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# SOME INVESTIGATIONS ON CHOLINE METABOLISM IN ASPERGILLUS NIGER

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## CHAPTER I

## LITERATURE SURVEY REGARDING CHOLINE

### 1. INTRODUCTION

Choline is one of the most important nitrogen compounds of the non-protein fraction of plants and animals. Synthesis and dissimilation of free choline form an integrating part of the metabolism of compounds with labile methylgroups. Acetylcholine and glycerophosphoric acid ester of choline belong to the watersoluble derivatives of choline; lecithin and sphingomyeline are choline containing compounds in fats.

Choline metabolism in plants has been only superficially investigated. With regard to synthesis HOROWITZ' work is of special importance (63, 64). By comparing the growth factors of a number of choline-less mutants of *Neurospora crassa* they were able to prove that the synthesis of choline is the result of the methylation of ethanolamine step by step.

The origin of the methylgroups of choline has been investigated by KIRKWOOD c.s. (99); they isolated choline with $C^{14}$ in the methylgroups after feeding $NaC^{14}OOH$ to sprouting barley.

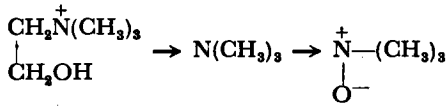
The greatest part of the experiments on the metabolism of choline has been made in animals (DU VIGNEAUD, BORSOOK, DUBNOFF); They proved that in rats methionine can provide a methylgroup for the synthesis of choline, whereas conversely choline can function indirectly as a methyldonor at the synthesis of methionine (140). This combined action of compounds with "biological labile methylgroups" is jointly termed transmethylation reactions.

During the dissimilation choline can be oxidised to betaine under the influence of a choline oxidising enzymsystem; the degradation of choline to trimethylamine has been shown too. The object of the investigation, the results of which have been described here, was to obtain a deeper insight into the metabolism of choline in *Aspergillus niger* of Tieghem.

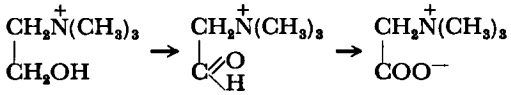
### 2. DISSIMILATION OF CHOLINE

The dissimilation of choline in the organism can proceed in two different ways:

*a.* the breakdown of choline to trimethylamine; in some cases trimethylamine is still oxidised to trimethylaminoxide.



*b.* Oxidation of choline via betaine-aldehyde to betaine.



## 2. 1 *The degradation of choline to trimethylamine*

The degradation of choline to trimethylamine has been described by CROMWELL (39) in leaves of *Chenopodium vulvaria L.* DYER and WOOD (49) discovered that in certain *Enterobacteriaceae* choline and acetylcholine break down into trimethylamine; betaine was not transformed. COHEN and his collaborators (36) observed a degradation of choline to trimethylamine, ethanolamine, ammonia and ethyleneglycol in some species of *Clostridium.* During their investigations on the creatine-synthesis with minced muscle of caviae BARRENSCHEEN and PANTLISCHKO (5) came to the conclusion that besides the oxidation of choline to betaine another way exists whereby choline is degradated; the end-product in this case would be trimethylaminoxide.

## 2. 2 *Oxidation of choline to betaine*

The oxidation of choline to betaine via betaine-aldehyde has been investigated by BERNHEIM and BERNHEIM (14, 15). After adding choline to an extract of rat-liver they determined the quantity of oxygen absorbed at different pH; it seemed that at pH 6.7 one and at pH 7.8 two atoms of oxygen per mol choline were used. In the faintly acid mixture the presence of betaine-aldehyde could be proved as a bisulphite-compound. In contrast to the alcaline the acid solution showed reducing properties. The presence of a choline-dehydrogenase which can oxidise choline via betaine-aldehyde to betaine was established.

After an elementary analysis of the reineckate compound of the oxidation product of choline MANN and QUASTEL (96) too came to the conclusion that choline is transformed enzymatically to betaine-aldehyde by rat-liver extract. In a subsequent publication they demonstrated that the enzymesystem was composed of two components viz.: choline-dehydrogenase which was not inhibited by small quantities of cyanide and of a cytochrome- and indophenol-oxydase. In anaerobic conditions the oxidation of choline could also be arrived at, provided a suitable hydrogen-acceptor (sodium-ferricyanide) was added. The fact that choline-dehydrogenase does

not oxidise alcohol (BERNHEIM c.s.), indicates that the alcohol-group does not obstruct the enzyme: in this respect the group-$NR_3$ is of importance because choline-dehydrogenase is inhibited by $NH_4^+$, $NH(CH_3)_3^+$ and betaine.

The presence of a choline-oxidising enzyme is not restricted to the animal organism. According to a recent publication by CROMWELL and RENNIE (40) this enzyme is also present in the roots of *Beta vulgaris;* the oxidation is accelerated in vitro by cytochrome *c*; the cyanide ion tends to retard.

The oxidation of choline is now of great importance. On the one hand the methylgroups of betaine are transferred by a transmethylation reaction to other compounds (2.2.1), on the other hand they can be oxidised to $CO_2$ via formate (2.3.).

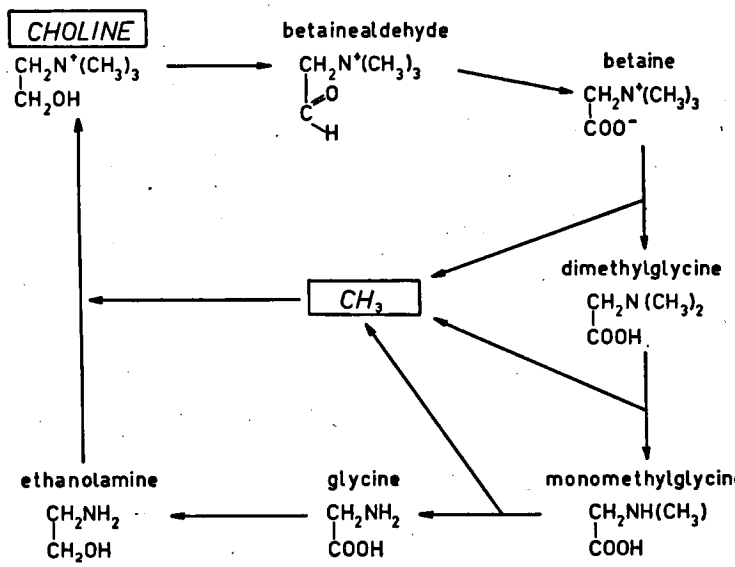
### 2.2.1 *The indirect methylation action of choline*

The experiments of ROSE (109) and DU VIGNEAUD (140) had shown that the amino acid methionine which is indispensable to rats could be replaced by homocysteine plus choline in their diet; BORSOOK and DUBNOFF (23) have demonstrated a synthesis of methionine from homocysteine and choline with homogenates of rat-liver, whereas SIMMONDS and others (119) with the aid of isotopes proved the transfer of a methylgroup of choline during the methionine-synthesis. To obtain a closer insight into the procedure of the reaction of homocysteine + choline → methionine, it proved necessary to investigate whether choline had a direct methylating action whether it is first transformed into another compound.

The experiments of BORSOOK and DUBNOFF (23) gave rise to the conjecture that choline was not directly involved in the transmethylation: under aerobic and anaerobic conditions the methylation of homocysteine in rat-liver homogenates by betaine proved to be quicker than by choline; a closer investigation moreover (42) showed that the methylation of homocysteine by choline in liver homogenates of different animals correlated with the contents of choline-oxidase of the livers of these animals. Thus the livers of caviae and rabbits contained no choline-oxidase, those of mice, chickens and rats did. The methionine-synthesis from choline and homocysteine by liver homogenates of caviae and rabbits was slight compared to those by liverhomogenates of mice, chickens and rats under similar conditions.

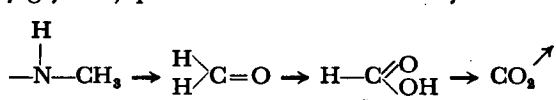
MUNTZ (106) provided a more direct proof of the methylating action of choline linked to an oxidation; he incubated a rat-liver homogenate under anaerobic conditions with homocysteine and choline containing $N^{15}$. With the aid of the "carrier-technique" it was found that after incubation the dimethylethanolamine present contained only slight quantities of $N^{15}$ and the dimethylglycine on the contrary considerable quantities of $N^{15}$. According to these experiments the transfer of a methylgroup of choline apparently requires in the first place the oxidation of the alcoholgroup of choline to the carboxylgroup of betaine.

The procedure of the transfer of a methylgroup of choline as formulated by MUNTZ (106) corresponds with the earlier experiments of STETTEN (128, 129). STETTEN fed rats betaine containing $N^{15}$ and discovered that after 3 days most of the $N^{15}$ appeared in glycine, slightly less in ethanolamine whereas choline contained the smallest quantity. DU VIGNEAUD and collaborators later repeated these experiments with betaine which besides $N^{15}$ contained deuterium in the methylgroups; they discovered betaine to be an extremely active methyldonor. Most of the $N^{15}$ was found in glycine, whereas a small quantity was also present in choline (141). The following scheme summarizes these reactions:




2.3. *Oxidation of the labile methylgroup.*

The action of the labile methylgroup of compounds as choline, betaine and methionine is not only limited to a transfer to a methylacceptor. The experiments of MACKENZIE (93) proved that the rat can oxidise a labile methylgroup of compounds as methionine and choline to $CO_2$. The oxidation of the methylgroup of sarcosine (monomethylglycine) proceeded via formaldehyde and formic acid.




FERGER and DU VIGNEAUD (53) have investigated the rate of oxidation of various compounds with labile methylgroups; the process of oxidation to $CO_2$ of the methylgroups of choline appeared to be much slower than that of betaine, dimethylthetine and dimethylpropiothetine.

The more rapid oxidation of the methylgroups of the last three compounds with respect to those of choline indicates a greater reactivity of the methylgroups; this coincides with the results of the experiments of MUNTZ which has already been mentioned before (106) on the indirectly methylating action of choline via betaine. Yet another indication of a link between the reactivity and the oxidability of the methylgroups became apparent from the experiments of MACKENZIE and DU VIGNEAUD (94) who discovered that rats do not produce radioactive $CO_2$ from creatine and creatinine which contain methylgroups with $C^{14}$; formerly DU VIGNEAUD and collaborators (138) had shown that the methylgroups of creatine and creatinine do not take part in the transmethylating reactions.

### 3. SYNTHESIS OF CHOLINE

The research on the metabolism of choline in animals with compounds not tagged with $C^{14}$ or D was founded on the observations of BEST (16, 17) on the lipotropic effect of choline, and on those of JUKES (69) on the ability of choline to prevent perosis. Such experimental data regarding the deficiency of choline in plants were not available; HOROWITZ (63) however succeeded in indirectly obtaining data on the synthesis of choline in plants in an indirect way.

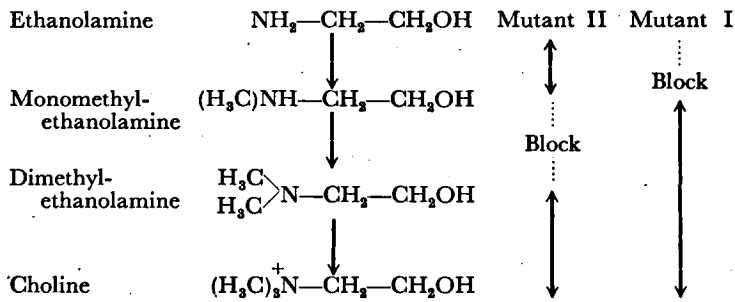
*Neurospora crassa* synthesizes choline during growth on a minimum nutritive-solution consisting of carbohydrates, salts and biotine.

By using ultra-violet ray treatment HOROWITZ and BEADLE (64) succeeded in obtaining a choline-less mutant (I) of *Neurospora crassa* which only grew when choline was also added to this minimum nutritive-solution. At a certain concentration the quantity of mycelium produced was proportional to the quantity of choline added; in this way choline could be determined microbiologically. This mutant was no longer able to synthesize choline out of the compounds present in the minimum nutritive-solution. Addition of ethanolamine to the nutritive-solution did not produce growth; the organism was apparently unable to synthesize choline from this compound. JUKES and DORNBUSH (71) proved however that mutant I was also able to grow if dimethylethanolamine was added to the nutritive-solution. We have to conclude therefore that mutant I is able to produce choline from dimethylethanolamine. The activity of this compound calculated per mol equalled that of choline.

Later HOROWITZ (63) acquired another mutant (II), which after adding choline showed a considerable increase in growth although growth was never completely retarded when choline was lacking. Closer investigation showed that a compound was formed by mutant II on a nutritive-solution without choline, which increased growth of mutant I; this compound proved to be monomethylethanolamine.

From this it was concluded that with mutant I formation of choline from ethanolamine was completely blocked at the first step (ethanolamine → monomethylethanolamine) whereas with mutant II the step of monomethyl → dimethylethanolamine was retarded. In

view of this it is probable that synthesis of choline in *Neurospora crassa* proceeds by methylation of ethanolamine into choline step by step.




With the aid of tracers these indirectly obtained results have been proved directly in animals. By feeding rats ethanolamine labelled with $N^{15}$, this was found to be present in the choline and phospholipoids of the tissues (STETTEN, 128, 129).

### 3.1. *Origin of the methylgroups*

When it became probable from the experiments of HOROWITZ (63, 64) and STETTEN (128, 129) that the synthesis of choline in plants and animals can be the result of methylation of ethanolamine, the question of the origin of the methylgroups immediately arose. In view of certain publications of which we will give a more detailed account later, it appears that two kinds of compounds can function as a methyldonor viz. on the one hand the compounds which comprise a biologically labile methylgroup (methionine, betaine, dimethylthetine and dimethylpropiothetine); on the other hand formate which can also function as a methyldonor according to recent investigations with tracers (1, 2, 13, 32, 99, 117, 118).

For a summary of the reactions which occur at the synthesis of choline we refer to the scheme on pag. 172; on account of a great deal of publications in connection with this, a certain curtailment proved necessary. Detailed summarizing articles regarding the metabolism of compounds with biologically labile methylgroups have been published by LUCAS and BEST (89), JUKES (70), DU VIGNEAUD (135) and BACH (4). In this summary we have especially laid stress on the metabolism of choline.

#### 3.1.1. *Transmethylationreactions.*

During the investigation on methionine-synthesis in rats it was found that choline can yield a methylgroup to homocysteine; this reaction can be reversed so that methionine can also yield a methylgroup at the synthesis of choline. DU VIGNEAUD c.s. (136, 137) observed that after administering rats for 3 weeks methionine with 3 deuterium atoms

in the methylgroup, 57 % of the deuterium could be recovered in the methylgroups of choline; after 94 days this became 88,6 %. This transfer of a methylgroup of methionine can occur not only in rats but also in human beings (120), in birds (26), in rabbits (115) and in dogs (30, 31, 77). Dimethylethanolamine, monomethylethanolamine and ethanolamine could function as methylacceptors. The earlier mentioned experiments of STETTEN (128, 129) proved that ethanolamine can function as a precursor in rats. After administering ethanolamine tagged with $N^{15}$ to rats, this N was found to be present in the choline and in the phospholipoids of the tissues.

In the plant the transmethylation has also been proved: KIRKWOOD (79, 99) found that when l-methionine labelled with $C^{14}$ in the methylgroup was administered to sprouting barley, the methylgroups of choline and hordenine contained $C^{14}$. Choline-methyl was not transferred to methionine in the barley plant.

CHALLENGER c.s. (32) showed in *Aspergillus niger* transmethylation by dl-methionine; they found that the methylation by this compound is about 20 times larger than by choline or betaine. Contrary to the rat it seems that in both plants the transmethylation of methionine to choline is irreversible.

As yet very little is known about the reaction-mechanism which takes place at the transfer of the methylgroup of methionine; literature on this subject indicates two ways in which this transfer can take place:

A. Transfer of the methylgroup of methionine via methionine-sulfoxide [BARRENSCHEEN and VON VALYI-NAGY (8)].

For their investigations they used a mash of crushed wheat seedlings to which they added glycine and methionine. After shaking 36 to 48 hrs while passing oxygen, they isolated choline and betaine; they found after adding glycine and methionine a larger quantity of choline than without addition of these 2 compounds. Unlike to BARRENSCHEEN (8) STEENSHOLT (126) could not discover any in vitro methylation of ethanolamine by methionine in a mash of wheat seedlings.

