THE METABOLISM OF AMINO ACIDS IN THE CENTRAL NERVOUS SYSTEM

by

Stephen Sved

A Thesis

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HISTORICAL INTRODUCTION

A. General Metabolism of the Nervous Tissue.

1. Carbohydrate Metabolism in Nervous Tissue.

Systematic studies on the metabolic activities of nervous tissue began with the classical studies of Warburg, Posener and Negelein (1). They measured oxygen consumption, aerobic and anaerobic glycolysis of several tissues of the rat in an attempt to investigate the differences between the metabolism of neoplastic and normal tissues. Among their normal tissues they included brain cortex slices and retina. The results of this investigation served as a basis for further studies on the metabolism of brain, which led to the discovery of a great number of metabolic pathways.

a. The Embden-Meyerhof scheme in the brain.

Warburg and co-workers found that isolated slices of brain tissues were able, when suspended in a suitable medium, to oxidize glucose to water and carbon dioxide, or to break it down anaerobically to lactic acid (1). The oxygen consumption, as well as the lactic acid production, in the presence of glucose was found by Krebs to be strongly in-

hibited by iodoacetate (2). According to Quastel and Wheatley, iodoacetate also inhibited the oxygen uptake by brain in the presence of fructose or mannose, but not when the above substrates were substituted by lactate, pyruvate, glutamate or succinate (3). These findings, together with that of Peters and Sinclair (4) in 1933, that sodium fluoride inhibits respiration with α-glycerophosphate as a substrate, strongly suggested that the break-down of glucose in the brain follows the same pathway as in muscle. This latter pathway was fully exposed by Embden and Meyerhof in 1932 (5) and is now known as the Embden-Meyerhof scheme. The full scheme was extensively reviewed by Meyerhof in 1937 (6).

The existence of the Embden-Meyerhof scheme in the brain was seriously questioned by Johnson in 1936 (7) when he found that lactate or pyruvate had no effect on the rate of disappearance of α -glycerophosphate, but in the same year Euler and co-workers (8) furnished conclusive evidence for its existence in calf, dog and rat brain homogenates. They showed that, when enolase is inhibited by fluoride, pyruvate and hexosediphosphate form lactate and phosphoglycerate in stoichiometric amounts. In the same paper they also showed the phosphate and cofactor requirements, as well as the substrates for glycolysis.

Another objection against the scheme in brain came from Ashford in 1934 (9), who could find no phosphorylation through glycolysis but his results were contradicted by Meyerhof in 1938 (10), when he demonstrated that phosphorylation was coupled to glycolysis in brain homogenates, dialysates and extracts.

b. The tricarboxylic acid cycle.

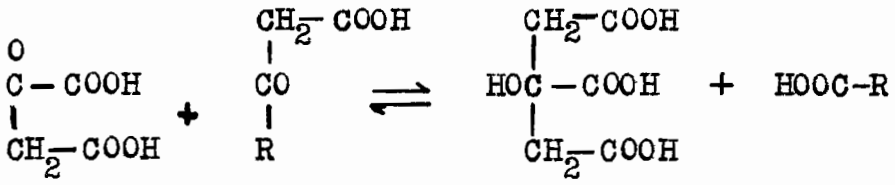
The existence of the tricarboxylic acid cycle in the brain has been accepted soon after Krebs proposed it in 1937 (14), Only its relative importance has been questioned by Breusch (19). However, work has been done on the formation of citrate from pyruvate. Lipman in 1937 showed that brain respiration was lower and glycolysis higher in thiamine deficient pigeon brain (20). Weil-Malherbe (13) demonstrated the anaerobic dismutation of pyruvate, to lactate and acetate.

$$2\ CH_3.CO.COOH \rightleftharpoons CH_3.CHOH.COOH + CH_3.COOH + CO_2$$

Long and Peters showed the same reaction to occur aerobically, accompanied by a further break-down of the products to carbon dioxide and water, and demonstrated the requirement for thiamine pyrophosphate as a coenzyme of decarboxylation

(21, 22). Banga, Ochoa and Peters confirmed the identity of cocarboxylase with thiamine pyrophosphate (23, 24). They also demonstrated, that a C_4 acid, especially fumaric, greatly stimulates the complete break-down of pyruvate, whereas malonate inhibits the process; citrate or α-ketoglutarate can partially replace fumurate. Similar results were obtained by Elliott and Libet (25) and Long (26).

Ochoa in 1941, using dialysed pigeon brain homogenates studied the phosphorylation coupled to the oxidation of pyruvate and fumarate (27). In the presence of fluoride (to inhibit ATP-ase) he found a P/O ratio of 4; succinate, when the further oxidation of pyruvate formed was inhibited by arsenite, gave a P/O ratio of 2. This has been taken as partial proof for the operation of the tricarboxylic acid cycle in the brain, although the P/O ratios found by Ochoa were lower than those found later. Further proof was provided by Breusch (15) when he partially purified the arsenite sensitive enzyme system "citrogenase" from the brain, catalysing the reaction



and by Coxon et al. when they demonstrated citrate synthesis, from pyruvate and fumarate, by washed particulate matters of brain in the presence of ATP and Mg^{++}(28).

The final proof for the mechanism of the tricarboxylic acid cycle in the brain was provided by Peters et al. They demonstrated (30) that fluoroacetate forms fluorocitrate, which acts as a specific inhibitor of aconitase. When they added fluorocitrate to brain homogenates respiring in a pyruvate-fumarate medium, the oxygen consumption was strongly inhibited and there was a simultaneous accumulation of citric acid. Injection of fluorocitrate into pigeons gave pronounced symptoms which resembled in many aspects the symptoms of thiamine defficiency, and the brains contained a great deal of accumulated citrate. Neither of these two phenomena occurred when citrate itself was injected (31).

c. The effect of potassium on brain metabolism.

A phenomenon peculiar to nervous tissue is that its metabolism <u>in vitro</u> can be stimulated by making the medium hypertonic with KCl (32, 33, 34). Apparently the intact neuron is necessary for stimulation, because extra potassium has no effect on homogenates of brain (35). The stimulation of rat or guinea-pig brain cortex slices is

especially marked when the slices respire in the presence of glucose: the oxygen consumption can double on addition of KCl to a final concentration of o.1 M (36). Other cations, with similar mode of action to potassium are rubidium, caesium and ammonium ions. These cations also stimulate aerobic glycolysis and inhibit anaerobic glycolysis.

Among other effects of potassium, Mann, Tennenbaum and Quastel (37) have found that it facilitates the release of the tissue-bound form of acetylcholine into the medium. They have proposed the following sequence of reactions:

$$\text{choline} \xrightarrow[\text{glucose, pyruvate, lactate}]{O_2} \text{"combined" acetylcholine} \longrightarrow$$

$$\text{acetylcholine} + \text{tissue} \xrightarrow[\text{retarded by } Ca^{++}]{K^+} \text{into medium}$$

Elliott in 1955 confirmed these results and suggested that the effect of elevated potassium in the medium may be exerted on certain cellular surfaces releasing occluded acetylcholine (29).

Lipsett and Crescitelli in 1950 undertook an extensive investigation of the mode of action of potassium (36). They measured the oxygen consumption of rat brain cortex slices with different substrates with and without added potassium, and found a stimulation with glucose, pyruvate and lactate; there was no stimulation with succinate, glutamate or α-ketoglutarate, nor in the absence of substrate. Furthermore, the extra oxygen uptake due to potassium in the presence of glucose was inhibited by citrate, glutamate, α-ketoglutarate and succinate, and the inhibition caused by succinate was reversed in the presence of malonate. These results suggest that the extra oxygen uptake due to potassium does not come through the tricarboxylic acid cycle. On the other hand, Kimura and Niwa found in 1953 that potassium stimulated respiration in the presence of glucose is much more sensitive to malonate inhibition than the unstimulated one (39). Malonate had little or no effect on endogenous or on glucose respiration, but brought down the potassium stimulated glucose respiration to the endogenous level. They concluded, that the tricarboxylic acid cycle is "masked" normally, but is opened up by potassium.

The high sensitivity to inhibitors of the extra oxygen consumption due to potassium, in the presence of glucose, has been reported by many workers (39,40,41,42,43) using such

substances as barbiturates, phenothiazines, ethanol and local anaesthetics. In all cases it was shown that the inhibitors at concentrations which had no effect on glucose respiration, abolished or at least considerably decreased the potassium effect. In many instances similar results were obtained by using other stimulants, such as high frequency electric current, protoveratrine and dinitrophenol (40, 41, 43, 44).

McIlwain and his co-workers demonstrated that while the oxygen consumption of the brain was greatly stimulated by potassium, its creatine phosphate and adenosine triphosphate content was considerably diminished (11, 12, 38). In accordance with these findings Findlay et al. in 1954 found that potassium inhibited the incorporation of radioactive phosphorus into the phosphate containing fractions of the brain (16). Rossiter pointed out in 1955 (17) that these phenomena may be due to an increased expenditure of energy by the cell membrane in an attempt to re-establish the disturbed potassium gradient accross the membrane.

2. Amino Acid and Protein Metabolism in the Nervous Tissue.

a. The intermediary metabolism of glutamic acid and glycine.

Studies on the metabolism of amino acids in the neuron were virtually limited to that of glutamic acid for almost two decades. The chief reason for this was that glutamate is the only amino acid that is capable of stimulating and maintaining oxygen uptake in brain preparations (45).

The concentration of free glutamate and glutamine in the brain is extremely high, much higher than that of any other amino acid (46), and should therefore fulfill an important metabolic role. One of these functions is glutamine synthesis from free ammonia:

$$\text{Glutamate} + NH_3 + \text{ATP} \longrightarrow \text{Glutamine} + \text{ADP} + H_3PO_4$$

The enzyme system has been isolated from sheep brain by Elliott (47, 48) and shown to be active with free ammonia only. This reaction is probably the sole means of removing free ammonia formed in the brain, since the "ornithine-urea cycle" does not operate there to any considerable extent (46). Another function of possible importance is the decarboxylation of glutamate to γ -amino-

butyric acid. The latter has been isolated from brain in considerable quantities (49, 50) and identified with factor I of Florey and Elliott (51, 60). It is inhibitory to a single sensory nerve cell preparation, the heart and the intestine of the crayfish and blocks the action of acetylcholine. The enzyme system glutamic acid decarboxylase, which forms γ-aminobutyric acid, was isolated by Roberts and Frankel in 1951 (52). It requires pyridoxal phosphate like all other amino acid decarboxylases. It is produced mainly in the grey matter of the brain after the "maturation of the central nervous system" (53).

Glutamic acid is also important in the brain in transamination reactions, especially in the formation of aspartic acid from oxalacetic acid (54). It can also transaminate with pyruvic as well as other α-keto acids, but at a much lower rate. The α-ketoglutarate formed this way, or by oxydative deamination of glutamate (45) can either act as an amino group acceptor in further transaminations, or be oxidized further via the tricarboxylic acid cycle. It is interesting to note, however, that the complete oxidation of glutamate does not generate any high energy phosphate, does not respond to electrical stimulation (55), and is not inhibited by malonate (56).

Very little is known about the intermediary metabolism

of glycine in the brain. With the help of carboxyl labeled C^{14} glycine, Nakada and Weinhouse in 1953 showed the liberation of $C^{14}O_2$ from rat brain slices at a rate which corresponded to 1.1 μmole glycine per gram dry weight per hour (57). Leeper et al. in 1953, also found liberation of the carboxyl carbon of glycine using rat brain homogenates; they found no label in the CO_2 using glycine-2-C^{14}, but the label was incorporated into glutamic acid and proteins (58).

Douglas and Mortensen in 1956 found that when they injected glycine-2-C^{14} into the cisterna magna of rats, the labeled glutathione formed contained all its label in the glycine residue; no C^{14} activity was found in either the glutamic acid or cysteine moiety of the peptide (59).

The metabolism of glycine, serine and related compounds in organs other than brain has been elucidated more fully and will be presented in section C. of this introduction.

c. The metabolism of proteins and peptides.

The first evidence for intensive protein metabolism in the brain was histological. Desclin in 1941 reported that the anterior pituitary cells of the rat accumulated RNA granules during pregnancy (61), and Hyden interpreted this as being due to an active protein synthesis (62). He also showed that proteins disappear, and the ribonucleoproteins increase in the nerve cells of animals upon exhaustion or

intense electrical stimulation. The opposite effect was observed by Brattgård (63): in the cells of retinal ganglia, the RNA content decreased to almost zero upon lack of stimulation (adaptation to dark), but the proteins already synthesized remained intact.

Similar indications of active protein metabolism in the brain came from the isolation of proteolytic enzymes by Kies and Schwimmer in 1942 (64). One, a cathepsin, was especially potent at pH 3.7, but inactive in neutral media. The concentration of proteases in the brain was found to be related to the number of cell bodies (65). Ansell and Richter in 1954 suggested that the cathepsin of Kies and Schwimmer, which was especially concentrated in the nerve cell nuclei, might be concerned with protein synthesis at neutrality (66). They also showed the presence of a highly unstable neutral protease (67).

Experiments on the incorporation of radioactive amino acids into the proteins of the brain, after intravenous administration, were not very successful until recently. Friedberg, Greenberg, Winnick and their co-workers published a series of papers in 1948 on studies with tyrosine (68), glycine (69) and methionine (70), all showing the same pattern of low incorporation and slow protein turnover in the brain, as compared with other tissues. However, they made reference

to an unpublished finding of Friedberg: methionine, when injected into the cisterna magna, was incorporated into the proteins of the brain faster even than into those of liver, kidney or plasma (70). This effect was interpreted as being due to the "blood-brain barrier". Lajtha et al. (71) repeated these experiments using labeled lysine, an amino acid that passes through the barrier with relative ease. They have shown that the most rapidly metabolized proteins of the brain, liver and muscle have a half-life of comparable magnitude (1-2 days).

Deluca et al. in 1953 showed that inorganic phosphorus was rapidly incorporated into phosphoproteins in cat brain slices in vitro, but pointed out that it could be a simple exchange of the phosphate groups (18).

B. Metabolism and Mental Disorders.

In spite of the low rate of metabolism of amino acids in the central nervous system, these compounds, directly or indirectly, have long been suspected to play an important role in mental disorders. Insofar, however, only one amino acid has been proven to be involved in such disorders, phenylalanine.

1. Phenylpyruvic Oligophrenia.

In some cases of oligophrenia it has been observed, that large amounts of phenylalanine, phenylpyruvic and phenyllactic acids were excreted in the urine (72, 73, 74). In these patients the free phenylalanine content of tissues, spinal fluid, blood, and sweat was also abnormally high (75, 76, 77), whereas the protein-bound amino acid level was normal (78). Jervis, in 1950, found that the amount of the three acids excreted in the urine of phenylpyruvic oligophrenics depended upon their level of protein intake, and concluded that there might be a block in the catabolism of phenylalanine (77). Similar results were obtained by Borek et al. in the same year (76): when they injected phenylalanine into normal subjects, there was no appearance of phenylpyruvic acid and only a very little increase in the phenylalanine level in the serum. However, when they injected the same amount of phenylalanine into phenylpyruvic oligophrenic patients, there was a tremendous increase in the serum level of both substances. Since tyrosine and p-hydroxyphenylpyruvic acid were not implicated, it was concluded that the step

$$\text{phenylalanine} + \tfrac{1}{2}O_2 \longrightarrow \text{tyrosine}$$

was blocked in these patients and the accumulated phenylalanine was deaminated to phenylpyruvic acid (79), which was

the detrimental agent.

Bickel et al. in 1953 kept a two year old phenylpyruvic oligophrenic child alternatively on a phenylalanine-free casein hydrolysate diet and a normal diet, and proved conclusively that, at this early age at least the syndrome is completely reversible by lowering the intake of phenylalanine (80).

2. Glycine Metabolism in Schizophrenics.

Quastel and Wales, in 1938, observed that schizophrenic patients do not excrete as much hippuric acid after administration of benzoate as do normal subjects (81). In the same year Strom-Olsen et al. (82) were unable to confirm these results; but further work on the subject showed that in certain classes of schizophrenia, the benzoic acid detoxication was definitely lower (83, 84, 85). Quastel proposed the theory that amines produced by intestinal bacteria and absorbed into the portal vein, are normally removed by the liver; in schizophrenics, however, liver fails to detoxicate at a normal rate, allowing some of these amines to reach the central nervous system via the blood. Quastel and Wheatley had previously shown that many amines strongly inhibit the metabolism of isolated nervous tissue (86). Thus, schizophrenics would be under

a constant "narcosis" of poisonous amines (84).

Georgi et al. in 1948 repeated the benzoic acid detoxication experiments in the absence (simple Quick test), and in the presence of an extra supply of glycine (modified Quick test) and concluded that faulty liver function exists in the most severe cases of catatonics only; in many other cases low values for the simple test, and a normal value for the modified test, pointed to a low level of available glycine in the body (87). Similar results were obtained by Levi and Savich (88), and Mall and Junemann (89), who observed a correlation between the clinical state of the patients and the difference between the two tests. Graetz et al. in 1954 confirmed these results, but pointed out that they were valid in chronic cases only, since in acute schizophrenics both the simple and modified Quick tests overlap the normal values (90).

The above results point to a low level of available glycine in the body of chronic schizophrenics, but Pond in 1950 (91) was unable to show any difference in the amino acid levels in the urine, plasma, or cerebrospinal fluid of mental patients and normal subjects. On the other hand, Orstrom (92) showed that a metabolite of glycine, phosphoglycolic acid, occurs in greater quantities and has a higher turnover in the erythrocytes of

chronic schizophrenic patients. This could indicate a higher turnover and thereby a lower availability of glycine. A further support to this view was given by Greig and Gibbons who found that adrenochrome and epinephine, two hallucinogenic amines (94) which may occur in higher concentrations in schizophrenics than in normal individuals (95, 96) are able to catalyze a rapid break-down of glycine to glyoxylic or glycolic acids (93).

3. The Effects of Glutamic Acid on Mental Activities.

Price et al. in 1943 administered glutamic acid dayly to patients with certain types of seizures (97). They noted, that besides the alleviation of the condition in some of the patients, there was a general improvement in the mental capacity of all the patients, shown especially by their increased alertness. Six years later Mayer-Gross and Walker (98) found that glutamic acid, injected into patients in an insulin coma, was able to lift the coma. There was a concomitant increase in blood sugar level, but not sufficient to explain the restoration of consciousness. Glutamic acid itself could not have provided the necessary energy because, although glutamic

acid is capable of maintaining respiration of isolated brain slices (45) it does not provide the high energy phosphate necessary for normal nervous activities (55).

Weil-Malherbe noted in 1949 that, while injection of glutamate is able to terminate the coma, it also produced typical adrenergic reactions: increase in blood glucose, pulse rate and blood pressure (99). He concluded that glutamate may act by stimulating the production of adrenaline. This hypothesis he proved in 1952 (100), when he found that the insulin coma greatly reduces the blood adrenaline (and noradrenaline) content, but glutamate is able to restore it to the normal level. Whether this stimulation of adrenaline production happens by a direct action of glutamate on the adrenal medulla, or through the mediation of the central nervous system, remains to be investigated.

4. The Effects of Chlorpromazine and other Tranquilizers.

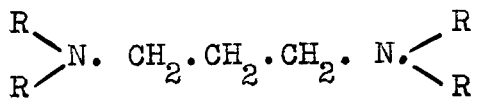
The discovery of two new tranquilizing agents, chlorpromazine and reserpine, has greatly stimulated studies on the metabolism of amines and amino acids in the nervous system in relation to mental disorders. The clinical

effects of these compounds are astounding: by careful administration of the two drugs, mental patients long since given up as incurables, have been restored to and maintained in a normal life. They also gave promising results in treating alcoholism (101) and reducing motor activity of morphine excitement (102). The clinical and physiological aspects of these treatments have been recently reviewed by Himwich (103). The biochemical effects are far from being elucidated in spite of the tremendous amount of work being done on them. Consequently, in this chapter, some of the major findings on the action of chlorpromazine will be given, without an attempt to show its exact mode of action.

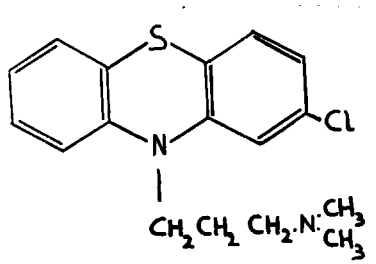
a. Chlorpromazine as a serotonin antagonist.

The significance of serotonin (5-hydroxytryptamine) for central nervous activities has been well established, although its action is still not known. In certain cases it acts as a stimulant (104, 105), but in others it seems to exert an inhibitory effect (106). Woolley in 1954 proposed the theory that hallucinogens act by antagonizing the effects of serotonin (105). This view was supported by the results of Welsh (104) when he found that LSD-25, a potent hallucinogen, antagonized the stimulation by serotonin on the heart of the clam. Gyermek in 1953 suggested that all serotonin antagonists should have the following

structure (107).



He discovered in 1955, that chlorpromazine, which also contains the same structure:



was effective in antagonizing the action of serotonin on isolated rat uteri (108). Similar inhibitions of the serotonin action by chlorpromazine were noted by Lecomte (105) and Benditt and Rowley (109) disagreeing with the theory of Woolley and Shaw (105). Shaw and Woolley in 1956 found that LSD-25 not only did not antagonize serotonin but it acted in the same manner in stimulating isolated clam heart and raising the arterial blood pressure in dogs (110).

b. Effect of Chlorpromazine on Tissue Metabolism. In spite of the unique action of chlorpromazine on

mental disturbances, its action on the metabolism of different tissues appears to be similar to that of narcotics. It depresses the uptake of iodine by the thyroid (111), inhibits a number of enzyme systems like cytochrome oxidase or ATPase (112), and decreases the oxygen consumption of mouse brain tissues at 37°C (113), although the latter action is not apparent at 30°C. This observation is of particular interest in view of the artificial potentiation of hibernation by chlorpromazine, to be described below.

