists of cylindrical vegetative hyphae with septae at intervals and about 20 nuclei in each segment. Growth occurs by branching and by elongation at the tips of the hyphae. After a few days of growth, chains of multinucleated asexual spores called macroconidia are cut off at the tips of some aerial hyphae and in addition, some uninucleated microconidia are produced. Vegetative reproduction may occur by microconidia, macroconidia or fragments of mycelium. Neurospora are heterothallic, i.e. strains of two sexes exist which are morphological identical but physiologically distinct. Fusion of hyphae of sex A and sex a results in the formation of a sexual fruiting body or perithecium in which meiotic division gives rise to 8 nuclei with 7 chromosomes. These develop into ascospores which may germinate and grow into vegetative hyphae. Alternatively, perithecia may arise from protoperithecia (developed under certain conditions from macroconidia) following fertilization by conidia or mycelial fragments of the opposite sex.

The applications of Neurospora to science have been along three general lines: genetic studies; cellular metabolism studies; and microbiological assay procedures. Although yeasts and bacteria have been utilized in microbiological assays for some time, molds as a group were not applied for this purpose until certain biochemically deficient mutants of Neurospora were developed about 10 years ago.

Biological Characteristics of Neurospora -- Neurospora is uniquely suited for such work for several reasons: Its life cycle is such as to facilitate genetic control and analysis. It is easily grown. Wild type strains require only a simple medium containing a carbon and energy source such as glucose, a nitrogen source and several organic salts. It is so biochemically versatile that it can synthesize all of its vitamin-like requirements

except biotin. It is not fastidious as regards cultural conditions and can tolerate wide limits of temperature, pH and oxygen supply. It lends itself to the development of mutant strains in response to various forms of ionizing radiation or chemical insult.

Mutants of Neurospora -- It was first noted about 10 years ago that some of these artificial mutants had lost the ability to synthesize one or other growth substance so that they could no longer live on minimal media unless this substance were supplied.

Thus began the mass treatment and testing of Neurospora strains in an attempt to discover further mutants with defects as regards synthesis of growth essentials. To date about 200,000 wild strains have been so treated and several dozen different mutants detected with altered nutritional requirements mostly with respect to vitamins of the B group, amino acids, purine and pyrimidine bases.

The general procedure for producing, detecting and studying artificial mutants was described by Beadle and Tatum in 1945. Dry conidia are exposed to ultraviolet, x-rays, neutron bombardment or nitrogen mustard and are then applied to protoperithecia of the opposite sex in culture tubes. When the fertilized perithecium matures, an ascospore is removed by micromanipulation and isolated by washing with a hypochlorite solution which destroys all other fungal elements. The single ascospore is heat activated and germinated on an adequate medium to produce a genetically homogeneous strain. Transfers are then made to several minimal media where growth depends on the ability to carry out normal synthesis. Any mutant which is unable to grow on these media is studied further by adding various growth substances in turn until the defect in biosynthesis is detected.

The concept has been developed (Tatum, 1946) that the biochemical

reactions in a given synthesis involve the cooperation of corresponding enzymes and genes. Production of each enzyme is controlled by a specific gene which is thereby in dynamic control of the biochemical reaction. In all of the mutants studied it has been found that a single defect in synthesis is associated with mutation of a single gene.

Neurospora in Cell Metabolism Studies -- The mutation of a gene controlling one or other enzymatically-catalyzed reaction within the cell results in the stopping of the metabolic process at this stage. As a result there may be an accumulation of an intermediate compound which ordinarily is so transient that it cannot be recognized. A naturally-occurring example of such a genetic block is that seen in alkaptonurics in which the breakdown of phenylalanine and tyrosine is blocked at the 2,-5 dihydroxy-phenyl acetic acid level. Similar genetically-controlled metabolic defects exist in congenital porphyrinuria, cystinuria, tyrosinuria, levulosuria, pentosuria and phenylpyruvic oligophrenia.

Neurospora mutants have aided in indicating the synthesis of methionine. Four single-gene methionineless mutants produced by radiation have been studied. The synthesis happens to be blocked at a different stage in each, hence

Methionineless-1 can utilize methionine only,
Methionineless-2 can utilize methionine or homocystine,
Methionineless-3 can utilize methionine or homocystine or cystathionine,
Methionineless-4 can utilize methionine, homocystine, cystathionine or cystine.

These findings published by Horowitz in 1947 suggest that cystine is converted into methionine by way of cystathionine and homocystine, each step being under genetic control.

This example of the use of Neurospora in cellular metabolism studies is one of many which could be cited. In theory, it should ultimately be

possible to produce mutants with blocks at every stage of every synthesis carried out by the organism. In this regard it is fortunate that wild strain Neurospora is remarkably versatile in a biochemical sense, being capable of a large number of syntheses which have counterparts in mammals. The further study of Neurospora mutants in this manner can therefore be confidently expected to provide a great deal more information regarding cellular metabolism.

Neurospora in Microbiological Assays -- Neurospora mutants are now available for the assay of all of the B vitamins except folic acid and B 12, as well as for the amino acids arginine, tryptophane, lysine, leucine, isoleucine, valine and proline. The growth response may be gauged by any of the following methods:

1. The rate of progression of the mycelial frontier in a horizontal tube of solid medium containing the material to be assayed. (Ryan, Beadle and Tatum, 1943).
2. The diameter of a colony on solid medium in a Petri dish as an index of the vitamin content of the medium. (Bacharach, 1947).
3. Microscopic observation of the rate of germination of conidia after the addition of vitamin-containing extract to the medium. (Ryan, 1946).
4. Dry weight of mycelium harvested from liquid medium after growth has ceased, i.e. the extent of growth relative to the added vitamin.
5. Dry weight of mycelium harvested from liquid medium after a given incubation period, i.e. the rate of growth relative to the added vitamin.

The last-mentioned method was the one employed by Horowitz and Beadle (1943) for the assay of choline in tissue extracts by the use of a 'choline-less' strain of Neurospora #34486.

Briefly stated, the principle of the assay is as follows: When 'cholineless' Neurospora is grown under standard conditions in a medium optimum with respect to all requirements except choline, the growth response is related to the quantity of choline present. Thus by simultaneously determining the growth response to known quantities of choline one may calculate the choline content of the 'unknown'.

TECHNIQUES OF NEUROSPORA MICROBIOLOGICAL ASSAY OF CHOLINE

The Method of Horowitz and Beadle is briefly as follows:

The assay is carried out in a series of 250 ml. Erlenmeyer flasks each containing 25 ml. of choline-free culture medium. To this is added appropriate amounts of the extracts to be assayed and the flasks are plugged and sterilized by heat. After cooling they are inoculated with a 'cholineless' strain of Neurospora and incubated at 25°C. for three days. The amount of mycelium which grows is in proportion to the amount of choline available for its nutrition. The mycelium forms a soft cohesive mat the size of which is measured by its dry weight. The quantity of choline in the 'unknown' flasks is calculated from reference to a standard curve prepared simultaneously by adding known amounts of choline to a series of flasks and plotting the mycelial yields obtained against the quantities of choline added.

Unfortunately Horowitz and Beadle did not describe their technique in sufficient detail to permit another worker to reproduce it with any exactitude. In the course of several months experience with the method a number of difficulties were encountered and several modifications were developed to improve the accuracy of the technique and to facilitate the manipulations.

Modifications of the Method of Horowitz and Beadle which have been evolved will be described in a step-by-step outline of the procedure and will be followed by a presentation of experimental results pertinent to the more important modifications.

(a) Preparation of Culture Media

The Choline-free 'Basal Medium' used by Horowitz and Beadle contained Fries #3 salt mixture (Fries, 1938) in three times the concentration finally required for the 'Assay Medium'. In this concentration

the salts tended to precipitate during storage in the cold but this difficulty was not encountered with double strength medium of the following composition in gms. per litre: Ammonium tartrate 10.0; ammonium nitrate 2.0; monobasic potassium phosphate 2.0; magnesium sulphate.heptahydrate 1.0; sodium chloride 0.2; calcium chloride 0.2; the following trace elements (in mg. per litre); boron 0.02; copper 0.20; iron 0.40; manganese 0.04; molybdenum 0.04; zinc 0.04. It was found that mixtures of salts (v.i.) containing these trace elements could not be stored in solution (due to precipitation on standing), or as the dried residue of an aqueous solution (due to relative insolubility) or as a mixture of dried powdered salts (because on intimate mixing, water of crystallization was released and the mixture became a paste). Since time did not permit investigation of numerous combinations of various salts of these elements in an effort to obtain an ideal mixture, the expedient was adopted of preparing individual solutions of the following salts and adding 0.25 ml. of each solution to each litre of double-strength 'basal medium' with the expectation that at this high dilution insoluble compounds would not be formed. The great importance of the trace elements to the growth of Neurospora is demonstrated in Fig. III, page 85.

The Trace Elements Solutions are respectively of the following concentrations expressed as mg. per 100 ml.: sodium borate 35; ammonium molybdate 15; ferric chloride.sexihydrate 365; zinc sulphate.heptahydrate 345; cupric chloride 80; manganese chloride.quadrihydrate 30.

The basal medium was routinely prepared in 6 litre batches, divided into 1,000 ml. Erlenmeyer flasks, plugged with cotton, sterilized by autoclaving, labelled, dated and stored in the refrigerator.

The Assay Medium which is used to charge the culture flasks has the following composition per 100 ml.: Double-strength 'basal medium', 50 ml.; sucrose, 2.0 gm.; biotin 0.5 micrograms (stored at 4 C as a solution containing 5.0 micrograms per ml.); and distilled water up to 100 ml. This medium is prepared freshly each day, the requirements being calculated on the basis of 25 ml. for each flask of the assay.