BARRENSCHEEN and others (7) have also investigated the methylation of ethanolamine in animal material that is in macerated slices of the rat; they found that the synthesis of choline is an aerobic process, during which the methylgroup of methionine by oxidation to methionine-sulfoxide will be made labile. According to these authors ethanolamine was methylated by methionine-sulfoxide under anaerobic conditions, but not by methionine. By using an extract of rat-liver STEENSHOLT (125) was also able to acquire the in vitro methylation of ethanolamine by methionine; he found that the methylating activity of methionine-sulfoxide amounts to approximately half of that of methionine.

During a later investigation STEENSHOLT (127) compared the activities of the d- en l-shape of methionine at the synthesis of choline. He discovered that the d(+)-methionine was much more active than the l(—)-methionine.

B. Transfer of the methylgroup of methionine by ATP.

In view of their in vitro experiments with rat-liver preparations

BORSOOK and DUBNOFF (22, 23) distinguished 2 types of transmethylation reactions *a*) methylation reactions which are dependent of $O_2$ (the synthesis of N-methylnicotinamide from nicotinamide and methionine) and *b*) methylation reactions which are independent of $O_2$ (the synthesis of methionine from homocysteine and betaine).

According to CANTONI (27, 28) the first type of methylation reaction needs an energy-rich phosphate for the transfer. Originally he supposed that phosphorylated methionine was the active agent. Further investigations showed however that the activated methionine consisted of a compound of methionine with the adenosine-part of ATP (29). This S-adenosylmethionine can function without ATP as a methyldonor.

SMITH c.s. (121) discovered that thiomethyladenosine can be enzymatically split off from S-adenosylmethionine; the linkage of methionine to adenosine does therefore not necessarily lead to the transfer of a methylgroup.

Finally it should be pointed out that the synthesis of choline is supposed to belong to the aerobic reaction type which necessitates the presence of ATP.

Besides methionine some other compounds with biological labile methylgroups can function as methyldonor; DU VIGNEAUD c.s. (141) discovered that at the synthesis of choline betaine was an extremely active methyldonor.

With regard to this type of methylation reactions DUBNOFF and BORSOOK (44, 45) like MAW and DU VIGNEAUD (101) link the lability of the methylgroup of betaine under anaerobic conditions with the structure of the betaine-molecule. In general, especially the compounds with an "onium" structure such as betaine and the S-analogue dimethylthetine under anaerobic conditions combine a methylating capacity with their growth-promoting properties. The reaction-mechanism of methylation by "onium" compounds as betaine is not known; further research will have to prove whether betaine is the methyldonor or whether it is first changed into other compounds.

Besides betaine the S-analogue of this compound can also function as a methyldonor at the methionine-synthesis (DU VIGNEAUD, 105, 139); when homocysteine plus sulfobetaine (dimethylthetine) is added to the diet of a rat which lacks choline and methionine, the animal will grow normally. MAW and DU VIGNEAUD (100, 102) have investigated some compounds related to dimethylthetine with regard to their growth-promoting action. Replacement of one methylgroup (methylethylthetine) resulted in a decrease of the growth and replacement of 2 methylgroups (diethylthetine) caused a cessation of growth. The homologue S-dimethylpropiothetine was as active as dimethylthetine; the first compound was isolated from algae by CHALLENGER and SIMPSON (33). DUBNOFF and BORSOOK (44, 45) investigated the in vitro methylation of homocystine by these S-compounds; they found that the transfer of the methylgroups of these S-compounds occur under the influence of a specific enzyme of the rat-liver. The methylating action of dimethylthetine and dimethylpropiothetine was several

times larger than that of betaine. The methylating action of the S-analogue of choline, sulfocholine has also been tested; this compound however was toxic to rats [MAW and DU VIGNEAUD (101)].

3.1.2. *Synthesis of methylgroups by the organism.*

In the introduction it has already mentioned that the methylgroup in plants and in animals are of different origin: in animals the methylgroup required for the synthesis of choline are for the greater part present in the diet. This is not the case in plants; that's why the plant must synthesize the labile methylgroups. In this connection the following experiments with animals are of special importance.

During his first fundamental investigations on the methylation of homocysteine by choline in rats on a diet lacking compounds with labile methylgroups DU VIGNEAUD (139) mentioned that now and again some animals, after first losing weight, started to grow again without the addition of choline to their diet. It was thought that this was caused by a possible synthesis of labile methylgroups by the microflora of the intestines. BENNETT (11, 12) later confirmed this incidental growth in some animals; after feeding them sulfasuxidine however, these animals ceased to grow. A new aspect presented itself when it became apparent that after cessation of growth due to sulfasuxidine growth would be resumed when a crude liver extract was administered. The formation of the methylgroups was probably not due to bacteria but to a vitamin factor of the liver extract.

BENNETT came to the conclusion that "There may be vitamin factors of either dietary or intestinal origin, the presence of which may enable the animal to compensate for the absence of dietary methyldonors by biosynthetic means of its own or of its intestinal bacteria."

Some time afterwards the vitamin $B_{12}$ was isolated in the liver. Later this appeared to be part of the A(nimal) P(rotein) F(actor). PATTON c.s. (107) proved, as did BIRD and others (18) that the APF-factor affected the need for methionine in chickens. Other observations regarding a relation between the metabolism of biologically labile methylgroups and the vitamin $B_{12}$ were those of SCHAEFER (114) and GILLIS and NORRIS (55).

In 1949 BENNETT (10) discovered that vitamin $B_{12}$ plus folic acid increased the growth of young rats in the same way as the formerly used crude liver extract. JUKES and STOKSTADT (72) found that in chickens the need of choline and methionine was reduced by vitamin $B_{12}$. These experiments clearly proved that labile methylgroups were synthesized under the influence of vitamine $B_{12}$; the question remained whether this synthesis was produced by the tissues of the rat rather than by the flora of the intestines. DU VIGNEAUD (142) in collaboration with REYNIERS of the Lobund Research Institute has solved this problem as follows: $D_2O$ was administered to completely sterile raised rats fed on a diet containing vitamin $B_{12}$ and folic acid. As controls non sterile animals raised on the same ration were used. After 10 days in the body fluids of the sterile animals 3.3 % of the total quantity deuterium was found in the methylgroups of choline,

after 23 days this amounted 6.4 %. The quantity of deuterium present in the methylgroups of choline was slightly more in non sterile animals which indicates a possibly extra synthesis of the methylgroups by the flora of the intestines.

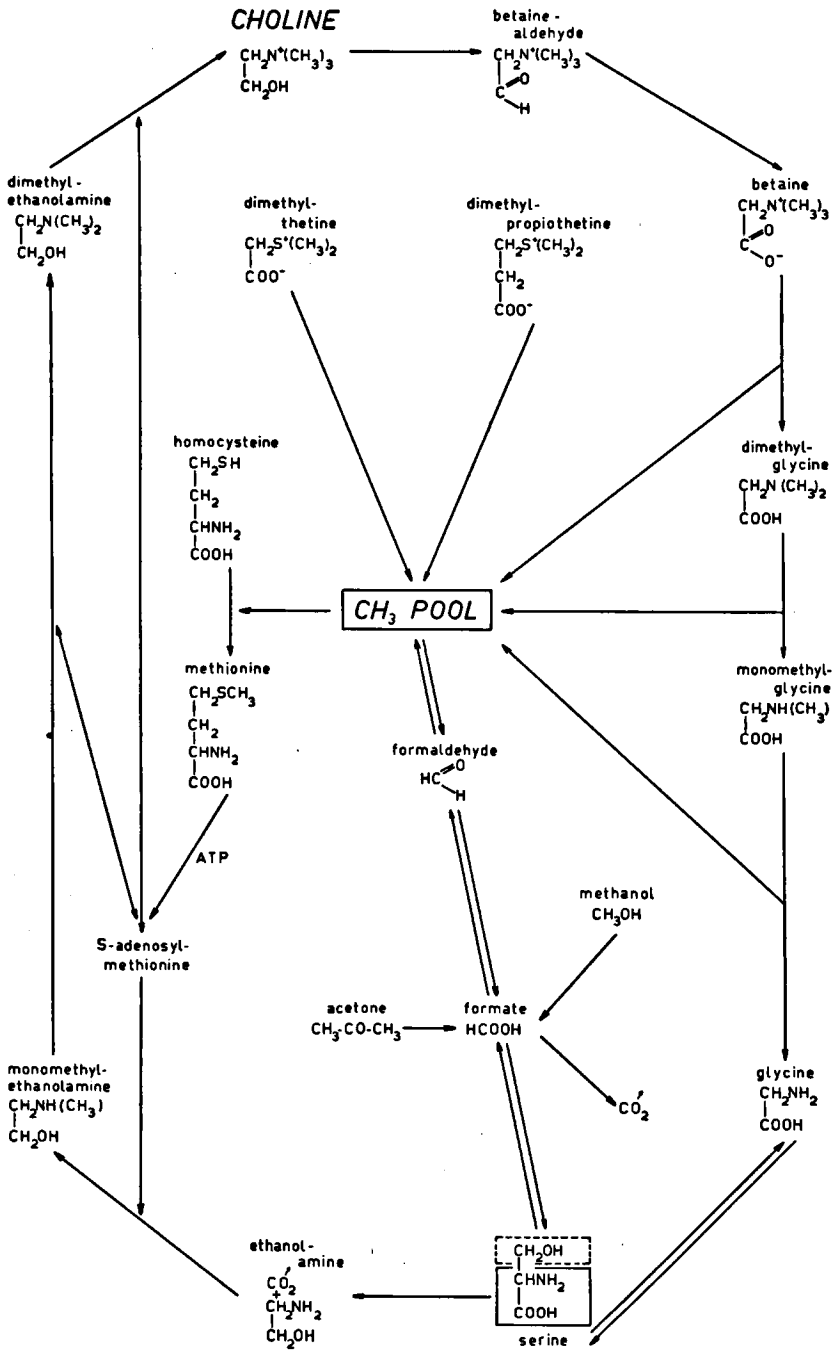
When it became apparent from these experiments by DU VIGNEAUD that, if vitamin $B_{12}$ was present, a rat could synthesize biologically labile methylgroups, all interest was centered on the compounds involved in this synthesis. In this connection MACKENZIE's experiments (93) on the oxidation of the labile methylgroup via formaldehyde and formic acid are of importance (see pag. 166); besides the methylgroup can be oxidised to $CO_2$. The function of formic-acid in the synthesis of the labile methylgroup can be gathered from the following experiments.

In 1948 SAKAMI (110) discovered that rats were able to form serine out of formic acid and glycine, whereas a year later it appeared that the methylgroup of choline labelled with $C^{14}$ reappeared as the $\beta$-carbon atom of serine (111)

(*a*) glycine $\mathrm{CH_2NH_2}$ (COOH) + HCOOH → $\mathrm{H_2COH}$ / $\mathrm{HCNH_2}$ / COOH serine

(*b*) choline $\mathrm{CH_2\overset{+}{N}(C^{14}H_3)_3}$ / $\mathrm{CH_2OH}$ → $\mathrm{H_2C^{14}OH}$ / $\mathrm{HCNH_2}$ / COOH serine

In view of these reactions it seemed probable that the C atom of the methylgroup of choline appeared in serine (reaction b) via formic acid. On closer investigation the reaction $—C^{14}H_3 \rightarrow HC^{14}OOH \rightarrow H_2C^{14}OH\text{-}CHNH_2\text{-}COOH$ proved to be reversible; thus SAKAMI and WELCH (113), SIEKEVITZ and GREENBERG (117, 118) and BERG (13) discovered that the carbon atom of formic acid reappeared in the methylgroups of methionine. These experiments were extented by ARNSTEIN (1, 2); JOHNSSON and MOSHER (68) and WEISSBACH (146), who used $C^{14}$ labelled aminoacids; thus the $\alpha$-carbon atom of glycine and the $\beta$-C atom of serine were recovered in the methylgroups of choline. If, for example, serine with a $\beta$-$C^{14}$ atom $C^{14}H_2OH\text{-}CHNH_2\text{-}COOH$ was fed to a young rat the methylgroups as well as the ethanolamine-part of the choline in the liver contained $C^{14}$; apparently serine does not only function as a methyldonor but is after decarboxylation a methylacceptor too. Besides formic acid (13, 113, 117, 118), glycine (2) and serine (2) are compounds as acetone (108), methanol and formaldehyde (138, 139) active in the synthesis of biologically labile methylgroups. The above mentioned reactions have been comprised in the following scheme:


CHOLINE
betaine-aldehyde
dimethyl-ethanolamine
dimethyl-thetine
dimethyl-propiothetine
betaine
homocysteine
dimethyl-glycine
CH3 POOL
methionine
monomethyl-glycine
formaldehyde
ATP
methanol
CH3OH
S-adenosyl-methionine
acetone
CH3-CO-CH3
formate
HCOOH
CO2
monomethyl-ethanolamine
glycine
ethanol-amine
serine

From the above it appears therefore that besides the labile methylgroups, which are at the disposal of the animal in its diet, a synthesis of methylgroups is possible under the influence of the vitamin $B_{12}$, whereby formic acid is an important precursor. Under normal conditions in animals the diet is the most important source of the labile methylgroups; the synthesized methylgroups are sometimes the cause of the non-appearence of certain deficiency phenomena such as the absence of growth when methionine is lacking.

The plant has no substances with labile methylgroups at his disposal. That is why the plant is practically always dependent on the synthesis of the labile methylgroups from substances like formaldehyde and formate. KIRKWOOD c.s. (99) and CHALLENGER (32) produced the experimental proof of this supposition; they isolated compounds with $C^{14}$ in the methylgroups after feeding $NaC^{14}OOH$ to sprouting barley resp. *Aspergillus niger.*

A comparison of formate and methionine as precursors of biologically labile methylgroups showed that the latter was much more efficient. This supports the idea that formate serves as a precursor of methionine-methyl.

With these results in mind we formulated a central "$CH_3$ pool" as shown in the scheme above from which a methylgroup can be transferred via methionine and S-adenosyl-methionine to the precursors of choline (ethanolamine, mono- and di-methylethanolamine).

## 4. INCORPORATION OF CHOLINE

To be able to acquire a more comprehensive idea of the metabolism of choline a survey of the synthesis and hydrolysis of the derivatives of choline should follow on the discussion of the dissimilation and synthesis of free choline. The choline-containing compounds of the organism can be divided in the phosphatides lecithine and sphingomyeline, in sinapine present in some plants and in acetylcholine found chiefly in animals.

### 4.1. *The phosphorus containing compounds of choline*

Biochemically and physiologically, the phosphatides appear to be involved in a multiplicity of functions of the greatest importance, about which knowledge is scanty. The phosphatides are active metabolic components of many organs and tissues, particularly the gastrointestinal tract, the liver and the blood. Very little is known about the course of phosphatide metabolism in plants, firstly because of the difficulty of the work involved and secondly because phosphatides are, for the most part, rather minor constituents of the members of the plants.

DUCET (46) proved that during the vegetative growth of soy a synthesis of phosphatides takes place at the expense of watersoluble choline. During the ripening of the seed a dissimilation of the phosphatides sets in whilst choline is liberated.

The hydrolysis of lecithine can proceed in various ways; there are four points of attachment (ester linkages), which should prove suscepti-

ble to enzymatic attack. These comprise the first and second fatty acid linkage, the phosphoric acid-glycerol linkage and the aminoalcohol-phosphoric acid linkage. Accordingly, the existence of lecithinases corresponding to each of the ester linkages of lecithin was postulated. These are defined as follows:

Lecithinase A: An enzyme which removes one fatty acid from lecithin.
„ B: An enzyme which removes the remaining acid from lecithin.
„ C: An enzyme with cholinephosphatase activity capable of splitting choline from lecithin.
„ D: An enzyme with glycerophosphatase activity which separates lecithin at its ester linkage between glycerol and phosphoric acid.