The inhibition of oxygen uptake by chlorpromazine is especially apparent with potassium stimulated rat brain cortex slices (43). This effect is very similar to those of narcotics and local anaesthetic agents (40, 41, 42).

Further indications for a decreased general metabolism in presence of chlorpromazine are: a decrease in renal clearance of creatine (114), although Drovanti and Peruzzo believe, that this is caused by changed neurovegetative and endocrine conditions rather than organic changes in the kidney; a delay in the expected fall in blood glucose after the ingestion of glucose (115), due to a depressed liver function; the high increase in the fasting blood sugar level in alloxan diabetic animals by chlorpromazine (116) pointing to a lower rate of utilization of glucose by the cells.

Turnover of phospholipid phosphorus in certain parts of the brain was found by Wase et al. to be stimulated at first, then decreased, after regular administration of chlorpromazine (117). They interpreted these results as an increase in cell permeability followed by an uncoupling of phosphorylation. Ansell and Dohmen have also reported a generalized depression of phospholipid, especially phosphatidyl choline, turnover in brain due to chlorpromazine (118).

c. Artificial Hibernation.

Laborit et al. in 1952 noted that when total anaesthesia was potentiated with chlorpromazine, the body temperature of the patient could be lowered to an extent where the organs of the body required considerably less blood than normally (119). Under these conditions, the blood supply to any organ could be cut off for a considerable length of time without causing any irreversible damage to that organ, a great advantage especially in thoracic operations. Dauri et al. (120) found that the basal metabolic rate hardly diminished until the external temperature reached 30°C, but at this temperature the drop in the basal metabolic rate was very sharp, except in hyperthyroidic patients who responded poorly to artificial hibernation. Similar results were found by Cocchia and

Cuocolo (121).

The function of chlorpromazine in artificial hibernation is not well known. Peruzzo in 1954 found that either cold (30°C) or chlorpromazine reduced the *in vitro* respiration of mouse brain cortex slices, but at 30°C chlorpromazine showed no additional inhibitory effect (113). Similarly, Bartlett and Register in 1955 showed that chlorpromazine decreased the total amount of sulfhydryl compounds in the liver at normal temperatures *in vivo*, but had no effect at low temperatures (122). Decourt believes that its function in artificial hybernation is a facilitation of the lowering of body temperature by its action as a hypotensive agent (123).

d. Other Effects of Chlorpromazine.

A small but definite proportion of the patients treated with chlorpromazine has shown signs of liver cirrhosis which could be an objection to its use. This finding may be connected with those of Wase et al. in 1956 (117), that the proteins of the liver of rats incorporated S^{35} activity from administered chlorpromazine-S^{35}. No other proteins showed any activity.

In many other cases, repeated administration of chlorpromazine caused a considerable increase in blood copper level accompanied by extrapyramidal nervous disorders.

The condition resembles Wilson's disease, but there is no liver cirrhosis, and recovery is complete a few days after suppression of the chlorpromazine treatment (124).

C. The Metabolism of Glycine and Serine *in Vivo*.

1. The Interconversion of Glycine and Serine.

a. Evidence in favour of the interconversion.

Studies on the metabolism of the two amino acids, glycine and serine, have always been complicated by their rapid interconversion. The mechanism of this interconversion was not elucidated until relatively recently. Shemin (125) in 1946 provided definite proof that the general reaction is:

$$\text{serine} \rightleftharpoons \text{glycine} + \text{1 carbon fragment}$$

He found that this reaction proceeded very rapidly in either direction in the rat and guinea-pig. Ehrensvärd et al. in 1947 incubated yeast with carboxyl labeled glycine, and found equal labeling in glycine, serine and proline carboxyl carbons isolated from the proteins of the cells (126). Winnick et al. incubated rat liver homogenates with methylene labeled glycine. The isolated proteins

contained the label in serine, glutamate, aspartate and arginine (127); again the activities of serine and glycine were almost equal (128). When they included a ten fold excess of unlabeled serine in the incubation medium, they observed an 88% dilution effect. A 90% dilution would indicate an "infinite rate of interconversation".

Elliott and Neuberger in 1950 found that the interconversion was extremely rapid in rats in vivo, and the amino nitrogen did not dissociate during the process (129).

Similar results were obtained by Elwyn and Sprinson in 1954, but they also found (130) that the ratio between C-3 and C-2 of serine remained unchanged, despite the rapid cleavage between the two carbon atoms.

b. The nature of the "one carbon fragment".

The origin or fate of the "one carbon fragment" has involved a great deal of research, but not until recently have the intermediates been established. At first, the "one carbon fragment" was believed to be formate. Results by Sakami in 1948 supported this view (131). He injected glycine 1-C^{13} and formate -C^{14} into rats and found that the liver proteins contained serine-1-C^{13}-3-C^{14}:

Similar results were obtained when the C^{14} formate was substituted by methyl-C^{14}-choline or glycine-2-C^{14}. He proposed that the methyl groups of choline and methionine and the α-carbon of glycine can form formate, which in turn might be oxidized to CO_2, or condense with glycine to form serine (132).

Siekewitz and Greenberg in 1949 confirmed these findings and showed that both the formation of formate from the α carbon of glycine and the oxidation of formate to CO_2 were irreversible reactions (133). Nakada and Weinhouse in 1953 provided further evidence to support the view that the one carbon fragment was formate (57) by isolating radioactive formate, after incubating rat liver homogenates with any one of glycine-2-C^{14}, serine-3-C^{14}, glyoxylate -2-C^{14} or glyocolate-2-C^{14}.

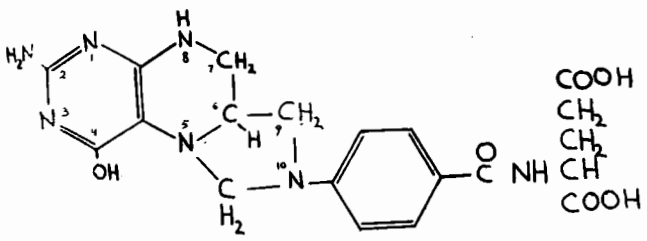
The first doubts of this theory came when Arnstein and Neuberger (134) found that synthesis of the methyl groups of choline from formate, or from the β-carbon atom of serine were of equal quantitative importance <u>in vivo</u> but the formate carbon gave rise to only very little of the β-carbon of serine. These were long term feeding experiments, and the distribution of activity reached almost a steady state value in the animals, thus relative activities were reflecting the importance of pathways rather than velocities. From the above results, the authors

concluded that formate was probably not an intermediate in methyl group formation from serine.

A non-enzymatic cleavage of serine was demonstrated by Metzler et al. in 1954 (135). He obtained as end products glycine and formaldehyde. In the same year Blakley demonstrated that pigeon liver extracts catalyzed condensation of glycine and formaldehyde to form serine (136). Formate was not able to replace formaldehyde. The system required ATP, DPN and either folic acid or tetrahydrofolic acid (THFA), the latter probably being the true cofactor.

Similarly, Doctor et al. found that chick liver acetone powders catalyze the incorporation of formaldehyde or the β-carbon of serine into methionine in presence of homocysteine and folic acid. Formate had very little activity (137).

It thus became evident that the "one carbon fragment" was a complex between THFA and formaldehyde. On the basis of his results Blakley (136) proposed that the active methyl donor was 5,10-methylene THFA:



The nature of this complex has recently been questioned by Blakley himself on the basis of some experiments on the non enzymatic combination between THFA and formaldehyde, and he proposed either bis (hydroxymethyl)THFA or N-5-hydroxymethyl THFA as the active methyl donor (138, 139). However Kisliuk (140) and Osborn and Huennekens (141) proved conclusively that it is the N-5, N-10-methylene bridge compound which is involved.

c. Cofactor requirements and reactions steps of interconversion.

The enzyme system responsible for the reaction:

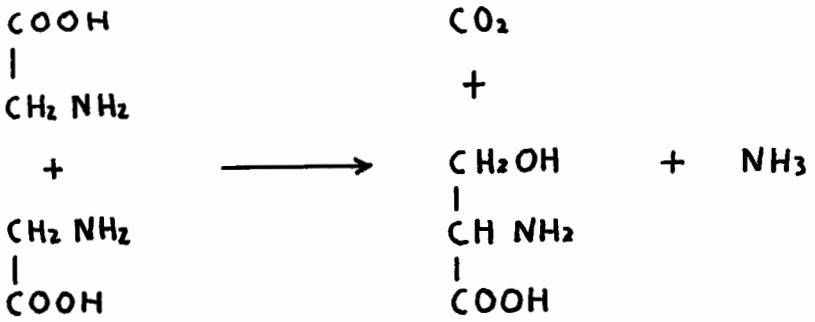
serine + THFA $\rightleftharpoons$ glycine + HCHO.THFA

has been partially purified from rabbit and sheep liver by Alexander and Greenberg, and Huennekens et al. (142, 143). It requires pyridoxal phosphate as a cofactor and is highly specific for serine in the forward reaction and for glycine and formaldehyde in the reverse: sarcosine does not replace glycine, and acetaldehyde or formate do not replace formaldehyde (144), and only one of the two optical isomers (due to the asymmetrical carbon 6) of THFA can participate in the reaction.

The enzyme system isolated by Huennekens et al. (143)

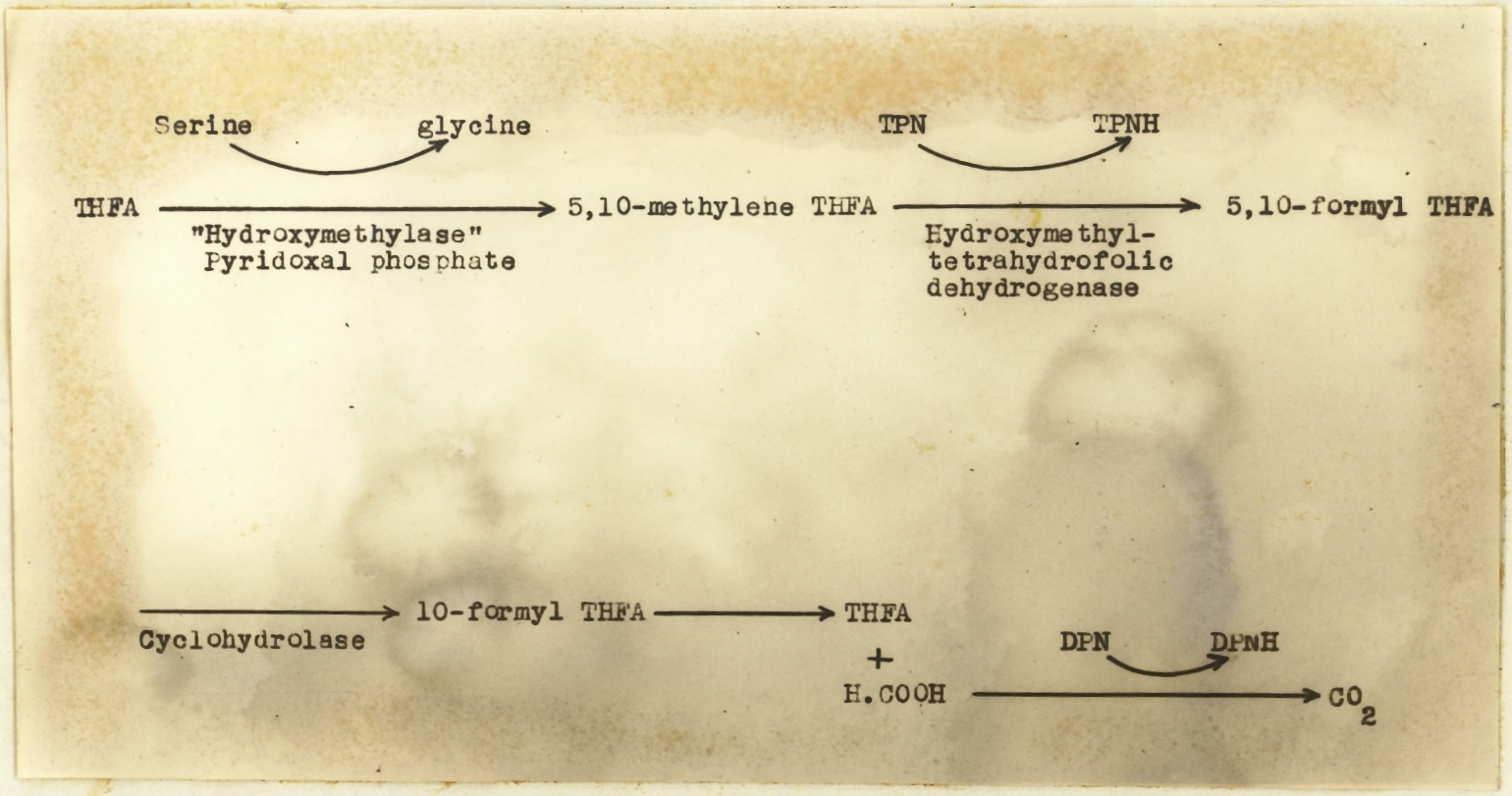
further oxidized the formaldehyde-THFA complex to N-10 formyl THFA, and by further purification they were able to demonstrate the sequence of reactions, (141, 145) represented in Scheme 1.

The role of other cofactors in the interconversion of glycine and serine have been worked out by Vohra et al. (146), Blakley (136, 144) and Doctor et al. (137). The different pathways with the sites of action of the cofactors proposed by these authors is represented in a combined map on Scheme 2. From this figure it is evident that glycine can give rise to serine by way of methylene-THFA, the overall reaction being:

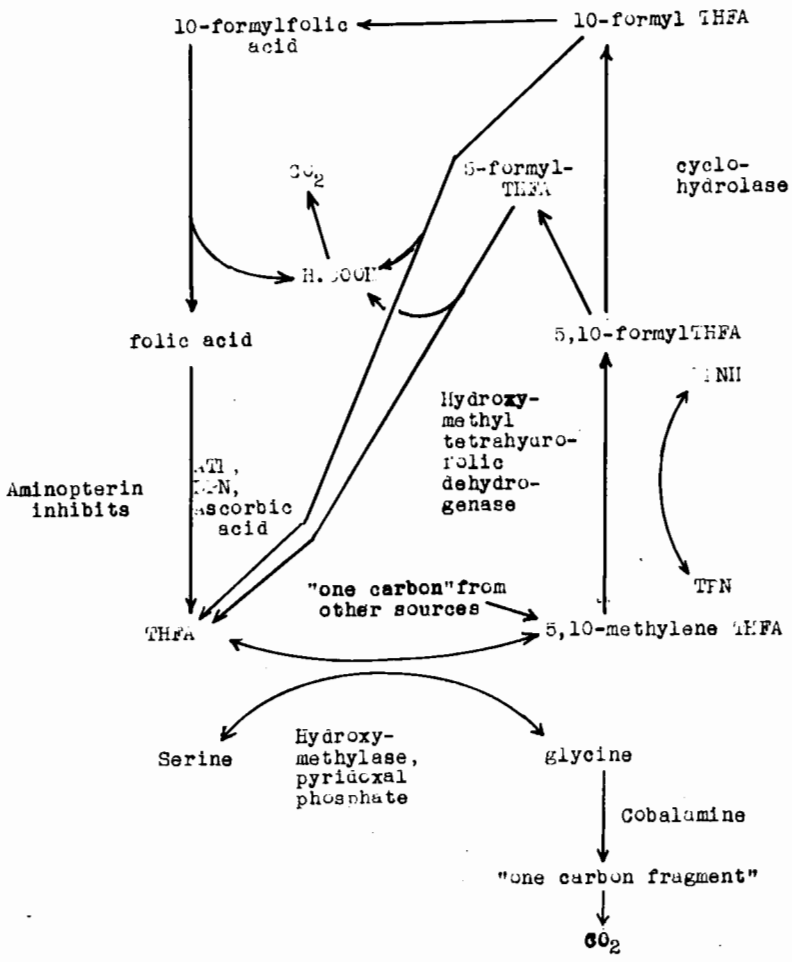


This reaction is in excellent agreement with the findings by Siekewitz and Greenberg (133) that carboxyl labeled glycine gives rise to serine labeled in the carboxyl position only, whereas α -labeled glycine gives rise to labeled serine with no label in the carboxyl carbon.

SCHEME 1


Serine
glycine
TPN
TPNH
THFA
5,10-methylene THFA
5,10-formyl THFA
"Hydroxymethylase"
Pyridoxal phosphate
Hydroxymethyl-
tetrahydrofolic
dehydrogenase
10-formyl THFA
THFA
Cyclohydrolase
+
DPN
DPNH
H.COOH
CO_2

SCHEME 2


10-formylfolic acid
10-formyl THFA
CO_2
5-formyl-THFA
cyclo-hydrolase
folic acid
5,10-formylTHFA
Hydroxy-methyl tetrahydro-folic dehydro-genase
Aminopterin inhibits
ascorbic acid
"one carbon" from other sources
THFA
5,10-methylene THFA
Serine
Hydroxy-methylase, pyridoxal phosphate
glycine
Cobalamine
"one carbon fragment"
CO_2

d. Quantitative aspects of the interconversion.

The reactions involved in the interconversion have been studied as to their biological importance by Arnstein and Neuberger in 1953 (147). They tried to establish the net synthesis of either amino acid by long term feeding of radioactive glycine and serine. They found that the amounts of these amino acids synthesized by the body did not depend either on intake level or on growth rate. However, the amount of glycine activity incorporated into the serine β-carbon depended on the glycine intake, and could be reduced to negligible levels by lowering the glycine intake. They concluded that the formation of serine from glycine was merely a regulating mechanism to keep the glycine at a constant body level.

Simkin and White obtained similar results (148, 149): conversion of glycine to serine was a rapid and first order reaction depending on the concentration of glycine, but not of serine. However, the reverse reaction was of zero order, proceeding at a constant rate regardless of the concentration of the reactants.

It is interesting to note that quantitatively the most important reaction for the break-down of serine was found to be the formation of glycine, with a simultaneous oxidation of the β-carbon to CO_2, in liver slices <u>in vitro</u> (150).

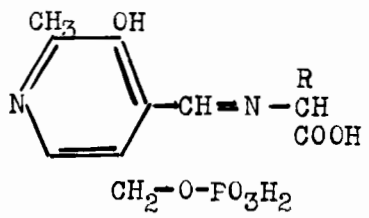
2. Biosynthesis and Break-down of Glycine and Serine.

a. Transaminations and the role of pyridoxal phosphate.

In mammals glycine and serine are non essential amino acids, thus there has to be a mechanism of formation from non-nitrogenous materials. They are also glucogenic (131, 156, 151). It was not until 1950, however, that the key step, the reversible deamination, or transamination, of either amino acid was discovered. As late as 1944, Edlbacher and Wiss stated that glycine was among the few amino acids which were not deaminated by pigeon or rat brain, liver, kidney or heart preparations (152, 153, 154). Feldman and Gunsalus in 1950 demonstrated a weak but definite transamination between glycine and α-ketoglutarate in bacterial cell preparations (155), and showed that pyridoxal phosphate was a necessary cofactor for this, as well as any other transamination reactions. About the same time, Camarata and Cohen (156) obtained similar results with aqueous extracts of pig heart, liver and kidney. Wilson, King and Burris in 1954 demonstrated the reverse reactions (157), synthesis of glycine from glutamate and either glyoxylic or glycolic acid.

In all these reactions pyridoxal phosphate was found to be a necessary cofactor, but its mechanism of action is still very strongly debated. Umbreit et al. (158) found that pyridoxamine phosphate can replace pyridoxal phosphate in certain preparations, but later showed that the amine is inactive with purified pig heart glutamic-aspartic transaminase (159). The amine was also inactive as a coenzyme for purified tyrosine decarboxylase. However, Wilson et al. in 1954 showed that a great number of transaminases in plant seedlings catalyze the transamination between pyridoxamine and α-ketoglutarate to form pyridoxal and glutamate in the presence of pyridoxal phosphate (157). In the same year Metzler et al. demonstrated a non-enzymatic reversible transamination between pyridoxal and a number of amino acids (160).

Whether or not pyridoxal phosphate acts as a nitrogen transport, it is well established that the first step in transamination reactions is the formation of a "Schiff base":



b. Conversion of glycine to glyoxylic acid.

Oxidative deamination of glycine to glyoxylic acid was first noted by Ratner et al. in 1944 (161). The enzyme system occurred in almost all organs and was able to oxidize both glycine and sarcosine. Due to the low activity of the system it was not investigated further until the radioactive carbon techniques became well established.

Weinhouse and Friedmann in 1951 undertook an extensive investigation of the oxidation of glycine *in vivo* (162). They found that glyoxylate and glycolate were readily incorporated into the glycine moiety of hippuric acid, but acetate and oxalate were inactive. All the substrates were oxidized mainly to CO_2. In 1953, Nakada and Weinhouse demonstrated that glycine is incorporated into glyoxylate and oxalate (57). They pointed out, however, that the formation of oxalate from glyoxylate took place only when the glyoxylate concentration in the body was extremely high. Otherwise only CO_2, and no oxalate, was formed.