The 'Stock Medium', a solid medium used to prepare slopes for the maintenance of the stock cultures of Neurospora, has the following formula: double-strength basal medium 50 ml.; glycerol (sp.gr. 1.25) 1.6 ml.; solution of hydrolyzed casein ('Larenamine', Stearns) 0.42 ml.; choline chloride 0.10 mg. (20.0 ml. of working standard solution); yeast extract Difco 0.5 gm.; malt extract Difco 0.5 gm.; agar 1.5 gm.; distilled water to 100 ml. After the agar is melted into solution in a boiling water bath, the medium is added to 13 x 100 mm. test tubes, the amount being adjusted so that there will be a short butt to the slopes. The tubes are plugged with cotton, sterilized by autoclaving, sloped, cooled and stored in the refrigerator until required.

(b) Stock Cultures of Neurospora

Of the four 'cholineless' mutants of Neurospora crassa available, #34486 is the most restricted as to its requirements for choline. (Horowitz, Bonner and Houlahan, 1945). A culture of this strain was kindly forwarded by air mail through the kindness of Dr. N.H. Horowitz of Stanford University in June, 1948 and has been maintained as a pure culture since that time.

The stock cultures are incubated at 25 C. and subcultured to a fresh slope of 'stock medium' every 7 days employing the usual aseptic precautions. The 7-day period is convenient since it means that a subculture

is made on each day of the week that assays are set up. In this way the inoculating suspension is always prepared from a 7-day-old culture thereby minimizing variations in growth rate related to the age of the culture. (At intervals reserve stock cultures are grown and stored at 4°C. against the contingency that current cultures might become contaminated.) Checks on the purity of the stock culture are afforded by observation of gross morphology on both solid and liquid media, microscopic examination of morphology, and by the finding that only very little growth is obtained in the absence of choline, as in the 'blank' assay flask.

(c) Preparation of Mycelial Suspension

After the subculture is prepared 5 ml. of sterile water is added to the donor tube and the remaining mycelium is scraped from the slope and emulsified with the aid of the wire to provide the turbid suspension of mycelial fragments and conidia which is used to inoculate the culture flasks.

(d) Culture Flasks

Limitation of incubator space enjoined the use of 125 ml. Erlenmeyer flasks rather than the recommended 250 ml. size. The mycelial yield is about 40% less in the smaller flasks due to the smaller surface area of medium but it is adequate for easy harvesting and accurate weighing.

It was repeatedly noted that flasks in which the cotton plug became moistened during sterilization gave unexpectedly low yields presumably due to interference with exchange of respiratory gases. The use of inverted 50 ml. beakers as caps to replace the plugs avoids this source of error and effectively guards against air-borne contaminants.

(e) The Incubator

The most commonly used temperature for incubation of Neurospora is 25°C. although a range up to 30°C. is fairly satisfactory. The incubator must incorporate facilities for both cooling below and warming above room temperature, since prevalent summer-time laboratory temperatures are often above 25°C.

A water-jacketed incubator can be cooled by running tap water through the jacket and the finer adjustments of temperature controlled by a thermostatically-regulated electric heating element. A small air-circulating fan within the incubator is desirable to aid in equalizing the temperature. The motor should be mounted outside the cabinet to exclude the heat generated and it should be borne in mind that vibration is said to effect the rate of growth of molds.

The importance of equalizing temperatures throughout the incubator was indicated by Assay VI in which it was found that increasing the incubating temperature from 28.8°C. to 31.0°C. decreased the yield of mycelium in comparable flasks as much as 20%. Temperature differentials between flasks of the same assay can be minimized by placing all flasks of a single assay on the same shelf. Even then radiant heating effects from the heating element are not affected by air currents and impinge most strongly on the nearby flasks. As a result of these factors it is difficult to obtain optimum conditions except in a refrigerating incubator of the 'constant temperature' type such as that employed for 'B.O.D.' (biochemical oxygen demand) determinations.

(f) Preparation of Standard Solutions of Choline

The following technique was evolved (see page 86) in order to ensure constancy of stock standard solutions from month to month: From 25 to 30 mg. of pure choline chlorid[illegible]

in a vacuumed desiccator until the weight is constant. The amount of choline is calculated (choline = 86.78% choline chloride) and dissolved in the volume of absolute ethanol required to produce a solution containing 250 micrograms of choline per millilitre. Exactly 1.0 ml. of this solution is placed in each of a dozen or more 50 ml. volumetric flasks which are stoppered and stored in the refrigerator.

Working Standard Solution containing 5 micrograms per ml. of choline as choline chloride is prepared at the beginning of each week by adding about 30 ml. of distilled water to one of the 50 ml. volumetric flasks containing 250 micrograms of choline, sterilizing by autoclaving, and when cool making up to volume with sterile distilled water. The solution is stored in the refrigerator and aliquots for use are removed aseptically by pouring from the flamed mouth of the flask into a separate container thus avoiding contamination with moulds or other choline-consuming organisms.

The accuracy of the weighing and preparation of solutions is checked by determinations of total nitrogen (11.56% of choline) and chloride (25.4% of choline chloride). The only check possible on the actual choline activity of a solution is by an acetylcholine or sensitive chemical assay although a crude check is afforded by noting in each assay the growth response to a certain known amount of choline under the standard conditions of the test. eg. using the technique to be described, 5 micrograms of choline per flask yields about 25 mg. of dried mycelium. A substantially smaller yield suggests decay of the solution and a larger yield suggests contamination with a non-cholineless organism.

(g) The 'Standard Series'

The amount of choline which gave rise to the found amount of mycelium in any given

plotted from 4 or 5 points determined in duplicate, or preferably in triplicate. Due to the effect of a number of 'build-in' variable factors which affect the growth of Neurospora it is necessary (see Fig. X, page 94) to prepare a new standard curve for each assay on the assumption that these physical variables will impinge equally on the standard and test flasks.

For the standard series five 125 ml. Erlenmeyer flasks are set up (in duplicate or triplicate) containing serially, 0.25, 0.5, 1.0, 1.5 and 3.0 ml. of working standard solution representing 1.25, 2.5, 5.0, 7.5 and 15.0 micrograms of choline respectively.

(h) The 'Test Series'

Appropriate extracts of the material to be assayed are similarly added to labelled culture flasks in duplicate or triplicate, usually in aliquots of 1.0 and/or 2.0 ml., the extract being so adjusted in concentration that these amounts contain between 2.0 and 10.0 micrograms in order that the resultant mycelial weights may be applied to the most suitable portion of the standard curve. Published methods are available for the preparation of extracts from urine, plasma, milk and milk products, solid foodstuffs and animal tissues. Preparation of extracts from human serum for free and total choline estimation will be described later.

(i) Addition of the Medium

Next, to each flask of both series is added 25 ml. of assay medium which is conveniently dispensed from a 100 ml. burette with a two-way stopcock to facilitate refilling from an overhead reservoir. The tip of the burette may be cut back to permit more rapid delivery.

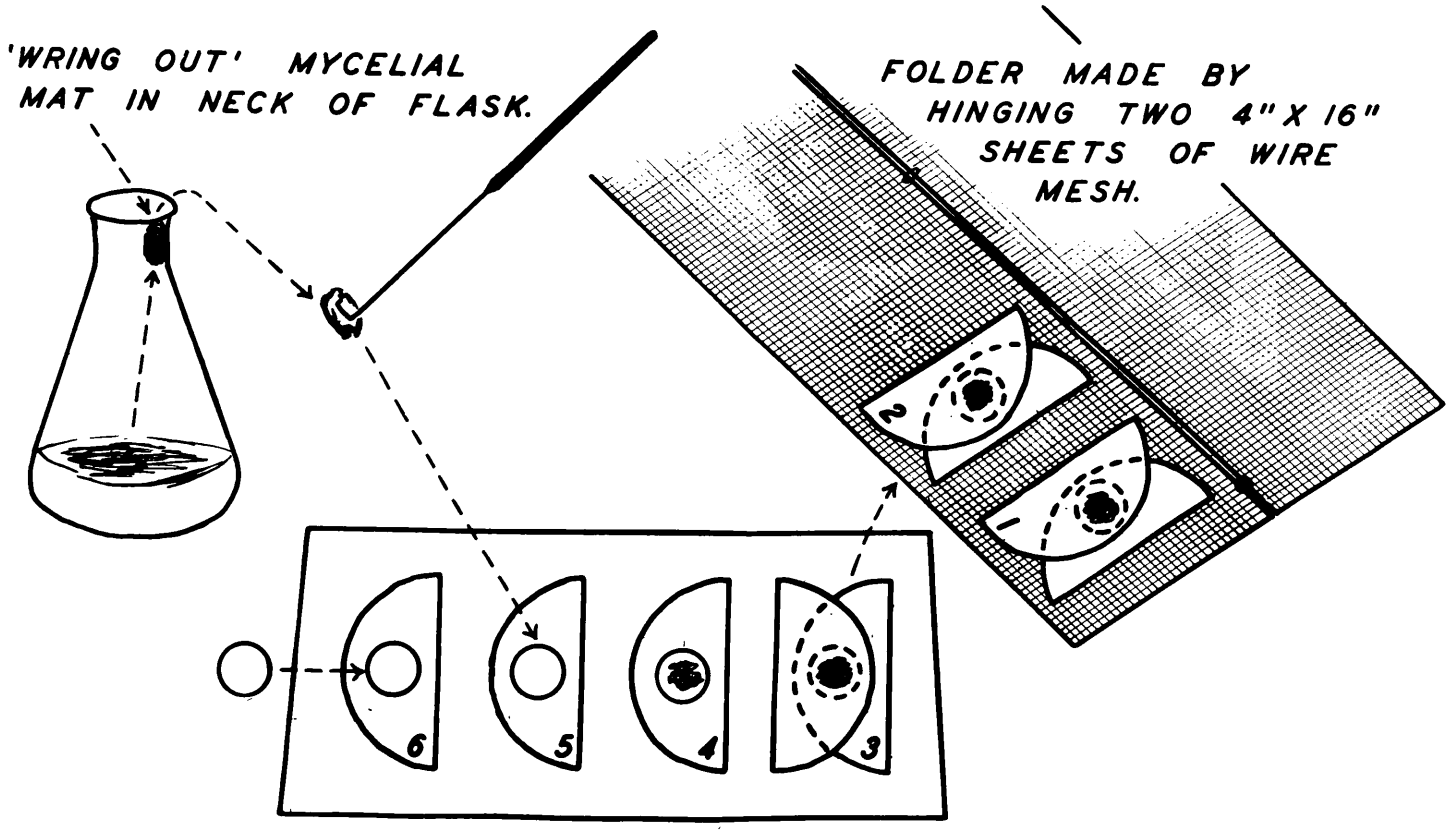
Of a number of techniques which were tried for this purpose the following proved most convenient and effective: flasks to be harvested are arranged in numerical order and a series of semicircles of filter paper (Whatman, #2, 9 cms.) are laid out in corresponding order and numbered in one corner. On the centre of each is placed a 1-inch disc of fine lens paper. (These discs, punched from books of lens paper by a local printing firm, were of such uniformity that weights of random samples agreed within 0.1 mg.). Using an L-shaped metal hook the mycelia are lifted from the flasks and excess liquid wrung out by pressing against the inside of the neck of the flask. The mycelial mats are then placed on the appropriate paper discs, covered with hemicircles of filter paper in such a way as to leave the identifying numbers visible, and as much liquid as possible is then pressed out by digital pressure. (Fig. II, page 82). A series of these 'sandwiches' are then arranged in a folder made by hinging together two 4-inch by 12-inch rectangles of coarse nylon mesh. The folder is closed by clipping the free edges together and placed in an automatic fill-and-drain pipette washer for two hours. By inverting the folder end-for-end at mid-time equal contact with the wash water is assured for all mycelial mats regardless of their position in the folder. At the end of the washing period the 'sandwiches' are turned out on towels, blotted, the upper layers of filter paper removed and discarded, and the lower numbered papers (bearing the mycelia on lens paper discs) are laid out in shallow cardboard boxes ready for drying.

