STUDIES ON THE SOURCE OF UREA CARBON

Thesis by

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ABSTRACT

A method has been devised for the testing of any substance as an intermediate in a reaction sequence. Using this technique, thirty-three substances have been tested as precursors of the urea carbon atom. Of the compounds tested, only citrulline and arginine were precursors.

Carbamyl L-glutamic acid was shown not to be a donor of the urea carbon, although it does have some function in urea synthesis.

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I. HISTORICAL INTRODUCTION

A. The Ornithine Cycle

Modern work on the problem of biological urea synthesis dates from the discovery by Krebs and Henseleit (1, 2) that ornithine acts catalytically in producing urea from ammonium lactate added to liver slices. It had previously been established that arginase is present only in the livers of animals in which urea was the major end product of nitrogen metabolism (3).

Putting these two facts together, Krebs and Henseleit proposed the scheme given in Figure 1, and as support for this hypothesis, showed that citrulline as well as ornithine acted catalytically in producing urea.

The scheme may be stated simply as the synthesis of arginine from ornithine, 2 NH_3, and CO_2, via citrulline. The arginine so formed breaks down to ornithine and urea under the action of the enzyme arginase, which had been discovered by Kossel and Dakin in 1904 (4). The ornithine obtained from the hydrolysis of arginine is now available to go around the cycle once more.

While many workers in the past have denied the existence of part or all of the ornithine cycle, most workers today hold the view that most, if not all, urea synthesis proceeds by way of the ornithine cycle.

Among early attempts to test the hypothesis was the work of Neber (5), London and Alexandry (6), Ikeda (7), and Isawa, Togo, and Kawabu (8). Neber found, using cat and guinea pig liver sections,

that the addition of keto acids and ammonia resulted in the formation of amino acids and urea in approximately equal amounts, and that ornithine acted as a regulator between the two. London and Alexandry, on the other hand, were unable to confirm the existence of the ornithine cycle and concluded that while it operated in liver slices, it did not in the intact animal. Using dogs, they injected various compounds into the portal vein and collected blood which was analyzed for urea, at one minute intervals from the hepatic vein. They found that the addition of small quantities of NH_4Cl resulted in increases of from 20-77 per cent in urea after 6 minutes; and further, that ornithine and citrulline under the same conditions had no effect. The addition of arginine stimulated less than did NH_3, and a longer time was required for the maximum effect to be obtained.

Ikeda and Isawa et al. were, on the other hand, able to confirm the catalytic effects of ornithine and citrulline. Ikeda, in perfusion experiments on dog livers, found that the addition of citrulline increased the urea obtained from NH_4^+ salts added to blood. Isawa et al. showed that the addition of ornithine doubled and citrulline tripled the Q urea of liver slices.

Trowell (9) again using perfused livers, was able to confirm the catalytic effect of ornithine, but not that of citrulline. At high concentrations, citrulline did stimulate somewhat, but this stimulation was transitory, while that from ornithine persisted for long periods of time. The results obtained with arginine were quite variable, while glutamic acid, glutamine, and alanine had no effect.

Trowell concluded that the results did not support the ornithine cycle theory and that the normal mechanism of urea formation remained to be discovered. Krebs (10) has criticized these results on the basis of the concentrations used and the general conditions prevailing in perfusion experiments.

More direct evidence for the existence of the ornithine cycle was obtained when the heavy isotopes of hydrogen and nitrogen became available. Clutton et al. (11) were able to show the conversion of ornithine to arginine using deuterium labelled ornithine. DL-ornithine containing 13.6 atom per cent excess was fed to mice. After nine days, arginine isolated from the total body protein was found to contain 1.02 atom per cent excess in the ornithine moiety, indicating that in this time period, 7.5 per cent of the total body arginine had been derived from ornithine.

When $N^{15}H_3$ was fed to rats and the total body amino acids isolated, glutamic acid and aspartic acid had the highest N^{15} concentrations, while arginine was next. All of the N^{15} in arginine was in the amidine group (12). If glycine (13) or tyrosine (14) labelled with N^{15} were fed, the level of isotope present in the liver arginine (all of the N^{15} being in the amidine group) was very nearly the same as that in the excreted urea, the arginine having the highest N^{15} content of any of the amino acids studied.

The first real criticisms of the ornithine cycle came from the work of Bach (15). Bach found that when citrulline was added to rat liver slices in high concentrations, the ratio of NH_3-N disappeared/urea N formed was unity, whereas the ornithine cycle as

postulated would predict a ratio of 0.5. This effect was later confirmed by Borsook and Dubnoff (16) and Gornall and Hunter (17). Bach also determined the rate of urea formation from ornithine, citrulline, and arginine, and as the theory would predict, found the rates to increase in that order.

A satisfactory explanation of the N ratio obtained was offered by Gornall and Hunter when they proposed that, if the step citrulline to arginine were the slowest one of the cycle, the citrulline which went to form arginine would be resynthesized from the ornithine produced, and hence for each mole of urea-N formed, one mole of NH_3-N would disappear. In support of this interpretation, they found that when ornithine was added in high concentrations, the ratio NH_3-N used/urea-N formed was 1.4 and that the extra N used (over the theoretical 1.0) was incorporated into citrulline which accumulated in the reaction mixture.