Similar results were obtained by Nakada, Friedmann and Weinhouse in 1955 using rat liver homogenates *in vitro* (163). Radioactive glycine or serine gave rise to labeled

CO_2, the activity being considerably diluted by including non active glyoxylate. However, no dilution was observed in the CO_2 from radioactive glyoxylate when either unlabeled glycine or serine were included. From these results they concluded that glyoxylate was an intermediate in the complete oxidation of serine or glycine:

c. Formation from glycine, and the fate of the "one carbon fragment."

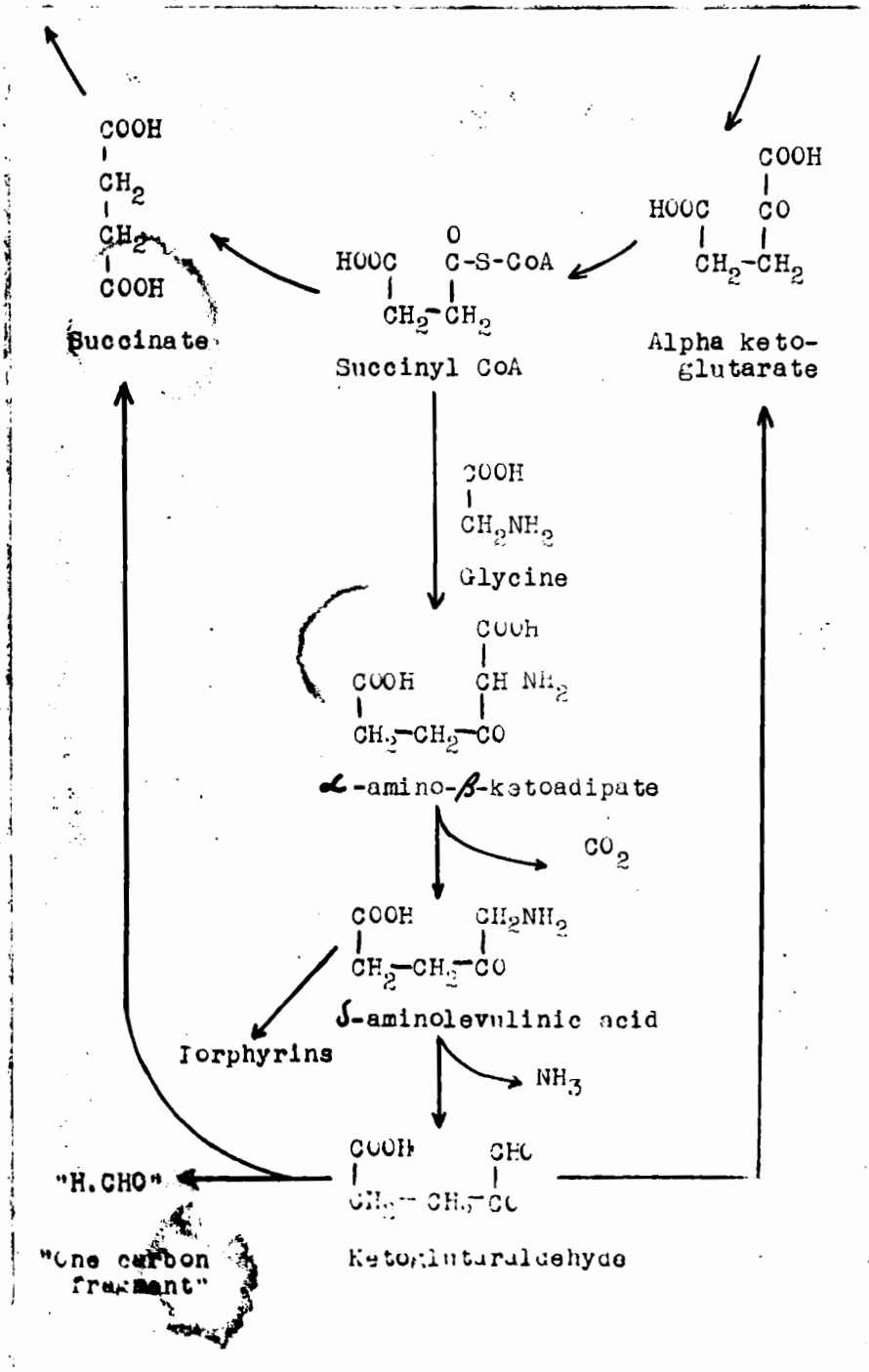
The nature, and formation from serine, of the "one carbon fragment" has been discussed earlier. There is also evidence, that it can be derived from the α-carbon of glycine (134, 146, 147). Nakada and Weinhouse (57) showed that the α-carbons of glycine, glyoxylic and glycolic acids are oxidized to CO_2 via a formate derivative. In view of the more recent findings of Huennekens et al. (143) the pathway of the oxidation of these αcarbons may proceed through the methylene-THFA sequence, although the mechanism of the transfer of the methylene group from the parent molecule to THFA is obscure.

One pathway for the formation of the "one carbon

fragment" from the α carbon of glycine was proposed by Shemin in 1956 (164). It is through a side reaction of the Shemin cycle (Scheme 3). According to this scheme, δ-aminolevulinic acid, containing the α-carbon of glycine at the δ position, can be oxidatively deaminated to keto-glutaraldehyde (succinylformaldehyde) which in turn will be hydrolyzed to succinate and formaldehyde. Unfortunately, Shemin gives no proof to this side reaction other than the fact that the α carbon of glycine gives rise to "one carbon fragment".

One of the most important roles of the "one carbon fragment" is the formation of methyl groups. DuVigneaud in 1941 demonstrated that the methyl group of methionine acts as a direct precursor of choline and creatine methyl-groups (165). In the reverse direction Sakami (132) found a considerable incorporation of the methyl groups of choline into serine, at the β position only. Arnstein and Neuberger in 1953 confirmed that methionine is the direct precursor of choline (134). They also showed that about 70-75% of the methionine methyl groups come from the β carbon of serine. Vohra et al. and Doctor et al. provided conclusive evidence that the formation of the methyl groups proceeds via the "one carbon fragment" sequence, when they demonstrated that these reactions do

SCHEME 3


COOH
CH2
CH2
COOH
Succinate
HOOC
O
C-S-CoA
CH2-CH2
Succinyl CoA
COOH
CO
CH2-CH2
Alpha keto-glutarate
COOH
CH2NH2
Glycine
COOH
CH NH2
COOH
CH2-CH2-CO
α-amino-β-ketoadipate
CO2
COOH
CH2NH2
CH2-CH2-CO
δ-aminolevulinic acid
Porphyrins
NH3
COOH
CHO
"H.CHO"
Ketoglutaraldehyde
"One carbon fragment"

not occur in the absence of folic acid (137, 146).

d. Synthesis of glycine and serine from carbohydrates.

The direct relationship between serine and the carbohydrates was first demonstrated by Sakami in 1948. He found (131) that formate-C^{14} and glycine-1-C^{13} gave rise to serine-1-C^{13}-2-C^{14} and to glycogen. The glucose moiety of the glycogen contained the C^{13} label in positions 3 and 4, and the C^{14} label in positions 1 and 6. Vernon and Arnoff (166) showed that labeled CO_2 was rapidly incorporated into the carboxyl carbon of serine and alanine during photosynthesis. They concluded that this occurred through the fixation of CO_2 onto a two carbon fragment, possibly acetate. However, Weinhouse and Friedmann (162) were unable to demonstrate conversion of acetate into glycine in rats. The reverse reaction, formation of acetate from serine or glycine was clearly demonstrated by Elwyn and Sprinson (130).

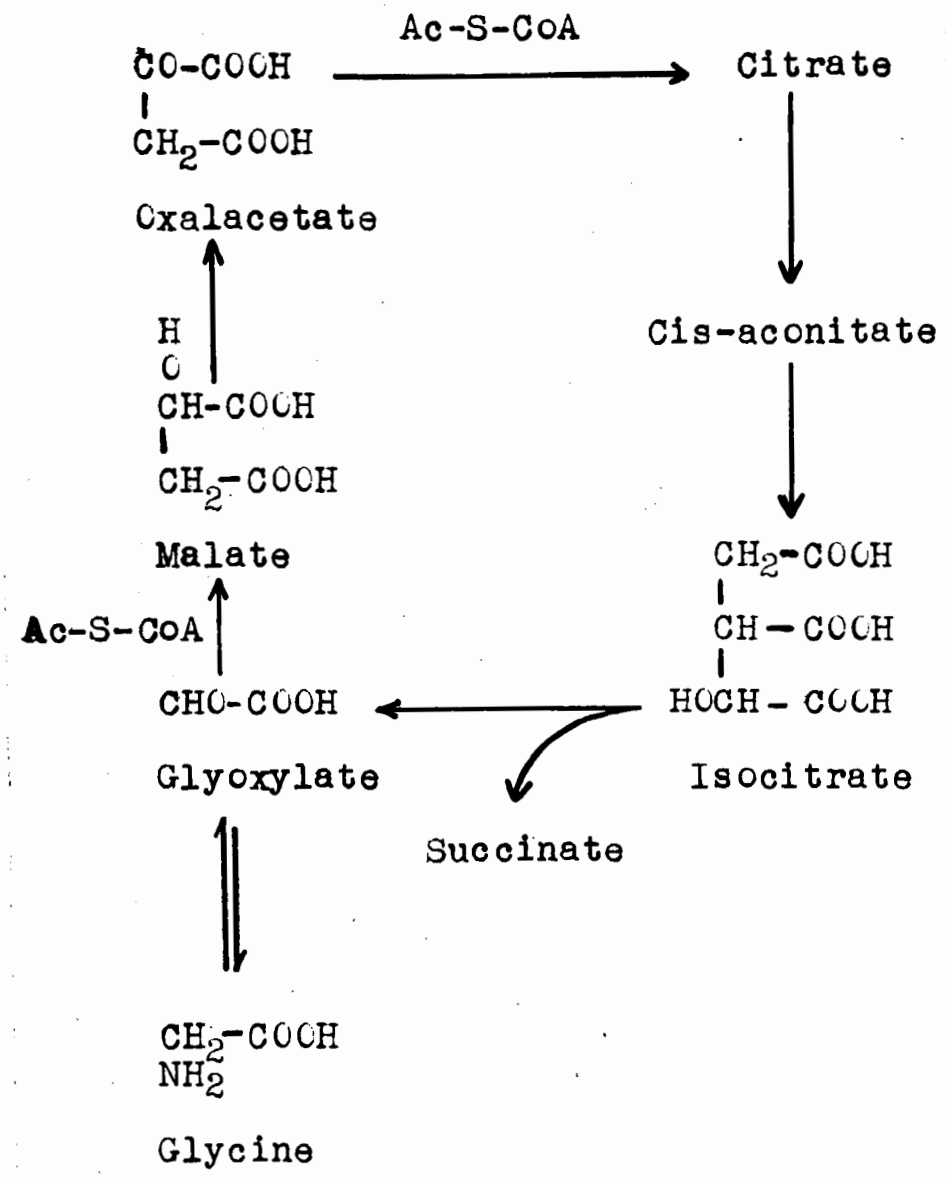
Proof for the net synthesis of serine in intact animals was obtained by Arnstein and Neuberger in 1953. On the basis of their experiments they have calculated that 3.5 millimoles of serine and 2.5 millimoles of glycine per day were synthesized in rats per 100 gm body weight.

This synthesis did not depend on either the growth rate or the level of intake of these amino acids (147). A net synthesis of serine from administered pyruvate was shown by Simkin and White (149). Koeppe et al. (167) showed that serine was formed from glycerol without randomization of the carbon atoms. They concluded that the key reaction of serine synthesis from carbohydrate intermediates occurs far from the tricarboxylic acid cycle and suggested 3-phosphoglyceric acid as a probable intermediate.

The reaction sequence involved in the formation of serine from carbohydrates in rat liver preparations has recently been worked out by Ichiara and Greenberg (168). Their evidence, although not conclusive, strongly suggests the following reactions: an irreversible oxidation of 3-phosphoglycerate to 3-phosphohydroxypyruvate; reversible transamination with glutamate to give phosphoserine; hydrolysis of phosphoserine to serine and inorganic phosphate.

Most of the glycine synthesized in the body comes from serine, but Meinhart and Simmonds (169) showed that in certain bacteria 33% of the glycine synthesized from glucose does not pass through serine. It may come from citrate through the action of "isocitricase", an enzyme

SCHEME 4


Ac-S-CoA
CO-COOH
CH2-COOH
Oxalacetate
Citrate
Cis-aconitate
H
C
CH-COOH
CH2-COOH
Malate
CH2-COOH
CH-COOH
HOCH-COOH
Isocitrate
Ac-S-CoA
CHO-COOH
Glyoxylate
Succinate
CH2-COOH
NH2
Glycine

of the "Glyoxylate Bypass" recently proposed by Kornberg and Krebs (170) (Scheme 4). Whether the same holds true in higher organisms has not yet been investigated.

Glycine can also be formed from choline by oxidation of the ethanolamine moiety via betaine and sarcosine (171, 172). This reaction, however, cannot be considered as a net synthesis since ethanolamine itself is derived from the decarboxylation of serine (134).

MATERIALS AND METHODS

Materials.

The Radioactive amino acids were obtained from Merck Co. Ltd. and from The Atomic Energy Commission of Canada. One sample of glycine-2-C^{14} was chromatographed monodimensionally using 80% pyridine in water and Butanol-Acetic acid-water. In neither solvent were any radioactive or ninhydrin positive impurities shown to be present. No further tests were done on the purity of subsequent batches.

Unlabeled amino acids were obtained from the Nutritional Biochemicals Corporation. All other chemicals used were "Reagent" grade.

The animals used in the experiments were adult rats ("hooded" strains) of either sex, weighing 150-250g, bred in our own laboratory.

Preparation of radioactive solutions.

The radioactive amino acid solutions were prepared by dissolving the required labeled and unlabeled amino acid in water to give a final concentration of 60mM and the desired activity. Aliquots of these solutions, dependent on the amino acid used, were diluted with distilled water, transferred on to aluminium plates and their radioactivities measured. The solutions were kept

under deep freeze, and only thawed during preparation of the experiments. Under these conditions they showed no sign of deterioration for several months.

Preparation of other solutions.

Solutions of respiratory substrates and inhibitors were prepared fresh each day.

The respiratory substrates were dissolved in distilled water, neutralized when necessary with 0.5 N HCl or NaOH and made up to ten times the required final concentration. 0.3 ml of the solution was added to the main compartment of the Warburg vessel together with 2 ml saline solution and distilled water to 3 ml.

The inhibitors were prepared in a similar manner to give neutral solutions thirty times the required final concentration. 0.1 ml was placed in the side arm and tipped after a preliminary incubation into 3 ml of medium.

Preparation of tissue slices.

The animals were killed by cervical dislocation and then decapitated, the cerebra rapidly removed, freed from the cerebellum, and chilled by submersion into ice

cold saline. The time lapse from killing the animal to cooling the brain never exceeded 30 seconds.

Cortex slices were cut with a chilled Stadie-Riggs tissue slicer (174) parallel to the dorsal and the lateral surfaces of the cerebral hemispheres using only one slice from each surface. The slices were quickly weighed on a torsion balance of±1mg accuracy and two slices, one dorsal and one lateral, weighing a total of 60-70 mg, placed in each vessel containing the ice cold medium.

In one set of experiments the cerebral cortex of a Rhesus monkey was used, kindly supplied by the University of Montreal. The animal was lightly anaesthetized and decapitated. The brain was quickly removed, wrapped in light aluminium foil, placed in crushed ice in a Dewar thermos bottle and transported to our laboratory. The cortex was divided into small cubes, freed from white matter and sliced with a chilled Stadie-Riggs slicer, cutting as many slices as possible from each cube.

Preparation of rat brain homogenates.

The rats were killed, decapitated, the brains rapidly excised, cooled in ice and weighed; 3g were placed in a solution containing 3ml. of each of the stock solutions for the modified McMurray-Reiner medium. The

system was homogenized in a Potter-Elvehjem glass homogenizer at 0°C. 3 ml of the homogenate was placed in the main compartment of the Warburg vessel together with 0.1 ml of a radioactive glycine solution. A small roll of filter paper together with o.15 ml of 40% KOH was placed in the centre well. The vessels were attached to the manometers and immersed in the bath at 37°C with air in the gas phase.

The incubation media.

Incubations were carried out using standard Krebs-Ringer saline (173) with 10 mM phosphate buffer at pH 7.4. Fresh stock saline was prepared bi-weekly, but the $CaCl_2$ excluded and prepared separately in order to prevent precipitation of $Ca_3(PO_4)_2$. This stock solution was prepared 1.5 times the normal concentration in order to allow other substances to be added without changing the final concentrations. The final concentrations of the medium were NaCl, 128 mM; KCL, 5 mM; $CaCl_2$, 3mM; $MgSO_4$, 1.3 mM and sodium phosphate buffer, 10 mM.

In one series of experiments with brain homogenates a modification of the medium described by McMurray et al. (175) was used. The final concentrations of this medium were glucose, 28 mM; hexosediphosphate, 5mM; adenosine triphosphate sodium, 0.7 mM; diphosphopyridine nucleotide, 1mM; cytochrome-c, 0.06 mM; sodium fumarate, 1.6 mM; nicotinamide, 40mM; $MgCl_2$, 8 mM; KCl, 80 mM and sodium

phosphate buffer at pH 7.4, 10 mM. 80 mM KCl was used to make the medium isotonic instead of hypotonic. Individual stock solutions of the components were prepared in ten times the final concentration, and stored individually under deep freeze.

Incubation of tissue slices.

The incubations were carried out using the standard Warburg manometric technique. The main compartment contained 2 ml of the concentrated stock saline and the side arm 0.1 ml of the radioactive amino acid solution. After placing the tissue slices in the main compartment, 0.3 ml of 0.3% $CaCl_2$ solution was added to the medium. 0.15 ml of 40% KOH solution was placed in the centre well together with a small roll of filter paper, and finally to the medium distilled water to a total volume of 3 ml. Unless indicated otherwise, respiratory substrates were placed in the main compartment and inhibitors and unlabeled suspected intermediates in the side arm.

The vessels were attached to the manometers, gassed with pure oxygen for 3-5 minutes and immersed in the bath. First readings were taken after 15 minutes of thermal equilibration and 20 minutes later the contents of the side arms were tipped into the main compartment. Incubation was for 90 minutes, after tipping, at 37°C. The reaction

was stopped by injecting, through the side arm, 0.3 ml of 30% trichloroacetic acid. The vessels were shaken for another 20-30 minutes in the bath in order to expel the dissolved CO_2 from the solutions.

Radioassay of CO_2.

The filter paper from the centre well of the Warburg vessel was transferred to a centrifuge tube containing exactly 1 ml of carrier Na_2CO_3, equivalent to 15-20 mg $BaCO_3$. The centre well was washed three times with distilled water and the washings added to the tube. The contents of the tube were mixed, stoppered and left overnight to elute the radioactive CO_2 from the paper.

The following day the paper was removed, rinsed and the combined solution and washings were treated with 0.1 ml 2 M NH_4Cl and 0.5 ml saturated $BaCl_2$. The $BaCO_3$ precipitate was centrifuged, washed twice with 10 ml portions of water, once with 10 ml acetone and finally resuspended in 0.5 ml acetone and transferred onto a tared aluminium plate for weighing and counting.

The total weight of $BaCO_3$ was taken as the sum of the carrier carbonate and the CO_2 produced during the incubation calculated from the O_2 consumed.

For the estimation of the $BaCO_3$ equivalent of the carrier NA_2CO_3 solution, 3 ml of the solution with

0.45 ml 40% KOH were left overnight in a stoppered test tube. The following day $BaCO_3$ was precipated with 1.5 ml of saturated $BaCl_2$, the precipitate was quantitatively transferred to a tared sintered glass crucible (medium porous), washed under suction with water and acetone, dried at 110°C for one hour and weighed.

Radioassay of proteins.

The procedure followed was essentially that of Siekewitz (176). The contents of the main compartments of the Warburg vessels were mixed with 5 ml of 30% trichloroacetic acid and homogenized with a teflon pestle for 3 minutes. The precipitate was centrifuged and extracted three times with 10 ml 6% trichloroacetic acid. The first extraction was carried out at 90°C for 10 minutes and the rest at room temperature. Subsequent extractions were carried out with 10 ml 95% ethanol; 10 ml of a mixture of ethanol and ether (3:1 v/v) at 60°C for 10 minutes; and 10 ml of ether. Finally, the proteins were resuspended in 1.0 ml of a mixture of chloroform and ether (4:1 v/v), transferred to aluminium plates, dried, weighed, and the protein radioactivities measured (177).

Chromatographic procedures.

The procedure is that of Du Ruisseau et al (46).

At the end of the incubation period, the tissue slices were immediately removed from the vessel, rinsed quickly in distilled water and transferred into a test tube containing 5 ml 80% ethanol. No trichloroacetic acid was added to these vessels. The mixture was homogenized with a teflon pestle, cooled and left at $0^{o}C$ for 2 hours to coagulate the proteins, then centrifuged. The supernatant was passed through a column of Cowex-50 resin, 2.5 x 0.7 cm^2 and the resin washed with water. The absorbed amino compounds were eluted with 8 ml N ammonia and the eluate evaporated at $60^{o}C$ in a stream of air.

The amino compounds were then dissolved in 0.1 ml H_2O, and chromatographed two dimensionally on Whatman No. 1 paper in buffered phenol and pyridine phases.

The first solvent, buffered phenol was prepared by saturating liquid phenol (Merck, Carbolic Acid) with a 10% solution of trisodium citrate. The solvent was placed in a cylindrical chromatography jar together with a small beaker containing 0.5 ml concentrated ammonia. The solvent front moved approximately 12 inches in 20 hours.