(n) Drying the Mycelia

The mycelia are placed in an oven at 60°C. for two hours at the end of which they will be dry and should be of a uniform off-white color if

Illustrating a convenient method for harvesting the mycelia and preparing them for the washing process.

(At the step represented by (3) digital pressure is used to press as much of the fluid out of the mycelium as possible.)



washing has been adequate, since traces of sugar impart a brown color after heating. It is important to expose the dried mycelia to room atmosphere for at least an hour before weighing because there is a considerable uptake of moisture by the mycelia in the first 30 - 45 minutes following removal from the oven.

(o) Weighing the Dried Mycelia

Weighing is greatly facilitated by this technique since the mycelia adhere firmly to the lens paper discs and are easily handled with forceps. Their weights may be read off directly if another paper disc is placed on the opposite scale pan as a counterweight. Mycelia are weighed to the nearest 0.2 mg. and duplicate weights that agree within 10% are averaged.

(p) Calculation of Results

The choline content of the test flasks is obtained by applying the mycelial yields to the standard curve prepared simultaneously.

(q) Reliability of the Method

Specificity -- Neurospora bioassay of choline is not entirely specific since the following substances also have growth activity for the choline-less strain: arsenocholine, phosphorylcholine, dimethylaminoethanal, monomethylaminoethanol, dimethylethylhydroxyethyl ammonium chloride, tri-ethyl choline, acetylcholine, methionine and choline-containing phospholipids, principally lecithin. Of these only the last two occur in significant concentrations in body tissues and fluids. Of 22 amino acids and 11 water-soluble vitamins tested by Horowitz and Beadle only methionine had growth-promoting activity for Neurospora and it was only 0.002 times as active as choline. In any case, where methionine is suspected of being present in quantity it can be removed by means of

an adsorption procedure. (Horowitz and Beadle, 1943).

Handler (1946) states that lecithin stimulates only very slow growth of Neurospora, while Horowitz and Beadle report that about 50% of the potentially available choline of lecithin is utilized by Neurospora during a three-day incubation period. The latter is in accord with our observations and emphasises the importance of complete removal of phospholipids before attempting to assay the free choline fraction of biological materials.

Sensitivity -- By the technique described, as little as 1.5 micrograms of choline per flask is within the accurate range of the assay and since there is no objection to the addition of 5 ml. or more of extract, concentrations as low as 0.3 micrograms per ml. can be measured.

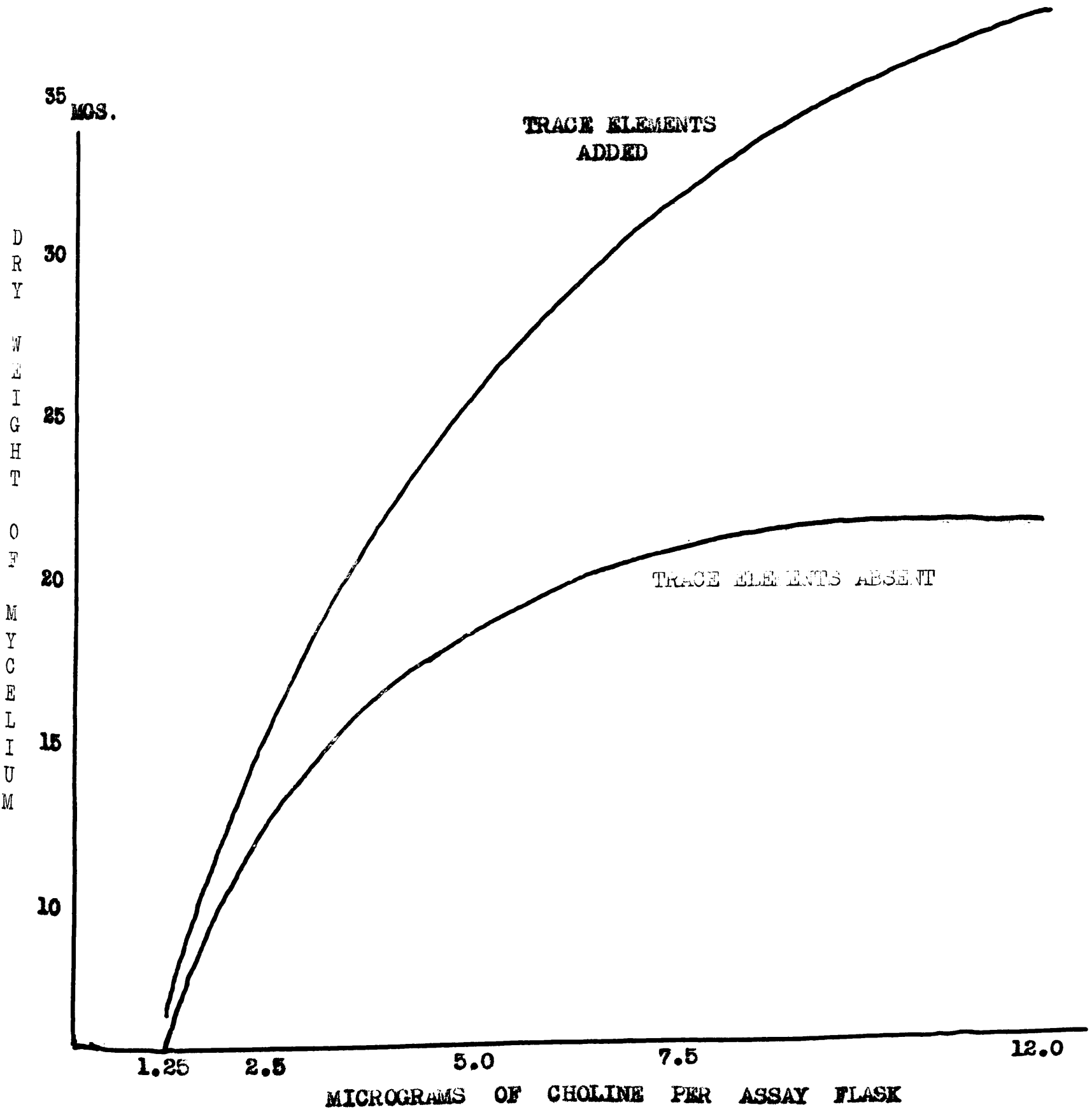
Reproducibility -- The high and low values of 60 consecutive duplicates were found to deviate from the mean by an average of 3.05%. However, as pointed out by Snell (1948) the non-random treatment of flasks results in 'excessively good agreement' which 'does not reflect the true variation encountered in the assay as a whole.' We have used deliberate random handling even to the point of incubating the two flasks of duplicate determinations in different parts of the incubator.

(r) Practicability of the method

Given the facilities described and appropriate extracts for assay it would be reasonable to expect a technician to carry out as many as 30 assays in duplicate every full working day. The time-consuming procedures are concerned with the preparation of the extracts of the material to be assayed.

EXPERIMENTAL RESULTS RELATING TO STANDARDIZATION OF VARIABLE FACTORS IN THE ASSAY PROCEDURE

(1) The Importance of Availability of Trace Elements can be appreciated from Figure III (below) which demonstrates the curtailment of growth resulting from absence of the trace elements from the medium.