Later work by Bach and Williamson (18) again questioned the validity of the ornithine cycle when these authors found that high concentrations of ornithine (1.6 per cent) would strongly inhibit urea formation from arginine, but not from ammonium lactate, arguing that urea production from ammonium lactate could therefore not go through arginine.

Krebs (10,19) argued that these experiments were not valid due to the thickness of the tissue slices used. Since the arginase activity observed in the experiments of Bach and Williamson was only approximately 1 per cent of that actually present, it would appear that the arginase inhibited was only that of the outer layer of cells

which had disintegrated, and that therefore the bulk of the arginase concerned in urea synthesis was not affected at all. In marshalling evidence for this view, Krebs was able to show that if the reaction medium was rendered more alkaline, where ornithine (because of its decreased ionization) would diffuse into the slices more easily, urea formation from ammonium lactate was actually inhibited by high concentrations of ornithine.

Further evidence in support of the existence of the ornithine cycle has come principally from two lines of work: the demonstration and isolation of the individual reactions concerned in cell free preparations (evidence in this connection will be discussed in detail under each reaction) and the discovery of reaction sequences, showing the characteristics of the ornithine cycle in non-mammalian organisms.

Srb and Horowitz (20) in a study of "argininelessness" mutants of Neurospora, concluded from the observed growth requirements, that one mutant was blocked in the step citrulline to arginine; two mutants were blocked in the step ornithine to citrulline; and four mutants were unable to synthesize ornithine. Neurospora also possesses the enzyme arginase, so that it would appear that a complete ornithine cycle exists in this mold. A similar case has been shown by Bonner (21) to exist for the mold Penicillium. "Argininelessness" mutants have been found there which are deficient in the ability to synthesize each of the members of the cycle. The mold also possesses the enzyme arginase.

Cohen and Lewis (22) have developed a technique for the force feeding of earthworms (Lumbricus terrestis). Using this technique

to study the nitrogen excretion of starved earthworms, they established that administration of arginine or citrulline, but not ornithine, glutamic acid, glutamine, glycine, alanine, histidine, hydantoin, or NH_4Cl resulted in increased urea excretion. The quantity of urea obtained from citrulline is increased by the simultaneous administration of amino acids, but this effect is not noted with ornithine. In a later paper (23), they demonstrate that a homogenate of earthworm intestine possesses arginase activity and that, interestingly enough, the arginase activity increases ten fold if the worms are starved 24-30 days.

B. Alternative Pathways

Alternative pathways of two types have been proposed by Bach (15): a cycle involving ornithine and citrulline, but not going through arginine, and a cycle involving glutamine. Bach (15) proposed that citrulline underwent a dismutation with keto acids to form glutamic acid, urea, and a hydroxy acid. However, in the opinion of this reviewer, the quantitative data upon which this concept is based do not warrant the formation of a new theory. The same author has also suggested (15) that urea is formed from glutamine directly.

C. Amides

Leuthardt and Glasson (24) in 1942, proposed that the whole amide group of a postulated half amide of oxalacetic acid was transferred intact to ornithine to form citrulline. This hypothesis was based on the observation that in the absence of ornithine, succinamide is more effective than succinic acid plus ammonia, or than

ammonia alone. However, these authors neglected to report what happens in the presence of ornithine. No further evidence has been advanced in support of this view.

Leuthardt (25,26) has also advanced the view that glutamine may play a specific role in urea formation. This was based on the following points:

(1) Liver slices form urea faster from glutamine than from ammonia.

(2) When glutamine is used, no oxidizable substrate is required.

(3) Muscle protein contains much glutamic acid; usually the free -COOH is present as the amide; and no glutaminase is present. A teleological validity therefore exists for forming urea from glutamine directly.

(4) Free energy relationships favor glutamine to urea in place of ammonia to urea.

Krebs (10) has compared the formation of urea in liver slices from glutamine, ammonia, and glutamic acid plus ammonia. He confirmed the greater effectiveness of glutamine as compared with ammonia, but also found that glutamic acid plus ammonia is as effective as glutamine. Other authors (16,27,28,29) have not been able to show urea formation from glutamine.

More recently, Leuthardt et al. (30) using homogenate, have found that glutamine is not utilized at low concentrations (less than 0.01 M.) and these authors have concluded that glutamine is therefore probably not an intermediate in urea formation.

D. Source of Urea Carbon

There is extensive evidence to show that the ultimate source of the urea carbon atom is CO_2. Krebs and Weil (31,32) have found that the rate of urea synthesis increases rapidly as the concentration of the bicarbonate buffer is increased, and that there is almost no synthesis if a phosphate buffer alone is used. These results have been confirmed in full by Leuthardt and Glasson (33). Gale (34) working with the Lancefield group D streptococci, has shown that they require arginine for growth, and that if arginine is replacedby ornithine and CO_2, the rate of growth then becomes a function of the CO_2 concentration.

Tracer studies have been conducted using C^{11} and C^{13} in liver slices. Evans and Slotin (35) found that when liver slices were incubated in the presence of ornithine, NH_4Cl, pyruvate, and $C^{11}O_2$, after 45 minutes the urea had a specific activity equal to 40 per cent of that present in the bicarbonate. Rittenberg and Waelsch (36) used liver slices with NH_4Cl, ornithine, and glucose as added substrates and found that after 2 hours, 50 per cent of the urea had been formed from the $C^{13}O_2$ added.