The second solvent, pyridine, was prepared by diluting distilled pyridine to 80% with water. The solvent front moved 16 inches in 18 hours.

The amino acids on non-radioactive chromatograms were located by ninhydrin. The papers were dipped into a solution of 0.04% ninhydrin in acetone, dried and heated at $110^{o}C$ for 5 minutes.

The radioactive spots were located by exposing the paper against a Kodak No Screen, Blue Base X-ray plate for 3-10 days. The activities of the spots were counted with a Geiger-Muller tube.

Measurements of amino acid uptake.

Essentially the same procedure was used as the preparation of the samples for chromatography. The tissue slices were removed from the vessels, rinsed quickly and homogenized with 80% ethanol. The mixture was cooled to $0^{o}C$, centrifuged and 0.6 ml aliquots of the supernatant were transferred onto aluminium plates, dried and counted.

Measurements of the radioactivities.

The radioactivities of the samples on the aluminium plates were measured with a thin mica window Geiger-Muller tube attached to a Tracerlab shielded sample changer and automatic scaler. The preset count was adjusted to 1280 counts except in the case of very low activity samples, where 640 counts were measured. The activity was corrected for self absorption, using a modified self-absorption curve for Ba_2CO_3 (182). Corrections were also applied for the initial activity of the amino acid and for the amount of tissue introduced into the vessels. It was found convenient to correct for an initial activity of 100,000 counts per

minute per micromole corresponding to 10^{-5} micromole per counts per minute, thus every count per minute activity obtained from one milligram tissue corresponds to one micromole of the original amino acid per 100 gram tissue. For this reason the C^{14} values obtained after incubating the amino acids with tissue slices are expressed as microatom C^{14} per 100 gm tissue dry weight, this unit being essentially the same as the "conversion capacity" of Nakada and Weinhouse (57).

Standardization of the Tissue Slices.

The metabolic activities of tissue preparations are usually expressed on the basis of mg dry weight of tissue. In the case of tissue slices, however, the ratio of wet weight to dry weight is not constant because of a variable amount of water carried onto the slice by the wet blade. There are four methods of overcoming this difficulty: slicing with a dry blade, estimation of water content on separate portions from the same organ as used for incubation, estimation of the water content of slices after incubation and estimation of the average water content on a series of animals. Of these four, the first two methods are not applicable to rat brain cortex slices because a dry blade causes too much damage to this tissue, and not enough material can be obtained from one animal for incubation and estimation.

In order to decide which of the latter methods

to follow, a series of investigations were carried out. The rat brain cortex slices were obtained as described previously, weighed on a torsion balance and divided into five parts.

1. Slices were placed in a drop of saline on a metric graph paper, flattened out and the contour carefully drawn in order to determine their surface area. By assuming that one milligram of wet tissue has a volume of one cubic millimeter, the average thickness was obtained by dividing the wet weight with the surface area. The slices were removed, rinsed quickly with distilled water and placed on tared aluminium plates, dried at 110°C for 1 hour and weighed.

2. Slices were incubated for 90 minutes in a hypertonic saline medium with 0.1 M KCl, in the presence of 10 mM glucose.

3. The medium used contained 10 mM glucose but was isotonic and the slices respired with normal activity throughout the experiment.

4. Slices were incubated as in (3), but at the end of the incubation period the medium was acidified with 0.5 ml of 0.1 M H_2SO_4 and the slices removed after 30 minutes.

5. Here the slices were incubated and treated as in (4), but in the absence of glucose.

The results are summarized in Table I.

From the results it can be seen that the content of non-volatile material of the brain slices depends on the type of incubation. The loss of non-volatile materials during incubation in the absence of potassium is in agreement with the findings of Elliott, in that potassium diffuses rapidly from slices into the medium (29). It was decided therefore, that the most reliable method to follow

TABLE I

Incubation	dry weight as per cent of wet weight	average thickness mm
1. None	17.5 ±0.5	0.41 ±0.03
2. Glucose-Saline-K^+	17.7	-
3. Glucose-Saline	16.8	-
4. Glucose-Saline-H_2SO_4	16.2	-
5. Saline-H_2SO_4	15.6	-

The conditions of the incubations are described in the text.

was to use the value of 17.5%. During the course of the

experiments this value was checked at regular intervals. The average deviation from 17.5 during the whole course of the experiments was 0.5, or 3% of the average.

CHAPTER I

THE EFFECT OF GLUCOSE ON THE METABOLISM OF AMINO ACIDS.

Results.

In 1953 Nakada and Weinhouse reported that slices of rat brain cortex, when incubated with glycine-1-C^{14}, were able to produce $C^{14}O_2$ (57) in vitro. However, the amount of glycine broken down was very small, and therefore

Table II

Effect of Glucose on the $C^{14}O_2$ Formation From Glycine-1-C^{14}.

Glucose concn. in mM	µl O_2 consumed per mg tissue	µmols $C^{14}O_2$ produced per 100 gm tissue
0	7.0	290
1	16.6	700
2	18.0	930
5	19.0	870
10	19.0	850

Rat brain cortex slices were incubated in 3 ml of Krebs-Ringer-phosphate medium at pH 7.4 for 90 minutes with various concentrations of glucose. The final concentration of glycine-1-C^{14} was 2 mM.

they did not further investigate the process in the brain. In a series of experiments, it was found that the break-down of glycine by rat brain cortex slices can be substantially increased by including glucose in the incubation medium. The results are represented in Table II. From these results it can be seen that glucose also increases the oxygen consumption of the tissue. The two phenomena, stimulation by glucose of the oxygen consumption and of the glycine break-down, proceed parallel to each other and amount to a three-fold increase under optimal conditions. The effect of glucose is not confined to the carboxyl carbon of glycine. From Table III it is evident that $C^{14}O_2$ production from either glycine-1-C^{14} or 2-C^{14} is stimulated by glucose to a similar extent. This stimulation is confined to the brain, in liver slices under identical conditions, the break-down of glycine is unaffected by the presence of glucose. In kidney cortex slices of the rat, however, glucose causes an inhibition of both the oxygen consumption and $C^{14}O_2$ production from glycine-1-C^{14} and 2-C^{14}, the inhibition being 21-23 per cent in all cases.

The animals used in these experiments were neither fasted nor exhausted, thus it is possible that the liver contained enough carbohydrate reserve to effect a maximal

TABLE III

The Rate of $C^{14}O_2$ Formation from Glycine-1 and 2-C^{14} in Different Tissue Slices.

Tissue	No Glucose			10 mM Glucose		
	µl O_2 per mg tissue	µmols $C^{14}O_2$ per 100 g tissue from glycine 1-C^{14}	2-C^{14}	µl O_2 per mg tissue	µmols $C^{14}O_2$ per 100 g tissue from glycine 1-C^{14}	2-C^{14}
Rhesus monkey cerebral cortex	5.5	160	25	11.8	320	54
Rat Cerebral Cortex	5.6	260	27	18.0	780	71
Rat Liver	13.0	620	300	13.0	690	300
Rat kidney cortex	30.0	12,200	1,050	23.4	9,600	830

The tissue slices were incubated at 37°C for 90 minutes with 2 mM glycine in a Krebs-Ringer-phosphate medium.

stimulation. It is also possible that either the break-down of glycine in the brain proceeds through a pathway different from that in liver and kidney, or that it is not glucose but a product of its metabolism which is implicated in the process. In all organs tested the rate of break-down of glycine is parallel to the rate of oxidation of glucose. However, the metabolic activities of nervous tissue are known to be highly specialized, thus we cannot exclude the possibility that the animo acid metabolism may also be specialized.

The effect of glucose on the amino acid metabolism in the brain is not confined to glycine. Of five amino acids tested, the break-down of glycine, serine and leucine was markedly stimulated, whereas the break-down of valine and phenylalanine was unaffected by the presence of glucose. The results of these experiments are shown in Table IV.

The amount of $C^{14}O_2$ produced from these amino acids in the absence of glucose can be divided into two groups: the CO_2 derived from the carboxyl groups, which corresponds to 300-450 micromole amino acid broken down in 90 minutes per 100 gm tissue, and the CO_2 derived from the α carbons (or β carbon of serine) which corresponds to 30-55 μmoles.

One possible explanation for this apparently uniform metabolism would be that there is a common mechanism of

TABLE IV

$C^{14}O_2$ Production from Different Labeled Amino Acids by Rat Brain Cortex Slices.

Amino Acid	Concn. in mM	µmoles $C^{14}O_2$ produced per 100 gm tissue		Extra $C^{14}O_2$
		No glucose	10 mM glucose	%
Glycine-1-C^{14}	2	300	800	170
Glycine-2-C^{14}	2	27	90	230
Serine-3-C^{14}	2	55	210	280
Serine-U-C^{14}	2	100	280	180
Leucine-1-C^{14}	1	410	1,350	230
	2	460	1,500	230
Valine-1-C^{14}	2	410	370	-10
Phenylalanine-2-C^{14}	2	30	30	0

Rat brain cortex slices were incubated in Krebs-Ringer-phosphate medium at pH 7.4 for 90 min.

oxidation for all the amino acids tested, and the presence of glucose opens up an additional pathway for certain amino acids. Glucose could either directly stimulate an enzyme system or provide a metabolite necessary for at least one step of the oxidation sequence, but in either case, if the system is saturated with respect to one amino acid, it cannot oxidize an additional one.

In order to test this hypothesis series of experiments were conducted using the isotopic dilution technique. The results are summarized in Table V. The enzyme system responsible for $C^{14}O_2$ production from glycine-1-C^{14} and leucine-1-C^{14} in the presence of glucose is saturated at 2 mM concentration of either amino acid. Very little additional $C^{14}O_2$ is produced on raising their concentration to 4 mM. However, the addition of unlabeled leucine to glycine-1-C^{14} or of unlabeled glycine to leucine-1-C^{14} does not alter the $C^{14}O_2$ production to any significant extent. The $C^{14}O_2$ produced by brain slices in the presence of glucose from a mixture of glycine-1-C^{14} and leucine-1-C^{14} at 2 mM each is nearly equal to the sum of the values obtained from the two amino acids individually. It is evident therefore that the metabolism of glycine and leucine proceeds through separate enzyme systems.

A similar series of experiments was conducted using valine as the diluent. The results show that the presence of unlabeled valine does not alter significantly the amount of $C^{14}O_2$ produced from glycine-1-C^{14} but reduces that produced from leucine-1-C^{14} (Table VI).

A stimulation by glucose of the metabolism of serine was expected in view of the close relationship between the metabolism of serine and glycine. This relationship between the

TABLE V

Isotopic Dilutions between Glycine and Leucine

Radioactive Amino Acid	Concn. in mM	Unlabeled Amino Acid	Concn. in mM	µmol $C^{14}O_2$ per 100 g tissue
Glycine-1-C^{14}	1	None	-	920
	2	None	-	900
	4	None	-	1,000
Leucine-1-C^{14}	1	None	-	1,330
	2	None	-	1,550
	4	None	-	1,700
Glycine-1-C^{14}	2	Leucine	2	900
Leucine-1-C^{14}	2	Glycine	2	1,500
Leucine-1-C^{14} Glycine-1-C^{14}	2 2	None	-	2,330

Each vessel contained 3 ml Krebs-Ringer phosphate medium with 10 mM glucose. Incubated for 90 minutes at 37°C.

TABLE VI

The Effect of Valine on the Metabolism of Glycine and Leucine

Radioactive Amino Acid	Concn. in mM	Unlabeled Amino Acid	Concn. in mM	μmol $C^{14}O_2$ per 100 g tissue
Glycine-1-C	2	none	-	900
		Valine	2	970
Leucine-1-C	2	none	-	1,550
		Valine	2	1,180

Each vessel contained 3 ml Krebs-Ringer-phosphate medium with 10 mM glucose. Incubated for 90 minutes at 37°C.

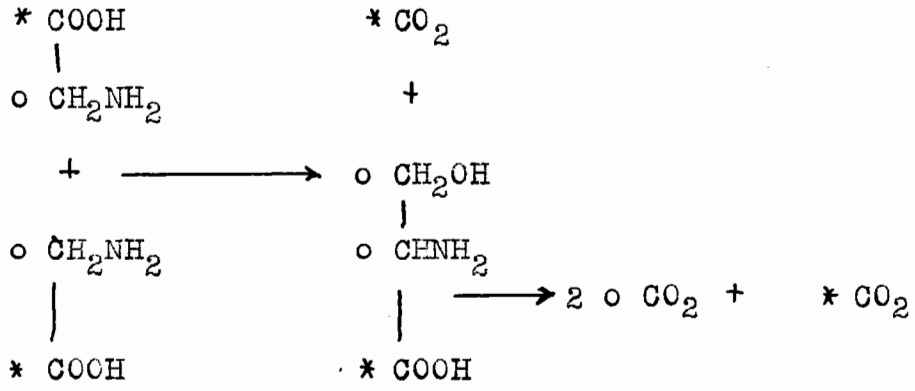
metabolism of the two amino acids in vivo and in surviving liver slices is well established through the studies of Shemin (125), Winnick et al. (127) and Arnstein and Neuberger (134). That a similar relationship exists between the metabolism of the two amino acids in brain also, can be seen from the results summarized in Table VII. The production of $C^{14}O_2$ from glycine labeled in either carbon atom with C^{14} is decreased by almost half the original value upon addition of unlabeled serine. Similarly, the $C^{14}O_2$ produced from serine-3-C^{14} is reduced by 74 per cent when unlabeled glycine is also included in the system.

Thus glycine has a stronger diluting power on the meta-

bolism of serine than serine has on that of glycine. In the course of a stimulating discussion on the subject, Dr. L. Booij suggested that this may indicate that glycine is an intermediate in the oxidation of serine and that at the same time there is a rapid interconversion of the two amino acids:

$$\text{Serine} \rightleftharpoons \text{glycine} \longrightarrow CO_2$$

The same view is supported by the fact that the diluting effect of serine is the same on either carbon atoms of the glycine molecule, because if the oxidation proceeded according to the reverse reaction:



half of the $C^{14}O_2$ produced from glycine-1-C^{14} should remain unaffected by the presence of unlabeled serine.

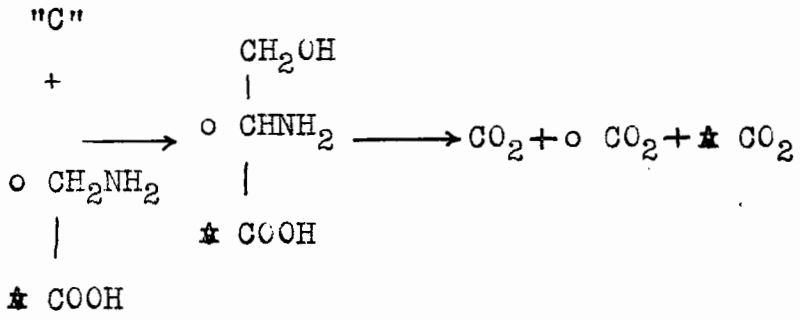
There are two important points however, which must be considered. Simkin and White (149) showed that the "one carbon

TABLE VII

Radioactive Amino acid	Concn. in mM	µmol $C^{14}O_2$ per 100 g tissue	Unlabeled Amino Acid	Concn. in mM	µmol $C^{14}O_2$ per 100 g tissue
Glycine-1-C^{14}	2	900	Serine	2	520(-42%)
Glycine-2-C^{14}	2	90	Serine	2	50(-44%)
Serine-3-C^{14}	2	210	Glycine	2	55(-74%)
Serine-U-C^{14}	2	280	Pyruvate	10	120(-57%)

Each vessel contained 3 ml Krebs-Ringer-phosphate medium with 10 mM glucose. Incubated for 90 minutes at 37°C.

fragment" produced from glycine in the intact animal is negligible as compared to the "one carbon fragment" from other sources. If this is true for isolated brain tissue, the serine formed from glycine will contain an equal number of carbon atoms from either positions of the parent glycine:



and therefore unlabeled serine will have an equal power of

dilution on the $C^{14}O_2$ produced from glycine labeled in either positions.

The second point is that while in the dilution experiments serine and glycine are added in equimolar concentrations to the suspending medium, their concentrations inside the cells are not necessarily equimolar. Experiments to be reported in a subsequent chapter show that the rate of transport of serine across the cell wall is more sensitive to the presence of glycine that is the rate of transport of glycine to the presence of serine.

The production of $C^{14}O_2$ from serine labeled uniform with C^{14} is markedly decreased by pyruvate in the presence of glucose (Table VII). This effect of pyruvate cannot be fully evaluated at this point, but it clearly indicates that pyruvate is involved in the metabolism of serine and glycine.

Summary

1. Rat brain cortex slices were shown to be able to metabolize glycine, serine, leucine, valine and phenylalanine. The metabolism of serine, glycine and leucine was strongly stimulated by the presence of glucose, the stimulation being parallel to the oxygen consumption. Glucose had no effect on the $C^{14}O_2$ production from valine-1-C^{14} or phenylalanine-2-C^{14}.

2. The stimulating effect of glucose on glycine metabolism was present in rat and rhesus monkey brain cortex slices. No stimulation could be observed using rat liver slices, and a 21-23% inhibition was observed with rat kidney cortex slices.

3. By using the isotopic dilution technique it was demonstrated that the glucose stimulated break-down of leucine proceeds by way of an enzyme system independent from the one responsible for the break-down of glycine and serine, but identical, in part at least, with the enzyme system responsible for the break-down of valine.

4. A close relationship was shown between the metabolism of glycine and serine in the brain in agreement with previous findings in the intact animal and in liver preparations.

5. The metabolism of glycine and serine in rat brain cortex slices may be connected to that of pyruvate.

CHAPTER II

THE METABOLISM OF GLYCINE-1-C^{14} IN SEPARATED RAT BRAIN CORTEX SLICES.

Throughout the experiments a considerable variation was observed in the rate of metabolism of glycine and serine, although the rate of oxygen consumption of the tissue preparations remained constant. The variation was especially large with the $C^{14}O_2$ production from glycine-2-C^{14} amounting to ± 40% throughout a two year period. However, variations encountered within each set of experiments were considerably smaller, usually below ± 10%, regardless whether the tissues were prepared from one or several animals. Low variations in the results were obtained within any shorter period of time, 2-4 weeks.

In order to eliminate the major source of error, the results reported in any one table were obtained within a short period of time. Each set of experiments included a complete set of controls together with the experimental determinations. Therefore, within any one table a change greater than 15% can be considered as significant. Care should be taken of the control values, however, when individual values rather than trends, reported in separate tables are compared.

The reason for this high degree of variation in the rate of metabolism of amino acids is not known, but could be due to the variable, and at times extremely high, concentration of NH_3 in the immediate atmosphere of the animals due to poor ventilation and the small size of the animal room. Richter and Dawson reported in 1948 (178) that the free ammonia content of the rat brain increases considerably under a variety of conditions, including even the act of decapitation. DuRuisseau et al. in 1957 have reported a change in the total amounts and distribution of some amino compounds in the brains of rats in vivo after the injection of amonium acetate (46) which may indicate a change in the amino acid metabolism.

The decapitation and excision of organs of the rats were routinely done in the animal room. The animals were able to smell the blood of decapitated rats and manifested obvious signs of deep fear (spontaneous profuse urination and defecation, intense efforts to hide under the saw-dust placed on the bottom of the cage). It is probable that the disturbed hormonal balance due to the frequent fear also contributed to the variability of glycine metabolism in the brain.

Results.

The amount of $C^{14}O_2$ produced from glycine-1-C^{14} at 2 mM concentration is almost ten times greater than

that produced from glycine-2-C^{14} at the same concentration. This finding suggested that the glycine molecule is not oxidized to CO_2 as an entity, but that two separate enzyme systems are responsible for the oxidation of either carbon fragments. Similar conclusions could be drawn from the results summarized in Table VIII and illustrated in Figure 1. The rat brain cortex slices

TABLE VIII

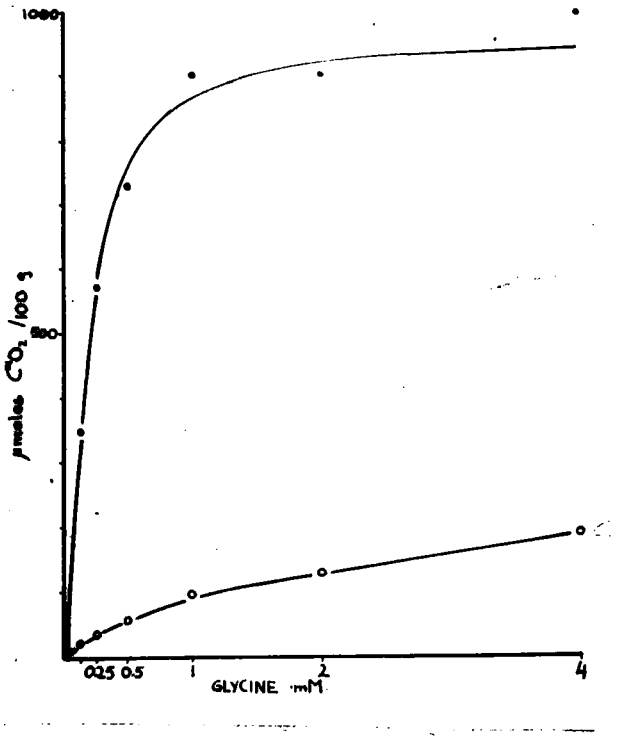
The Effect of Concentration on the Break-down of Glycine in Rat Brain Slices.