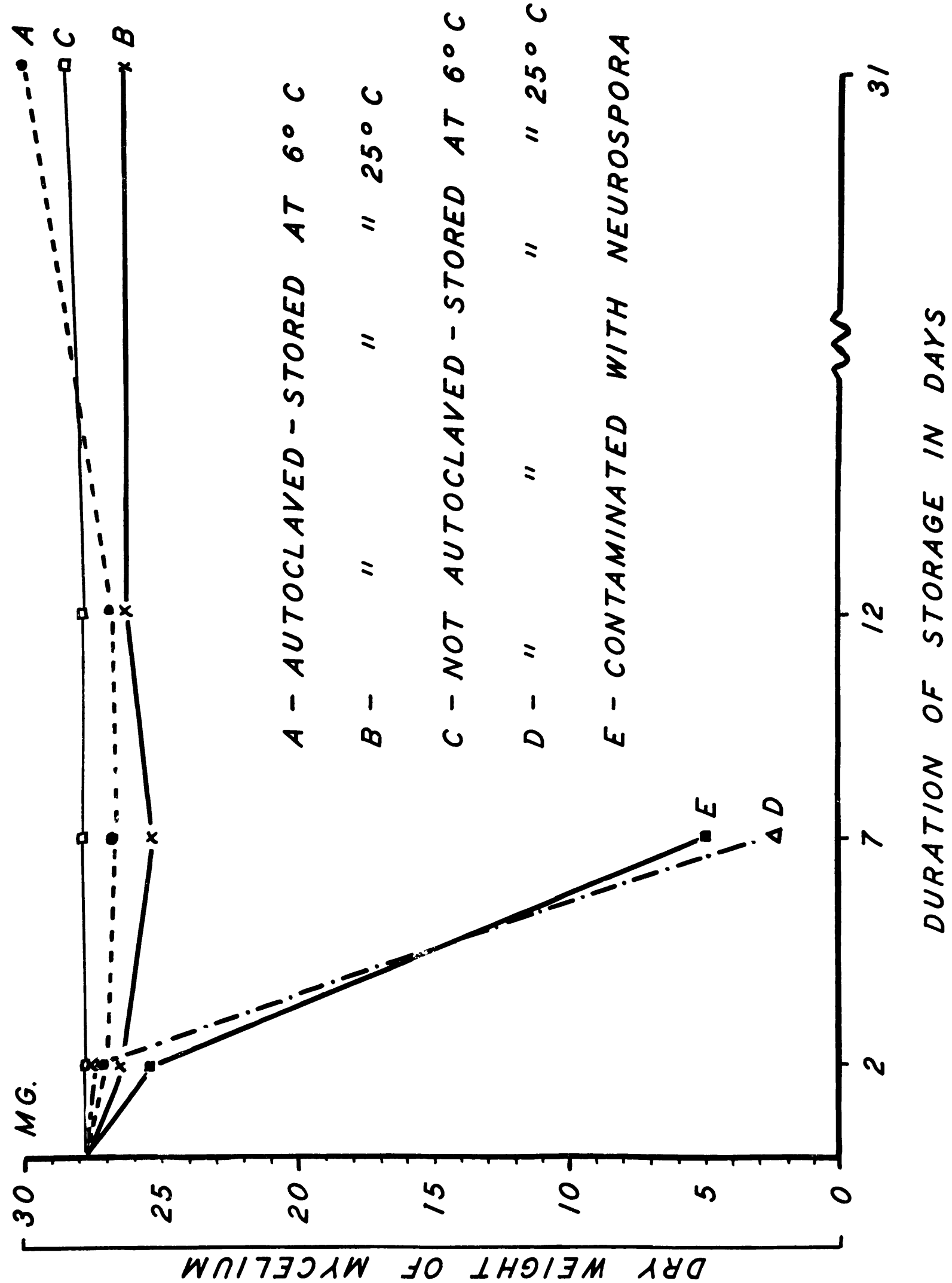

A – AUTOCLAVED – STORED AT 6° C
B – " " 25° C
C – NOT AUTOCLAVED – STORED AT 6° C
D – " " 25° C
E – CONTAMINATED WITH NEUROSPORA
DRY WEIGHT OF MYCELIUM
MG.
30
25
20
15
10
5
0
DURATION OF STORAGE IN DAYS
2
7
12
31
A
B
C
D
E

Figure IV

(4) <u>The Optimum Duration of Incubation</u> -- The incubation time suggested by previous authors for this mutant was 72 hours. In an attempt to determine the optimum period of incubation a series of standard curves were set up simultaneously and harvested after 2,3,4,5 and 6 days respectively. From Figure V on the following page it is evident that the 5 and 6 day curves are more nearly linear and therefore superior to those obtained with shorter incubation. The importance of utilizing only the steeply-rising linear portion of the curve has recently been emphasized because it avoids arithmetic exaggeration of technical errors, (Wood, 1947) and according to Hodson (1945) it minimizes the effects of interfering substances as well. Figure VI, page 90 illustrates this point by showing a 5-day standard curve projected by the use of up to 45 micrograms of choline per assay flask. From the profile of the curve it is evident that the measurements of choline above 10 micrograms per flask are less accurate due to the fact that the curve 'plateaus' in this range.

Figure VII, page 90 represents in a different manner the principle shown in Figure V, i.e., with longer incubation periods the growth potential is more nearly realized in those flasks in the middle and upper range of the standard series. Longer incubation thus has the effect of improving the standard curve by rendering it steeper and more nearly linear.

The disadvantages of the longer incubation is that more incubator space is required, results are obtained more slowly and if the extract contains phospholipids there is a possibility of more extensive 'spontaneous' hydrolysis and augmentation of growth due to the split-off choline. It was considered that these possibilities did not outweigh the advantages of the 5-day incubation period which was therefore adopted as standard procedure.

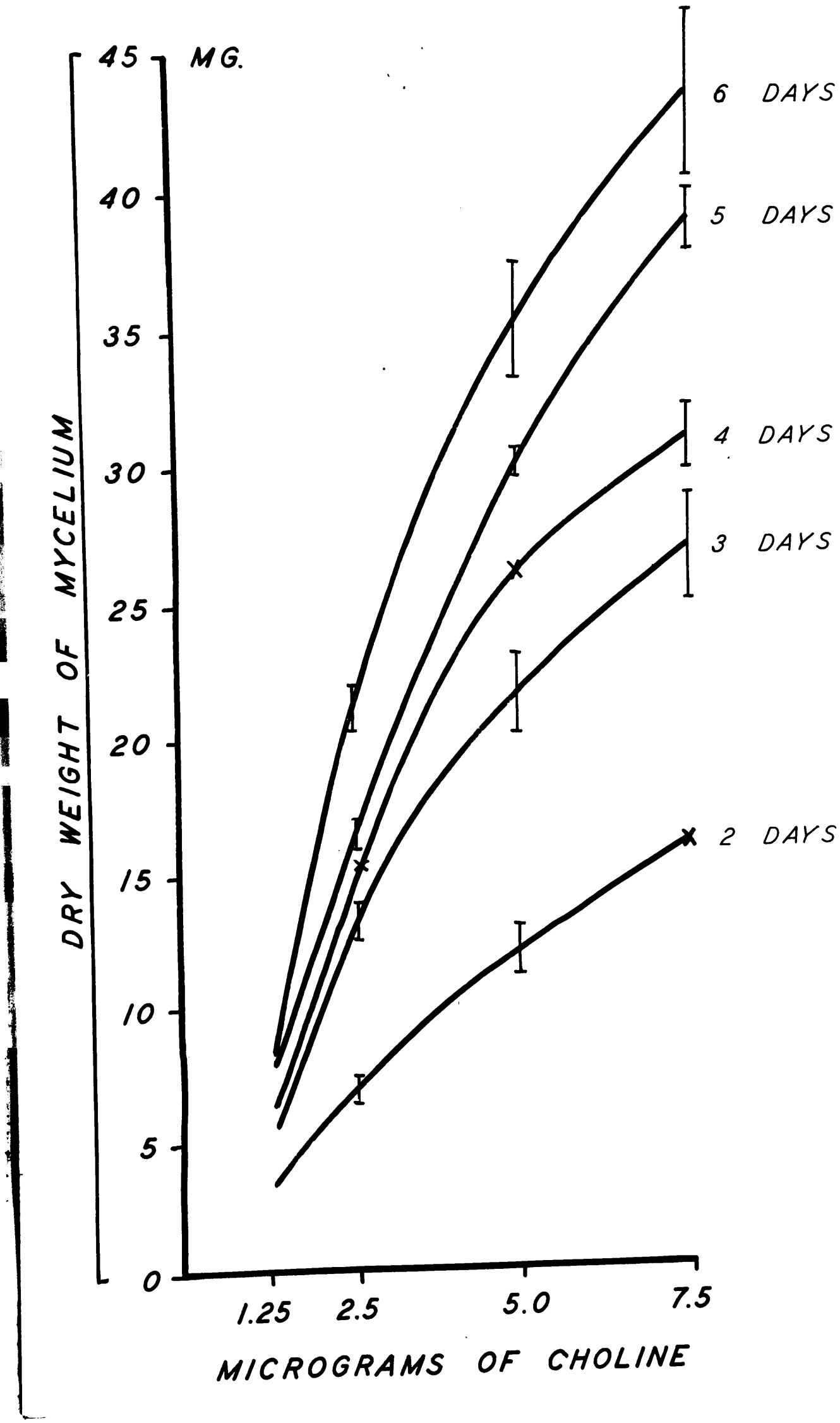

45
40
35
30
25
20
15
10
5
0
MG.
DRY WEIGHT OF MYCELIUM
6 DAYS
5 DAYS
4 DAYS
3 DAYS
2 DAYS
1.25
2.5
5.0
7.5
MICROGRAMS OF CHOLINE