MacKenzie and du Vigneaud (37) fed rats L-methionine with C^{14} in the methyl group. The excreted urea and the expired CO_2 had identical specific activities on both the first and second days, although on day two, urea excretion was up 35 per cent and CO_2 excretion down 13 per cent. Grisolia and Cohen (38) have followed citrulline formation from $C^{14}O_2$ in a semi-isolated enzyme system. The results obtained showed that the citrulline formed had a

specific activity identical with that of the bicarbonate, and furthermore, that urea produced from this citrulline by the action of whole homogenate, also had the same specific activity.

Biotin has been shown to play an important role in the CO_2 fixation reaction involved in urea synthesis, as well as in other reactions involving CO_2 fixation (39).

MacLeod and Lardy (40) have studied the uptake of injected $NaHC^{14}O_3$ into various body components in normal and biotin deficient rats. Their results show that normal rats incorporate C^{14} in liver protein arginine at six times the rate biotin deficient animals do. MacLeod et al. (41) compared the activity of the washed residue enzyme of Cohen (which converts ornithine to citrulline) in normal and biotin deficient rats. Biotin deficient animals had about 50 per cent of the activity possessed by normal rats. That this result is specific for biotin is shown by the fact that riboflavin, or pyridoxine deficient rats have the same activity as the control animals. Whether biotin functions as a coenzyme in this system, or is necessary for the production of the enzyme is not clear. Biotin or a heated extract of normal or deficient liver will not stimulate normal or deficient preparations. However, the addition of heated residue will stimulate the activity of whole homogenate from deficient rats, but not from controls.

E. Ornithine ⟶ Citrulline

Borsook and Dubnoff (42) first demonstrated that urea synthesis could be carried out in a cell free medium. Using guinea pig liver

homogenate, they found that the optimum reaction mixture included ornithine, ammonia, glutamic acid, oxalacetic acid, and ATP. The reaction was inhibited by arsenate and required the presence of oxygen.

Cohen and Hayano (28,43) discovered that by omitting Mg^{++} from the reaction mixture, the further conversion of citrulline could be inhibited, and thus the first step of the cycle isolated for study. In addition to ornithine, the reaction required glutamic acid, ammonia, CO_2, inorganic phosphate, and either adenylic acid or ATP, with ATP only 50 per cent as effective as adenylic acid. These authors were further able to isolate the first step in the cycle by their discovery that the enzymes concerned may be separated by centrifugation. If homogenate is centrifuged at 2000 g. for 10 minutes, the ornithine $\longrightarrow$ citrulline activity is in the precipitate (referred to as "washed residue") while the enzymes concerned with citrulline $\longrightarrow$ urea appear to have been separated into two fractions. The fraction present in residue is non-specific and can be supplied by a variety of tissues; it appears to be an insoluble H transport system including cytochrome. The fraction in the supernatent can only be obtained from liver and is apparently the specific enzyme system concerned.

Studying the washed residue preparation, they found that Mg^{++} is required for optimal activity in this step as well as in the further conversion of citrulline.

Cohen and Grisolia (29,44) in further studies on the washed residue preparation have succeeded in establishing the following facts:

(1) Carbamyl L-glutamate is 2-3 times more effective than glutamate in the conversion of ornithine to citrulline. Its effectiveness relative to glutamic acid is increased if CO_2 is excluded from the reaction mixture.

(2) Ammonia is required for the reaction, two moles being used if glutamic acid is the substrate, one mole if carbamyl glutamic acid is. Carbamyl glutamine and α-guanidoglutaric acid are less effective than carbamyl glutamic acid itself.

(3) In a footnote to their most recent paper, these workers report that they have been able to prepare, by fractionation of washed residue, a soluble enzyme which carried out anaerobic synthesis of citrulline from carbamyl glutamic acid and ornithine, but not from ornithine + glutamic acid + CO_2 + ammonia.

Leuthardt et al. (30,45) have confirmed that carbamyl glutamate reacts with ornithine to form citrulline. These workers also found that the reaction will not go unless ammonia and an oxidixable substrate are present.

Feldott et al. (46) report that biotin deficient rats which do not carry out the synthesis of citrulline from ornithine and glutamic acid as well as normal rats, show the same enzyme activity if carbamyl glutamic acid replaces glutamate in the test system.

Srb (47) has tested the ability of "arginineless" mutants of Neurospora to grow on carbamyl glutamate. In tests on all types of "arginineless" mutants, he found that carbamyl glutamic acid had no effect.

F. Citrulline ⟶ Arginine

This reaction was first demonstrated by Borsook and Dubnoff (48) using rat and guinea pig kidney slices. The reaction required the presenceof a dicarboxylic amino acid (glutamic acid and aspartic acid were equally effective) and was inhibited by arsenite, arsenate, and cyanide. The cyanide inhibition was partially relieved by the addition of hydrogen acceptors.