An example of the occurrence of the lecithinase C activity in the higher plant has been shown in the beautiful investigations of HANAHAN and CHAIKOFF (59, 60, 61). They found that the carrot phosphatides, which could be extracted with alcohol-ether mixtures from raw carrots were characterized by a low nitrogen content and by a virtual absence of choline. The same extraction when applied to steam-treated carrots, provided a more conventional phosphatide with a molar ration of 0.89 of nitrogen-phosphorus. The product extracted from raw carrots appeared to be similar to the phosphatidic acids; and it seemed logical, in view of the effect of prior treatment with steam, to postulate the presence of an enzyme which split the nitrogenous base from the phosphatide during the isolation process. This was substantiated in further work by HANAHAN and CHAIKOFF (60), who were able to isolate from carrots an enzyme extract, which possessed no choline-esterase activity, but which split the ester linkage between the nitrogenous base and the phosphoric acid grouping in phosphatides obtained from carrots and soybeans. The enzyme exerted its maximum activity at a pH range of 5.2 to 5.9; it showed a high degree of heat stability since it was not completely inactivated when exposed to a temperature of 95° C for 15 minutes.

A similar enzyme was shown to be present in cabbage leaves (61) and, as in the case of carrots, a normal phosphatide could be isolated only after the cabbage leaves had been subjected to steam treatment. From the fresh leaves, phosphatidic acids were isolated. The enzyme in cabbage leaves exhibited its maximum activity in the same pH range as the carrot enzyme and it was quite thermostable too.

The term lecithinase A was applied by CONTARDI and ERCOLI (37) to the enzyme which removes one fatty acid from lecithin; lecithinase A was originally found in cobra venom, where it was detected because it reacted with lecithin to yield a hemolytic substance. The enzyme was also found in the pancreas, dried heart, liver, spleen, muscle, brain, suprarenals thymus and prostate. The fresh organs contained only lecithin, indicating that the lecithinase A was in some manner held in check in the fresh organ by other enzymes or protective substances.

These materials were apparently inactivated during the drying process, leaving the lecithinase A free to act on the lecithin. Lecithinase A is also found in certain substances of vegetable origin, although its occurrence is apparently not as wide as in the animal kingdom. IWATA (66) found it in polished rice, and FRANCIOLI (54) obtained evidence for its occurrence in a fungus *Lycoperdon giganteum.*

The lecithinase B removes the remaining acid from lysolecithin; the enzyme appears to be present in higher fungi, in most animal organs and in blood. It is also found in some higher plants like soja (46), *Lolium perenne* (see chapter III) and leaves of potatoes. (46). The pH for the optimum activity of lecithinase B was determined by CONTARDI and ERCOLI (37) by noting the value at which the hemolytic activity of lysolecithin disappeared most rapidly. The value proved to be 3.5 which is quite different from the neutral value found to be the optimum for lecithinase A.

The enzyme which hydrolysed lecithin at the glycerol and phosphoric acid ester linkage may be referred to as lecithinase D. It was found by MACFARLANE and KNIGHT (95) in *Clostridium welchii;* the enzyme appears to be a component of the bacterial toxin elaborated by this organism.

The action of the lecithinases is summarized in the chart on page 176:

Besides lecithin sphingomyeline also occurs in the phosphatides of the brains and spinal cord; LEVENE (84) was the first to obtain relatively pure sphingomyelin and to report that the hydrolysis products consisted of phosphoric acid, choline, sphingosine and fatty acids (chiefly lignoceric acid).

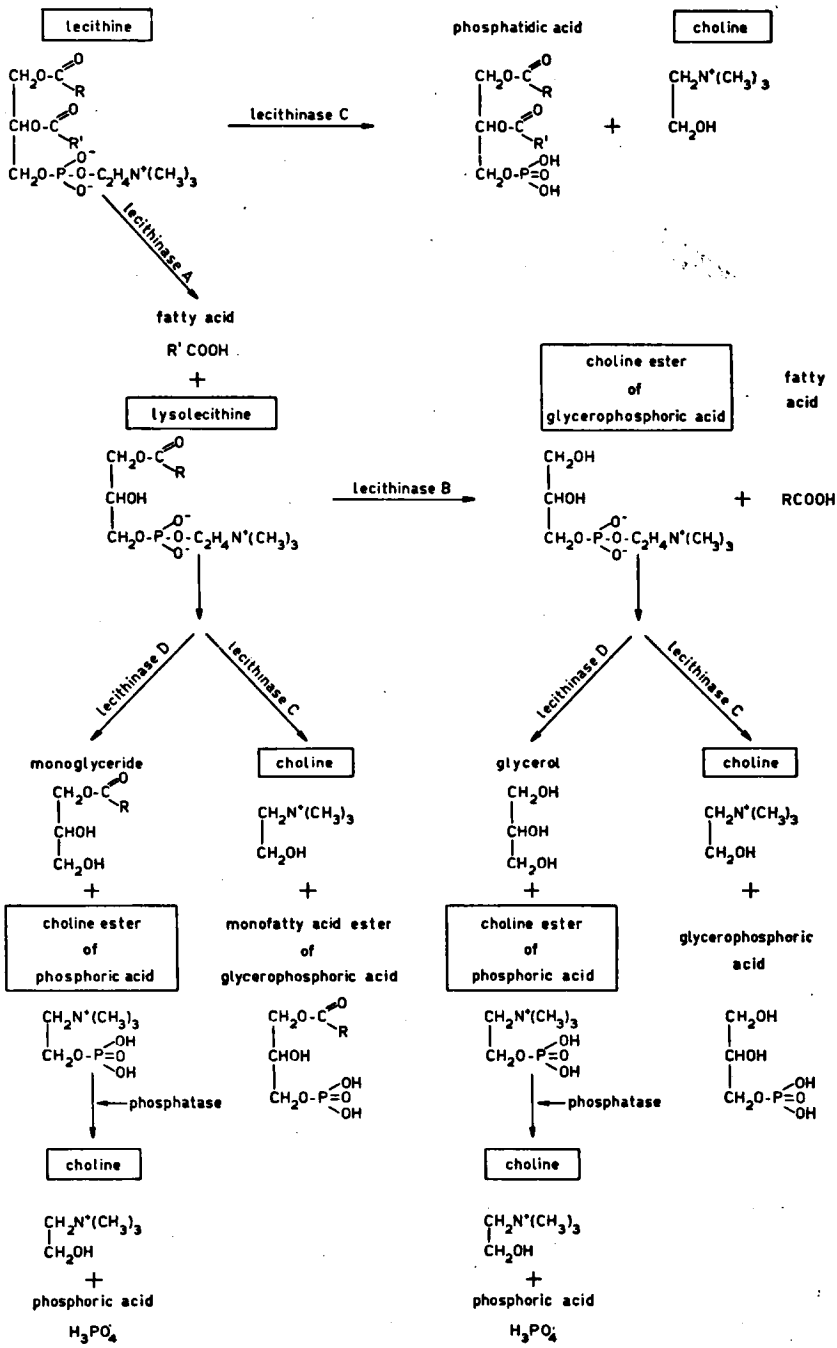
Very little is known about the biochemistry of sphingomyelin, save that in Niemann-Piek's disease the sphingomyelin content of the liver and the spleen increases greatly at the expense of the brain cerebrosides. Thus it appears that sphingomyelin plays a role in lipide metabolism (82, 133).

### 4.2. *Sinapine*

Sinapine is isolated from mustard (122); it occurs as the glycoside sinalbine which is composed of sinapine, glucose and p-oxybenzyl-mustardoil. Sinapine is the ester of choline and 3,5 dimethoxy-4-oxycinnamicacid (sinapine-acid); practically nothing is known about the metabolism of this compound.

### 4.3. *Acetylcholine*

Besides the role in the transmethylation reaction and in the lipide metabolism choline has still another function which is not yet too clearly understood. This involves the acetylation of choline in the organism to yield acetylcholine. The acetylation and deacetylation of choline seem to proceed at will in the animal, depending apparently on whether the blood pressure level is in need of lowering. DALE (41) showed that acetylcholine stimulates the parasympathic nerves as well as the sympathic ganglia and probably the autonomic ganglia. It seems to be the chemical agent involved in the transmission of nerve

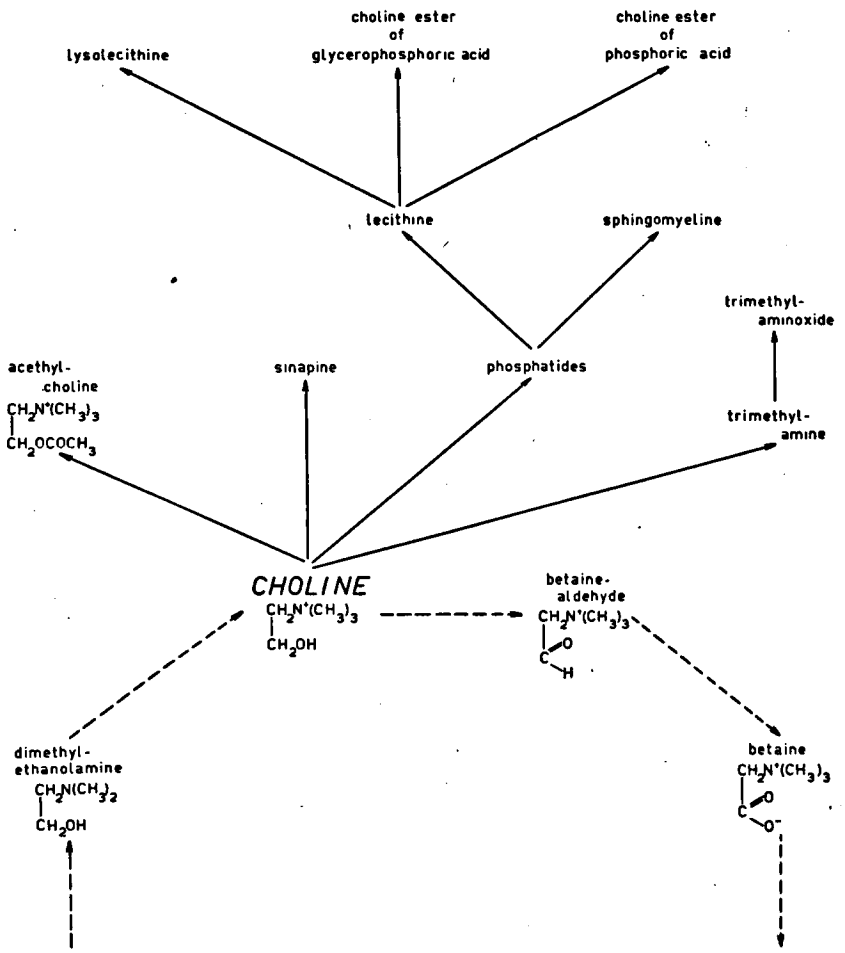

lecithine
lecithinase C
phosphatidic acid
choline
lecithinase A
fatty acid
R' COOH
lysolecithine
lecithinase B
choline ester of glycerophosphoric acid
fatty acid
RCOOH
lecithinase D
lecithinase C
monoglyceride
choline
glycerol
choline
choline ester of phosphoric acid
monofatty acid ester of glycerophosphoric acid
choline ester of phosphoric acid
glycerophosphoric acid
phosphatase
phosphatase
choline
choline
phosphoric acid
H3PO4
phosphoric acid
H3PO4

impulses to muscles. The enzyme cholineesterase is present in many tissues and readily hydrolyzes the acetylcholine, thereby controlling its action. A high concentration of the esterase is found at or near the surface of the nerve cell.

With regard to plants acetylcholine has been found in ergot (52) and in shepherd's purse (24), whereas it is also present in the squeezed juice of beets, turnips and gherkins. The presence of acetylcholine in the juice of sour gherkins and in the squeezed juice of silage would be caused by *Bacillus acetylcholini* (58, 76, 104).

## 5 Summary

Present knowledge regarding the metabolism of choline is summarized in the following chart:



## CHAPTER II

# DETERMINATION OF CHOLINE AND BETAINE IN VEGETABLE MATERIAL

### 1. INTRODUCTION

Choline may be found in plants either as free or bound choline; of the latter 2 forms are known viz. watersoluble bound and water insoluble bound or lipoid choline. SCHMIDT and collaborators (116) proved that watersoluble bound choline of the pancreas equals the choline ester of glycerophosphoric acid. On the analogy thereof one assumes that the watersoluble bound choline of the plant is the same compound; this has however not yet been proved. The water insoluble bound choline of the plant occurs as lecithin. Regarding the study of the metabolism of choline in plants it is desirable to be able to determine the quantities of free, watersoluble bound and of water insoluble bound choline, besides the quantity of total choline. Moreover, during the experiments on the dissimilation of choline by *Aspergillus niger*, it proved necessary to determine betaine also. This chapter will successively deal with the literature regarding the determination of free, watersoluble, waterinsoluble and total choline, the determination of betaine, after which a more detailed account will be given about the procedures of these determinations.

### 2. THE MOST IMPORTANT METHODS OF DETERMINING CHOLINE

Literature dealing with the determination of choline has been excellently summarized by GUGGENHEIM (57), LUCAS and BEST (89); we shall only confine ourselves at this stage to a short discussion on the various methods, at the same time referring to some later publications on this subject.

Choline can be determined either physiologically, microbiologically or chemically.

The oldest and most sensitive method is the physiological one [HUNT and DE TAVEAU (65)], which depends on the measurement of the contraction of a smooth muscle caused by acetylcholine. The activity of choline is far smaller than that of acetylcholine; the determination can be interrupted by alcohol, Ca, K, lecithin, traces of protein and cholesterol. By this method 0,1 $\gamma$ of choline can even be determined.

For the microbiological determination of choline HOROWITZ and BEADLE (64) use a "choline-less" mutant of *Neurospora crassa*. This they acquired from the wild form of this mould by radiation with ultraviolet light. On a nutritive-solution of sugars, salts and biotine the mutant showed very little growth unless small quantities of choline were added. At a certain degree of concentration the quantity of mycelium formed was proportional to the quantity of choline added to the nutritive-solution.

Several amino acids and water-soluble vitamins have been examined for their growth-promoting activity for this mutant; it appeared that

only methionine was able to promote growth slightly (approx. 1/500th part of the activity of choline); approx. 10 $\gamma$ choline can still be determined by this method.

The methods to determine choline chemically can be divided into:

*a*) determination of choline as trimethylamine. This method depends on the formation of trimethylamine out of choline by oxidation with potassium permanganate under alcaline conditions (87, 88, 131) or by boiling in concentrated lye (80, 81); after titration of the trimethylamine the quantity of choline can be calculated. The smallest quantity of choline to be determined by these methods approximates 1 mg; in that case however the average discrepancy is very large (approx 30 %) whereas quantities of 30–50 mg choline are necessary for a rather accurate estimation. To determine the percentage of choline present in moulds these methods are not suitable.

*b*) determination of choline as choline-reineckate. BEATTIE (9) uses this precipitate to determine choline colorimetrically; later JACOBI, BAUMANN and MEEK (67), ENGEL (50) and GLICK (56) introduced some modifications with the aim to increase the specificity of the determination (50, 67) and to counteract the loss of choline-reineckate caused by the washing of the precipitate (56). With this method choline is precipitated as choline-reineckate, the precipitate is sucked off, washed and after dissolving in acetone the intensity of the rose coloured solution is measured at 525 m$\mu$. Besides this maximum of absorption in the visible part of the spectrum a larger maximum of absorption exists at 327 m$\mu$ according to WINZLER and MESERVE (148) After measuring in the ultra-violet zone 50 $\mu$g of choline-chloride can still be determined, whereas the smallest quantity at 525 m$\mu$ is approx. 1 mg. The determination in biological material is not possible without pretreatment as besides choline other compounds may be precipitated. (48, 73, 130)

*c*) determination of choline as enneaiodide. The precipitation of choline with a solution of potassiumtriiodide has been described by STANEK (123); later several investigators (46, 47, 51, 108) used this reagens for the determination of choline. With these methods choline is precipitated with a surplus of the potassiumtriiodide under the formation of a greenish black cristalline precipitate of choline-enneaiodine. This is easily recognizable under a microscope by its characteristic cristalline shape of narrow obligue prisma's. After leaving some time in the refrigerator it is centrifuged and the above fluid is drained off. After rinsing some times with icewater the quantity I bound by choline is determined by titration with sodiumthiosulphate. The percentage of choline can be calculated from the formula of choline-enneaiodine [$I_8$. $IN(CH_3)_3.CH_2CH_2OH$]: per mol choline 8 atoms of I are titrated. With DUCET's method 0,5 mg choline-chloride can still be determined (46, 47); ERICKSON and collaborators (51) have increased the sensitivity of the determination 6 times by oxidising the I in the choline-enneaiodide to iodate.