Figure V

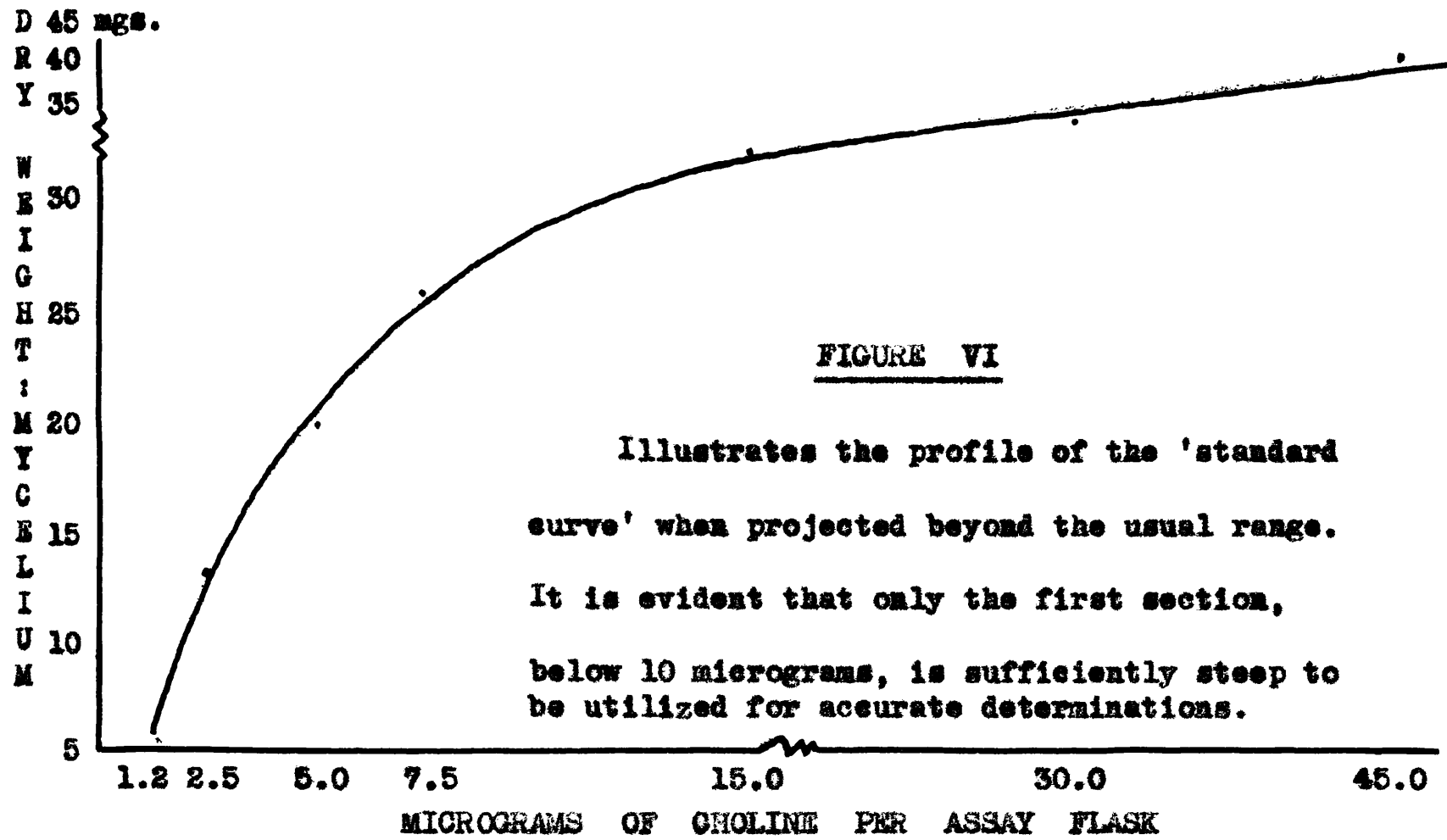


FIGURE VI

Illustrates the profile of the 'standard curve' when projected beyond the usual range. It is evident that only the first section, below 10 micrograms, is sufficiently steep to be utilized for accurate determinations.

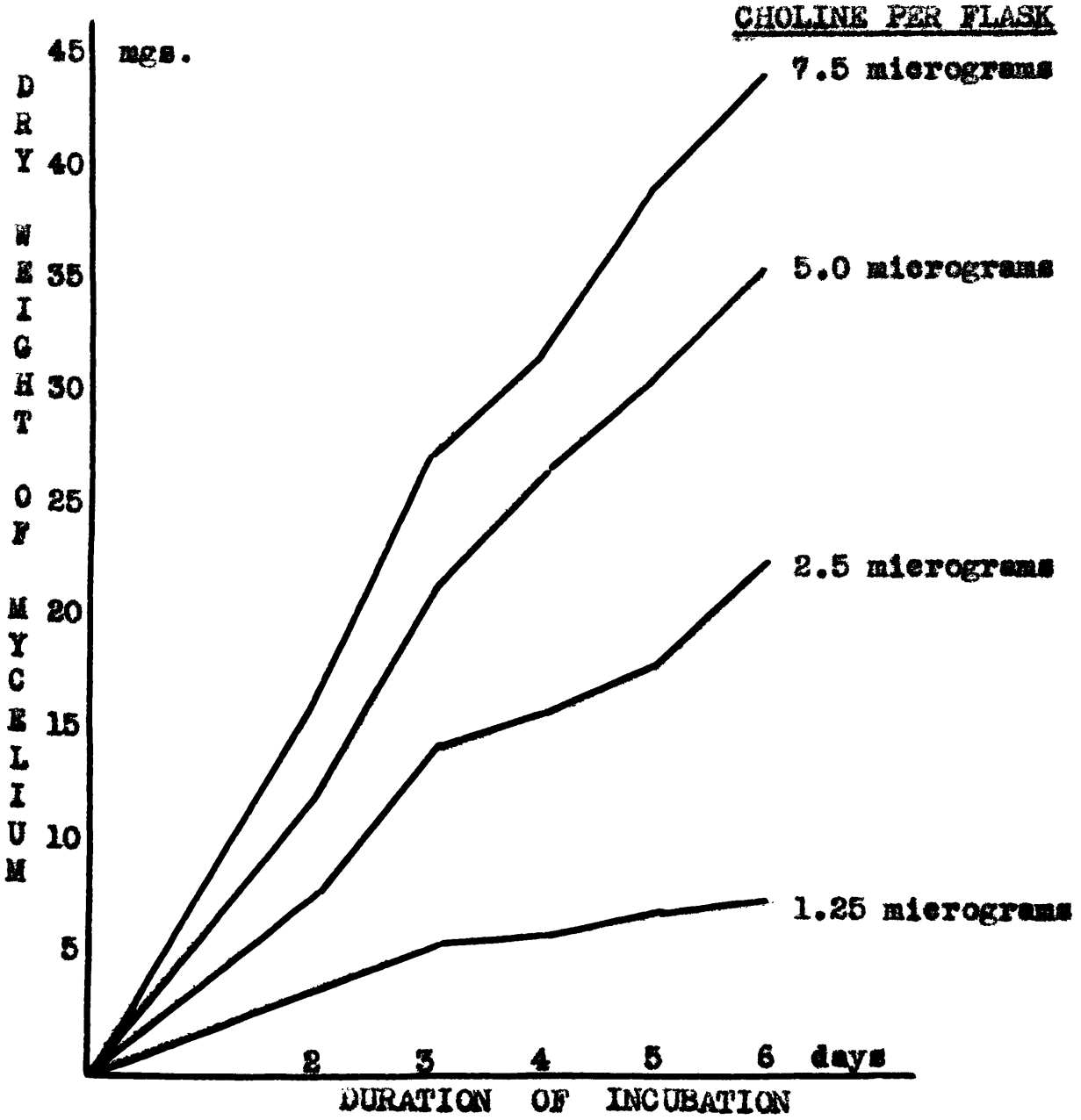


FIGURE VII

Illustrates the effect of the duration of incubation on the mycelial yield obtained with varying quantities of choline.

(5) <u>The Desirability of Washing the Mycelia</u> -- In connection with the gravimetric method of measuring the growth response, there has been insufficient emphasis placed on the desirability of washing the mycelium free of liquid medium which is held in the meshes of the mycelial mat and which because of its sucrose content, introduces a considerable gravimetric error. It is seen from Figure VIII, on the following page, that the presence of solids of the medium not only increases the height of the standard dose response curve but significantly alters its profile as well.

Neither Horowitz and Beadle (1943) nor Luecke and Pearson (1944) mention washing of the mycelia but Siegel (1945) suggested the use of fritted glass funnels in which the mycelia would be washed, dried and weighed. However, with large scale assays this method represents a considerable outlay for glassware, creates a cleaning problem and involves weighing relatively heavy containers in order to obtain, by difference, a result amounting to a few milligrams. The method outlined previously (page 81) has been found to be a simple and effective means of washing the mycelia.

The greater hygroscopicity of unwashed mats (Fig. IX) is a further argument for including the washing process. Figure IX also illustrates the weight gain shown by mycelia in the first 30 minutes after removal from the drying oven.