Cohen and Hayano (49,50) have also studied this reaction. They report that rat liver homogenates require ATP, citrulline, glutamic acid, cytochrome C, Mg^{++}, and O_2 for optimum activity. Aspartic acid was much less effective than glutamic acid and the reaction was inhibited by α-ketoglutaric acid and ammonia. Fahrländer et al. (51-53) have confirmed the inhibition by α-ketoglutaric acid, but find that contrary to the above workers, ammonia not only tends to reverse the α-ketoglutarate inhibition, but stimulates yields from glutamic acid as well. They also found that glutamic acid can be replaced by aspartic acid. They further report that the reaction is inhibited by malonate, methionine, and cysteine. The methionine inhibition is partially reversed by increasing the Mg^{++} concentration.

Ratner and Pappas (54-56) have succeeded in isolating a soluble enzyme free of pyridine nucleotides which catalyzes the anaerobic reaction:

Citrulline + ATP + Aspartate ⟶ Arginine + ADP + Malic

in the presence of Mg^{++} ions. Further purification has shown that two enzymes are actually concerned in the reaction. The first condenses citrulline and aspartic acid, while using up one high energy

phosphate bond, and the second cleaves the condensation product to arginine and malic acid. They explain the efficacy of glutamic acid in place of aspartic acid in whole homogenate as being due to the dual role glutamic acid can play by supplying both high energy phosphate and aspartic acid via oxalacetate and transamination.

G. Localization of Steps Within the Cell

Arginase has been shown by Dounce (57) to have the same concentration in the nucleus, and in the cytoplasm, while the preceding step (citrulline → arginine) has been found to be in the soluble cytoplasmic proteins (54,55). The first step (ornithine → citrulline) is apparently in the "washed residue" fraction (28). Since this fraction includes nuclei, cell fragments, unbroken cells, and cytoplasmic particles, it is not clear exactly in which part of the cell the first step is localized, though it is not in the soluble cytoplasmic fraction.

It is interesting tha t, although urea synthesis is a closely linked sequence of reactions, the individual steps do not appear to be spatially integrated within the cell.

H. Miscellaneous

Leuthardt and Fahrländer (58) report that methionine or methionine sulfoxide inhibits urea formation in liver slices. The methionine inhibition is relieved by the addition of pyruvate.

Bernheim and Bernheim (59) have found that certain amino acids inhibit urea production by liver slices from NH_4^+ salts, but not from alanine or glutamine. The inhibition can be overcome by adding orni-

thine, but not by Mg^{++}. 2,4-dinitrophenol has a similar effect. It inhibits urea production from ammonia strongly, has less effect when alanine is used, and has no effect when glutamine is used as the nitrogen source. The inhibitory action of 2,4-dinitrophenol is not relieved by ornithine, but can be overcome by the addition of pyruvate.

These same authors have also noted similar inhibitions by caffeine, theophylline, and theobromine (60). Ornithine and glutamine overcome the inhibition. Tests have shown that the point of inhibition is not arginase. The inhibitory effect of caffeine and theophylline has been confirmed (43).

Borsook and Jeffreys (61) have estimated the free energy change in the formation of urea from ammonia, acid and CO_2, under conditions existing in plasma to be +14,300 calories.

Leuthardt and Glasson (62,63) have found that in thiamin deficient rats and guinea pigs, the addition of thiamin to liver slices causes an increase in the rate of urea synthesis if pyruvate is the substrate, but not if oxalacetate is. They suggest that fixation of CO_2 into urea might be by way of the Wood-Werkman reaction. In this connection, Anfinsen et al. (64) have reported on the uptake of $NaHC^{14}O_3$ into protein by rat liver slices. The guanidine carbon of arginine had the highest specific activity of all substances tested. Oxalacetic acid was found to inhibit up to 50 per cent, whereas glutamic acid, aspartic acid, glutamine, asparagine, malate, pyruvate, and alanine had no effect.

Leuthardt et al. (45) have made the interesting observation that the addition of cis-aconitic acid inhibits the formation of citrulline from glutamic acid and ammonia, but has little effect when glutamine

is used instead. They also report that washing the homogenate removes a factor required for the reaction from glutamate and ammonia, but which is not required by glutamine.

II. PROBLEM AND PROCEDURE

A. Problem and Method of Approach

It has been shown that the primary source of the urea carbon atom is carbon dioxide (31-38). We are faced then with the problem of a reaction sequence in which we know the first substrate, carbon dioxide; several of the final steps, citrulline to urea; and the last product, urea. However, we do not know the following crucial points:

(a) What is the initial carbon dioxide acceptor?

(b) What metabolic transformations does the fixation product undergo?- leading to

(c) What is the substance, or substances, reacting with ornithine to form citrulline?

The point may be raised that if carbon dioxide reacts directly with the delta amino group of ornithine to form a carbamido compound as postulated by Krebs (65), the problem as stated above becomes meaningless. There are, however, several reasons for assuming that CO_2 does not react directly with ornithine. First, as has been pointed out by Leuthardt and Brunner (66), the pK of the delta amino group of ornithine is too high to permit of direct reaction at physiological pH's. Second, the energy required for the synthesis of urea from ammonia, CO_2, and acid, under conditions existing in plasma (which may be taken as a reasonable approximation of those existing inside a liver cell) is +14,300 calories (61). As a qualitative assumption, we can look at citrulline as a mono-substituted urea in which one hydrogen is replaced by the group $-CH_2 - CH_2 - CH_2 - CH(NH_2) - COOH$,

and the synthesis of citrulline as analogous to that of urea, but in which one of the ammonia molecules is replaced by the primary amine, ornithine. If these assumptions are correct, the formation of a carbamido ornithine should require in the neighborhood of 7,000 calories, an energy requirement which would be too high for a simple reaction between ornithine and CO_2. Third, there is the experimental evidence that under conditions which result in citrulline synthesis, there is CO_2 fixation if ornithine is omitted from the reaction mixture, but not if glutamic acid is omitted. And further, if enzyme and substrates with ornithine omitted, are pre-incubated, before the addition of ornithine, not only is there CO_2 uptake, but the yield of citrulline is higher than if no pre-incubation is carried out (29).