Glycine concn in mM	μ mol $C^{14}O_2$ produced per 100 g tissue from glycine	
	1-C^{14}	2-C^{14}
0.125	350	20
0.25	570	30
0.5	730	57
1.0	900	93
2.0	900	127
4.0	990	187

60-80 mg tissue incubated with various concentrations of glycine-1 and 2-C^{14} in the presence of 10 m$\underline{M}$ glucose in Krebs-Ringer phosphate medium. Incubation Time: 90 minutes.

FIGURE I

The Effect of Substrate Concentration on the Formation of $C^{14}O_2$ from Glycine-1 and 2-C^{14}.



Conditions: 90 minutes incubation in the presence of Glucose.

● - Glycine-1-C^{14}

o - Glycine-2-C^{14}

were incubated with varying concentrations of glycine-1-C^{14} and 2-C^{14} in the presence of 10 m<u>M</u> glucose for 90 minutes. The enzyme system responsible for the formation of CO_2 from the carboxyl carbon of glycine was saturated at a substrate concentration of 0.5-1.0 m<u>M</u>, but the enzyme system responsible for the oxidation of the methylene carbon was not saturated at 4 m<u>M</u> glycine concentration.

The liberation of the carboxyl carbon of the glycine could arise through a simple decarboxylation to give CO_2 and methylamine. If this was the case, radioactive methylamine should accumulate when the brain slices are incubated with glycine-2-C^{14}, but radioautography (Fig. 2) of total amino compounds after incubation showed that only serine contained radioactivity of comparable magnitude. Furthermore, decarboxylation reactions usually proceed anaerobically whereas the production of $C^{14}O_2$ from glycine-1-C^{14} is aerobic, as illustrated in Figure 3. The small amount of radioactive CO_2 produced in the absence of oxygen is probably due to traces of impurities in the commercial nitrogen used to flush the incubation vessels, since the ratio between $C^{14}O_2$ produced from glycine-1 and 2-C^{14} is the same as in the presence of oxygen, approximately 1:10.

Thus the CO_2 derived from the carboxyl carbon of

FIGURE 2

The Formation of Radioactive Serine from Glycine-2-C^{14}.

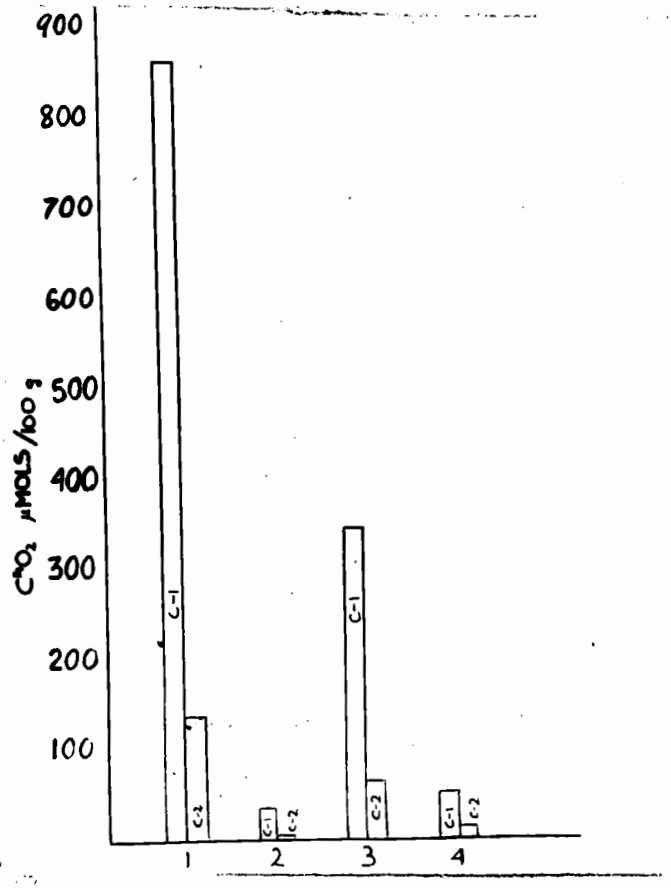
Radioautograph of a chromatogram of the amino compounds of the brain after incubation with Glycine-2-C^{14}, 2 mM, in the presence of Glucose, 10 mM.

1 - Glycine

2 - Serine

FIGURE 3

The Effect of Oxygen on the $C^{14}O_2$ Formation from Glycine-1 and 2-C^{14}.



Conditions:

1 - Aerobic in the presence of glucose, 10 m<u>M</u>.

2 - Anaerobic in the presence of glucose, 10 m<u>M</u>.

3 - Aerobic in the absence of glucose.

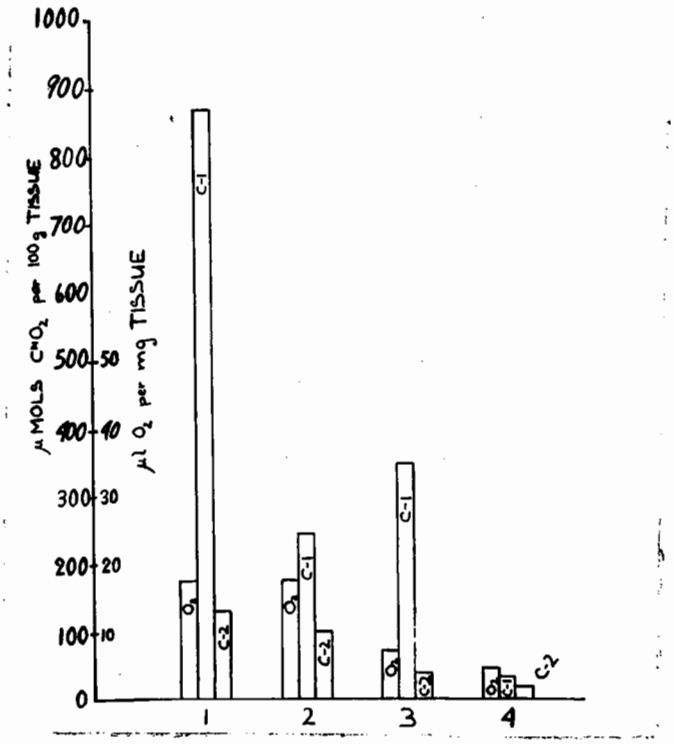
4 - Anaerobic in the absence of glucose.

glycine does not come through a simple decarboxylation. Pyridoxal phosphate, the co-decarboxylase, may be involved in the process since the liberation of the carboxyl carbon of glycine as CO_2 is extremely sensitive to the presence of semicarbazide. A series of experiments showed that when rat brain cortex slices were incubated with 2 mM glycine in the presence of glucose, 850 micromols CO_2 was formed from the carboxyl carbon of glycine per 100 g tissue in 90 minutes. The amount of CO_2 derived from the methylene carbon of glycine was 125 micromols. When 10 mM semicarbazide was included in the system, the production of CO_2 from the carboxyl carbon of glycine dropped from 850 to 240 micromols which corresponds to a 72% inhibition, whereas the CO_2 produced from the methylene carbon of glycine dropped to 100 micromols, or a 20% inhibition. At this concentration semicarbazide did not alter the rate of respiration.

In the absence of glucose, semicarbazide had an even greater effect on the metabolism of the carboxyl carbon of glycine, the CO_2 production being inhibited by 91%. However, the oxidation of methylene carbon of glycine to CO_2 and the oxygen consumption of the rat brain slices were also inhibited by 40 and 30% respectively. These results are illustrated in Figure 4.

FIGURE 4

The Effect of Semicarbazide on the $C^{14}O_2$ Formation from Glycine-1 and 2-C^{14}.



Conditions: Aerobic

1 - Glucose, 10 m<u>M</u>.

2 - Glucose, 10 m<u>M</u> and Semicarbazide, 10 m<u>M</u>.

3 - No glucose.

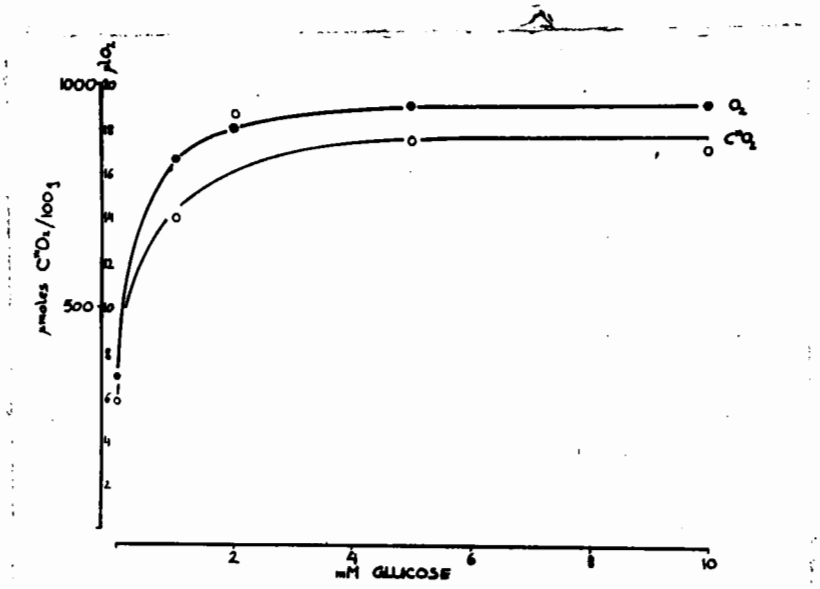
4 - No glucose, semicarbazide, 10 m<u>M</u>.

Semicarbazide is a very potent conjugating agent of aldehydes and ketones, giving stable semicarbozones of low solubility. Many of the intermediates of carbohydrate metabolism are aldehydes or ketones, therefore it could act by removing from the system the intermediates necessary for the metabolism of glycine. However, the $C^{14}O_2$ produced from glycine-1-C^{14} in the presence of semicarbazide and glucose is less than the $C^{14}O_2$ produced in the absence of the two. Furthermore, the stimulation by glucose of the oxygen consumption and $C^{14}O_2$ production from glycine-1-C^{14} at different concentrations of glucose are parallel to each other (Fig. 5 and Table II). By removing the intermediates of glucose metabolism, semicarbazide should also inhibit the oxygen consumption and the $C^{14}O_2$ production from glycine-2-C^{14} to a similar extent, whereas from Figure 3 it is evident that the oxygen consumption remains unaltered, and the methylene carbon metabolism of glycine is inhibited to a very small degree only.

Semicarbazide is usually used as a potent inhibitor of reactions requiring pyridoxal phosphate as a cofactor. Such reactions are the transport of amino acids across the cell wall, transaminations with α-ketoglutarate as nitrogen acceptor, decarboxylations of amino acids to amines and the formation of serine from glycine and 5,10-methylene

FIGURE 5

The Effect of Glucose on Oxygen Consumption and Formation of $C^{14}O_2$ from Glycine-1-C^{14}



• μl O_2 consumed per mg tissue (dry weight).

o μmol $C^{14}O_2$ produced per 100 g. tissue (dry weight).

tetrahydrofolic acid (143, 145, 156).

The rate of transport of glycine across the cell membrane cannot be altered significantly by semicarbazide. In Table VIII and Figure 1, it was shown that the enzyme system responsible for the liberation of the carboxyl carbon of glycine as CO_2 was saturated at much lower concentrations of glycine than the one responsible for the oxidation of the methylene carbon. If the rate of transport across the cell wall was altered, the change in the concentration of glycine inside the cell would be reflected by a greater change in the metabolism of the methylene carbon than that of the carboxyl carbon.

It has also been shown that the production of $C^{14}O_2$ from glycine-1-C^{14} cannot come through simple decarboxylation. In order to investigate the possible transamination of glycine with α-ketoglutarate, the two compounds were incubated with dialysed supernatants of rat brain homogenates in the presence of versene. The 10% aqueous homogenate of rat brains was centrifuged for 10 minutes in a refrigerated centrifuge at 2°C, and the supernatant dialysed against running tap water buffered by dilute phosphate, for 8 hours at 4°C, to remove free amino acids present in the soluble fraction of brain. Finally versene (1m<u>M</u>) was added to inhibit the liberation of free amino acids by the proteolytic enzymes, (kindly suggested by Dr. R. Johnstone).

Aliquots of the preparation were incubated alone; with glycine; with α-ketoglutarate; and with glycine and α-ketoglutarate. After 2 hours of incubation at 37°C the systems were analysed for glutamic acid by chromatography. The chromatograms are shown on Figure 6. There was no visible increase in the concentration of glutamate due to the presence of glycine, but any slight transamination between glycine and α-ketoglutarate may have been masked by the large amount of glutamate formed from traces of residual amino acids upon addition of α-ketoglutarate alone.

To test for the formation of glycolate or glyoxylate from glycine, brain slices were incubated with glycine-1-C^{14} and unlabeled glycolate, glyoxylate and glycolaldehyde. Of the three substances, glyoxylate slightly inhibited the respiration (Table IX) but none altered the formation of $C^{14}O_2$ significantly either in the presence or absence of glucose. The two aldehydes, glyoxylate and glycolaldehyde, actually stimulated the formation of $C^{14}O_2$ from glycine-1-C^{14} in the absence of glucose, but the significance and mechanism of this stimulation has not been investigated.

The lack of an isotopic dilution effect on glycine-1-C^{14} by any of its three derivatives did not necessarily mean that they are not intermediates of glycine oxidation, but

FIGURE 6

Formation of Glutamate from α-Ketoglutarate
and Serine or Glycine in
Rat Brain Extracts.

1. α-Ketoglutarate and serine
2. α-Ketoglutarate and glycine
3. α-Ketoglutarate
4. Serine
5. Glycine
6. None

Chromatographed in 80% pyridine

TABLE IX

The Effect of Glycolate, Glyoxylate and Glycolaldehyde on the Metabolism of Glycine-1-C^{14}

Diluent	Concn in mM	No Glucose		10 mM Glucose	
		µl O_2 per mg tissue	µmol $C^{14}O_2$ per 100 g tissue	µl O_2 per mg. tissue	µmol $C^{14}O_2$ per 100 g. tissue
None	-	5.6	260	18.0	780
Glycolate	10	5.5	250	18.0	745
Glyoxylate	5	4.5	290	14.5	745
Glycolaldehyde	2	5.6	350	16.8	770

60-80 mg rat brain cortex slices were incubated in 3ml of Krebs-Ringer-phosphate medium (pH 7.4) at 37°C for 90 minutes. Concentration of glycine-1-C^{14} was 2mM final, other concentrations as indicated.

could have been due to a lack of uptake of these substances into the cell. This possibility was eliminated however by using homogenates of rat brain. In the series of experiments, presented in Table X, 10% homogenates of whole rat brains were incubated in modified Reiner-McMurray medium with 2mM

TABLE X

The Effect of Glyoxylate on the $C^{14}O_2$ Production from Glycine-1 and 2-C^{14} by rat brain homogenates

Na-glyoxylate	µl O_2 consumed per mg tissue	µmol $C^{14}O_2$ produced per 100 gm tissue from glycine	
		1-C^{14}	2-C^{14}
None	7.9	29.4	0.73
2m<u>M</u>	7.7	39.0	0.61

Whole rat brain homogenates, 10% in modified Reiner-McMurray medium. Concentration of glycine: 2m<u>M</u>, sodium glyoxylate, as indicated. Incubation: 1 hour at 37°C.

glycine-1 and 2-C^{14} with and without 2m<u>M</u> glyoxylate. The $C^{14}O_2$ produced from glycine labeled in either position did not exceed 1-3% of the value obtained with whole slices of brain cortex. The presence of glyoxylate caused no significant amount of isotopic dilution and, in the case of glycine-1-C^{14} a marked stimulation was again observed.

<u>Discussion</u>

Arnstein and Neuberger reported in 1953 that the α carbon of glycine is a precursor of biological methyl groups. The β carbon of serine was found to be an intermediate in

this process (134). In a subsequent communication, they proposed that the transformation of the α carbon of glycine into the β carbon of serine was merely a regulating mechanism to keep the body glycine at a constant level, as it depended on the concentration of glycine only and was independent on the serine concentration (147). Similar findings were reported by Simkin and White in 1957 (148-149).

During the process of incorporation of the methylene carbon of glycine into the β carbon of serine, the glycine molecule must split and the carboxyl carbon is given off as CO_2. We have made a study of the mechanism of incorporation by measuring the $C^{14}O_2$ produced from glycine-1-C^{14}. That incorporation occurs in the brain at all we have been able to show by isolating radioactive serine from brain after 90 minutes incubation with glycine-2-C^{14}.

The metabolism of glycine in the brain may be quite different from that in other organs. Of three organs tested, only in brain was there a stimulation of glycine metabolism in the presence of glucose. This stimulation was not simply "an improved metabolic condition" even though it paralleled the stimulation of oxygen consumption, as in this case the metabolism of all amino acids should have been stimulated. The fact that out of the five amino acids tested three showed a three-fold stimulation and two showed no stimulation at all, with no intermediate degrees, also points to a specific effect of glucose on the metabolism of certain amino acids.

Isotopic dilution experiments with glycine and serine also indicated a more complex mechanism of interconversion of the two amino acids in brain than in other organs. Unlabeled serine diluted equally the $C^{14}O_2$ produced from glycine-1-C^{14} and -2-C^{14}. If it is assumed that glycine is converted to serine by the addition of a "one carbon fragment", which itself comes from the oxidation of glycine (133, 146), the diluting power of serine should be less with glycine-1-C^{14} than with glycine-2-C^{14}. In this case the CO_2 formed from the carboxyl carbon of glycine, when the carbon is converted to a "one carbon fragment", should be independent of the concentration of serine (147, 149). The results presented could be explained by a great excess of available "one carbon fragment" from sources other than the α carbon of glycine. The nature of these sources is obscure, choline and methionine, the usual sources of labile methyl groups (165) are not present in the brain in concentration high enough to supply the carbon requirement. Furthermore, the exchange of the methyl groups of these compounds is reversible (132) and no substantial incorporation of radioactivity into choline or methionine from either glycine or serine could be shown.

The strong diluting power of glycine on the metabolism of serine and the equal diluting power of serine on the metabolism of either carbon of glycine could be explained, however, by another pathway: a fast and efficient inter-

conversion of glycine and serine and a direct oxidation of glycine which does not involve serine as an intermediate.

Ratner et al. in 1944 demonstrated the presence of glycine oxidase in many organs (161). This enzyme oxidizes glycine to glyoxylic acid and NH_3. Weinhouse and Friedman (162) found that glyoxylate and glycine is broken down by the intact animal to CO_2 at the same rate and in a similar manner; the carboxyl carbon of the two compounds gave three times more CO_2 than the α carbon. Nakada et al. (57, 163) have proven conclusively the interconversion of glycine and glyoxylate in rat liver homogenates. They have also obtained evidence that the oxidation of serine and glycine does not involve lactate or acetate.

The direct oxidation of glycine in rat brain cortex, if present, does not involve free glyoxylic, glycolic acids since there was no dilution effect when these substances, unlabeled, were added to the system, using either slices or homogenates. The coenzyme of oxidative deamination, pyridoxal phosphate, was definitely shown to take part in the liberation of the carboxyl carbon of glycine, the $C^{14}O_2$ formation from glycine-1-C^{14} being very strongly inhibited in the presence of semicarbazide.

The addition of the two aldehydes, glyoxylic acid or glycolaldehyde stimulated the liberation of $C^{14}O_2$ from glycine-1-C^{14} by brain homogenates and by cortex slices in the absence of glucose. A possible explanation of this phenomenon is that the reaction requires the presence of an aldehyde. The aldehyde is slowly broken down in homogenates and brain slices when glucose is not present. Glycolaldehyde and glyoxylate could competitively inhibit the enzyme system responsible for the breakdown of the cofactor.

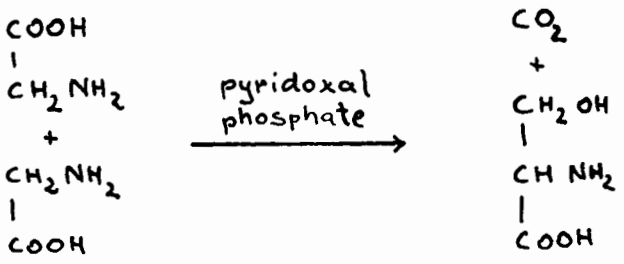
The effect of semicarbazide is very difficult to explain. Blakley in 1957 demonstrated that the "one carbon fragment" is formaldehyde combined with tetrahydrofolic acid, a complex which dissociates very easily to give free fromaldehyde (139). The addition of semicarbazide to a system containing the formaldehyde-tetrahydrofolic acid complex should therefore cause a dissociation of the complex and conjugate the free formaldehyde formed into a stable semicarbazide complex. If glycine is metabolized to formaldehyde and CO_2, the addition of semicarbazide should cause a strong inhibition of the oxidation of the α carbon of glycine, and the accumulation of free tetrahydrofolic acid should cause a stimulation of the oxidation of the carboxyl carbon.

Semicarbazide, however, inhibited the formation of

$C^{14}O_2$ from glycine-1-C^{14} and did not alter the formation of $C^{14}O_2$ from glycine-2-C^{14}. Thus the incorporation of the α carbon of glycine into serine does not involve free formaldehyde.

The inhibition of the $C^{14}O_2$ formation from glycine-1-C^{14} by semicarbazide suggests that pyridoxal phosphate is involved in splitting glycine, but a reduction in the amount of glycine split to CO_2 and the "one carbon fragment" should also reduce the oxidation of the α carbon, if this latter process involves the "one carbon fragment" as an intermediate.