*d*). The determination of choline with paperchromatograms. To procure the separation of the N-containing substances of the

lipoid fraction from animal material LEVINE and CHARGAFF (85, 86) used the paperchromatographic method; after hydrolysis of the lipoid fraction and removal of the fatty acids serine, ethanolamine and choline are separated paperchromatographically by developing with a mixture of butanol, diethyleneglycol and water. The place of choline on the paperchromatogram can be found after spraying with a solution of phosphomolybdic acid; the surplus of phosphomolybdic acid is removed and the choline-phosphomolybdate is reduced with stannous chloride after which a bluish spot appears. According to BRANTE (25) choline can also be located on the chromatogram by holding the paper in I-fumes; in accordance with this preliminary statement, the position of the amines, ethanolamines and betains can be located on the paperchromatogram. For the quantitative determination of the choline LEVINE and CHARGAFF (86) measured the surface of the blue choline spot on the chromatogram; between the logarithm of the choline concentration and the surface of the choline spot they found a linear relationship. The concentration of the choline solutions should be approximate 0.05–0,75 %. The exactitude of this method is limited by the accuracy with which the surface of the choline-spot can be measured.

## 3. DESCRIPTION OF THE METHODS USED

### *a* Determination of choline as reineckate.

Choline is precipitated from its solution by adding 6 ml of a freshly prepared 2% solution of ammonium-reineckate in methanol. After standing for 4 h in a refrigerator the formed choline-reineckate is sucked off on a fritted glass (SCHOTT $G_3$) filter and rinsed 3 × with 2.5 ml amylalcohol. Afterwards the choline-reineckate is dissolved in a known volume of acetone and the intensity of the rose coloured solution measured in the stufenphotometer with a S 53 filter.

The amount of choline is calculated by using a calibration-curve. According to ENGEL (50) the extinction is not proportional to the amount of choline-chloride; other investigators (56, 67, 98, 147, 148) found however that Beer's law was applicable, although the values found for the specific extinction-coefficients were not the same. Proceeding from choline-reineckate (prepared from pure choline-chloride by precipitating with ammonium-reineckate and recristallizing from a watery acetone-solution) we calculated the specific extinction-coefficients and found the values mentioned in table I.

The specific extinction-coefficient found by us shows the greatest equivalence with that found by WILLSTAEDT c.s. (147) who also used a stufenphotometer.

### *b* Determination of choline as enneaiodide.

To determine choline as enneaiodide 5 ml of a solution is transferred into a centrifuge-tube with a long drawn-out point, after which 1,5 ml potassiumtriiodide-solution (160 g $I_2$ and 240 g KI per liter) is added. Upon standing 4 h in a refrigerator it is centrifuged for 3 minutes and the surplus reagens drawn off over the precipitate with a double-bent capillary tube which at one end is fitted with a quick filtering filterpaper. The diameter of the capillary tube is such that the end connected with the small filterpaper can be brought within close range of the precipitate in

the extreme point of the centrifuge-tube, without however touching the precipitate. The inside of the centrifuge-tube is again rinsed with ice-water and care should be taken that no air is sucked through in order to prevent as much as possible the dissolution of the choline-enneaiodide. After washing, traces of enneaiodide which have been sucked up, are dissolved in alcohol from the filter and squirted into the centrifuge-tube. Upon solution of the choline-precipitate in more alcohol the quantity of bound I is titrated with 0,05 n sodiumthiosulphate. The calculation is based on 8 atoms I per mol choline-chloride i.e. 1 ml 0,050 N potassiumthiosulphate equals 0,875 mg choline-chloride.

TABLE 1

*Determination of the specific extinction-coefficient*

| mg choline-chloride per ml | mg choline-reineckate per ml | k 1 cm | Specific extinction-coefficient | |
|---|---|---|---|---|
| | | | calculated | Literature-values |
| 1,12 | 3,388 | 0,806 | 0,72 | 0,86 (143) |
| 0,84 | 2,541 | 0,596 | 0,71 | 0,80 (54, 95) |
| 0,56 | 1,694 | 0,400 | 0,71 | 0,736 (142) |
| 0,42 | 1,271 | 0,302 | 0,72 | 0,66 (65) |
| 0,21 | 0,635 | 0,154 | 0,73 | |
| 0,14 | 0,424 | 0,103 | 0,74 | |
| | | Average | 0,72 | |

## 4. The preparation of biological materials before the determination of total choline

Before determining total choline an extraction and hydrolysis should first take place.

The extraction of free and bound choline from biological materials has been investigated by Engel (50). He acquired his highest results after 24 hours of extraction with methanol. He then saponified the residue of the methanol extract by heating to 100° for 2 hours with a saturated solution of barite; after neutralising with acetic acid and filtrating choline is determined as reineckate.

It appears however that with this method as with the modification of Glick (56) choline-reineckate is greatly polluted by the accompanying materials; after dissolving the reineckate in acetone one obtained a cloudy solution which did not clear after filtration. The method was therefore altered. By clearing with lead-acetate after saponifying with barite a precipitate of fine crystalline choline-reineckate was obtained which was not polluted by other materials and when dissolved in acetone gave a completely clear solution. The determination of total choline proceeded as follows:

1 g dried and powdered mycelium of *Aspergillus niger* is extracted for 20 h in a sohxlet with absolute methanol. Upon evaporation of the methanol the residue is saponified with a 30 ml of a saturated barite solution on heating for 2 h at 100°; after cooling the pH is brought at 4 with acetic acid and cleared by adding ½ g lead-acetate dissolved in a little water. The precipitate is then sucked off over a asbestos filter and rinsed; surplus lead in the filtrates is removed with 10 ml of a 10% solution of secondary sodiumphosphate. Afterwards the pH is put at 8 with lye and the precipitate of lead- and bariumphosphate is sucked off over an asbestos-filter. After washing the precipitate the combined filtrates are evaporated and finally filtrated; its volume then amounts to approx. 40 ml. With this solution

choline can only be determined as a reineckate; not as an enneaiodide as the presence of disturbing materials prevents this.

A completely different method was used by DUCET (46); it is based on the fact that choline and betaine in comparison with various other organic N-compounds are much more stable with regard to boiling mineral acids. The material is boiled with diluted nitric acid which liberates the bound choline; moreover a number of organic compounds which interfere with the determination of choline, are decomposed. A clarification then follows with calciumcarbonate and ferrisulphate acc. to LUNEAU (90).

The determination of total choline proceeds as follows:

1 g dried and pulverized mycelium of *Aspergillus niger* is refluxed 4 h with 2½ ml nitric-acid and 5 ml water; about ½ g of norit *) is added, the mixture neutralised with calciumcarbonate and after heating cleared with 10 ml of a 50% ferrisulphate solution. After boiling again some calciumcarbonate is added and the precipitate which consists chiefly of calciumsulphate, calciumcarbonate, ferrihydroxide and norit is drawn off. The precipitate resuspended in water, again boiled and drawn off after which the procedure is repeated another time. After evaporation of the filtrates on a waterbath the concentrate is boiled with some norit, drawn off, whereafter the precipitate of norit is boiled 3 times with warm water; the filtrates are filled to 25 ml. In an aliquot part of the solution choline can be determined as reineckate or as enneaiodide.

## 5. DETERMINATION OF THE DIFFERENT CHOLINE-FRACTIONS

As far as we know KLEIN and LINSER (80, 81) were the first to try to determine the choline-fractions in plants; to accomplish this they made use of the slight solubility of free choline in dry ether. According to this method an extract of alcohol is shaken with ether and water; after separation part of the ether-layer is evaporated and the lipoid bound choline determined. One of the objections to this method is that a correction must always be made for the free choline dissolved in the moist ether. This difficulty is avoided by DUCET's method (46); the material is boiled with water and the resulting suspension is cleared with calciumcarbonate and ferrisulphate.

The filtrate contains free and watersoluble bound choline. In this filtrate free choline can be directly determined; upon hydrolysis with diluted nitric acid the total amount of watersoluble choline can be found. Moreover it is possible after adsorption to silicagel on determine the watersoluble bound choline directly.

Lipoid bound choline can be determined by eliminating the surplus calciumcarbonate and ferrihydroxide from the clearing-precipitate, treating the residue with nitric acid or sulphuric acid and determining the freed choline. The separation was made as follows:

1 g dried and powdered mycelium and a little calciumcarbonate are boiled with water until every particle is properly moistened. This mixture is then cleared by adding 5 ml of a 50%-solution of ferrisulphate and some calciumcarbonate; after boiling a moment the solution is drawn off. The precipitate is resuspended in water and again drawn off after boiling. This procedure is once more repeated. Water soluble choline can be found in the combined filtrates whereas the lipoid bound

*) Medicinal norit of the General Norit Co. Ltd. at Amsterdam showed a marked discoloration whilst no choline was adsorbed.

choline is present in the clearings-precipitate. In order to fraction free and water-soluble choline the combined filtrates are evaporated on the waterbath up to approx. 2 ml. Then choline is separated from the calciumnitrate present by an alcohol-procedure: 40 ml of alcohol are added, heated and shortly afterwards drawn off. The precipitate of calciumnitrate is again partly solved with a little water and after addition of 40 ml alcohol heated and drawn off. This procedure is again repeated. The combined alcoholic filtrates are then evaporated to dryness and the residue taken up in water and brought to a certain volume (generally 11 ml).

To 5 ml of this volume 1,5 ml potassiumtriiodide-reagens is added in order to determine free choline.

The quantity of total water soluble choline is determined by saponifying 5 ml of this solution by boiling for 1 h with 2,5 ml concentrated nitric acid, then neutralising it with calciumcarbonate and after drawing off, washing the precipitate 3 times with warm water; on evaporation of the filtrates on the waterbath the water soluble choline can be determined as enneaiodide in the remaining liquid.

Determination of the lipoid bound choline is a rather tedious procedure. For routine determinations it is sufficient to take the difference between the total and water soluble choline. It is possible to check this however:

The clearings-precipitate is treated with concentrated hydrochloric acid until the surplus of calciumcarbonate and ferrihydroxide are dissolved. On filtrating through a plyfilter the precipitate is washed free of acid and the filter together with the precipitate are then placed in a glass dish into which sulfuric acid is poured. After standing overnight at 105° water is added to the mixture under stirring; the mash obtained is neutralized with calciumcarbonate. On drawing off the precipitate is suspended in water and drawn off after boiling; this procedure is repeated once again. The combined filtrates are evaporated, boiled for 8 h with 5 ml of concentrated nitric acid, cleared with calciumcarbonate and evaporated after which the lipoid bound choline can be determined in an aliquot part.

## 6. Determination of betaine in the presence of choline

Glycine-betaine $C_5H_{11}O_2N$ $[(CH_3)_3\,N.CH_2COO^-]$ crystallizes with one mol of crystalwater; owing to a great similarity in the formulas of choline and betaine several reagentia show the same precipitation-reactions with these two compounds. In principle, however, it is possible to separate the reineckate- or the periodide compounds from the choline and betaine, as betaine has a potential carboxylgroup and choline an alcoholgroup; on account of this in a weakly alcaline or neutral solution only the choline-reineckate or -periodide compounds are precipitated, whereas in an acid environment the betaines are also precipitated. A technical difficulty on determining the betaines arises however owing to the fact that the reineckate and the periodide compounds of the betaines are more easily soluble than the corresponding compounds of choline; owing to this the separation and rinsing of the betaine-precipitates becomes more difficult.

The separation of choline from the betaines via the periodide has already been described by Stanek (124); whereas later Blood and Cranfield (20) and also Reifer (108) have worked out a determination of glycine-betaine as periodide besides choline.

According to our experience the reineckate method is not very suited for the determination of betaine owing to the relatively great solubility of the betaine-reineckate. Walker and Erlandsen (145) also could not isolate the betaine-reineckate quantitatively. Quantities of 2 to 20 mg of betaine can be determined by this method

with an accuracy of 4 %. Large quantities of mycelium of *Aspergillus niger* are required in order to determine betaine. That is why we have used REIFER's periodide method for our investigation. When betaine in the presence of choline was determined in the mycelium of *Aspergillus niger* it appeared that after a treatment with nitric acid according to DUCET (46) no clearly defined endpoint of titration with thiosulphate could be obtained. The reason is probably that this determination takes place in an acid environment and the traces of nitric acid still present in the clarified solution have a disturbing influence. To avoid this difficulty we neutralised with $BaCO_3$ instead of $CaCO_3$ after the nitric acid treatment; the solubility, namely of bariumnitrate is considerably less than that of calciumnitrate (resp. 8.7 at 20° and 341 at 25°). In order to remove the still present bariumnitrate we used an alcohol-treatment after the cleared filtrates had been evaporated. The determination then proceeds as follows:

1 g of dried mycelium of *Aspergillus niger* is treated with nitric acid and the extract cleared; bariumcarbonate however is used instead of calciumcarbonate to neutralize. On evaporation of the cleared liquid an alcohol treatment is used as with the determination of free choline (see page 183). After evaporation of the alcohol the residue is taken up in water, discolored with norit and filled to a given volume. In an aliquot part of this liquid choline is then determined as an enneaiodide.

To determine choline plus betaine 2 ml of the liquid is poured into a centrifuge-tube, then 1 ml of a 30% sodiumchloride solution is added, in order to reduce the solubility of betaine-periodide, and mixed. Next 0,5 ml of concentrated phosphoric acid is slowly added at the wall of the tube so that this settles as a separate layer at the bottom. This protects the subsequently formed periodide-precipitate during the rinsing. Then 1 ml of a potassiumtriiodide solution according to REIFER (108) (60 g $I_2$, 120 g KI and 100 g NaCl per liter water) is added and the top layer stirred with a glass rod. On standing for 3 h at approx. — 10° C, it is centrifuged as a result of which, the betaine-periodide precipitate joins the phosphoric acid layer; removal of- and rinsing of the excess reagens in the upper layer proceeds as in the choline determination. After dissolving the precipitate in alcohol the I bound by choline and betaine is titrated with sodiumthiosulfate. According to the formula of the betaine-periodide $C_5H_{11}O_2N.HI.I_5$ 5 atoms of I correspond with 1 mol betaine-HCl or 1 ml 0,0500 N thio equals 1,535 mg betaine-HCl.

On interpreting the results of the betaine-determination of REIFER (108) various difficulties arisé which we will explain more fully on page 189.

## CHAPTER III

# SOME RESULTS WITH THE DETERMINATION OF CHOLINE AND BETAINE IN VARIOUS MATERIALS

### 1. COMPARISON BETWEEN THE CHOLINE-DETERMINATION VIA REINECKATE AND VIA ENNEAIODIDE

We deemed it advisable to determine the quantity of total choline in a number of products according to the method of ENGEL (50) as well as to the method of DUCET (46), because a comparison between these methods is lacking in the literature. In the first place we

investigated whether the same value was acquired with the determination via reineckate in a solution of pure choline-chloride as with the determination via the enneaiodide (Table 2). Choline-chloride of Hoffmann-Laroche and choline-chloride that we isolated from the dregs of soya were used as reference substances. As choline-chloride is extremely hygroscopic the concentrations of these solutions were calculated from the N-percentages determined according to Kjeldahl. It should here be pointed out that REIFER (108) only succeeded in finding part of the theoretical quantity of nitrogen; prolongation of the destruction-period or the use of other catalysts brought no improvement. STANEK (123) and KIESEL (78) obtained the theoretically expected quantities of N in the precipitates of ennea-iodide; TERMEULEN and RAVENSWAAY (103) too found the right values. As a catalyst for the destruction we used a mixture of Se, $CuSO_4$ and $Na_2SO_4$; we found only a part of the total amount of nitrogen in betaine or choline. However when 200 mg glucose was added before the destruction the theoretical values were obtained.

The isolation of choline from the dregs of soya was executed as follows:

140 gr of dregs of soya was extracted with chloroform and the extract of chloroform poured out in a sixfold volume of acetone. After standing for 1 h. in the dark the lecithin was drawn off and hydrolysed by boiling 4½ h. with diluted hydrochloric acid. This hydrolysed mixture was evaporated to dryness on the water-bath and the residue extracted with 100% alcohol. After treatment with norit this filtrate was concentrated in vacuo, then diluted with water and the pH of the solution adjusted at 8. After this ammonium-reineckate (dissolved in alcohol) was added until the supernatant liquid turned dark-red. After standing in the refrigerator the choline-reineckate formed was drawn off and washed with cold alcohol. After drying the meltingpoint of the reineckate was 254° under decomposition. The choline-reineckate was decomposed by adding the calculated quantities of silver-sulphate and bariumchloride [KAPFHAMMER c.s. (19)]. The choline solution was brought to a certain volume.