The problem may then be stated: Through what sequence of compounds does the carbon atom of CO_2 pass before reacting with ornithine to form citrulline?

At the time this work was begun, it was felt that what was needed most for any successful attempt to solve this problem was a reasonably rapid technique for testing compounds as possible carbon donors in urea synthesis. The technique finally worked out was in response to this requirement.

It depends upon the principle that if there is a reaction sequence $S \longrightarrow X \longrightarrow P$, and we start with radioactive S, then the specific activity of the product P will be depressed in the case where X is added to the reaction mixture. In practice, the experiment is performed by incubating a mixture of enzyme, buffer, and the necessary substrates, and then determining the yield and specific activity

of the product. These values are now compared with data from a similar set of vessels to which a substance, suspected of being X, has been added. If the substance is X, the specific activity of the product would be depressed; the yield may or may not be increased. If it is not X, the yield may or may not be affected, and the specific activity would not be lowered.

The starting substrate used (S in the sequence $S \longrightarrow X \longrightarrow P$) has been C^{14} labelled $NaHCO_3$, and urea has been the product (P) which has been analyzed. Urea was chosen as the product to follow in preference to citrulline for the reason that, as was mentioned in the introduction, there has been work on the urea synthesis problem which suggested that the ornithine cycle might not be the only way to make urea. If this is the case, important facts might now show up if citrulline were chosen as the end product.

The enzyme system used was rat liver homogenate. This was used in preference to the semi-isolated enzyme system used by Cohen's group (28) since, as was mentioned above, the aim was to follow urea formation not citrulline. It was also felt that the removal of enzyme systems might reduce the chances of identifying X. If a suspected compound is not X, but is metabolically converted to X by an enzyme present in whole homogenate, but not in an isolated enzyme system, the tested compound would be expected to cause some depression of the specific activity in a test using whole homogenate as an enzyme source, but not in a test using a partially isolated enzyme system.

B. Validity of the Method of Approach

Experimental proof of the above technique can be obtained by testing citrulline and arginine, the two known intermediates in the urea cycle. The results of testing these substances are shown in Table 1.

TABLE 1.

EFFECT OF ARGININE AND CITRULLINE ON THE INCORPORATION OF $NaHC^{14}O_3$ INTO UREA

Additions to Control	Urea Formed	Total Urea Radioact.	Urea Spec. Activity	Per Cent Control
	(μm.)	(cts./min.)	(cts./min./μm.)	
None	0.89	64	72	100
L-citrulline (20 μm.)	3.43	7	2	3
L-arginine (20 μm.)	17.2	138	8	11

Conditions given in text (Section II-C). 0.03 M. fumarate present in all vessels. Glutamate was .05 M. $NaHCO_3$ added had 252 cts./min./μm. Enzyme concentration was 21 mg. (wet weight) liver/ml. reaction mixture.

As would be expected, both citrulline and arginine greatly stimulate the yield of urea and markedly depress the specific activity. The figures on total urea radioactivity are interesting because they confirm several facts about the ornithine cycle. Arginine behaves almost as though equimolar quantities of urea and ornithine had been

added; it actually stimulates the total number of counts fixed. This is in agreement with the known rapid hydrolysis of arginine to ornithine and urea, which is the fastest step of the cycle (17). Citrulline, on the other hand, not only causes a marked depression in the specific activity of the urea, but a strong inhibition of the total counts fixed. The step citrulline to arginine is known to be the slowest of the cycle (17). It is at such a step that one would expect the greatest decrease in specific activity of the product, since great quantities of synthesized citrulline would have to pile up for an appreciable number of counts to go through.

Further evidence on this point was obtained from experiments in which the enzyme concentration was increased 2.5 fold. Since it has been found that, with increased enzyme concentration, less citrulline tends to pile up, and its conversion to arginine is more rapid (43), it would be expected that under these conditions, there would be an increase in the yield of urea from citrulline, in the total urea radioactivity, and in the urea specific activity with respect to the control. This is exactly what was found. With added citrulline, in the presence of a higher enzyme concentration, the total urea radioactivity becomes greater than the control (though still less than when arginine is added), and the urea specific activity approaches the value obtained in the presence of arginine.

C. Experimental Methods

Preliminary experiments were carried out in the experimental vessels shown in Figure 1. A mixture of substrates and enzyme were

placed in the reaction vessel and $Na_2C^{14}O_3$ added to a final volume of 5.0 mls. The tops were placed on with both stopcocks closed and incubated at 38° C with shaking. Following the incubation period, 1.0 ml. of 3 M. acetate buffer (pH 4.8) was added through stopcock A under suction to bring the pH to 5.0. The vessels were then evacuated to 45 - 50 mm. pressure for 5 minutes, after which air was admitted and sucked through for 5 minutes under slight vacuum. The vessels were now re-evacuated and CO_2 permitted to enter. A total of five evacuations were carried out alternating air and CO_2. After the addition of 4.0 mls. of water, the vessels were boiled 5 minutes on a boiling water bath, and filtered through Whatman No. 5 paper.