One possible explanation for the process should be to suppose that there are two pathways operating simultaneously. The major pathway would be the condensation of two glycine molecules to form serine in the presence of pyridoxal phosphate:



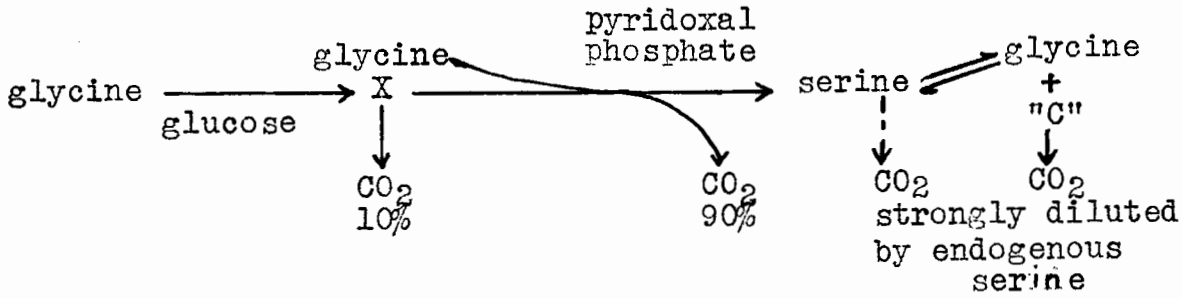
The maximal rate of this reaction cannot exceed the rate of formation of $C^{14}O_2$ from glycine-1-C^{14}, which is 900 micromols per 100 g tissue (dry weight) in 90 minutes. According to DuRuisseau et al. (46) the total serine content of rat brain is 19 mg per 100 g fresh weight or 4000 micromols per 100 g dry weight. Since the rate of

oxidation of serine itself is low, 200-300 micromols per 100 g tissue per 90 minutes, practically all the α carbon of glycine incorporated into the serine by the above reaction would therefore be trapped and contribute only very little to the $C^{14}O_2$ formed from glycine-2-C^{14}.

Simultaneously, a minor pathway would operate, in order to account for the oxidation of the α carbon of glycine. The relative importance of this would be ten times less than that of the major pathway. In favor of this hypothesis is that the amount of $C^{14}O_2$ produced from glycine-1-C^{14} in the presence of semicarbazide is always greater than the $C^{14}O_2$ produced from glycine-2-C^{14} (Fig. 4).

The CO_2 production from both carbons of glycine is stimulated by glucose to the same extent, it is therefore possible that at least one step is common to both. The second pathway would oxidize glycine completely to CO_2, however pyridoxal phosphate is not a cofactor and glyoxylic and glycolic acids are not necessarily intermediates. The proposed reactions are represented in Scheme 5.

SCHEME 5



Summary.

1. The two carbon atoms of glycine were shown to be oxidized to CO_2 at different rates, the carboxyl carbon atom being liberated ten times faster. It was also shown that the oxidation of the carboxyl carbon was optimal between 0.5 - 1.0 mM substrate concentration, whereas no optimal concentration was found for the oxidation of the methylene carbon of glycine.

2. The production of CO_2 from the carboxyl carbon of glycine is strongly stimulated by glucose, requires oxygen, and is inhibited by semicarbazide.

3. The effect of semicarbazide in the presence of glucose is limited to the carboxyl carbon; the $C^{14}O_2$ production from glycine-2-C^{14} remains unaffected. In the absence of glucose, semicarbazide nearly abolished the $C^{14}O_2$ production from glycine-1-C^{14}, reduced oxygen consumption of the brain slices by 30%, and $C^{14}O_2$ production from glycine-2-C^{14} by 40%.

4. From the evidence presented pyridoxal phosphate was suggested as a possible cofactor for the formation of $C^{14}O_2$ from glycine-1-C^{14}. However, pyridoxal phosphate did not act in the system as a coenzyme of decarboxylation, transamination or transport across the cell wall.

5. The presence of glyoxylic acid did not dilute the $C^{14}O_2$ produced from glycine-1-C^{14} by either rat brain cortex slices or rat brain homogenates. No dilution of the $C^{14}O_2$ production, from glycine-1-C^{14} by rat brain cortex slices, was observed using either glycolate or glycolaldehyde.

CHAPTER III

THE METABOLISM OF GLYCINE-2-C^{14} IN SEPARATED RAT BRAIN CORTEX SLICES.

Elwyn and Sprinson in 1954 found that when serine-3-C^{14} was administered to rats for 1-3 days, the glycine from the hydrolyzed proteins of 7 internal organs contained very little activity (130). When glycine-2-C^{14} was administered, serine contained a medium amount of C^{14} in the β position, and much less in the α position (128, 130). These results indicated that the α carbon of glycine was capable of giving rise to a "one carbon fragment", but the amount produced was much less than that formed from other sources. Similar results were obtained <u>in vitro</u> by Siekevitz and Greenberg (133) using rat liver homogenates. Vohra et al. showed that the process required cobalamine (146).

Huennekens et al. (143) demonstrated a complete oxidation of the "one carbon fragment" to CO_2.

In view of these findings a series of experiments were undertaken to study the mechanism of CO_2 formation from the α carbon of glycine.

The rate of oxidation.

The amount of $C^{14}O_2$ formed, in relation to the time of incubation, from glycine-1 and 2-C^{14} and from serine-3-C^{14} is summarized in Table XI and represented in Figure 7. As can been seen, the evolution of $C^{14}O_2$ from glycine-2-C^{14}

TABLE XI

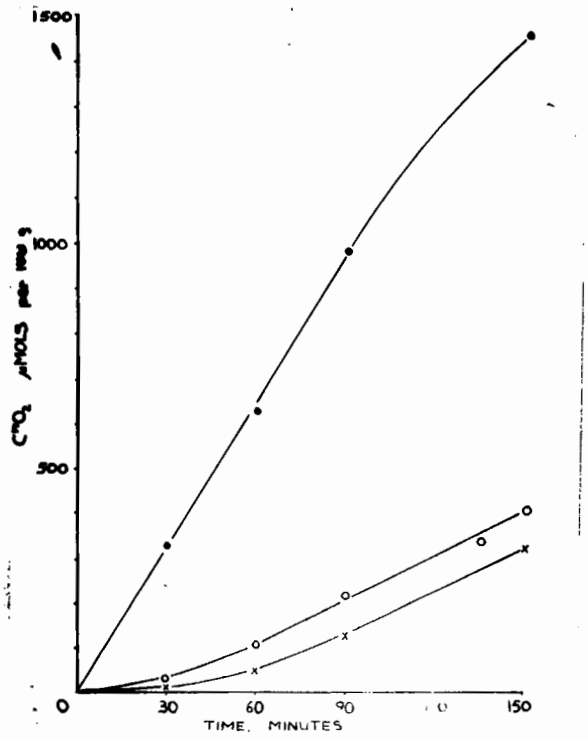
The Rate of $C^{14}O_2$ Formation from Serine-3-C^{14}, Glycine-1 and 2-C^{14} in Rat Brain Slices.

	µmols $C^{14}O_2$ formed per 100 g tissue		
	Glycine		Serine
TIME in min.	1-C^{14}	2-C^{14}	3-C^{14}
30	329	9.1	31
60	632	44	107
90	987	128	217
135	-	-	337
150	1,463	330	406

All vessels contained 60-80 mg tissue slices in Krebs-Ringer-phosphate medium, 10 mM glucose, and 2mM amino acid. Incubated at 37°C with O_2.

FIGURE 7

The Rate of $C^{14}O_2$ Formation
from Glycine-1 and 2-C^{14} and Serine-3-C^{14}



- ● Glycine-1-C^{14}
- × Glycine-2-C^{14}
- o Serine-3-C^{14}

Conditions as in Table XI

starts after a lag period of 30-60 minutes, then proceeds linearly for 90 minutes. During the lag period unlabeled intermediates, preformed from glycine or from other sources, have to be saturated with C^{14}; that is, if glycine gives rise to CO_2 by way of A and B, the addition of radioactive glycine will have to give rise to radioactivity in A and in B before radioactive CO_2 is produced. The lag period depends on the rate of transformation and on the amounts of unlabeled A and B present in the tissue.

The evolution of $C^{14}O_2$ from serine-3-C^{14} starts after a shorter lag period than that of glycine-2-C^{14}, then proceeds linearly parallel to the $C^{14}O_2$ from glycine-2-C^{14}. This would indicate that serine is an intermediate in the breakdown of glycine. It may also indicate that both serine and glycine can give rise to the same intermediate, the rate of transformation of serine being greater than that of glycine, and the rate limiting reaction of CO_2 production being beyond the intermediate.

The rate of $C^{14}O_2$ production from glycine-1-C^{14} is linear for 100 minutes. There is no appreciable lag period of $C^{14}O_2$ formation, which indicates that either no intermediates are involved in the liberation of the carboxyl group of glycine or

their concentration is negligible as compared to their rate of formation from glycine.

The effect of respiratory substrates.

Glycine, when incubated with brain slices, did not affect the endogenous respiration. However, the presence of glucose at concentrations which supported an increased rate of respiration, strongly stimulated the metabolism of glycine. It was therefore necessary to investigate the metabolism of glycine in the presence of respiratory substrates other than glucose. The results of these investigations are summarized in Table XII. The amount of $C^{14}O_2$ produced from glycine-1-C^{14} in the presence of glutamate, α-ketoglutarate and a mixture of pyruvate and fumarate (10:1) was found to be very nearly the same as in the presence of glucose. Glutamate gave slightly lower values than α-ketoglutarate; this may be because it has to be deaminated before it can act as a stimulant.

The presence of a mixture of pyruvic and fumaric acids had a strong stimulating effect on the $C^{14}O_2$ production from glycine-1-C^{14}, whereas either compound alone had very little effect. Fumarate, at 10 mM concentration, produced a 30% stimulation but had no effect on the oxygen consumption. Pyruvate supported a high rate of respiration,

TABLE XII

$C^{14}O_2$ Production from Glycine-1 and 2-C^{14} in Rat Brain Cortex Slices with Different Substrates for Respiration.

Substrate	Concn. in mM	µl O_2 per mg tissue	µmols $C^{14}O_2$ per 100 g tissue from glycine-1-C^{14}	%	2-C^{14}	%
Nil	-	6.1	300	0	27	0
Glucose	10	17.2	900	200	90	233
Glutamate	10	14.5	600	100	5.0	-81
α-ketoglutarate	10	13.3	800	166	43	59
Pyruvate + Fumarate	10 1	22.2	900	200	28	4
Fumarate	10	5.7	400	33	11	-59
Pyruvate	10	17.4	250	-17	8.5	-69
Acetate	10	5.8	190	-37	9.5	-65
Succinate	30	16.7	400	33	18	-33

Incubation medium, Krebs-Ringer-phosphate, pH: 7.4, at 37°C.
Incubation time, 90 minutes.
Concentration of glycine, 2mM; respiratory substrates, as indicated.

but inhibited slightly the $C^{14}O_2$ production. These facts indicate, that citrate, formed from pyruvate and fumarate, may be the actual compound which causes an increased liberation of CO_2 from the carboxyl group of glycine.

Succinate, similar to pyruvate, supported a high rate of oxygen consumption but had little effect on the $C^{14}O_2$ production. Acetate did not support respiration and caused a decrease in the $C^{14}O_2$ production.

In the formation of $C^{14}O_2$ from glycine-2-C^{14}, none of the substances tested gave a stimulation comparable to that of glucose. The presence of α ketoglutarate gave a 60% stimulation, as compared to the 230% stimulation by glucose. The mixture of pyruvate and fumarate did not alter the production of $C^{14}O_2$, and all other substrates tested inhibited from -33 to -80%. These inhibitions could have been due to isotopic dilution effects of the substances used.

Three important points arise from Table XII. The first is that acetate, pyruvate, and fumarate, three substances that can give rise to acetyl-CoA, strongly inhibit the oxidation of the methylene carbon of glycine. The second point is that the metabolism of the carboxyl carbon is stimulated by all substrates which are a part of the glucose oxidation sequence, as far as α-keto-

glutarate. These facts indicate that the metabolism of glycine may proceed, partly at least, through the Shemin cycle (164) represented on Scheme 3 of the Introduction.

The third important fact is that glutamate, while stimulating the oxidation of the β carbon, strongly inhibits the oxidation of the α carbon of glycine, whereas α-ketoglutarate stimulates the oxidation of both carbons. This fact suggests that a transamination reaction, involving α-ketoglutarate as nitrogen acceptor, may be part of the pathway responsible for the formation of $C^{14}O_2$ from glycine-2-C^{14}. However, attempts to demonstrate this transamination, described in Chapter II (Figure 6), provided no conclusive evidence.

The effect of competitors for coenzyme-A.

Shemin in 1956 described the cycle for porphyrin synthesis. The first step of the cycle involved a condensation of succinyl-CoA with glycine to give α-amino-βketoadipic acid (Scheme 3). He provided evidence (164) that substances which can give rise to Coenzyme-A derivatives decreased the rate of the reaction, since free Coenzyme-A is required for the oxidative decarboxylation of α-ketoglutarate to succinyl-CoA.

Since acetate, and its precursors inhibit the oxidation of the methylene carbonof glycine, it was felt that at least

part of the oxidation proceeds through the Shemin cycle. In this case the α-amino-β-ketoadipic acid would decarboxylate spontaneously to give δ-aminolevulivic acid and CO_2. The CO_2 produced in this reaction would come from the carboxyl carbon of glycine.

To test this possibility, a series of fatty acids were incubated with glycine-1-C^{14} in the presence of glucose, and their effects on the $C^{14}O_2$ production measured. The results of these experiments are summarized in Table XIII.

TABLE XIII

$C^{14}O_2$ Production from Glycine-1-C^{14} in the Presence of Fatty Acids.

Fatty acid	Concn. in mM	No Glucose		10 mM Glucose	
		µl O_2 per mg tissue	µmol $C^{14}O_2$ per 100 gm tissue	µl O_2 per mg tissue	µmol $C^{14}O_2$ per 100 gm tissue
None	--	6.2	170	16.1	640
Hexanoate	20	--	---	16.1	550
Decanoate	10	2.4	33	5.0	42
Benzoate	20	--	--	16.1	610
Phenylacetate	20	--	--	14.6	675
Phenylpyruvate	20	--	--	14.5	675

60-70 mg rat brain cortex slices incubated with 2 mM glycine-1-C^{14} for 90 minutes. Medium: Krebs-Ringer-phosphate, pH, 7.4. $BaCO_3$ isolated without carrier carbonate.

The presence of hexanoic and benzoic acids was insignificant, on both the oxygen consumption and $C^{14}O_2$ production; phenylacetic and phenylpyruvic acids inhibited slightly the oxygen consumption but did not alter the $C^{14}O_2$ production; decanoic acid strongly inhibited both. These results suggest that coenzyme-A is not involved in the metabolism of the carboxyl carbon of glycine to any significant extent.

The effect of decanoic acid on the glycine metabolism is probably due to a general inactivation of the enzyme systems of the brain. Scholefield in 1956 provided evidence that the site of inhibition of oxygen consumption by decanoate is not at the formation of acetyl-CoA, since it does not inhibit the formation of acetate from pyruvate, but probably at the phosphorylating reactions (180, 181).

Isotopic dilution of labeled glycine.

Although coenzyme-A is not necessary for the production of $C^{14}O_2$ from glycine-1-C^{14}, it was felt that the Shemin cycle may be of greater importance for the oxidation of the α carbon of glycine. According to the cycle, the α carbon of glycine is incorporated into the δ position of δ-aminolevulinic acid. This carbon could then be oxidized to CO_2, either through the α carboxyl group of α-ketoglutarate, or through a "one carbon fragment"

according to Scheme I. In either case, ∫-aminolevulinic acid would be a necessary intermediate.

Isotopic dilution experiments with glycine-1-C^{14} and unlabeled glycolic, glyoxylic acids and glycoaldehyde have been described in Chapter II. A similar series of experiments have been carried out to determine the effect of these compounds on the methylene carbon of glycine. The tissue slices were incubated with glycine-1 and 2-C^{14} in the presence of an excess of unlabeled compound in order to trap the radioactivity. The results of these experiments are summarized in Table XIV.

As can be seen from the Table ∫-aminolevulinic acid, at a concentration of 1 mM, had no effect on the oxygen consumption or the rate of glycine metabolism in the absence or presence of glucose. The $C^{14}O_2$ production from glycine-2-C^{14} in the presence of glucose was not diluted even at 5 mM concentrations of ∫-aminolevulinic acid. Since this substance can diffuse into the cell with relative ease (164, and Dr L. Booij, personal communication) it was concluded, that the Shemin cycle did not operate in the brain to an extent comparable to the rate of oxidation of glycine.

Glycolic acid had no effect on either oxygen consumption or $C^{14}O_2$ production. The presence of glyoxylic acid and glycolaldehyde caused a significant drop in the $C^{14}O_2$

TABLE XIV

Isotopic Dilution of Glycine with Possible Intermediates in the Rat Brain Cortex.

"Diluent"	Concn. in mM	No Glucose			10 mM Glucose		
		µl O_2 per mg tissue	µmol $C^{14}O_2$ produced per 100 g tissue from glycine-1-C^{14}	µmol $C^{14}O_2$ produced per 100 g tissue from glycine-2-C^{14}	µl O_2 per mg tissue	µmol $C^{14}O_2$ produced per 100 g tissue from glycine-1-C^{14}	µmol $C^{14}O_2$ produced per 100 g tissue from glycine-2-C^{14}
Nil	--	5.6	260	13	18.0	780	71
Glycolic acid	10	5.6	250	14	18.0	745	96
Glyoxylic acid	5	4.5	290	4.8	14.5	745	36
Glycolaldehyde	2	5.6	350	8.7	16.8	770	55
∫-aminolevulinic acid	1	5.6	270	12	18.0	850	90
	5	-	-	-	18.0	-	70

Incubation for 90 minutes in Krebs-Ringer-phosphate medium with 2 mM glycine. "Diluents" mixed with glycine in the side arm except for ∫-aminolevulinic acid at 5 mM which was included in the saline medium.

production from glycine-2-C^{14}. These two compounds also inhibited the oxygen consumption of the brain slices, but had no effect on the $C^{14}O_2$ formation from glycine-1-C^{14}. The effect of glycolic acid and glycolaldehyde on the metabolism of the α-carbon of glycine may be due to isotopic dilution, although there are two points against this argument.

Glyoxylic acid is not oxidized to CO_2 to any significant extent, since it does not stimulate the oxygen consumption, thus, if it is an intermediate, it should be able to trap almost all labeled carbon atoms. However, the drop of $C^{14}O_2$ formation amounts to only -50% in the presence of glucose and -63% in its absence.

Both glyoxylic acid and glycolaldehyde inhibit respiration thus they may act as simple inhibitors of the oxidation of glucose.

The effect of inhibitors.

The type of enzymes and cofactors involved in the metabolism of any substance can be best studied by the use of specific inhibitors. One series of experiments was therefore designed to study the effect of some of the more common inhibitors on the metabolism of glycine. The results of these experiments are summarized in Table XV and XVI.

TABLE XV

The Formation of $C^{14}O_2$ from Glycine-1 and 2-C^{14} in the Presence of Inhibitors.

Inhibitor	Concn. in mM	No Glucose			10 mM Glucose		
		µl O_2 per mg tissue	µmol $C^{14}O_2$ produced per 100 g tissue from glycine-		µl O_2 per mg tissue	µmol $C^{14}O_2$ produced per 100 g tissue from glycine	
			1-C^{14}	2-C^{14}		1-C^{14}	2-C^{14}
Nil	--	7.3	350	36	17.5	850	126
(Anaerobic)	--	--	32	4	--	21	3
Semicarbazide	10	4.6	30	17	17.5	240	100
p-Chloromercuri-benzoate	0.2	5.8	210	20	17.5	510	26
Potassium	100	4.6	290	25	27.0	850	70
2.4-dinitrophenol	0.025	4.8	280	18	32.8	900	110
n-Dichloroacetyl-glycine	2	7.3	320	34	17.5	780	100
4-aminopteroyl-glutamic acid	1	7.3	390	34	17.5	750	120

Incubation of the rat brain cortex slices proceeded for 90 minutes at 37°C. N-dichloroacetylglycine and 4-aminopteroylglutamic acid was included in the Krebs-Ringer-phosphate medium, others tipped with glycine from the side arm.

TABLE XVI

Per Cent $C^{14}O_2$ Formed from Glycine-1 and 2-C^{14} in the Presence of Inhibitors.

Inhibitor	Concn. in mM	No Glucose			10 mM Glucose		
		O_2 consumed	$C^{14}O_2$ from glycine 1-C^{14}	2-C^{14}	O_2 consumed	$C^{14}O_2$ from glycine 1-C^{14}	2-C^{14}
Nil	--	100	100	100	100	100	100
(Anaerobic)	--	-	9	11	--	2	2
Semicarbazide	10	63	9	47	100	28	80
p-Chloromercuri-benzoate	0.2	79	60	55	100	60	21
Potassium	100	63	83	70	154	100	56
2.4-dinitrophenol	0.025	66	80	50	187	106	87
n-Dichloroacetyl-glycine	2	100	91	94	100	92	80
4-aminopteroyl-glutamic acid	1	100	110	94	100	89	95

Conditions as described in Table XV.

The CO_2 formation from glycine is almost completely abolished in the absence of oxygen and the small amounts produced can be explained by traces of oxygen, approximately 3%, in the commercial N_2 used.