TABLE 2

*Estimation of choline as a reineckate or as an enneaiodide in a solution of pure choline-chloride*

| choline-chloride preparation | mg choline-chloride calculated from N-content according to Kjeldahl | mg choline-chloride precipitated as | |
|---|---|---|---|
| | | reineckate | enneaiodide |
| isolated from the dregs of soya | 1,71<br>1,77 | 1,70<br>1,72 | 1,76<br>1,74 |
| Hoffmann-Laroche | 2,06<br>2,08 | 2,06<br>2,10 | 2,03<br>2,07 |

Table 2 shows that the choline-estimation via the reineckate and via the enneaiodide produces equal values in a pure solution.

Thereafter we compared the hydrolysis and clearing according to DUCET (46) with the process according to ENGEL (50).

It appeared impossible to estimate choline as an enneaiodide after a pretreatment according to ENGEL as the precipitate produced by

adding the potassium triiodide-reagens, did not completely dissolve in alcohol. In the liquid after the pretreatment according to ENGEL the with potassiumtriiodide interfering compounds are not sufficiently eliminated.

Table 3 shows the results acquired with a number of products. Both methods of estimation produced similar results.

TABLE 3

*Quantities of total choline in some feeding stuffs and in the mycelium of Aspergillus niger* (mg. choline-chloride per 100 g. air-dried material)

| Products | Intake (g) | Pretreatment acc. to ENGEL (48) and estimated as reineckate | Pretreatment acc. to DUCET and KAHANE (45) and estimated as | |
|---|---|---|---|---|
| | | | reineckate | enneaiodide |
| Lolium perenne . . . | 1 | 173 | 169 | 171 |
| potatoes . . . . . | 1 | — | 120 | 120 |
| steamed potatoes . . . | 1 | 98 | 100 | 100 |
| livermeal . . . . . | 0,5 | 857 | 851 | 821 |
| tankage . . . . . | 1 | 270 | 284 | 273 |
| dried carrots. . . . . | 1 | — | 88 | 89 |
| dried yeast . . . . . | 0,5 | — | — | 459 |
| dried mycelium of *Aspergillus niger*. . . . | 0,5 | — | 650 | 668 |

The results of a number of determinations where choline has been added to dried mycelia of *Aspergillus niger* are shown in table 4; mycelia without the addition of choline served as controls. On account of the fact that the total quantity of choline added is recovered it follows that the method of determination employed produces reliable and reproductive figures. The deviation of the quantities recovered varies from —4 to + 2 %.

TABLE 4

*Recovery of the quantity of choline added to dried mycelium of Aspergillus niger.* (3 ml of a solution of choline-chloride was added (1,05 mg/ml)).

| Intake mycelium (mg) | mg choline-chloride in mycelium | | Recovery | |
|---|---|---|---|---|
| | with addn. of choline (3,15 mg) | without addn. of choline | mg/ml soln. | % |
| 500 | 5,34 | 2,30 | 1,01 | 96 |
| 400 | 5,05 | 1,88 | 1,06 | 101 |
| 400 | 5,76 | 2,68 | 1,03 | 98 |
| 400 | 6,32 | 3,17 | 1,05 | 100 |
| 400 | 6,10 | 2,89 | 1,07 | 102 |

## 2. DETERMINATION OF THE CHOLINE FRACTIONS IN HIGHER AND LOWER PLANTS

As has been mentioned before it was desirable in order to obtain some insight in the metabolism of choline in *Aspergillus niger* to determine the forms of choline which occur in the mycelium.

This separation has been made not only in the *Aspergillus niger* but

also in grass, soy-flour and dried yeast; the results have been laid down in table 5:

TABLE 5

*Determination of choline-fractions in grass, soy-flour, dried yeast and mycelium of Aspergillus niger.* (mg choline-chloride per 100 g air-dry material)

| Products | Lolium perenne | | Soyflour | | Dried yeast | | Dried mycelium of Asp. niger | |
|---|---|---|---|---|---|---|---|---|
| | mg | % | mg | % | mg | % | mg | % |
| Total choline . . . . | 175 | 100 | 231 | 100 | 459 | 100 | 572 | 100 |
| Lipoid bound choline . | 98 | 56 | 22,5 | 10 | 17 | 4 | — | 22 |
| Total watersoluble choline . . . . . | 76,5 | 44 | 183 | 81 | 432 | 94 | 447 | 78 |
| Free choline . . . . . | 65,5 | 37 | 127 | 59 | 15 | 3 | 18 | 3 |
| Watersoluble bound choline (calculated) | — | 7 | — | 22 | — | 91 | — | 75 |

On studying the values of watersoluble bound choline a marked difference is seen: it appears that the mycelia examined contain a large quantity of watersoluble bound choline (91 % of yeast and 75 % of the total amount of *Aspergillus niger*) whereas in *Lolium perenne* and soy-flour these values are 6 resp. 22 %. In so far as one is allowed to draw conclusions from these few analyses one would be inclined to suppose that in the higher plants choline is generally present in a lipoid bound form (lecithin), whereas in mycelia choline is mostly present in a watersoluble bound form. KAHANE and LEVY (74) point out that the foot of the snail *Patella vulgata* especially contains watersoluble bound choline; they have tried to isolate this form of choline in this material, but their material was lost owing to the war (75). To start a new investigation on the exact composition of watersoluble bound choline the lower plants provide a material which is easier obtained. The high percentage of watersoluble bound choline in yeast and in *Aspergillus niger* equals a low percentage of lipoid bound choline. In *Lolium perenne* we found 56 % of the total amount of choline to be lipoid bound and in soy-flour only 10 %. This low figure is probably due to the action of lecithinase C described by HANAHAN and CHAIKOFF (61) (See Chapter I,4) through which choline is separated from lecithine. We have tried to verify this in *Lolium perenne* by assessing the different fractions of choline in the fresh and artificially dried material. For comparison the fractions in an old sample dried with hot air have also been mentioned (table 6).

It appears that a similar enzyme is active in *Lolium perenne*, in fresh material the quantity of lipoid choline averages 78 %, after drying quickly at 105° 56 % and after drying more slowly in a hot current of air 27 % of the total quantity, whereas in an old sample of 1941 this only averaged 18 %.

DUCET (46) has also shown an activity of lecithinase C in potato and chestnutleaves and in the soy-plant by determining the quantity of free and lipoid bound choline in fresh and in dried material.

To investigate the action of lecithinase C in the mycelium of *Aspergillus niger* we have determined the choline fractions in fresh mycelium and in mycelium dried at 105°; the results are mentioned in

TABLE 6

*Influence of lecithinase on the choline fractions in Lolium perenne.*
(mg choline-chloride per 100 g air-dry material)

| Date Samples | 30-9-1948 | | | | | | 1941 | |
|---|---|---|---|---|---|---|---|---|
| Pretreatment | no (fresh) | | dried with hot air of ± 70° C | | dried in oven at 105° C | | dried with hot air of ± 70° C | |
| | mg | % | mg | % | mg | % | mg | % |
| Total choline . . . . | 171 | 100 | 171 | 100 | 175 | 100 | 179 | 100 |
| Lipoid choline . . . . | 134 | 78 | 47 | 27 | 98 | 56 | 32½ | 18 |
| Total watersoluble choline . . . . . | 37 | 22 | — | 73 | 76 | 44 | 146½ | 82 |
| Free choline . . . . . | — | — | — | — | 66 | 37 | 116 | 65 |
| Watersoluble bound choline (calculated) . | — | — | — | — | — | 7 | — | 17 |

table 7; the quantities of free choline are small in both cases (6 resp. 12 % of the total). The fractioning of choline during the investigation of the synthesis of choline (Chapter V) was therefore performed by us in dried mycelia because here the action of lecithinase has not been found.

TABLE 7

*The proportional relationship of the choline fractions in fresh mycelium and in mycelium dried at 105° C of Aspergillus niger of Tieghem*

| Days incubated | 4 | 7 | 9 | 12 | |
|---|---|---|---|---|---|
| | Fresh mycelium | Fresh mycelium | Fresh mycelium | Fresh mycelium | Dried mycelium |
| Total choline . . . | 100 | 100 | 100 | 100 | 100 |
| Lipoid choline (calculated) . . . | 42,5 | 22 | 21 | 20 | 17 |
| Total watersoluble choline . . . . | 57,5 | 78 | 79 | 80 | 83 |
| Free choline . . . | 5 | 3 | 16 | 6 | 12 |
| Watersoluble bound choline (calcd.) . | 52,5 | 75 | 61 | 74 | 71 |

### 3. Determination of betaine in the presence of choline

In the first place the exact degree of accuracy with which small quantities of betaine can be determined was investigated. For this purpose 307,0 mg of betaine-HCl was dissolved in 50 ml of a 30 % solution of sodium chloride; 5 ml of this solution determined by titration with lye contained 29,6 mg and calculated from the percentage of nitrogen according to Kjeldahl contained 30,7 mg betaine-HCl. Subsequently the stock solution was diluted 10 times and the quantity betaine determined according to Reifer (108) in 1,2 and 3 ml of the

diluted solution (table 8). From this it is apparent that betaine-HCl can be determined up to 2 mg with a difference of 0.08 mg.

TABLE 8

*Determination of betaine according to* REIFER (*108*)

| Intake (mg betaine-HCl) | mg betaine-HCl calculated | | |
|---|---|---|---|
| | acidimetric titration | N-determination acc. to Kjeldahl | iodometric determination acc. to REIFER |
| 30,7 | 29,6 | 30,7 | — |
| 0,61 | — | — | 0,61 |
| 1,23 | — | — | 1,15 |
| 1,84 | — | — | 1,81 |

Subsequently betaine in the presence of choline was determined in the mycelium of *Aspergillus niger*; in order to check this we also investigated whether betaine-HCl added before treatment with nitric acid and before the precipitation with potassium triiodide-reagens were recovered.

With reference to table 9 the following may be noted:

1° The quantity of betaine present in the mycelium is minute in respect to that of choline (resp. 0,77 and 4,38 mg per 400 mg dried mycelium).

2° The 1,23 mg of betaine-HCl added before precipitation was recovered quantitatively.

3° On addition of 3,69 mg betaine-HCl before the nitric acid treatment 3,64 mg has been recovered; if before the precipitation another 1,23 mg of betaine-HCl is added, the recovery amounts to 103 %.

4° The determination of betaine in the presence of choline according to REIFER's method (108) can only be used accurately if the quantity of betaine is large compared with that of choline, for betaine is found as the difference of the titration-values after precipitation in acid (betaine plus choline) and in alcaline conditions (choline). When the quantity of betaine is small in comparison with choline, the betaine is calculated from the difference between two large numbers; the result is a limited accuracy. Table 9 shows that the titration value of betaine-HCl amounts to 0,04 ml 0,05 N thio per 2 ml. A deviation of 0,02 ml in the titration value would already have given rise to a change of 50 % in the percentage of betaine of the mycelium. Moreover the interpretation of the results of the betaine estimation according to REIFER (108) gives rise to several difficulties. Besides the betaine of glycine other betaines (e.g. trigonelline) might be present. This is difficult to prove because the periodides of the betaines,—in contrast to that of choline—, do not have a typical crystalform. The presence of several betaines could be proved by way of paperchromatography. When more betaines are present the calculated value of glycine-betaine will be too high. Glycine-betaine is precipitated as a periodide with 5 titratable I-atoms, trigonelline with 3. When more betaines are present nothing can be calculated from the titration figures. We

TABLE 9

*Determination of betaine and total choline in mycelium of Aspergillus niger*

(400 mg mycelium pretreated acc. to DUCET (44); endvolume 25 ml; 1 ml 0,05 N thio ~ 0,875 mg choline-chloride or 1,535 mg betaine-HCl)

| Intake | Choline-chloride ml thio titrated for 3 ml of the endsoln. | Choline-chloride + betaine-HCl ml thio titrated for 2 ml of the endsoln. | Betaine-HCl ml thio titrated for 2 ml of the endsoln. | mg choline-chloride per 400 mg mycelium | Mg betaine-HCl | | Recovery betaine-HCl | |
|---|---|---|---|---|---|---|---|---|
| | | | | | per 2 ml of the endsoln. | per 400 mg mycelium | mg | % |
| | I | II | III = II - 2/3.I | IV=I. 0,875 $\frac{25}{3}$ | V=III. 1,535 | VI=III. 1,535 $\frac{25}{2}$ | | |
| A mycelium . . . . . | 0,60 | 0,44 | 0,04 | 4,38 | 0,06 | 0,77 | — | — |
| B mycelium + 1,23 mg betaine-HCl added to 2 ml before precipitation | 0,60 | 1,25 | 0,85 | 4,38 | 1,30 | — | 1,24[1]) | 101 |
| A mycelium . . . . . | 0,60 | 0,44 | 0,04 | 4,38 | 0,06 | 0,77 | — | — |
| B mycelium + 3,69 mg betaine-HCl before nitric acid attack . . . . | 0,61 | 0,635 | 0,23 | 4,45 | 0,35 | 4,41 | 3,64[2]) | 99 |
| C mycelium + 3,69 mg betaine-HCl before nitric acid attack + 1,23 mg betaine-HCl added to 2 ml before precipitation . . . . . | 0,61 | 1,465 | 1,06 | 4,45 | 1,63 | — | 1,28[3]) | 104 |

[1]) $V_B - V_A$. [2]) $VI_B - VI_A$. [3]) $V_C - V_B$.

used this method, however, for the investigation of the dissimilation of choline in *Aspergillus niger*, because under these conditions the quantities of betaine are of the same order as those of choline (see table 12 and 13); moreover under normal conditions the quantity of betaine in this mould is very small.

The results of some measurements of the quantities of choline and betaine in vegetable stuffs are given in table 10.

TABLE 10

*Quantities of choline and betaine in mycelium of Aspergillus niger, in Lolium perenne and in molasses*

| | Choline-chloride | Betaine-HCl |
|---|---|---|
| Mycelium A. niger on normal nutritive solution | 6,3 mmol per 100 g dry matter | 0 mmol per 100 g dry matter |
| id. on nutritive solution [1]) 12,8 mmol added choline-chloride | 6,7 mmol per 100 g dry matter | 8,4 mmol per 100 g dry matter |
| id. on nutritive solution [2]) 11,5 mmol added betaine-HCl | 6,0 mmol per 100 g dry matter | 8,3 mmol per 100 g dry matter |
| Lolium perenne . . . . . | 1,2 mmol per 100 g dry matter | 4,0 mmol per 100 g dry matter |
| Molasses. . . . . | 0,3 mmol per 100 g | 9,3 mmol per 100 g |

[1]) In the nutritive-solution still 1,9 mmol choline-chloride and 0,9 mmol betaine-HCl present.

[2]) In the nutritive-solution still 0,0 mmol choline-chloride and 3,9 mmol betaine-HCl present.

## CHAPTER IV

# THE METABOLISM OF CHOLINE IN ASPERGILLUS NIGER: CHOLINE-OXIDASE

### 1. CHOICE OF ORGANISM; METHOD OF CULTIVATION; CHOLINE-CONTENTS AND AGE OF MYCELIUM

In order to study the metabolism of choline in plants we chose *Aspergillus niger* of Tieghem. Preliminary investigations had shown that the percentage of choline present in this mycelium was rather high (approx. 0,6 % of the dry stuff).

The stock cultures of the mould were cultivated on an agar medium of the following composition: malt-agar (Difco) 45 g, asparagine 2 g, peptone 10 g, water 1 l. After inoculation the tubes were incubated during 3 weeks at 25° C. Afterwards a sporesuspension was made by collecting the spores of about 20 tubes in 120 ml sterile water. Cultures on a basal medium of the following composition were used: saccharose 40 g, glucose 10 g, ammonianitrate 10 g, primary potassium phosphate 5 g, magnesiumsulfate 7 $H_2O$ 2,5 g, zincsulphate 7 $H_2O$ 50 mg; ferrichloride trace; water 1 l. In each erlenmeyerflask of 100 ml capacity 25 ml of basal medium was brought together with 2 ml of the sporesuspension; temp. 25° C.

In very young mycelia of about 3 days old the average deviation

of the dry stuff amounted to approx 6 %; in older cultures (5–7 days) the average difference is smaller (approx. 1 %). For choline-determinations mycelia of three or more flasks were dried together at 105° C.