A 3.0 ml. aliquot of the filtrate was taken for manometric analysis of urea, the sidearm containing 0.3 mls. of a mixture of 3 vols. 3 M. acetate buffer and 1 vol. of a glycerol extract of jack bean meal.* Temperature equilibration was 20 minutes. As a check on the analytical method, recovery experiments were performed by adding known quantities of urea to enzyme buffer mixtures and carrying them through the procedure. The results are shown in Table. 2.

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* The extract was prepared according to the method of Koch (67). It has the advantage over commercial urease preparations in that it is extremely active and shows no loss in activity on standing several years in the refrigerator. To test the specificity of this enzyme preparation, a 50 mls. batch of reaction mixture was run and the product analyzed using this urease, a commercial urease (Nutritional Biochemicals Corp. #1607), and a twice-re-crystallized, 100 times purified commercial urease obtained through the courtesy of Prof. H. Borsook. The results obtained were 1.00, 0.96 and 0.97 µm./ml. of de-proteinized solution, respectively.

TABLE 2.

RECOVERY OF UREA ADDED TO HOMOGENATES

Experiment No.	Urea Added	Urea Found	-Blank
	(µm.)	(µm.)	(µm.)
A	0.0	2.20	0.0
	6.0	8.18	5.98
	12.0	14.4	12.2
B	0.0	2.94	0.0
	6.0	9.22	6.28
	12.0	15.1	12.2

All values are averages of duplicates.

The total urea radioactivity was determined by incubating a 3.0 ml. aliquot of the filtrate with 1.0 ml. of the glycerol-acetate mixture four hours at 38° C. The vessels were now aerated 10 minutes with CO_2 free air into two glass bead columns as shown in Figure 3. Each column contained 8 mls. of 0.7 N. CO_2 free NaOH.

Following the aeration, the columns were washed down twice with 8.0 mls. of CO_2 free water. The NaOH and washings were pooled, and 1.0 ml. of a 20 per cent $BaCl_2$ solution added. They were stored overnight in the refrigerator and the $BaCO_3$ filtered the next day onto tared papers and dried 20 minutes at 100° C. They were weighed and fastened to aluminum plates for counting. Since the self-absorp-

tion of samples having a total weight over 20 mg. becomes high, all samples were kept to below 20 mg. in weight. In cases where the $BaCO_3$ precipitate weighed more than 20 mg., only part of the sample was used. A new plate was made by suspending part of the original sample in alcohol and refiltering.

In these experiments, the standard reaction mixture was 5.0 mls. and was composed of:

Compound	Concentration
KCl	.145
$MgSO_4$	.0033
KH_2PO_4	.0125
L-glutamate	.05
DL-ornithine	.0067
NH_4Cl	.0067
Adenylic acid	.001

The reaction mixture contained 1.0 ml. of a 25 per cent homogenate of rat liver in isotonic KCl. Starting pH was 7.4 and the reaction was carried out for 90 minutes at 38° C with shaking.

After preliminary experiments were carried out as described above, the procedure was abandoned as being unsuited for routine work. The procedure and conditions described below applies to all experiments reported except those otherwise noted as "Prelim".

Reactions were carried out in 15 ml. serum bottles (Kimble No. 15105) which were incubated thirty at a time in a Dubnoff apparatus (69). The reaction time was 60 minutes at 38° C with shaking and air as a gas phase. The buffer was of the following composition:

Solution A.		gm./liter	conc. (M.)	Final Conc. (M.)
	KCl	23.2	.312	.145
	K_2HPO_4	7.3	.042	.0125
Solution B.				
	$MgSO_4$	24.4	.099	.0033

It was stored in the refrigerator and just before use, 9 vols. of Solution A. were mixed with 1 vol. Solution B and the pH adjusted to 7.2 with HCl; 1.25 mls. of buffer were used per reaction vessel, and the figures given in the last column represent the final concentration of substances in the reaction mixture.

The standard substrate mixture was of the following composition:

Compound	Mgs.	Final Conc. (M.)
L-glutamic acid	868	.050
DL-ornithine • HCl	122.4	.0067
NH_4Cl	42.4	.0067
Adenylic acid	43.2	.0010

It was made up to 30 mls., the pH adjusted to 7.2, and 1.0 ml. used per vessel. In any experiments in which the concentration of glutamic acid was varied, the rest of the substrate mixture was made up to half volume and 0.5 ml./vessel used. 24 µm. of bicarbonate were added per vessel, the bicarbonate solution being prepared fresh for each experiment by adding water plus a standard solution of $Na_2C^{14}O_3$ to dry $NaHCO_3$.*

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* The standard $Na_2C^{14}O_3$ solution was prepared in the following manner. A 2.5 mg. sample of $Ba\ C^{14}O_3$ was placed in the reaction vessel shown in Figure 1. The C^{14} was supplied by Carbide and Carbon Chemicals Corp., Oak Ridge, Tenn., and obtained on allocation from the U.S. Atomic Energy Commission. The gas inlet was connected to a source of tank N_2, the gas outlet to a round bottomed Peligot flask, with 10 bulbs containing 20 mls. of 0.0984 N. NaOH. The outlet from thePeligot flask

(continued on next page)

For practical purposes, the concentration of carbonate in the C^{14} standard was zero. Compounds tested were made up fresh and adjusted to pH 7.2; in a few cases, a test solution was stored in the deep freeze for use in more than one experiment. In all cases, water was added to a final volume of 3.8 mls.