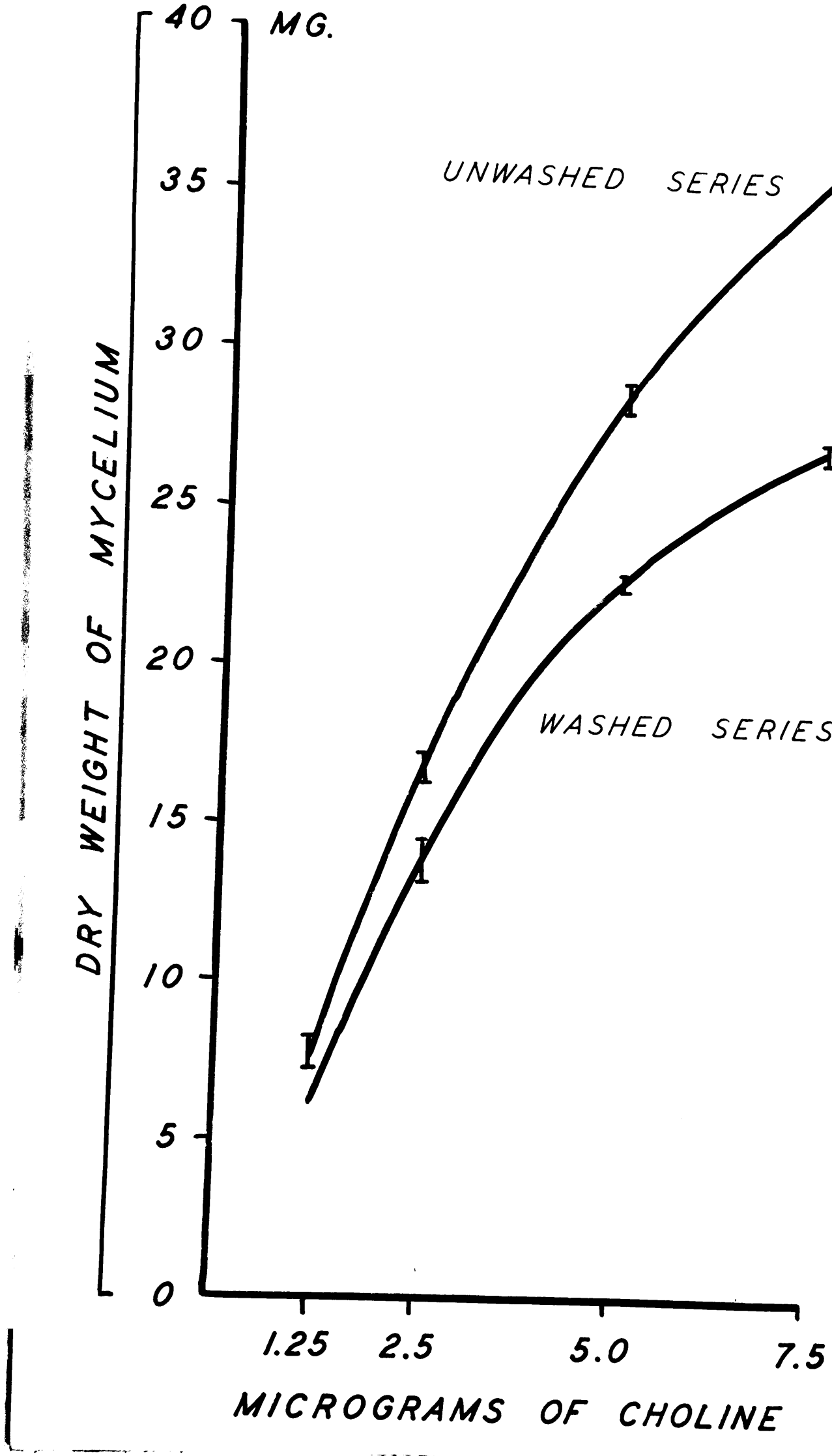

40 MG.
35
30
25
20
15
10
5
0
DRY WEIGHT OF MYCELIUM
UNWASHED SERIES
WASHED SERIES
1.25
2.5
5.0
7.5
MICROGRAMS OF CHOLINE

Figure VIII

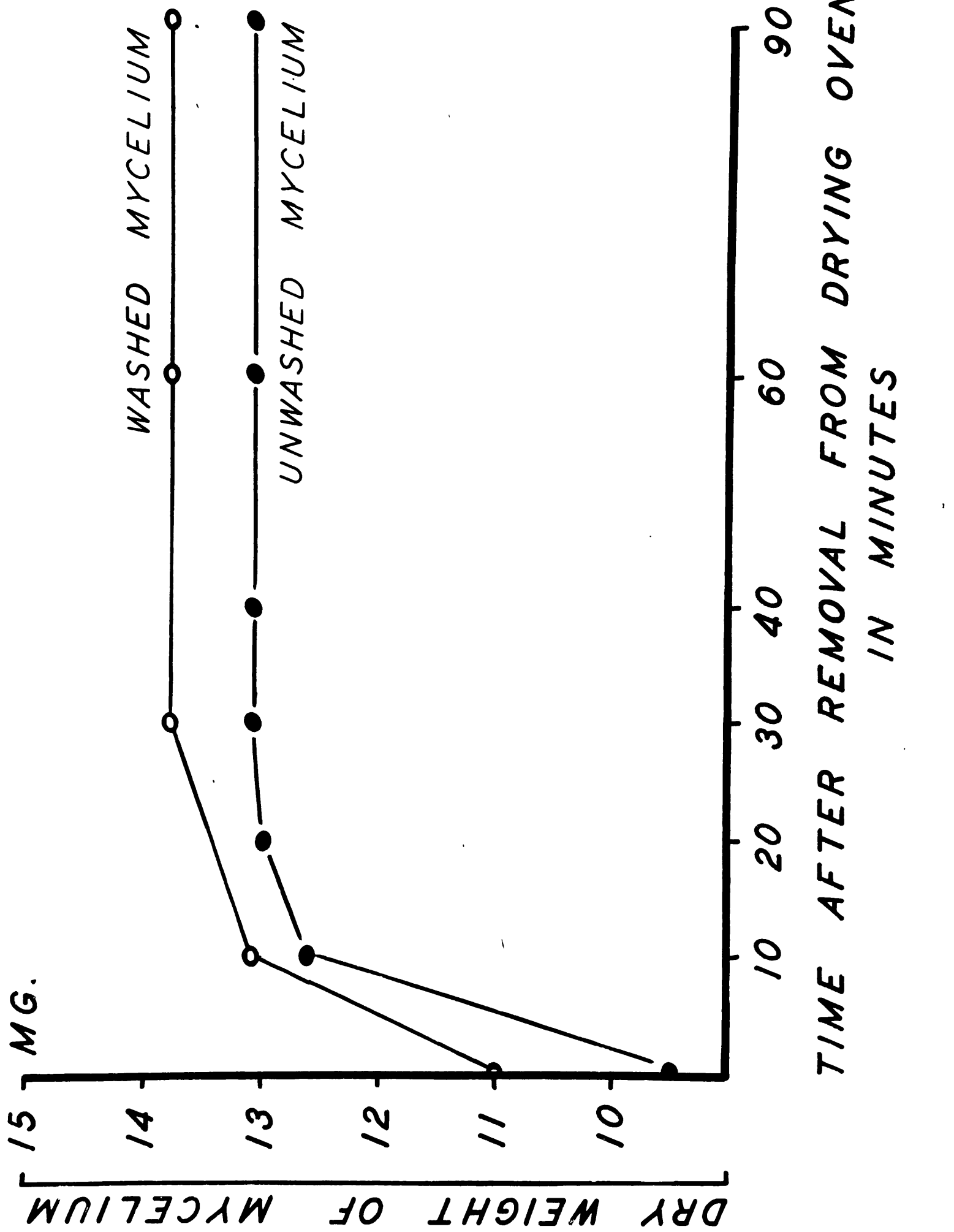

WASHED MYCELIUM
UNWASHED MYCELIUM
DRY WEIGHT OF MYCELIUM
MG.
15
14
13
12
11
10
10
20
30
40
60
90
TIME AFTER REMOVAL FROM DRYING OVEN
IN MINUTES

Figure IX

FIGURE X

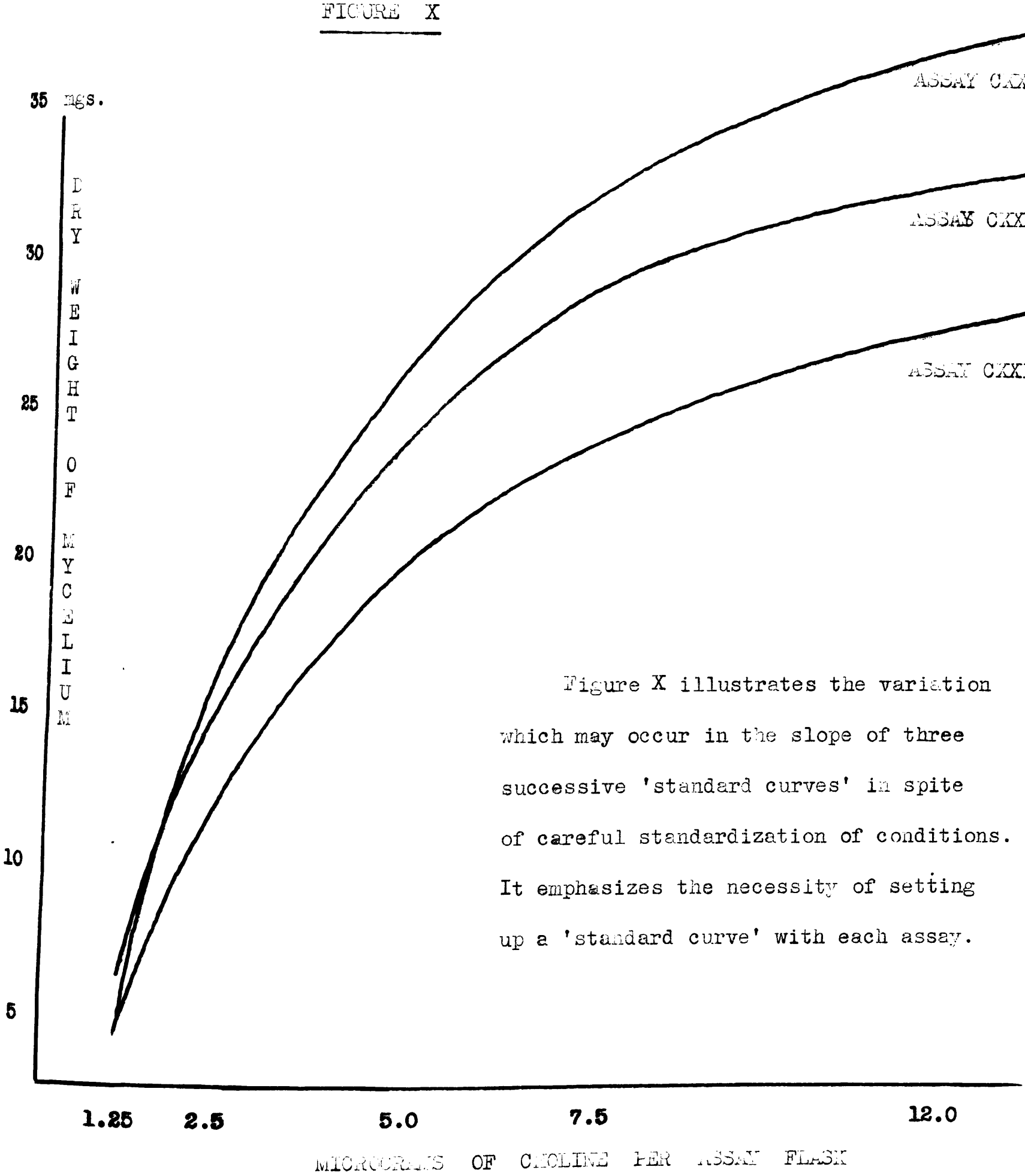


Figure X illustrates the variation which may occur in the slope of three successive 'standard curves' in spite of careful standardization of conditions. It emphasizes the necessity of setting up a 'standard curve' with each assay.