Semicarbazide, at 10 mM concentration, strongly inhibits the CO_2 formation from the carboxyl carbon of glycine, but does not affect the oxidation of the α carbon. This indicates that the break-down of glycine to CO_2 in the brain is strictly aerobic, and the pathway responsible for the liberation of the α carbon as CO_2 requires pyridoxal phosphate as cofactor. These implications have been discussed in greater detail in Chapter II.

p-Chloromercuribenzoate is a potent inhibitor of enzymes which have active free -SH groups. There are many enzymes of this type involved in the metabolism of glucose, therefore the inhibitor was used at a concentration of 0.2 mM which does not affect the oxygen consumption, in the presence of glucose. p-Chloromercuribenzoate caused a strong inhibition of the $C^{14}O_2$ formation from both glycine-1 and 2-C^{14}, the inhibition being especially great in the presence of glucose.

One important point arises from the inhibition by parachloromercuribenzoate, which can be seen from Table XV. The presence of this inhibitor almost abolishes the effect

of glucose on the oxidation of the α carbon of glycine (20:26 as compared to 36:126 in the case of the controls), but does not alter the effect of glucose on the production of CO_2 from the carboxyl carbon (210:510 as compared to 350:850).

This result implies that for the complete break-down of glycine, an -SH enzyme, as well as a glucose intermediate are required, whereas for the mechanism responsible for the liberation of the carboxyl carbon of glycine, only a product of glucose metabolism is required. Although the oxygen consumption in the presence of glucose is unaffected by the inhibitor, the formation of some of its products may be inhibited slightly.

Potassium at 100 mM concentration strongly stimulates the respiration of brain cortex slices in the presence of glucose. The respiration in the absence of glucose is inhibited by 37%. No stimulation of glycine metabolism was observed in either case. The formation of $C^{14}O_2$ from glycine-1-C^{14}, was not altered significantly, although a slight decrease was observed in the absence of glucose, The $C^{14}O_2$ formation from glycine-2-C^{14} was inhibited in both cases. This effect could be due to a decrease in the available ATP content of potassium stimulated brain slices (17), however, experiments to be reported later indicate

a lowering of the concentration of glycine inside the cell due to potassium (Table XVII).

2,4-Dinitrophenol is a known uncoupler of oxidative phosphorylation (41). In the absence of glucose, it inhibits strongly the $C^{14}O_2$ formation from glycine-2-C^{14}, but only slightly the $C^{14}O_2$ formation from glycine-1-C^{14}. It also inhibits the oxygen consumption. This fact points to a requirement of ATP for the oxidation of the α carbon of glycine. In the presence of glucose the oxygen consumption is stimulated, but the oxidation of either carbons of the glycine molecule is unaffected, as enough ATP is generated through the glycolytic, non oxidative phosphorylations.

N-dichloroacetyl glycine and 4-aminopteroylglutamic acid had no effect at the concentrations used.

The uptake of glycine by tissue slices.

Incubation of glycine-1-C^{14} with tissue slices produced a very active concentration of radioactivity. The radioactivity was assumed to be due to glycine itself as its slow metabolism could not permit a substantial accumulation of labeled products. This assumption was verified later by chromatography.

The amount of glycine taken up by the cells was strongly

stimulated by glucose and inhibited by potassium. The results are represented on Table XVII.

The slices were incubated for 30 minutes, during which time the amount of glycine taken up in the presence of glucose was 10.6 micromols per gram tissue, fresh weight. By assuming that 1 gram tissue contains 1 ml water, this would correspond to a concentration of 10.6 mM. The assumption is only a limiting value and the concentration obtained is the minimal concentration.

The efficiency of active concentration of glycine is expressed as a concentration ratio. From Table XVII it is seen that potassium interferes with the transport of glycine across the cell wall, and the presence of glucose facilitates it.

It was shown previously (Table VIII) that the rate of $C^{14}O_2$ formation from glycine-1-C^{14} was independent of the substrate concentration within the range of concentrations used, however, the formation of $C^{14}O_2$ from glycine-2-C^{14} depended, at all concentrations, on the concentration of glycine. Thus it is probable, that the effect of potassium on the break-down of glycine occurs at the uptake level. The effect of glucose, however, cannot be due to a facilitation of uptake, since it affects equally the CO_2 form tion from either carbon atoms of glycine.

TABLE XVII

The Effect of Glucose and Potassium on the Uptake of Glycine by Rat Brain Cerebral Cortex Slices.

Glucose Concn. in mM	Potassium concn. in mM	Glycine concn. inside the cell.	Concn. ratio: (inside) (outside)
0	0	5.0	2.65
0	100	3.3	1.72
10	0	10.6	6.05
10	100	8.3	4.58

Glycine inside concentration expressed as micromoles per gram tissue, fresh weight. The tissue slices were incubated with 2 mM glycine, original concentration, in 3 ml Krebs-Ringer phosphate medium for 30 minutes.

Glucose was included in the medium, glycine and potassium were mixed in the side arm and tipped after thermal equilibration.

The uptake of glycine in the presence of glucose has been further investigated in relation of the initial outside concentration of glycine and the time of incubation. The results are represented in Table XVIII.

The uptake of glycine is expressed as μmol glycine-1-C^{14} per gram tissue, fresh weight, and the concentration ratios are included in parentheses.

At all concentrations used, the influx of glycine from the medium into the slice was still active after 90 minutes.

TABLE XVIII

Rate of Uptake of Glycine by
Rat Brain Cortex Slices in Presence of Glucose.

Glycine introduced µmols per ml medium	Duration of Incubation 5 min.	15 min.	30 min.	60 min.	90 min.
	Concentration of glycine inside the cell.				
0.5	--	--	3.6 (8.7)	4.3 (10.7)	6.0 (7.4)
1.0	--	--	5.5 (6.3)	8.4 (10.4)	11.2 (15.1)
2.0	3.0 (1.55)	7.2 (3.9)	10.6 (6.0)	13.9 (8.3)	17.7 (11.1)
4.0	--	--	18.2 (5.1)	22.8 (6.4)	26.2 (7.7)

The figures are expressed as micromol glycine per gram tissue, fresh weight.

The figures in brackets represent concentration ratios: $\frac{\text{(glycine) inside the slice}}{\text{(glycine) in the medium}}$

60-80 mg rat brain cortex slices were incubated in 3 ml Krebs-Ringer-phosphate medium at 37°C in the presence of glucose, 10 mM.

The rate of influx at 2 mM initial concentration of glycine is shown in Figure 8. The effect of the outside concentration of glycine on the uptake and the concentration ratio after 90 minutes of incubation is shown in Figure 9. As can be seen, the amount of glycine taken up in 90 minutes by the slice was increased by increasing the initial concentration of glycine introduced into the medium. At 4.0 mM initial outside concentration, the concentration inside the cell was 26.2 micromols per gram tissue, fresh weight, which corresponds to a minimal concentration ratio of 7.1. The concentration ratios increase with time at any given initial concentration, but decrease with increasing initial concentrations. This may indicate an "overload" on the transport mechanism.

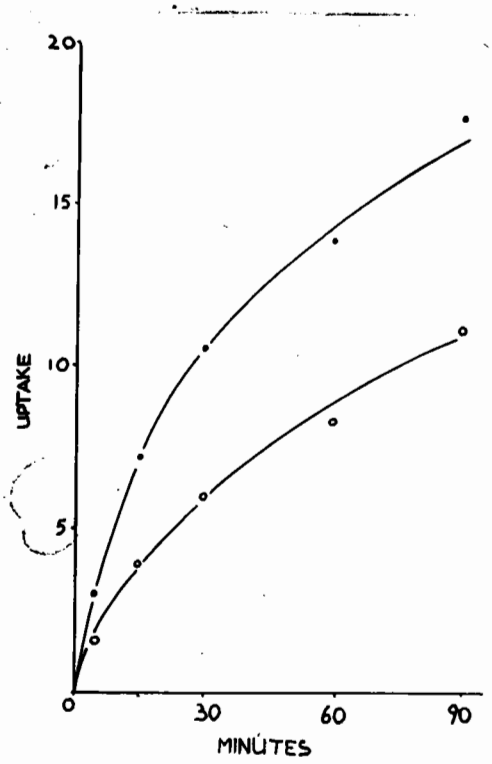
The incorporation of glycine and serine into other amino acids.

In order to determine whether the radioactive uptake was due to glycine, the soluble fractions of the slices were analysed for radioactive materials after incubation.

The tissue slices were incubated with glycine-1 and 2-C^{14} and serine-U-C^{14}, 2 mM each, for 90 minutes, and assayed as described previously. The radioactivity in all cases was 1,000,000 counts per minute per vessel.

FIGURE 8

The Rate of Uptake of Glycine by Rat Brain Cortex Slices.



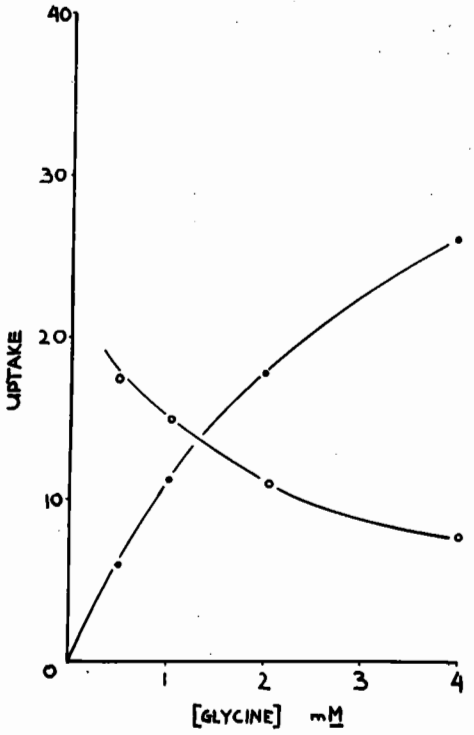
● Concentration of glycine inside the slice (μmol per gram tissue, fresh weight).

o Concentration Ratio: $\frac{\text{(Glycine) inside}}{\text{(Glycine) outside}}$

All vessels contained 10 mM glucose and 2 mM glycine.

FIGURE 9

The Uptake of Glycine by Rat Brain Cortex.



- • Concentration of glycine inside the slice, expressed as μmol glycine per gram tissue, fresh weight.
- o Concentration Ratio: $\frac{\text{(Glycine) inside}}{\text{(Glycine) outside}}$

All vessels contained 10 mM glucose.
Incubation time: 90 minutes.

After passage of the ethanol extract through the Dowex 50 column and elution with ammonia, the total amino compounds in the eluate contained 90% of the total radioactivity of the original extract. The remaining radioactivity was in the first effluent containing neutral and anionic compounds. This first effluent was not tested further.

The eluate was chromatographed and the radioactive spots were located by radioautography and counted. Due to the unknown amount of self absorption of the paper, the activities are expressed as counts per minute per spot after correction for the amount of tissue introduced into the vessels. Thus, although the figures do not indicate the concentration of each compound, they do indicate the relative rate of incorporation. The results are summarized in Table XIX. Both glycine-1- and 2-C^{14} are incorporated into serine, the rate of incorporation being twice as great for the α than the carboxyl carbon. This provides a partial proof for the hypothesis that two glycine molecules give rise to one serine molecule with the liberation of a carboxyl group as CO_2.

In the slices incubated with glycine-1-C^{14}, the activity of glycine corresponds to 98% of the total radioactivity. A small amount of radioactivity was present in

TABLE XIX

The Incorporation of Radioactivity into Amino Acids from Glycine-1 and 2-C^{14} and Serine-U-C^{14}.

Isolated amino acid	Parent amino acid: Glycine 1-C^{14} c.p.m.	Glycine 2-C^{14} c.p.m.	Serine U-C^{14} c.p.m.
Glycine	52,100	46,100	1,350
Serine	680	1,550	26,800
Glutamic acid	170	100	364
Glutamine	49	78	0
γ-amino butyric acid	16	21	30
Alanine	108	64	96
Aspartic acid	61	45	82

70 mg rat brain cortex slices incubated in Krebs-Ringer-phosphate medium with 2 mM labeled amino acid, 10^6 counts per minute per vessel each in the presence of 10 mM glucose. Procedure of chromatography as described previously.

glutamic acid and its derivatives, glutamine and γ amino-butyric acid, in aspartic acid and alanine.

A similar pattern of incorporation was observed in the slices incubated with glycine-2-C^{14} and serine-U-C^{14}.

Since serine was labeled equally in all carbons, the high activity observed in the glycine indicates that the formation of glycine from serine is faster than the reverse reaction. It is of interest that, while serine gave rise to 2-3 times greater radioactivity in glutamic acid than did glycine, labeled in either carbon, there was no activity detected in glutamine. Since the formation of glutamine from glutamic acid in the brain depends mainly on the rate of formation of free ammonia (46, 47, 48), a large fraction of the amino group of the glycine metabolized is liberated as free ammonia rather than transaminated.

In all three experiments radioactivity was detected in glutamic acid, alanine and aspartic acid, all of which are closely associated with the Tricarboxylic acid cycle, whereas leucine, valine, tyrosine, methionine and arginine, although detected by ninhydrin, showed no radioactivity.

Very low radioactivity was also detected in five ninhydrin negative spots, which have not been identified.

Discussion.

The stimulation by glucose of the metabolism of glycine is unique in that no other respiratory substrate can fully reproduce its action. The intermediates of glucose metabolism as far as α-ketoglutarate, have an action similar

to glucose on the CO_2 formation from the carboxyl group of glycine, but not on the oxidation of the α group. Thus it is possible that the metabolism of the two carbon atoms of glycine is stimulated by two separate substrates, both of which are derived from glucose, but only one can be formed from other respiratory substrates. In view of this, glucose stimulates the metabolism of the two carbon atoms of glycine by two separate mechanisms: that the extent of stimulation is the same for both carbons has no special significance.

The effects of pyruvate, fumarate and acetate are interesting. Pyruvate, in the absence of fumarate, is readily decarboxylated to acetate in rat brain mitochondria (181). There are indications, that at least 50% of the pyruvate metabolized in rat brain slices is also transformed into acetate (Mr. M.M. Kini, personal communication). Fumarate can also give rise to acetate by way of oxal-acetate and pyruvate. It therefore seemed justified to assume that acetate was involved in the metabolism of glycine. Weinhouse and Friedman (162), however, could not demonstrate a relationship between the metabolism of the two compounds <u>in vivo</u>.

The action of fumarate on the carboxyl carbon of glycine can be explained by a stimulation masked by a dilution

effect, since it strongly dilutes the CO_2 produced from the α carbon.

The inhibition caused by acetate cannot be true inhibition since other fatty acids, with the exception of decanoate, do not inhibit the metabolism of glycine. It must be assumed that in the brain, acetate and glycine can give rise to the same intermediate. Glyoxylic acid and glycolaldehyde probably give rise to the same intermediate, although they themselves are not intermediates of glycine metabolism.

Glutamate stimulates the formation of CO_2 from the carboxyl carbon of glycine, but inhibits the formation of CO_2 from the α carbon. α -ketoglutarate stimulates both oxidations. The stimulation by glutamate and α -ketoglutarate, of the liberation of the carboxyl group of glycine as CO_2, shows that a substance near to α -ketoglutarate in the Tricarboxylic acid cycle is the actual stimulating agent.

Takagaki et al. showed that in the presence of high concentrations of glutamate, most of the free ammonia of the guinea-pig brain was transformed into glutamine (56). Elliott isolated the glutamine-forming enzyme system and and showed that it was active with free ammonia (47, 48) and results by DuRuisseau et al. (46) indicated that this reaction

is the sole means of removal of free ammonia in the brain.

The results in Table XIX show that the formation of glutamine from glutamic acid is greatly enhanced by the presence of glycine, thus free ammonia must be formed during the incubation. Therefore the reaction responsible for the liberation of the carboxyl carbon of glycine as CO_2 also liberates ammonia which, if not removed as glutamine, accumulates and inhibits the reaction.

The stimulation by glucose of $C^{14}O_2$ production from glycine-2-C^{14} is two fold. Studies with inhibitors proved that ATP is required for the process; 2,4-dinitrophenol in the absence of glucose was inhibitory. The stimulation by α-ketoglutarate and inhibition by glutamate point to another process: transamination with α-ketoglutarate as a nitrogen acceptor. It is difficult to explain why this reaction is not sensitive to the presence of semicarbazide. An inhibition of only 20% was observed when semicarbazide was included into the system. This may indicate that pyridoxal phosphate has a greater affinity for the enzyme system than for semicarbazide.

The enzyme system which is responsible for the complete break-down of glycine involves an enzyme with an active, free -SH group, since the $C^{14}O_2$ production from glycine-2-C^{14} is strongly inhibited by p-chloromercuribenzoate. The

inhibition is especially apparent in the presence of glucose, amounting to 80%.

There was no inhibition when 4-aminopteroylglutamic acid was included into the system. This substance is a strong antagonist of the folic acid, in vivo, but Blakley demonstrated that its site of action is the inhibition of the hydrogenation of folic acid to tetrahydrofolic acid (136). Although tetrahydrofolic acid is involved in the formation of serine from glycine, the amount of this substance normally present in the brain is sufficient to keep the reaction at a maximal rate during the course of the in vitro experiments.

N-dichloroacetyl glycine was also inactive. It is very likely that the dichloroacetyl group makes this substance inaccessible to the enzymes responsible for the metabolism of glycine.

Amino acids, which are near the Tricarboxylic acid cycle, incorporate radioactivity from glycine and serine. The incorporation proceeds at a slow rate. Part of the label comes from a fixation of CO_2 onto unlabeled pyruvate to give radioactive oxalacetate. However, the radioactivity observed in alanine indicates that a small amount of labeled pyruvate is also formed. The labeled carbon is then distributed among the compounds of the tricarboxylic acid cycle.

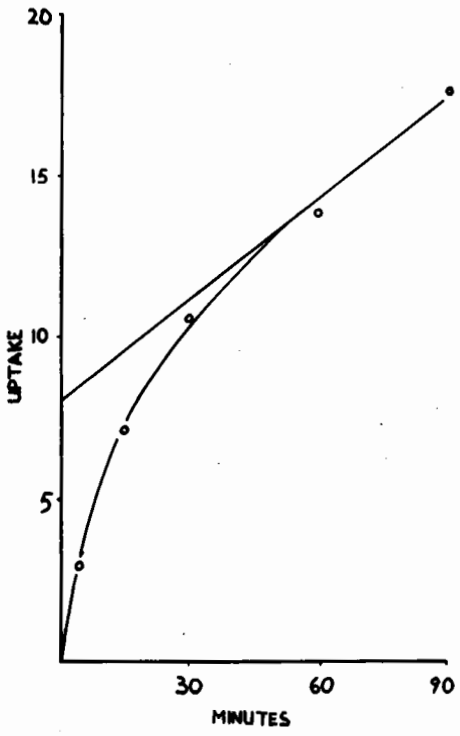
The rate of uptake of glycine into the nerve cell was presented in Figure 8. The uptake approaches a constant positive value with time, which means that at any concentration of glycine inside the cell there is still an active uptake. Although chromatography showed no substantial accumulation of any substance other than glycine, it can be assumed that simultaneously with the transport, a zero or first order reaction transforms glycine into a labile intermediate which breaks down to give free glycine during the extraction procedure. (Figure 10).

Thus, the uptake curve can be resolved into a straight line, representing the formation of the intermediate and a curve of the uptake itself. By extrapolating the straight portion of the curve to 0 time, the value at the intersection of the line and the ordinate will represent the true uptake of glycine by the cell at equilibrium. The value comes to approximately 8 μmol glycine per gram fresh weight, or a concentration ratio of 4 - 5. This concentration ratio is near to the values reported for other organs (183).

The effects of glucose and of potassium on the uptake of glycine indicate that the transport is an ATP requiring process. However potassium may inhibit the transport by competition for the active sites of adsorption on the cell surface (177).

FIGURE 10

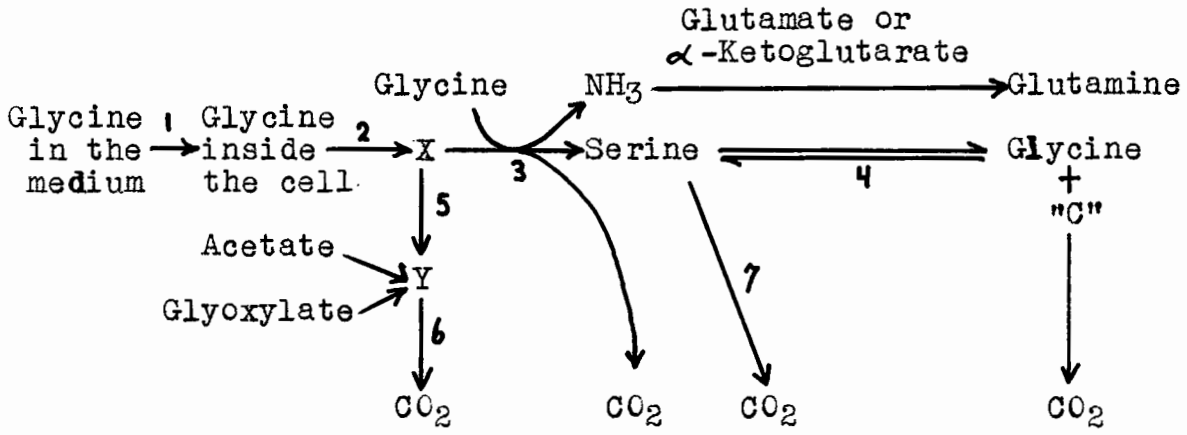
The Rate of Uptake of Glycine by
Rat Brain Cortex Slices.



Uptake expressed as μmol glycine per gram tissue, fresh weight.
All vessels contained 10 mM glucose and 2 mM glycine.