In an actual experiment, all additions except the enzyme and bicarbonate solutions were added the night before and frozen. The enzyme was prepared by killing a male albino rat by stunning and rapidly excising 4 to 8 grams of liver. This was placed in a 20 ml. beaker which had been packed in ice and frozen. The liver was weighed and the weight adjusted to give a final homogenate concentration of 20 per cent. The weighed liver was now minced and added to a cold solution of isotonic KCl; for each gram of liver, one drop of N. KOH was added and the liver homogenized in a glass tube using a lucite pestle with a stainless steel rod. The pH at the end of homogenization was from 8.4 to 8.8 and the temperature from 11-13° C. The pH was adjusted to 7.3 and 0.4 ml. of this used per vessel, to give a final concentration of 5.5 mg. (wet weight) liver/ml. of reaction mixture.

The enzyme was added to the reaction vessels which had been thawed out, and following this, the bicarbonate solution. The vessels

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* (Footnote continued from page 24). was connected to a NaOH safety trap. 5 mls. of 1 per cent perchloric acid was added to the reaction vessel and N_2 passed through for 2 hours. At the end of the aeration, the contents of the Peligot flask were washed out with a total of 30 mls. of water. The pooled washings and N/10 NaOH are the standard solution. It contained 176,500 counts/min./ml. with a final base concentration of 0.04 N.

were stoppered and incubated.* At the end of the incubation period, 0.4 mls. of 5 M. KOH were added through the stopper using a 1.0 ml. hypodermic syringe with a 23 gauge needle.** The vessels were shaken 20 minutes closed to absorb all the CO_2 into the solution; and then the caps removed and the vessels shaken 90 minutes with a fan blowing across the top. This procedure removes all ammonia present.

Stoppers fitted with center wells*** were put on, each center well containing 0.5 mls. 0.7 N. NaOH. 0.25 mls. of 41 per cent acetic acid were then injected past the center well and into the main body of the solution to bring to pH to 5.2, at which pH all the CO_2 is liberated. The vessels were shaken at 38° C for 30 minutes to effect complete transfer of CO_2 to the center well. The center well contents were discarded and the vessels placed in a boiling water bath for 10 minutes to coagulate the protein.

Following this treatment, fresh center wells containing 0.5 mls. 0.7 N. CO_2 free NaOH are prepared and placed in the vessels immediately following the addition of 5 drops of urease**** and shaken 60 minutes at 45° C. The center wells now contain the CO_2 obtained from urea hydrolysis and their contents were washed into 50 ml.

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* The pH of the reaction mixture after the addition of the bicarbonate solution is 7.4 - 7.5. The pH at the end of the reaction is 7.0 - 7.1.

** A 23 gauge needle upon being withdrawn leaves a gas-tight seal.

*** Center wells are constructed from 13 x 100 mm. test tubes by cutting off a 15 mm. length from thebottom and attaching this to a 5 cm. length of 4 mm. glass rod. The rod is passed through a hole in the stopper to suspend the center well inside the vessel.

**** The urease is the glycerol extract of jack bean meal mentioned previously.

Erlenmeyers and 0.5 mls. of a solution of $BaCl_2$ and NH_4Cl added.* The flasks were allowed to stand overnight stoppered and then filtered onto filter paper discs,** washed with water, alcohol, and ether. The funnels were washed between each filtration to insure no contamination from sample to sample. Samples prepared had an area of 2.85 $cm.^2$ and were taped to aluminum plates for counting.

Samples were counted using an end window (mica) counter with a counting efficiency of 5 per cent. All counts reported are corrected for background and have been corrected to a standard sample.

The serum bottles which now contain ammonia derived from the hydrolysis of urea have one drop of lauryl alcohol and one drop of Turkey red oil added.*** 2.0 mls. of a saturated solution of potassium metaborate were added and the vessels aerated in a train into 10 mls. of N/10 sulfuric acid, contained in a 16 mm. i.d. test tube. The aeration is carried out 90 minutes and following this nesslerized using 2.0 mls. of Nessler's solution.**** Colors were read in a Coleman Jr. Spectrophotometer. A composite picture of the experimental details is shown on the flow sheet in Figure 4.

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* The solution is prepared by adding 15 gms. $BaCl_2$ and 3 gms. NH_4Cl to 100 mls. water. The NH_4Cl neutralizes the NaOH present and gives better precipitates.

** The discs were circles 2.1 cm. in diameter cut from Whatman No. 1. paper.

*** The addition of lauryl alcohol and Turkey red oil is necessary to prevent foaming during the aeration. It is also necessary to use metaborate as base; KOH will not work due to excessive foaming.