In order to investigate the metabolism of choline during the development of *Aspergillus niger* quantities of total, watersoluble bound, lipoid and free choline were determined in a series of cultures (fig. 1).

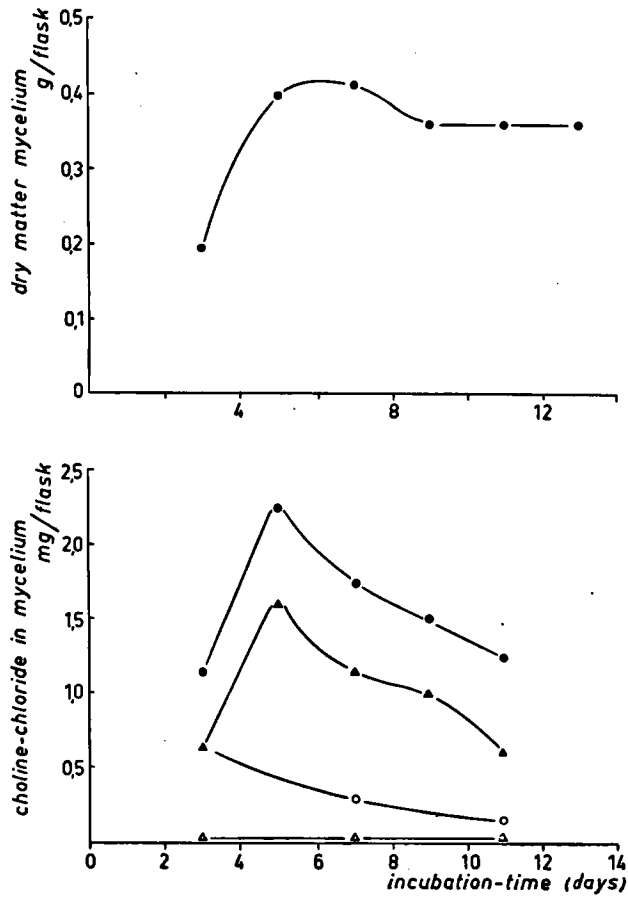



Fig. 1. Relation between the choline contents and the age of mycelia of *Aspergillus niger* (•——• total choline; ▲——▲ watersol. bound choline; O——O lipoid choline; △——△ free choline)

The quantities of total and watersoluble bound choline attain their maximum in the mycelia of this series after 5 days, whereafter a rapid decrease takes place. The lipoid bound choline also decreased when the mycelia grow old; the free choline is only a small part of the total choline present. Even after an incubation of 12 days the solution contained only very small quantities of choline. That is why in many cases we only determined the quantities of choline in the mycelia.

## 2. Investigation to the occurrence of a choline-oxidase in Asp. niger

In order to study the dissimilation of choline we took young actively growing cultures. This was done in order to prevent that part of the contents of the cell would permeate into the basal-solution. This is one of the difficulties with older cultures caused by the dying-off of part of the mycelium. The nutritive solution was always replaced by a basal solution lacking carbohydrates. The influence exerted by this impoverishment is shown in fig. 2; 24 hours after the impoverishment a synthesis of choline still takes place owing to the reserve-substances present in the mycelium. Later on the amount of total choline decreases.

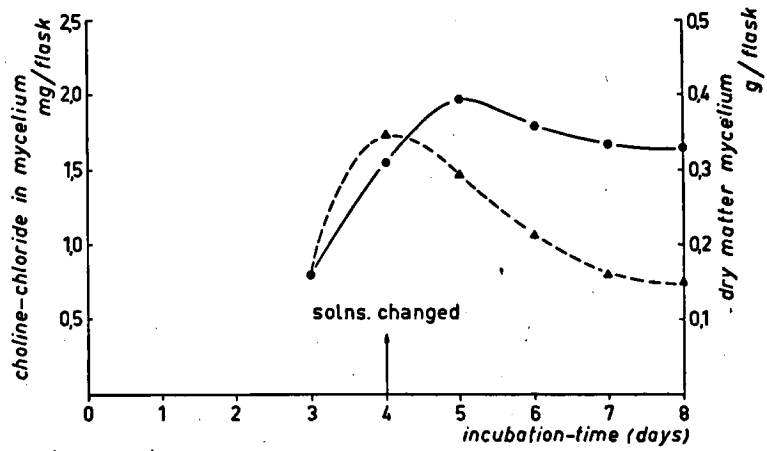



Fig. 2. Influence of changing the culture-soln. by a basal-soln. lacking carbohydrates on the choline contents of mycelia of *Aspergillus niger* (•——• total choline; ▲----▲ dry matter)

As during the impoverishment the quantity of dry material of the mycelium decreases much more than the amount of choline it is probable that this group of compounds is not easily taken up in the metabolism.

We investigated the dissimilation of choline by adding free choline to the basal solution without carbohydrates. After an incubation period of 97 hrs. the culture liquid was replaced by a basalsolution without carbohydrates; moreover choline was added to some of the cultures. Fig. 3 shows that the greater part of the choline added (1,9 mg) disappeared from the solution after 25 hours, whereas the total percentage of choline of the mycelium is only slightly more (0,3 mg) than that of the control cultures. The added choline is apparently not aerobic converted into watersoluble- or lipoid bound choline.

It has been seen from literature mentioned in Chapter I that the most likely reactions during the dissimilation of choline are an oxidation to betaine or a degradation under separation of trimethylamine. We

have first investigated whether the added quantity of choline was converted into betaine.

Some preliminary experiments showed that this was indeed the case.

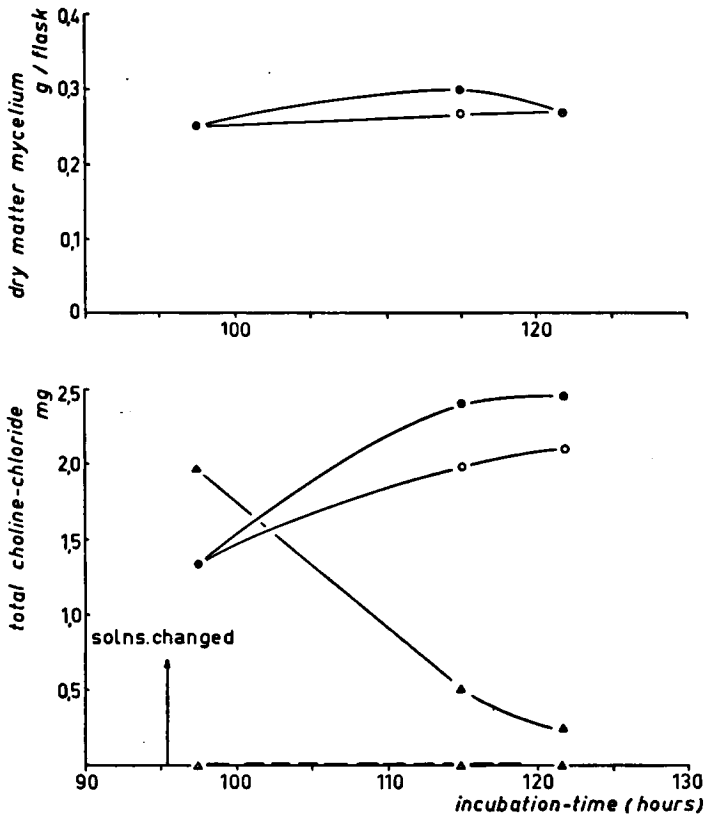



Fig. 3. Choline contents of mycelia and nutritive-solns. of cultures of *Asp. niger* after impoverishment in carbohydrates at 97 h. (•——• with choline; O——O without choline; ▲——▲ choline in nutr.-soln. after addn.; Δ----Δ id. without addn.)

Table 11 shows the results of a series of experiments performed with the aim to investigate the conversion of choline in betaine through *Aspergillus niger*. The liquid under the mycelium in a number of flasks was replaced after an incubation of 120 hrs. by a solution without carbohydrates; moreover in the series II, III and IV resp. 0,055 mmol choline-chloride, 0,050 mmol betaine-HCl and 0,055 mmol choline-chloride plus 0,050 mmol betaine-HCl was added. The addition of betaine was necessary in order to investigate the possibility that the betaine formed from choline was converted afterwards into other compounds. Besides choline betaine was determined too in the mycelium and in the culture medium.

With regard to table 11 the following may be noted:

1. The quantity of choline plus betaine has increased in 24 hours

of incubation after the solution has been renewed from 0,021 to 0,026 mmol per flask (series I). It is not absolutely certain that this is also the case in series II, III and IV where choline and (or) betaine was added. For simplicity's sake we based our calculation of the total

TABLE 11

*Conversion of choline into betaine by A. niger on a basalsolution lacking carbohydrates.* (After 120 hrs. of incubation a new basalsolution without carbohydrates was brought under the mycelium; moreover in series II, III and IV resp. choline-chloride, betaine-HCl and choline-chloride plus betaine-HCl was added, thereafter incubated for 24 hrs.; quantities in mmol per flask; for each determination 4 flasks were taken)

| | dry matter (g) | choline-chloride (mmol) | betaine-HCl (mmol) | total mmol per flask | total mmol calcd. with series I |
|---|---|---|---|---|---|
| Quantities when the solutions are changed (120 h). | | | | | |
| mycelium . . . . | 0,423 | 0,018 | 0,000 | | |
| nutritive solution . . | — | 0,000 | 0,003 | | |
| | | | | 0,021 | |
| *Series I:* quantities 24 h after changing the nutritive solutions. | | | | | |
| mycelium . . . . | 0,397 | 0,024 | 0,000 | | |
| nutritive solution . . | — | 0,000 | 0,002 | | |
| | | | | 0,026 | — |
| *Series II:* as series I plus 0,055 mmol choline-chloride per flask | | | | | |
| mycelium . . . . | 0,431 | 0,029 | 0,036 | | |
| nutritive solution . . | — | 0,008 | 0,004 | | |
| | | | | 0,077 | 0,081 (0,026 + 0,055) |
| *Series III:* as series I plus 0,050 mmol betaine-HCl per flask | | | | | |
| mycelium . . . . | 0,436 | 0,026 | 0,036 | | |
| nutritive solution . . | — | 0,000 | 0,017 | | |
| | | | | 0,079 | 0,076 (0,026 + 0,050) |
| *Series IV:* as series I plus 0,055 mmol choline-chloride plus 0,050 mmol betaine-HCl per flask | | | | | |
| mycelium . . . . | 0,415 | 0,034 | 0,036 | | |
| nutritive solution . . | — | 0,030 | 0,029 | | |
| | | | | 0,129 | 0,131 (0,026 + 0,055 + 0,050) |

number mmol in the last three series on the figures of series I; we suppose therefore that the normal weak choline synthesis is not influenced by additions. If this supposition should not be satisfactorily complied with, its influence is not of great importance because the

largest deviation which might result can at most be 10 % of the added quantities of choline or betaine. When we compare the results in table 11 of the last two columns it follows that on acceptance of the above suppositions within the experimental deviations the quantities of choline and betaine balance. This is the reason why we stuck to this supposition as a basis for future investigation.

2. On addition of 0,055 mmol choline-chloride the total quantity of choline plus betaine per flask amounts to 0,077 mmol; on the basis of series I the calculated amount is 0,081 mmol. We may therefore conclude that choline is oxidised by *Aspergillus niger* to betaine exclusively and that no demonstrable degradation to trimethylamine takes place. We can therefore limit our studies on the dissimilation to the determination of choline and betaine. After addition of 0,055 mmol of choline-chloride 0,040 mmol of betaine-HCl is found in the mycelium and nutritive solution, whereas this quantity amounts to 0,002 for the control series. Within 24 hours *Aspergillus niger* can convert approx. 70 % of the choline into betaine.

3. The fact that the experiment described in this table produces a square balance for the dissimilation of choline proves that given these chosen experimental conditions betaine is not further converted by *Aspergillus niger*. For in series III 0,050 mmol of betaine was added, whereas $0{,}053 - 0{,}002 = 0{,}051$ mmol (per flask) was recovered.

4. In series IV, where choline and betaine are added, the oxidation of choline is smaller than after addition of choline alone. The choline taken up amounts to $0{,}055 - 0{,}030 = 0{,}025$ mmol, whereas this quantity amounts to $0{,}055 - 0{,}008 = 0{,}047$ mmol in series II. This difference might be explained by supposing that the wall of the cell becomes less permeable to choline when betaine has been added simultaneously; or that owing to the presence of betaine the surface of the choline-oxidising enzymesystem is already so covered by materials which have a quaternaire ammonia-group that the choline has less chance of oxidising. MANN c.s. (97) thus account for the reduced oxidation of choline by an enzyme preparation from ratliver in the presence of materials with $-NH_3$ groups as $NH_4^+$, $NH(CH_3)_3^+$ and betaine.

Then we investigated if cultures, who were not impoverished in carbohydrates, oxidized the added choline in the same way (table 12)

Owing to the presence of carbohydrates in the new basalsolution the mycelia have grown rapidly after the renewal, whereas in 48 h. after renewal the quantity of choline was doubled. If one wishes to suppose that normal synthesis is not influenced by the addition of choline or betaine, the added choline is practically recovered as choline + betaine, in other words, no other reaction would take place except the conversion of choline into betaine. The increase of betaine in this series compared to the control determination amounts to $0{,}046 - 0{,}003 = 0{,}043$ mmol, which is a conversion of approx. 93 % of the quantity of choline taken up. In series III where 0,040 mmol betaine-HCl per flask has been added this is recovered practically unchanged (0,044 mmol). It seems that in the presence of carbohydrates betaine does not react under the experimental conditions.

We may conclude from table 12 that notwithstanding the presence of carbohydrates oxidation proceeds more rapidly than the incorporation to watersoluble bound choline. Possibly the glycero phosphoricacid is not present in sufficient quantities to bind the choline

TABLE 12

*Oxidation of choline by Asp. niger to betaine on a basal-solution containing carbohydrates.* (after 144 hrs of incubation a new basalsolution containing carbohydrates was brought under the mycelium (series I) which in series II and III also contained choline-chloride and betaine-HCl. Thereafter incubated for 48 h; quantities in mmol per flask; for each determination 3 flasks were taken)

| | dry matter (g) | choline-chloride (mmol) | betaine-HCl (mmol) | total mmol per flask | total mmol calcd. with series I |
|---|---|---|---|---|---|
| Quantities when the solutions are changed (144 h) | | | | | |
| mycelium . . . . | 0,491 | 0,013 | 0,003 | | |
| nutritive solution . . | — | — | — | | |
| | | | | 0,016 | — |
| *Series I:* quantities 48 h after changing the nutr. solns. | | | | | |
| mycelium . . . . | 0,905 | 0,027 | 0,003 | | |
| nutritive solution . . | — | — | — | | |
| | | | | 0,030 | — |
| *Series II:* as series I plus 0,066 mmol choline-chloride per flask | | | | | |
| mycelium . . . . | 0,898 | 0,026 | 0,020 | | |
| nutritive solution . . | — | 0,020 | 0,026 | | |
| | | | | 0,092 | 0,096 (0,030 + 0,066) |
| *Series III:* as series I plus 0,040 mmol betaine-HCl per flask | | | | | |
| mycelium . . . . | 0,911 | 0,027 | 0,011 | | |
| nutritive solution . . | — | — | — | | |
| | | | | 0,074 | 0,070 (0,030 + 0,040) |

at the right time. A relatively greater binding could be ensured if choline was added in equal quantities as with which glycerine-phosphoricacid is produced during the metabolism.

As has already been mentioned in chapter I MANN and QUASTEL (95) have observed the oxidation of choline under anaerobic conditions. They found that the choline-oxidising enzymesystem in rat-liver consisted of at least 2 parts: a cytochrome—and indolphenoloxidase and a choline-dehydrogenase, which is not inhibited by small quantities of cyanide. Nevertheless they succeeded in obtaining an oxidation of the choline under anaerobic conditions through this dehydrogenase when a suitable hydrogen acceptor i.c. sodium-ferricyanide was added. Analogous with these experiments we have investigated the oxidation of choline by *A. niger* under anaerobic conditions. For this experiment

the flasks were placed in a vacuum-exsiccator after the nutritive solution had been renewed. After evacuation $N_2$ was induced after having removed all traces of oxygen by rinsing in a solution of sodium-hydrosulphate. The evacuation and inducement of nitrogen was once more repeated and after incubating for 24 h the quantity of choline was determined. Table 13 and 14 show the results of these experiments.