PREPARATION OF EXTRACTS OF BLOOD FOR ESTIMATION OF TOTAL AND 'FREE' CHOLINE

1. CHOICE OF BLOOD FRACTION FOR STUDY

From the tabulated data on blood choline values (page) it is noted that several different blood fractions have been utilized for choline assays by different workers, viz. whole blood, defibrinated blood, serum, citrated, oxalated and heparinized plasma. At the beginning of the experiments serum was arbitrarily chosen as being convenient and easy to handle. One possible disadvantage in the use of serum is that it requires about an hour for adequate clot retraction, centrifugation and separation before the extraction process can begin. The question arose as to the possibility of enzymatic hydrolysis of lecithin during this period so the use of whole blood, oxalated and heparinized plasma was explored since these forms permit prompt initiation of the extraction procedure. But certain theoretical and practical disadvantages exist for these media also. We noted that whole blood forms a coarse, gummy curd in alcohol and Boyd (1936) showed that lipid extraction of whole blood was difficult; heparin, being a tissue extract, may contain lecithinase although we found that it did not affect the growth rate of Neurospora per se; oxalate, being a precipitant of calcium, might remove this necessary trace element from solution in the assay flasks.

However some comparative assays were carried out the the following results: (Values represent 'free' choline, micrograms per millilitre of serum).

Number of Assay	Time Relation to meal	Whole Blood (extracted at once)	Serum (extracted after 1 hr)	Oxal. Plasma (extracted at once)	Heparinized Plasma (at once)	Heparinized Cells (56 min)
XLVII	fasting	1.0	2.5			
LIV	post-prandial		15.2	7.5	17.0	17.6
LIX	" "				16.5	2.6
XIII	" "		43.0	30.0		
XIII	" "		256.0	216.0 (total choline)		

The smaller values with oxalated plasma may be due to a toxic effect on the test organisms, or to removal of ionized calcium, but it is more likely that there is another explanation since the same effect is noted in chemical methods. Schmidt (1935) found phospholipid content of oxalated plasma to be 13% lower than that of heparinized plasma and Boyd (1935) reported lower values in oxalated plasma than in defibrinated blood, a discrepancy attributed to shrinkage of the erythrocytes by the oxalate with resultant dilution of the plasma. Others have suggested that oxalates combine with some of the phospholipids (MacLean and MacLean, 1927). Similar objections exist for the use of citrate since Kirk, Page and Van Slyke (1934) found values for phospholipids 13% higher in heparinized than in citrated plasma.

The erythrocyte choline is seen to be small in amount compared to the 'free choline' of plasma, (viz. 2.6:16.5). Luecke and Pearson (1945) found the proportions to be similar -- 5.0:36.0 and Hunt (1915) noted that the addition of a 10% volume of red cells to a sample of serum did not alter the choline content appreciably. Rubin (1939) concluded erythrocyte phospholipids were inert in relation to fat transport when he found no change in either pathological hyperlipemia or alimentary lipemia.

Permitting heparinized plasma to remain at ice-box temperature for an hour before beginning the extraction resulted in no significant increase in the 'free' choline content and in Assay LXVI no appreciable change occurred in the 'free' choline content of serum up to 3 hours after venepuncture.

These findings disposed of the chief objection to the use of serum which was employed almost exclusively in subsequent determinations.

2. PREPARATION OF SERUM EXTRACT FOR THE ESTIMATION OF TOTAL CHOLINE

Hydrolysis of Phospholipids -- In view of the fact that 95% or more of the total choline of serum exists in the phospholipid molecules it is necessary to break down these compounds by hydrolysis in order to free choline in a form that can be assayed. In consideration of a technique for splitting off the choline moeity of serum lecithins and sphingomyelins, the problem is to effect complete hydrolysis without destroying any of the freed choline and without leaving any substance in the final extract which interferes with the assay of choline by Neurospora.

A. ACID HYDROLYSIS

Sulphuric Acid -- Luecke and Pearson (1944) used an unstated volume of 3% sulphuric acid and autoclaved at 15 pounds pressure for 2 hours. When an enquiry regarding the amount of acid used failed to bring forth a reply, a series of experiments was undertaken to determine the optimum conditions for acid hydrolysis. After a number of orienting assays, a large assay was set up testing 36 different combinations of acid concentration, acid volume and duration of autoclaving. In all, 79 combinations of conditions were tested and it was found that the largest yield resulted from the use of 3 volumes of 3% sulphuric acid autoclaved at 15 lbs. for 75 minutes. Excess sulphuric acid is removed as barium sulphate by adding saturated aqueous barium hydroxide until neutral to Congo red. (Assays XI A B C D E F G & H). The results of Assay XI H to determine the optimum conditions of acid hydrolysis are tabulated:

Duration in minutes of autoclaving at 15 lbs.		30	45	60	75	90	120
	1	13.5	14.2	14.0	14.4	13.7	14.5
Volumes of	2	13.1	14.0	15.0	15.3	13.4	14.4
3% H_2SO_4	3	14.9	14.0	14.3	16.0	14.7	13.7
per volume	4	14.1	14.3	14.0	14.1	14.7	13.0
of serum	5	13.0	13.2	15.0	14.6	14.5	14.0
	6	12.1	14.0	14.0	13.6	14.7	13.6

(Values indicate weight of dried mycelium after 3 days incubation.)

Nitric Acid -- Ducet and Kahane (1946) refluxed the phospholipid-containing material with 30% nitric acid and neutralized by the addition of powdered calcium carbonate.

Hydrochloric Acid hydrolysis followed by neutralization with glacial acetic acid as proposed by Glick (1944) was attempted but the resulting extract is toxic for Neurospora and no growth resulted.

Gaseous Hydrogen Chloride in Absolute Methanol -- Thannhauser, Benotti and Reinstein (1939) found that this method produced complete hydrolysis, even of sphingomyelins, and had the advantage that the reagent could be driven off after the period of hydrolysis obviating the necessity of neutralization and consequent contamination of the extract with salts.

B. ALKALI HYDROLYSIS

Sodium methylate)
Barium hydroxide in water-alcohol) Tested by Thannhauser, Benotti and Reinstein (1939) with poor results.

Sodium Hydroxide -- Found by Williams et al (1938) to yield incomplete recoveries.

Barium Hydroxide Hydrolysis of dried methanol extract using saturated aqueous barium hydroxide at boiling temperature for 2 hours followed by

neutralization to phenolphthalien by acetic acid. (Engel, 1942). This technique was found more satisfactory than similar treatment carried out at 80°C. as proposed by Jacobi, Baumann and Meek, 1941.

Saturated Aqueous Barium Hydroxide -- Reports in the literature as reviewed by Bull (1936) led Williams et al (1938) to critically evaluate the effectiveness of barium hydroxide hydrolysis of phospholipids. The technique they evolved was to add 1 ml. of saturated barium hydroxide to about 25 volumes of an alcoholic extract of serum phospholipids and carry evaporation to dryness on a steam bath. Hydrochloric acid was then used to acidify the residue. This method was later modified by using 5 ml. of saturated barium hydroxide and more prolonged heating. It was found to give yields comparable to the gaseous hydrogen chloride method of Thannhauser. (Erickson et al, 1940).

Entenman, Taurog and Chaikoff (1944) used 15 volumes of saturated aqueous barium hydroxide to 5 ml. of methanol:ether solution of phospholipids and heated on a steam bath for 2 hours. The mixture was then acidified with hydrochloric acid. They found that barium hydroxide hydrolysis by this technique had no destructive effect on added choline chloride.

C. DETERMINATION OF OPTIMUM CONDITIONS FOR HYDROLYSIS OF SERUM PHOSPHOLIPIDS

From the foregoing survey it appears that the most promising agents for hydrolysis appear to be gaseous hydrogen chloride in methanol, barium hydroxide or sulphuric acid. Erickson et al (1940) compared the first two methods and found no essential difference in effectiveness. Having determined optimum conditions for acid hydrolysis (Page 97) it was decided to make a comparison with alkali hydrolysis. Initial experiments indicated that hydrolysis of whole serum with 5 volumes of saturated aqueous barium

hydroxide in a steam bath for 2 hours yielded higher values than sulphuric acid hydrolysis:

ASSAY LII

Serum Specimen	1	2	3	4	5	6
Average Mycelial Yields, (mg.) Sulphuric acid hydrolysis	37.8	34.3	34.2	34.8	30.9	34.6
Barium hyrdox. hydrolysis	38.1	36.6	35.2	36.2	32.8	37.2

From these results, which were corroborated in Assays LVIII and LIX, barium hydroxide appeared to be superior to sulphuric acid as a hydrolytic agent. The effect of using varying amounts of barium hydroxide was then explored in assays LXIII and LXVII:

Hydrolysis at Steam-bath Temperature for 120 Minutes

Hydrolytic Agent	3% H_2SO_4	Saturated $Ba(OH)_2$		
Volumes/Volume of Serum	3	3	5	10
Average Mycelial Yield (mg.)	23.3	21.7	23.6	22.1

The use of 5 volumes of saturated barium hydroxide in conjunction with varying degrees of heating were then tested:

5 volumes of barium hydroxide (saturated) plus 1 volume of serum

Method of Applying Heat	Steam bath (90-95°C.)		Autoclave 15 lbs. (120°C.)	
Duration (minutes)	30	60	30	60
Av. Mycelial Yield (mg.)	22.1	22.6	25.8	28.3

It was apparent that more prolonged heating might increase the degree of hydrolysis so Assay LXVII was carried out with the following results:

Hydrolysis at 120°C. (15 lbs. pressure). Serum #94.

Hydrolytic agent	3 vols. 3% H_2SO_4	5 vols. saturated $Ba(OH)_2$			
Duration of heating (mins.)	60	60	90	120	180
Av. Mycelial Yield (mg.)	25.4	29.3	<u>34.0</u>	31.2	28.7

In view of the considerable differences between the calculated values for total choline resulting from varying conditions of hydrolysis it was desirable to determine as closely as possible the optimum conditions. Therefore tests were made at 15 minute periods within the most promising range of heat exposure:

Assay LXXII -- Hydrolysis with 5 vols. sat'd $Ba(OH)_2$. Serum pool #101.