Conclusion.

On the basis of the results, the following reaction sequence is proposed:



Reaction 1 is stimulated by glucose, possibly through ATP, and is inhibited by potassium. Reaction 2 forms a labile intermediate of glycine. This reaction may also require ATP. Reaction 3 probably consists of more than one step, pyridoxal phosphate and ATP are cofactors, and formaldehyde may not be involved. The carboxyl group of one glycine molecule is liberated as CO_2 and free NH_3 formed is removed as glutamine. Reaction 4, the formation from serine of glycine and a one-carbon fragment, is possibly identical with the sequence reported by Blakley (136). The reaction is reversible and strongly favors the formation of glycine. Reaction 5, involves a transamination,

with α-ketoglutarate as the nitrogen acceptor, free glyoxylate is not formed. This reaction is ten times slower than reaction 3. Reaction 7 is the complete oxidation of serine by way of pyruvate and the tricarboxylic acid cycle.

Summary.

1. In the presence of glucose, glycine-1-C^{14} was shown to give rise to $C^{14}O_2$ at a constant rate. No lag period was observed. The $C^{14}O_2$ formation from glycine-2-C^{14} started after a lag period of 30-60 minutes, then proceeded at a rate lower than for glycine-1-C^{14}. Serine-3-C^{14} gave rise to $C^{14}O_2$ at a rate similar to glycine-2-C^{14} but showed a shorter lag period.

2. The presence of glucose caused a three-fold increase in the metabolism of both carbons of the glycine molecule. A similar increase in the CO_2 formation from the carboxyl group of glycine was observed in the presence of α-ketoglutarate, glutamate and a mixture of pyruvate and fumarate. No effect was observed in the presence of pyruvate, fumarate and succinate, whereas acetate was inhibitory.

3. The formation of $C^{14}O_2$ from glycine-2-C^{14} was slightly stimulated by α-ketoglutarate, inhibited by

glutamate, pyruvate, fumarate, succinate and acetate. No change was observed in the presence of a mixture of pyruvate and fumarate.

4. Alphatic and aromatic acids as well as δ-amino-levulinic acid had no effect on the metabolism of glycine, thus the Shemin cycle does not operate to any significant extent in the brain. Glycolic acid had no effect on the metabolism of glycine, whereas glyoxylic acid and glycolaldehyde inhibited the oxidation of the α carbon to CO_2.

5. The CO_2 formation from glycine is abolished in the absence of oxygen. The aerobic oxidation of the α carbon of glycine is inhibited by p-chloromercuribenzoate and high concentrations of potassium. It is also inhibited by 2,4-dinitrophenol but only in the absence of glucose. p-Chloromercuribenzoate abolishes the effect of glucose on the oxidation of the α carbon of glycine. Semicarbazide strongly inhibits the $C^{14}O_2$ formation from glycine-1-C^{14} but does not affect glycine-2-C^{14}.

6. Glycine was shown to be actively concentrated by rat brain cortex slices, the extent of concentration depending on the initial concentration of glycine in the incubating medium and the incubation time. The uptake is stimulated by glucose and partially inhibited by potassium.

7. The rapid interconversion of serine and glycine was confirmed in the rat brain by chromatography and radioautography. The amount of the α carbon of glycine incor-

porated into serine is twice the amount of the carboxyl carbon. Labeled glycine and serine also give rise to labeled glutamic acid, glutamine, δ-aminobutyric acid, aspartic acid, alanine and five ninhydrin negative amino compounds. No label was found in leucine, valine, tyrosine, methionine or arginine.

8. The formation of glutamine from glutamate was found to be greater in the presence of glycine than in the presence of serine.

CHAPTER IV

THE EFFECT OF CHLORPROMAZINE ON THE METABOLISM OF GLYCINE-1-C^{14} IN THE RAT BRAIN.

The use of chlorpromazine in psychotherapy is now generally accepted because of its unique action as a tranquilizer without narcosis. However, its exact site of action is still obscure, despite the large amount of work being done to elucidate it.

In order to study the mode of action of chlorpromazine a series of investigations were undertaken using the metabolism of glycine-1-C^{14} as an index of the activity of rat brain cortex slices.

All the results reported in this chapter have been obtained in collaboration with Dr. O. Lindan under the supervision of Professor J. H. Quastel, and published previously (43, 177).

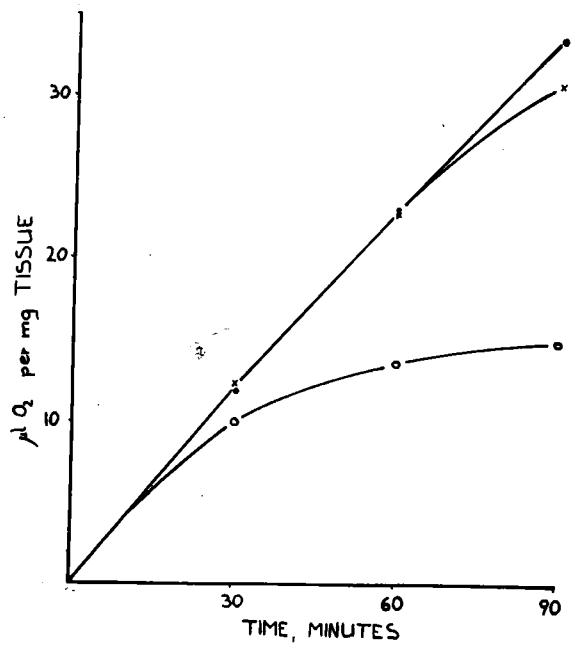
Results.

The inhibition of oxygen consumption by chlorpromazine follows a pattern different from that of narcotics in that it is a progressive type of inhibition. Typical results are represented in Figure 11. The inhibition

FIGURE 11

EFFECT OF CHLORPROMAZINE ON THE

OXYGEN CONSUMPTION BY RAT BRAIN CORTEX SLICES.



- ● No chlorpromazine
- × 0.3 mM chlorpromazine
- o 0.6 mM chlorpromazine

Incubated in Krebs-Ringer-phosphate medium at 37°C in the presence of 10 mM glucose.

caused by 0.3 mM chlorpromazine is not apparent for 60 minutes. 0.6 mM chlorpromazine shows a slight inhibition after 30 minutes, then the oxygen consumption drops sharply reaching 10% of its original value within a further 60 minutes.

A pattern of inhibition similar to that of the oxygen consumption can be observed with the formation of $C^{14}O_2$ from glycine-1-C^{14} (Table XX).

TABLE XX

The Effect of Chlorpromazine on the Incorporation into Proteins and Break-Down of Glycine-1-C^{14}.

Chlor-promazine mM	Time	µl O_2 per mg tissue	µmol $C^{14}O_2$ per 100 g tissue	µmol glycine incorporated per 100 g protein
0	30	6.4	172	7.2
	90	18.5	560	23.7
0.3	30	6.2	193	5.1
	90	15.8	480	11.1
0.6	30	5.5	110	3.0
	90	8.1	145	3.4

Rat brain cortex slices were incubated in a Krebs-Ringer-phosphate medium at 37°C in the presence of 10 mM glucose, 2 mM glycine-1-C^{14} and chlorpromazine as indicated.

Chlorpromazine at any concentrations has very little effect after 30 minutes, but strongly inhibits at 0.6 mM concentration after 90 minutes incubation. However, the incorporation of glycine into proteins is markedly decreased within the first 30 minutes in the presence of 0.6 mM chlorpromazine.

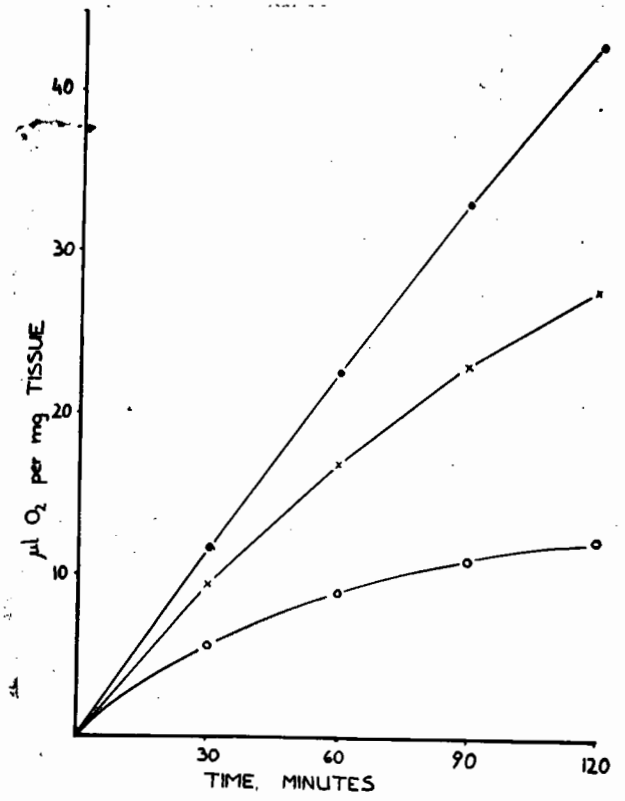
Although the oxygen consumption and $C^{14}O_2$ formation are not affected within 30 minutes, the inhibition of incorporation of glycine into proteins indicates that chlorpromazine is rapidly taken up by certain parts of the tissue and slowly diffuses to the sites responsible for oxidation. This hypothesis was confirmed by a series of experiments illustrated in Figure 12.

The rat cerebral cortex slices were incubated at 37°C in the presence of 10 mM glucose and 0.6 mM chlorpromazine for 5, 15 and 30 minutes. At the end of this period the slices were rapidly removed from the vessels, rinsed in saline and transferred to other vessels containing chlorpromazine-free Krebs-Ringer-phosphate medium with 10 mM glucose and incubated for 2 hours. Control slices were treated similarly, except that the first medium of incubation did not contain chlorpromazine.

From Figure 12, it can be seen that a marked and progressive inhibition occured in the oxygen consumption

FIGURE 12

THE IRREVERSIBLE ADSORPTION OF CHLORPROMAZINE



Tissue slices incubated with 0.6 mM chlorpromazine, for different times, then rinsed and transferred into inhibitor-free medium.

- • Pre-incubated in the absence of chlorpromazine
- × Pre-incubated for 5 minutes
- o Pre-incubated for 15 minutes.

of slices treated with chlorpromazine for 5 or 15 minutes. The slices treated for 30 minutes with the inhibitor were completely inhibited at the beginning of the second incubation.

Chlorpromazine, at concentrations which have no effect on the rate of unstimulated respiration of rat brain cortex slices, depresses the extra oxygen consumption due to potassium (Table XXI). The inhibition

TABLE XXI

The Effect of Chlorpromazine on Potassium Stimulated Respiration.

Chlorpromazine mM	Q_{O_2} in 30 minutes intervals		
	1st	2nd	3rd
0	18.8	16.8	12.6
0.2	15.4	15.0	12.0
0.4	15.8	14.0	13.0
0.6	11.0	7.4	3.4

The rat brain cortex slices were incubated in a Krebs-Ringer-phosphate medium at 37°C in the presence of 10 mM glucose, 100 mM potassium and chlorpromazine as indicated.

is especially apparent during the first 30 minutes of incubation. At higher concentration of chlorpromazine (0.6 mM) the potassium effect is completely abolished. In view of this finding it was of interest to investigate the rate of formation of $C^{14}O_2$ and incorporation into proteins of glycine-1-C^{14} in the presence of stimulants and of chlorpromazine. The results are summarized in Table XXII.

TABLE XXII

The Effect of Chlorpromazine on the Glycine-1-C^{14} Metabolism by Stimulated Brain Slices.

Stimulant added	Concn. in mM	Chlor-promazine in mM	µl O_2 per mg tissue	µmol $C^{14}O_2$ per 100g tissue	µmol glycine incor-porated per 100g protein
None	--	0	18.5	560	23.7
		0.3	15.8	480	11.1
		0.6	8.1	145	3.4
KCl	100	0	28.6	450	6.4
		0.3	21.8	445	4.9
		0.6	13.2	222	2.9
DNP[1]	0.025	0		530	13.0
		0.3		277	4.1
		0.6		102	2.5

[1] DNP 2,4-dinitrophenol
Rat brain cortex slices were incubated in Krebs-Ringer phosphate medium at 37°C for 90 minutes in the presence of 10 mM glucose, 2 mM glycine and chlorpromazine, potassium and 2,4-dinitrophenol as indicated.

In the absence of stimulants the oxygen consumption and $C^{14}O_2$ formation is only very slightly affected by 0.3 mM chlorpromazine, but the rate of incorporation is strongly inhibited. 0.6 mM chlorpromazine markedly inhibits all three phenomena.

The presence of potassium increases the oxygen consumption, and this is abolished in the presence of chlorpromazine. The pattern of inhibition of the $C^{14}O_2$ production by chlorpromazine is unaltered by the presence of potassium, indicating that the site of potassium stimulation is different from the site of the glycine break-down and of the resting oxygen uptake. The incorporation of glycine into proteins is inhibited 75% in the presence of potassium, and is further decreased by chlorpromazine. Similar results were obtained when potassium was substituted by 2,4-dinitrophenol.

Discussion.

The strong inhibition by potassium, of the incorporation into proteins of glycine-1-C^{14} in the rat brain cortex slices, is partly due to the decreased uptake of glycine by the cells. The high degree of inhibition suggests another site of action. Unpublished experiments by Bickis (Mr. I. Bickis, personal communications) indicate that the incorporation of amino acids into

proteins *in vitro* is proportional to the amount of available ATP in the tissue. Thus, if potassium stimulates respiration by removing available ATP from the system, through an increased ATPase activity (17), a concomitant decrease in incorporation is expected. When chlorpromazine is added to the system, the extra oxygen consumption is abolished but the inhibition of incorporation is unaffected, instead, an additional inhibition due to chlorpromazine is superimposed. This indicates that the drug inhibits, not the ATPase activity, but the enzyme system which forms the ATP.

Formation of $C^{14}O_2$ from glycine-1-C^{14} is inhibited by chlorpromazine to the same extent as the oxygen consumption. The inhibition is progressive and does not occur at concentrations of the drug less than 0.5 mM, thus the break-down of the glycine must occur at a site remote from the sites of attachment of chlorpromazine. During the course of the experiments the drug diffuses from the sites of attachment into the cell, causing a generalized inhibition of the enzymes concerned with the metabolism of glucose and glycine.

Incorporation and active protein synthesis in the brain *in vivo* has been reported by Desclin (61), Hyden (62) and Lajtha et al. (71), and the *in vitro* incorporation of glycine-1-C^{14} into the proteins probably

occurs through a similar protein synthesis. The incorporation is more sensitive to chlorpromazine than is the oxygen consumption of the resting neurone.

Results reported by Hyden and Brattgård (62, 63) indicate that the protein synthesis is increased by intense stimulation of the nerve cell, whereas a lack of stimuli decreases the protein synthesis. The effect of chlorpromazine on the incorporation of glycine into the proteins of brain cortex slices may be related to the tranquillizing effects of the drug, although it is difficult to determine whether the protein synthesis is decreased by an inhibition of the mechanism responsible for nervous activity, or vice versa. It is certain, however, that the concentrations of the drug that are effective in the inhibition of the incorporation of glycine are nearer to physiological concentrations than those effective in the inhibition of the oxygen uptake and glycine break-down.

Summary.

1. Chlorpromazine at 0.6 mM concentration progressively inhibits the oxygen consumption by rat brain cortex slices. No inhibition was observed during the first 30 minutes. After the 30 minutes lag period, the oxygen consumption

drops sharply to reach 10% of its original value within a further 60 minutes. Lower concentrations of the drug caused no inhibition within 90 minutes.

2. Chlorpromazine is irreversibly absorbed onto the slices, during the lag period and diffuses into the tissue to cause progressive inhibition.

3. The "extra" oxygen consumption by brain slices, due to the presence of potassium, is more sensitive to the drug than to the "resting" oxygen uptake. Significant inhibitions of the "extra" oxygen uptake were obtained within the first 30 minutes with 0.2 mM chlorpromazine.

4. The rate of $C^{14}O_2$ production from glycine 1-C^{14} is inhibited by chlorpromazine, the extent and pattern of inhibition being similar to "resting" oxygen uptake. The presence of potassium did not alter the rate of break-down of glycine nor the extent of inhibition caused by the drug.

5. The incorporation of glycine-1-C^{14} into the proteins of brain slices in vitro is more sensitive to chlorpromazine than the break-down of glycine-1-C^{14}. The extent and pattern of inhibition is similar to that of the "extra" oxygen uptake in the presence of potassium.

6. The incorporation of glycine into the proteins is strongly decreased in the presence of potassium; chlorpromazine decreases the incorporation further.

CLAIMS TO ORIGINAL RESEARCH

1. Rat brain cortex was shown to be able to oxidize glycine, serine, valine, leucine and phenylalanine *in vitro*. The $C^{14}O_2$ production from glycine-1-C^{14} is ten times faster than that from glycine-2-C^{14}.

2. Chlorpromazine was shown to inhibit the oxygen consumption, the $C^{14}O_2$ production, and incorporation into proteins of glycine-1-C^{14} in the rat brain *in vitro*. The inhibition is of the "progressive" type.

3. It was shown that chlorpromazine is rapidly and irreversibly adsorbed on the tissue slices and slowly diffuses from the site of adsorption into the cell.

4. The incorporation of glycine-1-C^{14} into the proteins of the brain is inhibited by chlorpromazine at concentrations which have no effect on the oxygen consumption and $C^{14}O_2$ production. Potassium at 0.1M also inhibits the incorporation and the two inhibitions are additive.

5. The inhibition of the $C^{14}O_2$ production from glycine-1-C^{14} in the presence of chlorpromazine follows the same pattern as the inhibition of oxygen consumption.

6. The extra oxygen consumption of rat brain cortex slices in the presence of potassium is inhibited by chlorpromazine at concentrations which have no effect on the

"resting" oxygen consumption.

7. The metabolism of glycine, serine and leucine in the brain is strongly stimulated by the presence of glucose. Glucose has no effect on the metabolism of glycine in the liver and kidney cortex nor on the formation of $C^{14}O_2$ from valine-1-C^{14} and phenylalanine-2-C^{14} in the brain, in vitro.

8. The metabolism of glycine and serine in the brain are closely related. Pyruvate was shown to be connected with the process.

9. It was shown that the metabolism of leucine is not related to the metabolism of glycine in the rat brain.

10. The rate of $C^{14}O_2$ formation from glycine-1-C^{14} in the presence of glucose was shown to be maximal at 0.5 - 1.0 mM substrate concentration. The maximal liberation of $C^{14}O_2$ from glycine-2-C^{14} was indeterminable.

11. The rate of formation of $C^{14}O_2$ from glycine-1-C^{14} in the rat brain in vitro, was shown to be constant and to start without a lag period.

12. The rate of formation of $C^{14}O_2$ from glycine-2-C^{14} reaches a constant value after a 30-60 minutes lag period, and was shown to be lower than for glycine-1-C^{14}.

13. The rate of formation of $C^{14}O_2$ from serine-3-C^{14} is similar to that of glycine-2-C^{14} but with a shorter lag period.

14. The effect of glucose in the brain in causing a

three fold increase in the CO_2 production from either carbons of glycine is unique in that no other respiratory substrates can fully reproduce its action.

15. The presence of intermediates of glucose metabolism, as far as α-ketoglutarate, were shown to stimulate the CO_2 formation from the carboxyl carbon of glycine to a rate similar to that obtained in the presence of glucose. Glutamate stimulates the CO_2 formation to a lesser extent.

16. The formation of $C^{14}O_2$ from glycine-2-C^{14} in the rat brain is stimulated by α-ketoglutarate and inhibited by glutamate, pyruvate, fumarate, succinate and acetate. No change was observed in the presence of a mixture of pyruvate and fumarate.

17. With the aid of the isotopic dilution technique it was shown that free formaldehyde, glycolate, glyoxylate, glycolaldehyde and δ-aminolevulinic acid are not intermediates of the metabolism of glycine in rat brain cortex slices.

18. The CO_2 formation from glycine is abolished in the absence of oxygen. The aerobic oxidation of the α carbon of glycine is inhibited by p-chloromercuribenzoate and high concentrations of potassium. It is also inhibited by 2,4-dinitrophenol but only in the absence of glucose.

p-Chloromercuribenzoate abolishes the effect of the glucose on the oxidation of the ∝ carbon of glycine.

19. Semicarbazide strongly inhibits the $C^{14}O_2$ formation from glycine-1-C^{14} but does not affect glycine-2-C^{14}.

20. It was demonstrated that when rat brain cortex slices are incubated in the presence of glycine-1-C^{14}, the radioactivity is actively and rapidly concentrated. The rate of concentration depends on the initial concentration of glycine, it increases with time, but equilibrium is not reached within 90 minutes. The concentration ratios obtained exceed those reported for other organs including neoplastic tissue.

21. Although chromatography revealed no other substance containing substantial radioactivity, the shape of the curve for the concentration of glycine-1-C^{14} indicates that part of the glycine inside the cell is transformed into a labile intermediate.

22. The rapid interconversion of serine and glycine was shown to occur in the brain <u>in vitro</u>, in agreement with results from studies <u>in vivo</u>. The radioactivity from glycine-1 and 2-C^{14} and serine-U-C^{14} was shown to be incorporated into glutamate, ɣ -aminobutyrate, aspartate and alanine, but not into leucine, valine, tyrosine, methionine or arginine.

23. The formation of glutamine from glutamate was found to be greater in the presence of glycine than in the presence of serine.

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