The results of the experiments described in table 13 prove that

TABLE 13

*Oxidation of choline by A. niger under aerobic and anaerobic conditions.* (nutritive solution lacking carbohydrates)

| | dry matter (g) | choline-chloride (mmol) | total mmol choline-chloride per flask | increase compared with the quantities when the solns. are renewed |
|---|---|---|---|---|
| Quantities when the solutions are changed. (incubated for 97 h) | | | | |
| mycelium . . . . . | 0,271 | 0,009 | | |
| nutritive solution . . | — | — | | |
| | | | 0,009 | — |
| Aerobic. | | | | |
| 1. Quantities 25 h after changing the nutritive solutions. (0,014 mmol choline-chloride added per flask) | | | | |
| mycelium . . . . . | 0,277 | 0,018 | | |
| nutritive solution . . | — | 0,002 | | |
| | | | 0,020 | 0,011 |
| 2. as 1 but without choline-chloride-addn. | | | | |
| mycelium . . . . . | 0,262 | 0,015 | | |
| nutritive solution . . | — | 0,000 | | |
| | | | 0,015 | 0,006 |
| | | | mmol choline disappeared | 0,009 |
| Anaerobic. | | | | |
| 1. Quantities 25 h after changing the nutritive solutions. (0,014 mmol choline-chloride added per flask) | | | | |
| mycelium . . . . . | 0,272 | 0,011 | | |
| nutritive solution . . | — | 0,011 | | |
| | | | 0,022 | 0,013 |
| 2. As 1 but without choline-chloride-addn. | | | | |
| mycelium . . . . . | 0,258 | 0,010 | | |
| nutritive solution . . | — | 0,001 | | |
| | | | 0,011 | 0,002 |
| | | | mmol choline disappeared | 0,003 |

TABLE 14

*Oxidation of choline by A. niger under aerobic and anaerobic conditions* (nutritive solutions lacking carbohydrates)

| | dry matter (g) | choline-chloride (mmol) | total mmol choline-chloride per flask | increase compared with the quantities when the solns. are renewed |
|---|---|---|---|---|
| Quantities when the solutions are changed (149 h) | | | | |
| mycelium . . . . . | 0,544 | 0,023 | | |
| nutritive solution . . | — | — | 0,023 | — |
| Aerobic. | | | | |
| 1. Quantities 24 h after changing the nutritive solutions (0,046 mmol choline-chloride added per flask) | | | | |
| mycelium . . . . . | 0,482 | 0,027 | | |
| nutritive solution . . | — | 0,002 | | |
| | | | 0,029 | 0,006 |
| 2. As 1 but without choline-chloride-addn. | | | | |
| mycelium . . . . . | 0,467 | 0,024 | | |
| nutritive solution . . | — | — | 0,024 | 0,001 |
| | | | mmol choline disappeared 0,041 | |
| Anaerobic | | | | |
| 1. Quantities 24 h after changing the nutritive solutions (0,046 mmol choline-chloride added per flask) | | | | |
| mycelium . . . . . | 0,420 | 0,016 | | |
| nutritive solution . . | — | 0,050 | | |
| | | | 0,066 | 0,043 |
| 2. As 1 but without choline-chloride-addn. | | | | |
| mycelium . . . . . | 0,410 | 0,012 | | |
| nutritive solution . . | — | 0,011 | | |
| | | | 0,023 | 0,000 |
| | | | mmol choline disappeared 0,003 | |

after incubation during 25 h from the added 0,014 mmol of choline-chloride under aerobic conditions $0{,}020 - 0{,}015 = 0{,}005$ mmol are still present; under anaerobic conditions rests $0{,}022 - 0{,}011 = 0{,}011$ mmol. This means that 0,009 resp. 0,003 mmol of choline-chloride have disappeared.

Still clearer figures were obtained with the experiment mentioned in table 14, which was conducted in a similar way as that in table 13; the quantity of choline-chloride added however is much larger

viz. 0,046 mmol. After incubating for 24 h. under aerobic conditions 0,006 mmol is left as choline-chloride, whereas under anaerobic conditions this amounts 0,043 mmol; resp. 0,041 and 0,003 mmol choline-chloride disappeared.

A strong oxidation of choline therefore occurred under aerobic conditions. The decline observed under anaerobic conditions (0,003

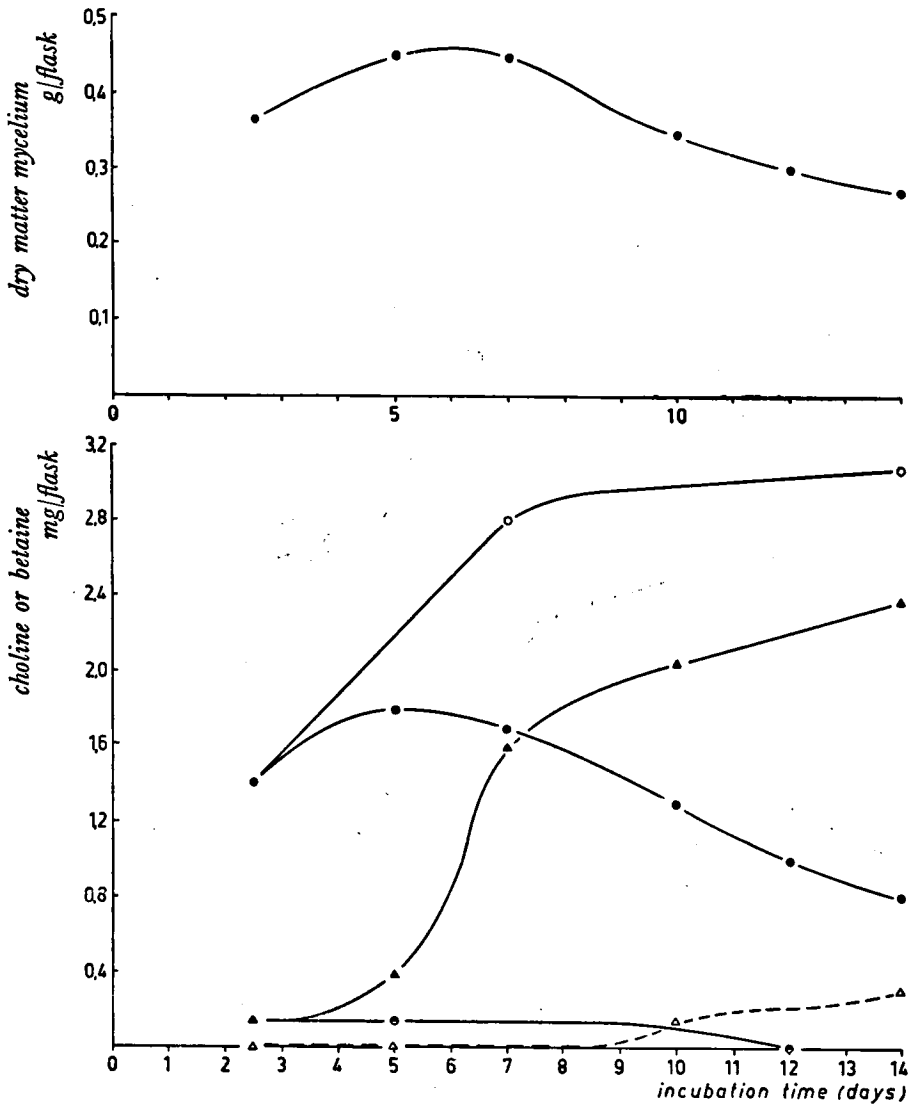



Fig. 4. Relation between the choline and betaine contents and the age of cultures of *A. niger* (•——• choline per flask; ◓——◓ id. in soln. per flask; ▲——▲ betaine per flask; △- - - - △ id. in soln. per flask; ○——○ choline and betaine per flask)

mmol) can not be considered as significant because this figure is of the same magnitude as the experimental deviation. During the experiments shown in table 14, the quantity of choline in the mycelia under anaerobic conditions was reduced half of the original amount, where as in the experiments of table 13 the quantity even slightly increased under anaerobic conditions. Both cases showed no clear dehydrogenation of choline under anaerobic conditions.

Finally it should be noted that we tried to obtain an anaerobic oxidation of the choline by adding potassium-ferricyanide as a hydrogen-acceptor. This compound had a poisonous effect in the concentrations used (0,55 and 0,09 %) owing to which the largest part of the choline ($\pm$ 75 %) disappeared from the mycelium into the nutritive solution. No oxidation of choline could be established.

On investigating the metabolism of choline in relation to the development of *A. niger* we found that the quantity of total choline attained its maximum after 5 days, whereafter a rather rapid decrease occurs (fig. 1). The preceding pages have shown that choline can be practically quantitatively oxidised to betaine. Next we investigated whether the decrease of the amount of choline on the fifth day during normal growth was compensated by an increase of the quantity of betaine. The results of such an experiment* are mentioned in fig. 4 where the quantity of choline and betaine present in the mycelium and in the nutritive solution were determined after $2^1/_2$, 5, 7, 10, 12 and 14 days. The quantity of choline and betaine present in the nutrition solution was again very small. The experiment shows that the decrease of the quantity of choline correlates with an increase of the quantity of betaine. The quantity of choline + betaine after 7 days is larger than after 5 days, whereas the amount of choline has already been decreased: apparently between the 5th and 7th day choline is still synthesized under simultaneously oxidation to betaine.

The total quantity of choline and betaine remains more or less constant after the 7th day. At this stage the metabolism of choline results in a liberation of choline from the watersoluble bound choline by lecithinase and in an oxidation of the choline to betaine.

## CHAPTER V

# THE METABOLISM OF CHOLINE IN ASPERGILLUS NIGER: SYNTHESIS

### 1. SYNTHESIS FROM ETHANOLAMINE AND METHIONINE

The investigation of the synthesis of choline in plants has been limited to 2 groups of investigations: the methylation of ethanolamine by methionine in a mash of wheat seedlings, whereby BARRENSCHEEN and collaborators (8) meant to establish a synthesis of choline. STEENSHOLT however (126) could not confirm this. HOROWITZ (63,

* For the execution of these experiments we are especially grateful to MR. W. GRAAFLAND, biol. drs.

64) investigated the in vivo synthesis of choline in *Neurospora crassa* by comparing the growth of choline-less mutants. Recently KIRKWOOD (79, 99) showed that when $HC^{14}OONa$ or l-methionine labelled with $C^{14}$ in the methylgroup was fed to sprouting barley, the methylgroups of choline contained $C^{14}$.

During our investigation on the synthesis of choline we replaced the old nutritive solution for a new one to which ethanolamine or methionine had been added. We started with the investigation of the activities of these two compounds as precursors in the synthesis of choline. Table 15 clearly shows that *Aspergillus niger* does not synthesize choline from these 2 compounds on a solution without carbohydrates.

When however the solution under the mycelium is replaced by a nutritive-solution containing carbohydrates, addition of methionine plus ethanolamine produces a distinct synthesis (Table 16).

On addition of methionine plus ethanolamine approx. 0,039 mmol of choline has been recovered in the mycelium; without addition 0,025 mmol, so that the synthesis is 0,014 mmol choline per flask, i.e. approx. 14 % of the theoretically possible quantity. Besides the quantity of choline in the nutritive solution has been determined in a number of these series; this was very slight however, so that these values are not mentioned in a number of experiments.

After we had succeeded in obtaining a synthesis from methionine plus ethanolamine it was of importance to investigate whether it was sufficient for this synthesis to add one of these compounds; this might indicate a surplus presence of the other compounds in the cell.

Moreover it could be decided whether the influence of methionine and ethanolamine is additive or whether the quantity of choline produced from both compounds is larger than the sum of the synthesis acquired on adding each of these compounds separately (table 17)

TABLE 17

*Choline-synthesis by A. niger on a nutritive-solution containing carbohydrates after adding methionine, ethanolamine, methionine plus ethanolamine*

| Added compound | Synthesis (mmol choline-chloride per flask) | | |
|---|---|---|---|
| | average | maximum and minimum value | number of series |
| 0,248 mmol methionine . . . . . | + 0,001 | — 0,003; + 0,003 | 5 |
| 0,098 mmol ethanolamine . . . . . | — 0,001 | — 0,005; + 0,003 | 4 |
| 0,248 mmol methionine + 0,098 mmol ethanolamine . . . . . | + 0,013 | + 0,010; + 0,018 | 7 |

The added quantities of methionine and ethanolamine are the same as those in table 16. Owing to the rather large deviation we were obliged to repeat these experiments several times. The final column shows the number of series out of which the averaged figure has been calculated. It appears that on addition of methionine or of ethanolamine

TABLE 15

*Synthesis of choline from methionine and ethanolamine by Aspergillus niger on a nutritive-solution lacking carbohydrates.*
(every figure originates from at least 3 flasks).

| Series | h incubated before changing nutritive-soln. | h incubated after changing nutritive-soln. | added per flask | | dry matter of mycelium (g per flask) | | choline-chloride (mmol per flask) | | Synthesis |
|---|---|---|---|---|---|---|---|---|---|
| | | | ethanol-amine mmol | methionine (mmol) | flasks with addn. | flasks without addn. | flasks with addn. | flasks without addn. | |
| I | 132 | 30 | 0,014 | 0,042 | 0,239 | 0,264 | 0,012 | 0,012 | 0,000 |
| | 132 | 72 | 0,014 | 0,042 | 0,167 | 0,197 | 0,012 | 0,012 | 0,000 |
| II | 140 | 24 | 0,076 | 0,225 | 0,398 | 0,407 | 0,024 | 0,026 | —0,002 |
| III | 143 | 48 | 0,098 | 0,242 | 0,408 | 0,425 | 0,015 | 0,014 | +0,001 |
| IV | 147 | 37 | 0,098 | 0,242 | 0,440 | 0,465 | 0,014 | 0,016 | —0,002 |

TABLE 16

*Synthesis of choline from methionine and ethanolamine by Aspergillus niger on a nutritive-solution containing carbohydrates.*
(every figure originates from at least 3 flasks)

| Series | h incubated before changing nutritive-soln. | h incubated after changing nutritive-soln. | added per flask | | dry matter of mycelium (g per flask) | | choline-chloride (mmol per flask) | | Synthesis |
|---|---|---|---|---|---|---|---|---|---|
| | | | ethanol-amine mmol | methionine mmol | flasks with addn. | flasks without addn. | flasks with addn. | flasks without addn. | |
| I | 148 | 39 | 0,098 | 0,248 | 0,727 | 0,742 | 0,042 | 0,027 | 0,015 |
| II | 143 | 38 | 0,098 | 0,248 | 0,913 | 0,825 | 0,041 | 0,023 | 0,018 |
| III | 147 | 37 | 0,098 | 0,248 | 0,904 | 0,806 | 0,037 | 0,024 | 0,013 |
| IV | 144 | 48 | 0,098 | 0,248 | 1,018 | 0,905 | 0,037 | 0,027 | 0,010 |
| | | | | | | Average | 0,039 | 0,025 | 0,014 |

no choline synthesis occurs. Both methionine and ethanolamine are necessary to produce a synthesis of choline.

In order to acquire data on what form choline synthesized from methionine and ethanolamine is present, we have determined several choline fractions in mycelia acquired from a synthesis-experiment. Choline appeared to be present in a practically equal percentage in the watersoluble form as in the mycelia of control-cultures (table 18). The additional synthesized choline is present in at least 70–80 % as watersoluble bound choline in the mycelium.

If we beær in mind the possibility of a slight deviation, we see that in the newly formed choline practically the same percentages of free choline and watersoluble bound choline are present as originally in the mycelium.