Duration in mins. of autoclaving at 15 lbs.	60	75	90	105	120	150
Av. Mycelial Yield (mg.)	35.7	<u>36.5</u>	34.7	34.9	35.5	33.8

Dr. R.G. Sinclair (personal communication) found somewhat more complete hydrolysis from the use of barium hydroxide solution saturated at boiling temperature rather than at room temperature. This was tested in only two sera in one of which the boiling saturated solution was equally effective as the cold saturated while in the other it was less effective. The boiling saturated solution is less convenient to handle and requires large quantities of acid for its neutralization so that this modification would require clear-cut evidence of superiority to warrant its use.

The impression gained from studies on the hydrolysis of choline-containing phospholipids is that apparently some of the phospholipids are readily hydrolyzable and others can only be split with difficulty. The sphingomyelins are said to be more difficult to hydrolyze than the

lecithins. In Assay XVIII it was found that in 6 days at room temperature about half the phospholipids of a sample of serum underwent spontaneous hydrolysis. Longer storage did not result in more complete hydrolysis, however.

During experiments on the use of acetone to isolate the 'free' choline fraction of serum it was observed that the phospholipid fraction which was acetone insoluble and not extractable with petroleum ether (about 15% of the total choline-containing phospholipids in this case) was also readily broken down during storage, with liberation of about 65% of the contained choline in three days. A further 60% of the choline-containing phospholipids were acetone insoluble but petroleum ether soluble and this fraction hydrolyzed during 3 days storage only to the extent of 25%. (The remaining 25% of phospholipids were acetone-soluble.)

These observations serve to indicate the heterogeneous character of the phospholipids and the inconstancy of their physical properties and solubilities, at least when encountered in mixtures. Further discussion of this point is found in the section on the preparation of extracts for assay of 'free' choline.

D. TECHNIQUE FOR PREPARATION OF EXTRACTS FOR THE DETERMINATION OF THE TOTAL CHOLINE CONTENT OF SERUM

In accordance with the foregoing findings, the following conditions were adopted for hydrolysis of the choline-containing phospholipids of serum and for the preparation of an extract suitable for Neurospora bioassay:

5.0 ml. of saturated aqueous barium hydroxide solution is added to exactly 1.0 ml. of serum in a 50 ml. centrifuge tube which is capped with an inverted beaker and autoclaved at 15 lbs. pressure for 75 minutes.

On removal from the autoclave, about 20 ml. of distilled water is added to the mixture in the tube and the contents are neutralized to Congo red paper by the addition of 3% sulphuric acid. (about 2 ml. is required). Distilled water is then added until the tube is nearly filled. (The use of a generous amount of diluent in this way reduces the amount of choline which remains in the interstices of the barium sulphate precipitate and obviates the need for washing the precipitate.)

After centrifuging, the clear supernatant is decanted into a 100 ml. volumetric flask and made up to volume. The addition of 1.0 ml. of this extract per assay flask yields mycelial weights within the applicable range.

In spite of the unavoidably large dilution factor (1:100) which magnifies technical errors in inverse degree, it has been noted that remarkably good agreement is obtained between values of multiple determinations on the same sample of serum. In 18 assays of total choline in duplicate or triplicate, the individual values deviated from the mean by an average of 1.3%. Poorest agreement occurred in Assay LXVIII where triplicate values on Serum #96 were 285.0, 305.0 and 295.0 with an average

of 295.0 micrograms per millilitre. Assay of a fourth aliquot to which 65 micrograms of choline was added gave a value of 365.0. (Calculated 360.0

The completeness of recovery of added choline chloride suggests that no choline is destroyed by the process of hydrolysis, but it does not prove this point, since it could be interpreted as indicating the possibility that the addition of choline chloride to the mixture permitted more thorough hydrolysis of phospholipids. Therefore choline chloride solution was subjected to the physical conditions of the hydrolysis procedure and no loss occurred. (Assay XLVII). In addition it was shown that a 'blank' extract prepared in this manner did not stimulate or depress the growth rate of Neurospora. (Assay LXII).

Finally, it is seen in Figure XI (Page 105) that the dose response curve of serial dilutions of total choline extract parallels quite closely the curve of mycelial weights resulting from the use of corresponding dilutions of choline chloride. This constitutes a fairly rigid test of suitability of an extract for Neurospora bioassay since the presence of interfering substances would give rise to 'drift' of the curve away from that plotted from dilutions of the pure vitamin.

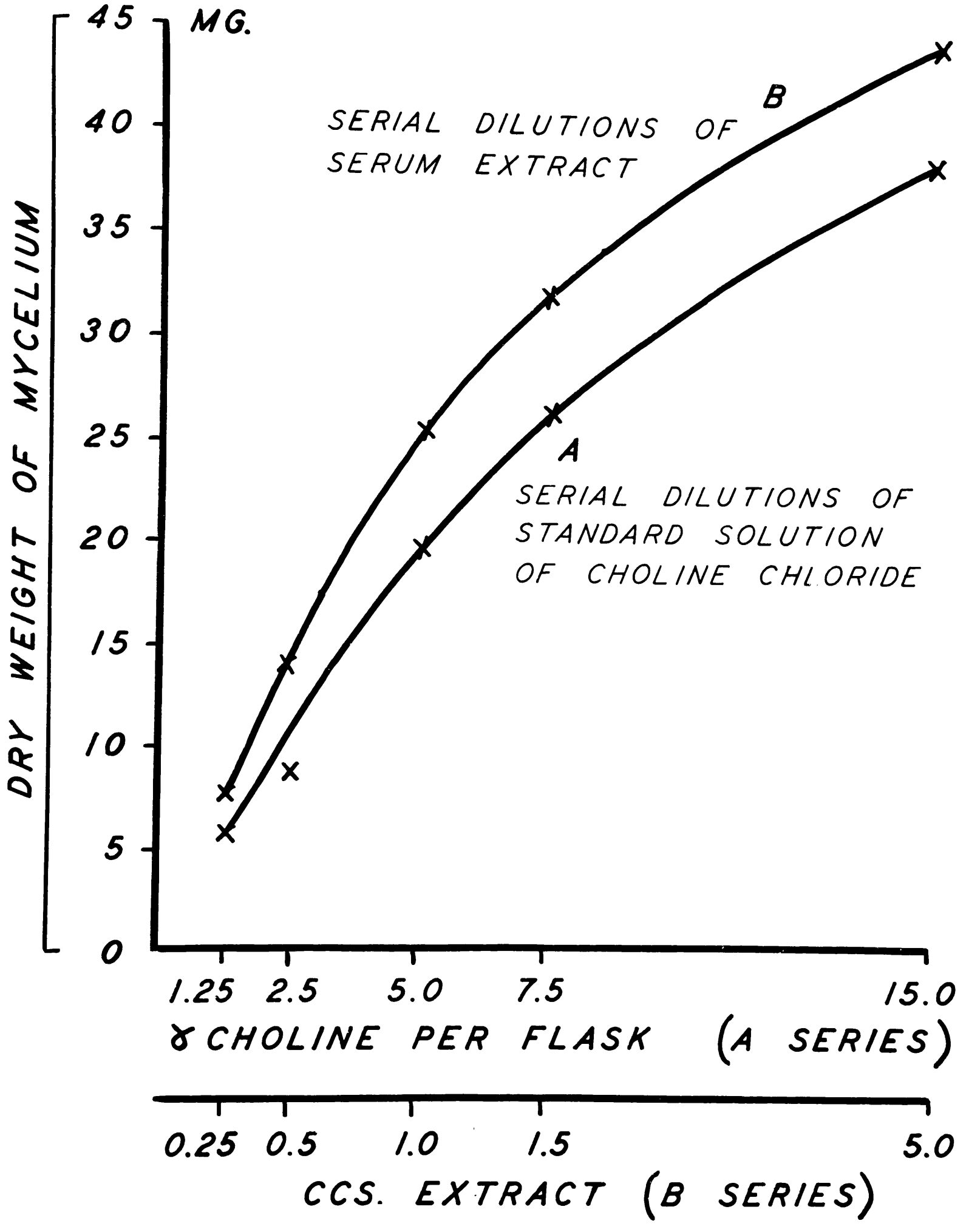

DRY WEIGHT OF MYCELIUM
MG.
45
40
35
30
25
20
15
10
5
0
SERIAL DILUTIONS OF SERUM EXTRACT
B
A
SERIAL DILUTIONS OF STANDARD SOLUTION OF CHOLINE CHLORIDE
1.25 2.5 5.0 7.5 15.0
γ CHOLINE PER FLASK (A SERIES)
0.25 0.5 1.0 1.5 5.0
CCS. EXTRACT (B SERIES)

FIGURE XI

EFFORTS TO PREPARE AN EXTRACT FOR THE ASSAY OF FREE CHOLINE IN SERUM

Separation of the small 'free' choline fraction of blood from the choline-rich phospholipids, rapidly enough and gently enough to avoid contamination with choline from inadvertent hydrolysis of the phospholipids, proved to be a difficult and discouraging problem. This difficulty was first recognized by Hunt (1915) who used ether washing of the dried residue of an acetone extract in an effort to remove the phospholipids, although he admitted he had no idea of the completeness of the removal.

There are no well-substantiated claims in the literature for a technique to effect clean separation of the 'free' choline of blood. Most of the published values are vitiated by failure to consider the large error introduced by incomplete removal of phospholipids and some authors have seriously questionned whether 'free' choline exists as such or whether choline found in that form has, in fact, been split off from phospholipids during or preceding extraction.

The efforts described in the following sections to prepare a 'free' choline extract did not meet with unqualified success but are included in this presentation for a number of reasons: First, because discrepancies in certain published methods were noted; secondly, a brief discussion of negative results obtained during various unsuccessful approaches to the problem may be indicated to aid in avoiding or modifying these approaches in future; finally there is included the description of a promising technique which was explored and tested but has not yet been sufficiently tried to fully establish its value.