TABLE 18

*Division of choline in mycelia of A. niger during synthesis-experiments.*
(each figure originates from 4 flasks)

| | Total choline | watersoluble bound choline | | During the experiment newly formed choline | | |
|---|---|---|---|---|---|---|
| | | | | Total choline | watersoluble bound choline | % total choline |
| | mmol | mmol | % total | mmol | mmol | |
| without addn. . . . . | 0,027 | 0,025 | 93 | — | — | — |
| after addn. (0,248 mmol meth. + 0,098 mmol ethanolamine) . . . . | 0,042 | 0,036 | 86 | 0,015 | 0,011 | 73 |
| after addn. (0,248 mmol meth. + 0,098 mmol glycine) . . . . . | 0,042 | 0,037 | 88 | 0,015 | 0,012 | 80 |

The fact that on a nutritive-solution lacking carbohydrates no increase of the quantity of choline can be seen after adding methionine and ethanolamine might be explained by supposing, that choline would have been formed but that owing to a lack of glycerophosphoric acid it is not transformed into the watersoluble bound form but directly oxidised to betaine. We have tried to verify this in two ways: 1) by determining betaine in the presence of choline 2) by adding glycero-phosphoric acid.

ad 1) Table 19 proves that the total quantity of choline plus betaine without addition of methionine and ethanolamine in cultures on a solution without carbohydrates is practically equal to the quantity of choline and betaine after addition of these compounds. It should be noted that the betaine-determination can only be used here with restriction; betaine is found as a difference (see chapter III, 3) and as the quantity of choline in this experiment is large with regard to that of betaine, the values for betaine are less accurate than was the case in the investigation on choline-oxidase. The difference of 0,003 mmol therefore lies within the limit of experimental errors. The fact that no increase of the quantity of choline was noted on a nutritive-solution

with methionine and ethanolamine but without carbohydrates is not due therefore to a possible further oxidation to betaine but to the fact that choline was not formed at all.

TABLE 19

*Synthesis of choline by A. niger on a nutritive-solution lacking carbohydrates*

| | choline-chloride mmol per flask | betaine-HCl mmol per flask | total mmol per flask | increase compared with the control-flasks |
|---|---|---|---|---|
| Incubated for 140 h | | | | |
| mycelium . . . . . | 0,021 | 0,007 | | |
| nutritive-solution . . . | — | — | 0,028 | — |
| Quantities 24 h after the nutritive-solutions are changed (0,025 mmol ethanolamine + 0.076 mmol methionine) | | | | |
| mycelium . . . . . | 0,026 | 0,005 | | |
| nutritive-solution . . . | 0,001 | 0,001 | 0,033 | — 0,003 |
| Quantities 24 h after the nutritive-solutions are changed without addn. | | | | |
| mycelium . . . . . | 0,024 | 0,011 | | |
| nutritive-solution . . . | 0,001 | 0,000 | 0,036 | — |

ad 2) A nutritive-solution in which the carbohydrates were substituted by glycerophosphoric acid was used. Choline synthesized from methionine and ethanolamine could possibly be converted to the watersoluble form of choline (table 20); the result is clearly negative. With carbohydrates the synthesis amounts to 0,013 mmol per flask, with glycerophosphoric acid the total quantity of choline is 0,001 mmol and without carbohydrates 0,002 mmol per flask. During the experiments we added the glycero-

TABLE 20

*Synthesis of choline by A. niger on a nutritive-solution without carbohydrates, id. on a nutritive-solution with glycerophosphoric acid and on a nutritive-solution with carbohydrates.* (incubated for 147 h., after renewal of nutritive-solution incubated for another 37 h., mmol choline-chloride in mycelium per flask)

| Nutritive-solution after changing | Quantities when the nutritive-solutions are changed (147 h) | 37 h after changing nutritive-solution | | |
|---|---|---|---|---|
| | | 0,248 mmol methionine + 0,098 mmol ethanolamine | without addition | difference |
| — | 0,014 | — | — | — |
| without carbohydrates. . | — | 0,016 | 0,014 | + 0,002 |
| glycerophosphoric acid [1]) | — | 0,016 | 0,015 | + 0,001 |
| with carbohydrates [2]) . . | — | 0,037 | 0,024 | + 0,013 |

[1]) 55 g sodium-salt of glycerophosphoric acid per l.
[2]) 40 g sucrose + 10 g glucose per l.

phosphoric acid in two parts, because the possibility that this compound is dephosphorylated by the phosphatases in *A. niger* could not be ruled out: one part during the renewal of the nutritive solution and the remainder 24 h. later.

## 2. Synthesis from compounds other than methionine and ethanolamine

Experiments on the synthesis of choline in animals had shown that other compounds than methionine and ethanolamine may function as a methyldonor and a methylacceptor. Thus ethanolamine might be replaced by monomethylethanolamine, dimethylethanolamine, glycine and serine.

This last compound according to Arnstein (42) might function not only as a methylacceptor but also as a methyldonor; methionine can be replaced by betaine, dimethylthetine and dimethylpropiothetine.

Several of these compounds have been examined for their activity with regard to the synthesis of choline in *A. niger*. Table 21 shows the results of synthesis-experiments on a nutritive-solution with carbohydrates to which serine, ethanolamine + serine, serine + methionine, glycine + methionine have been added.

TABLE 21

*Synthesis of choline by Aspergillus niger on a nutritive solution with carbohydrates after addition of serine, ethanolamine + serine, serine + methionine, glycine + methionine*

| Added compound | Synthesis (mmol choline-chloride per flask) | | |
|---|---|---|---|
| | average | maximum and minimum value | number of series |
| 0,098 mmol serine . . . . . | — 0,001 | — 0,005; + 0,003 | 3 |
| 0,098 mmol ethanolamine + 0,248 mmol serine . . . . . | + 0,005 | + 0,003; + 0,006 | 3 |
| 0,098 mmol serine + 0,248 mmol methionine . . . . . | + 0,002 | + 0,000; + 0,006 | 4 |
| 0,098 mmol glycine + 0,248 mmol methionine . . . . . | + 0,010 | + 0,008; + 0,015 | 2 |

The synthesis due to serine amounted —0,001 mmol; after addition of serine and methionine +0,002 mmol (after decarboxylation of serine ethanolamine arises), while after addition of ethanolamine and serine (serine as methyldonor) it was 0,005 mmol. These increases are too small to conclude to a synthesis.

Addition of methionine and glycine resulted in a synthesis of the same magnitude as that of methionine and ethanolamine (resp. 0,010 and 0,014 mmol). This can be explained by the supposition that *Aspergillus niger* is able to reduce glycine to ethanolamine. Another explanation is that a possible oxidation of ethanolamine to glycine is inhibited by addition of this to the basalsolution.

### 3. HYPOTHESES REGARDING THE RELATION BETWEEN THE SYNTHESIS OF CHOLINE AND THE CARBOHYDRATE METABOLISM

In chapter I we discussed the reaction-mechanisms for the transfer of the methylgroup of methionine to ethanolamine.

A. According to BARRENSCHEEN c.s. (5) this transfer would only take place after the oxidation of methionine to methionine-sulfoxide.

In order to investigate the course of the methylation-reaction in *Aspergillus niger* we have added methionine-sulfoxide, ethanolamine plus methionine-sulfoxide[1] and ethanolamine plus methionine to a number of cultures on a nutritive solution with carbohydrates. Table 22 shows that the quantity of choline due to the addition of methionine-sulfoxide and of ethanolamine plus methionine-sulfoxide is too small (resp. 0,004 and 0,003 mmol) to decide on a synthesis.

TABLE 22

*Synthesis of choline by Aspergillus niger on a nutritive-solution with carbohydrates after addition of methionine-sulfoxide and methionine-sulfoxide plus ethanolamine*

| Added compound | Synthesis (mmol choline-chloride per flask) | | |
|---|---|---|---|
| | average | maximum and minimum value | number of series |
| 0,248 mmol methionine-sulfoxide . . . | + 0,004 | + 0,003; + 0,006 | 3 |
| 0,098 mmol ethanolamine + 0,248 mmol methionine-sulfoxide . . | + 0,003 | + 0,002; + 0,005 | 2 |
| 0,098 mmol ethanolamine + 0,248 mmol methionine . . . . . | + 0,013 | + 0,010; + 0,018 | 7 |

Following the experiments of BARRENSCHEEN on muscle extract from the rat (7) we have tried to obtain an anaerobic synthesis of choline from methionine-sulfoxide plus ethanolamine. In the event of a positive effect this would prove the reaction-mechanism formulated by BARRENSCHEEN. Besides this it was also necessary to demonstrate that under these conditions no choline synthesis resulted from the addition of methionine plus ethanolamine. Under anaerobic conditions however the fungus died in the course of this series of experiments.

The fact that choline cannot be synthesized from methionine-sulfoxide plus ethanolamine under aerobic conditions (table 22) may be regarded as an argument against the course of the choline-synthesis according to BARRENSCHEEN.

B. CANTONI (29) has shown that the transfer of the methylgroup of methionine occurs after this compound is converted by ATP in S-adenosyl-methionine. We have tried to prove the influence exerted by ATP during the methylation of ethanolamine as follows: According

[1]) Methionine-sulfoxide was prepared by oxidation of methionine with $H_2O_2$ according to TONNIES and KOLB (132); the product acquired had a melting-point of 225° C (lit. 225-230° C).

to CHUGTAI and WALKER (35) the carbohydrate consumption of *Aspergillus niger* is increased after addition of sodium-arsenite. Analogous to experiments with yeast they assume that in the presence of sodium-arsenite the balance of the enzymatic activities would be deflected towards citrate formation. The results of their experiments prove that 0,004 M sodium-arsenite decreases respiration whereas the quantity of citric acid is considerably larger than in cultures without arsenite. Owing to this change in the metabolism a smaller quantity of ATP may be expected than in cultures without arsenite. An indirect indication to the reaction course according to CANTONI, might be acquired if it could be proved that the choline-synthesis of methionine and ethanolamine in the presence of sodium-arsenite was smaller than in cultures without arsenite. Table 23 shows the results of two experiments regarding the influence of sodium-arsenite on the synthesis of choline.

In series I the decrease caused by sodium-arsenite of the synthesis from ethanolamine and methionine amounted to 0,008–0,003 = 0,005 mmol i.c. 62 % The quantity of choline synthesized via the control cultures is considerable larger than when arsenite is present (resp. 0,033 and 0,020 mmol). Even without addition of ethanolamine and methionine sodium-arsenite apparently retards the synthesis of choline; in this case the fungus has, as N-source for the choline to be synthesized, ammonia-nitrate in the nutritive-solution at its disposal. In order to keep the choline-synthesis from the N-compounds of the nutritive-solution as small as possible, the ammonia-nitrate has been omitted from the new solution, brought under the mycelium after 102 and 118 h of incubation. It appeared then that the quantities of choline of the control cultures deprived of N are much less (resp. 0,021 and 0,019 mmol); the inhibition of the choline-synthesis from ethanolamine and methionine by sodium-arsenite amounts to 0,012–0,007 = 0,005 mmol or 42 %. The complication to series I viz. that control cultures with choline synthesized from the nutritive-solution have been used to calculate the inhibition of the choline-synthesis from methionine and ethanolamine, does not apply to series II.

The fact that arsenite inhibits the synthesis of choline, indirectly supports the view that in *Aspergillus niger* the transfer of the methylgroup of methionine is accomplished via S-adenosyl-methionine.

Another way to demonstrate the relation existing between the trans-methylation and ATP can be deduced from BONNER's observation (21), that 3,4-dinitrophenol (DNP) functions as inhibitor of ATP in *Avena* coleoptiles. BACCARI and GUERRITORE (3) thus proved that the methylation of guanido acetic acid in slices or homogenates of rat-livers is inhibited by 0,004 M DNP. By administering DNP to the nutritive-solution of cultures of *Aspergillus niger* we now tried to reduce the action of ATP; after the addition of ethanolamine and methionine this would result in finding no or only a minute choline-syntheses. The results of these experiments however varied so much that we were unable to draw any conclusions.

TABLE 23

*The influence of 0,004 M sodium-arsenite on the synthesis of choline from methionine and ethanolamine on a nutritive-solution containing carbohydrates* (mmol choline-chloride in mycelium per flask; each figure originates from 3 or 4 flasks).

| Series | | 0,098 mmol ethanolamine + 0,248 mmol methionine | | Without methionine + ethanolamine | | Synthesis mmol | Inhibition of the synthesis | |
|---|---|---|---|---|---|---|---|---|
| | | dry matter (g) | mmol choline-chloride | dry matter (g) | mmol choline-chloride | | mmol | % |
| I | Nutr.-soln. changed after 103 h; incubated for 47 h, again new nutr.-soln. and still incubated for 92 h | — | — | 0,709 | — | — | — | — |
| | without sodium-arsenite | 0,873 | 0,041 | 0,896 | 0,033 | 0,008 | | |
| | with sodium-arsenite | 0,828 | 0,023 | 0,819 | 0,020 | 0,003 | | |
| | | | | | | | 0,005 | 62 |
| II | Nutr.-soln. changed after incubating for 102 h, impoverished in N for 16 h; again new nutr.-soln. without N and still incubated for 48 h | — | — | 0,591 | — | — | — | — |
| | without sodium-arsenite | 0,849 | 0,033 | 0,848 | 0,021 | 0,012 | | |
| | with sodium-arsenite | 0,818 | 0,026 | 0,727 | 0,019 | 0,007 | | |
| | | | | | | | 0,005 | 42 |

## DISCUSSION

The experiments in chapter IV and V have shown that *Aspergillus niger* oxidises added free choline to betaine; when carbohydrates are present the addition of free choline does not result in an incorporation into a water-soluble bound form. The dissimilation of choline is apparently not influenced by the metabolism of carbohydrates.

This does happen, however, at the synthesis of choline. The formation of choline from methionine and ethanolamine occurs only in the presence of carbohydrates whereafter choline is converted into the water-soluble form. Without carbohydrates no increase of the quantity of choline or secondary formed betaine was observed. The addition of glycerophosphoric acid which would promote the binding of eventually formed choline, was of no effect.

The choline synthesis can schematically be divided in 2 phases: the methylation of ethanolamine and the incorporation of choline to watersoluble bound choline. What connection exists between the metabolism of carbohydrates and each of these phases?

For the transfer of the methylgroup to ethanolamine two reaction-mechanisms have been described [BARRENSCHEEN (5) and CANTONI (29)]; regarding the formation of the watersoluble bound choline no data are known.

It might be possible to find a connection existing between the metabolism of carbohydrates and the choline synthesis if one regards the oxidation of methionine to methionine-sulfoxide [BARRENSCHEEN (5)] linked with the reduction of phosphoglyceraldehyde to glycerophosphoric acid. In view of the negative results obtained with methionine-sulfoxide in table 22 the course of the reaction formulated by BARRENSCHEEN in *Aspergillus niger* is not very probable.

Although we are only able to prove indirectly the connection between ATP and the transfer of the methylgroup of methionine to ethanolamine as described by CANTONI (29), this view is in preferable to that of BARRENSCHEEN. BACCARI and GUERRITORE (3), with regard to the results obtained with slices and homogenates of livers of rats and caviae arrive at the conclusion that the transfer of the methylgroup of methionine to guanidoacetic acid proceeds under influence of ATP.

The investigation on the synthesis of choline has shown that methionine is an active methyldonor. Methyldonors are not administered to the normally raised cultures of *Aspergillus niger*. It is therefore necessary that the fungus synthesizes the required methylgroups. MATCHETT, MARION and KIRKWOOD (99) have proved that sprouting barley synthesizes the methylgroup of choline from sodium formate.

SUMMARY

1. Chapter I gives a survey of the literature on the metabolism of choline in plants and animals. The synthesis of choline appears to progress via methylation of ethanolamine. The methylgroups required in animals are mainly supplied by compounds with biologically labile methylgroups present in the ration. The existence of the synthesis

of methylgroups from compounds like formic acid is found only when special conditions prevail. In plants the methylgroups are synthesized from "one C-fragments". The synthesized choline can be built in (lecithin); during dissimilation it can be converted into trimethylamine and into betaine.

2. Chapter II proceeds to describe the methods of determining total, free, lipoid and watersoluble bound choline and betaine.

3. Chapter III mentions the results of a comparative investigation on the determination of choline according to 2 different methods. By the determinations of the choline fractions in plants it appeared, that in the higher plant the amount of total choline consisted mainly of lecithin and in the lower plant mainly of watersoluble bound choline. Lecithinase C present in the higher plant might give a wrong impression of the constitution of the choline fractions.

4. Chapter IV describes the dissimilation of choline in *Aspergillus niger:* the fungus appeared to oxidise added choline to betaine; oxidation under anaerobic conditions is small in comparison to oxidation under aerobic conditions. No relation between the dissimilation of choline and the metabolism of carbohydrate could be found.

5. Chapter V mentions the investigation of the synthesis of choline in *Aspergillus niger:* In the presence of carbohydrates a synthesis of choline from methionine and ethanolamine takes place. We have tried to obtain more data on the relation between the metabolism of carbohydrates and the synthesis of choline; CANTONI's supposition that the transfer of the methylgroup of methionine occurs by means of ATP is to be preferred to that of BARRENSCHEEN, who supposes the transfer takes place after oxidation of methionine to methionine-sulfoxide.

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