****The Nessler's solution was a modification of that published by Koch and McMeekin (70). In the preparation used, the solution was allowed to become as hot as possible after the addition of mercury.

All procedures in the analytical scheme have been checked by control experiments, and the results are given in the summary below.

Addition of 5 M. KOH - As shown below, CO_2 transfer from the main solution to the center well is quantitative in 20 minutes. Under these conditions, CO_2 will be completely absorbed into the main volume of the solution which has a surface area ten times that of the center well. Efficiency of ammonia removal (after the stoppers are removed) was tested by determining the quantity of ammonia left in a mixture of homogenate and buffer after various shaking times. After 20 minutes shaking, 13 per cent was left; and after 60 minutes shaking, no residual ammonia was found. Ninety minutes was the time used throughout to provide a margin of safety.

Addition of 41 per cent Acetic Acid - In the first eight experiments, it was found that the addition of 0.25 mls. of acetic acid solution always resulted in a pH between 5.1 - 5.3. After this, 0.25 mls. were added routinely. The time for CO_2 diffusion was determined by putting a standard carbonate solution in serum bottles and liberating the CO_2. Trapped CO_2 was analyzed gravimetrically as $BaCO_3$. It was found that diffusion was complete in less than 20 minutes. Thirty minutes was therefore adopted as a routine time. Tests on the recovery and retention of $C^{14}O_2$ showed 100 per cent recovery and no detectable retention.

Urease - Activity of the enzyme was tested by the time required to effect 100 per cent hydrolysis of 5 µm. urea at 38° C. It was found that 3 drops of enzyme caused complete hydrolysis in less than 20 minutes. Routinely a 60 minute incubation at 45° C (the

temperature optimum) with 5 drops of enzyme has been used to assure complete hydrolysis and diffusion.

Aeration - The time required for complete aeration was found to be 55 minutes. A 90 minute aeration has been used throughout. Absorption of ammonia by one trap containing 10 mls. of N/10 H_2SO_4 is complete as shown by an average recovery of 100.2 per cent on standard nitrogen samples, and a complete lack of color in a second trap if it is placed in series after the first.

In addition to the above controls, two tests of the analytical procedure were conducted periodically throughout the course of the investigation. The first of these were recovery experiments on standard samples of urea added to reaction mixtures at time zero and carried along with the experimental vessels. Recoveries as determined in this fashion ranged from 94 - 101 per cent. The second test was the determination of the radioactivity of the urea formed during the enzyme incubation in blank vessels (vessels in which the substrate mixture was omitted). In no case did the urea formed under these conditions show any trace of radioactivity, proving that none of the urea radioactivity measurements were affected by incomplete removal of CO_2.

This point is interesting in that it also shows that none of the urea formed when enzyme and buffer are incubated in the absence of added substrates, is derived from CO_2. All of the carbon of this urea, then, comes from organic compounds already present in the homogenate. Probably the principal source of this urea is arginine, which would be continually made available during protein breakdown.

Note: - All values reported are the averages of duplicate

vessels, and, unless specifically noted, all results have been confirmed using more than one batch of enzyme.

III. RESULTS

A. Enzyme System

Before entering into a discussion of the results obtained in testing various substance, some data on the nature of the system will be reported.

The relationship between enzyme concentration and yield, as found in one experiment, is shown in Figure 5. The curve goes through a maximum , which occurs at a concentration of approximately 0.5 mls. homogenate in 3.8 mls. of reaction mixture. Two things are of interest concerning the shape of the curve. First, at low concentrations of homogenate, the rate is an exponential function of the concentration (a plot of log activity v. log concentration indicates a limiting slope of 1.8); and second, the curve goes through a maximum. These two facts suggest that the observed rate is the resultant of two factors: (a) an increase in rate per unit enzyme with rising concentrations of homogenate, due to the increase in concentration of interacting enzymes and co-factors; and, (b) an inhibiting reaction whose rate is a higher exponential functions of the concentration of homogenate than the first set of reactions. No evidence is available for determining the particular reaction concerned with the inhibition. It is possible, however, that it may be involved with the phosphate metabolism of the enzyme preparation.

Urea formation is known to be dependent on a supply of phosphorylative energy (28,56), and Ball and Cooper (71) have shown that the enzyme systems supplying high energy bonds are attacked by other enzymes present in liver homogenate. These enzymes appear to be phos-

phatases. In a series of experiments designed to discover which of the four substrates reacted with CO_2, it was observed that the omission of adenylic acid had a unique effect on the stability of the system. Substrates were omitted singly and then added 10 minutes after the start of the experiment; if adenylic acid was omitted in this manner, the enzyme system was totally inactivated. Such a marked effect was not observed as a result of the omission of other substrates.

It was observed in early experiments that the pH of the homogenate must at all times be kept above 7 for maximum activity. If the pH is allowed to drop below 7 during preparation of the enzyme, not only is there a decreased yield of urea, but the pH of the reaction mixture following incubation is 6.75 - 6.85 instead of 7.0 - 7.1. A possible explanation for this effect is that nucleoprotein breakdown is in some way triggered by a prior drop in pH to the acid side of neutrality, and the resulting pH of approximately 6.8 is the consequence of the buffering action of the liberated phosphoric acid.

As seen from Figure 5., the maximum yield of urea obtained in 60 minutes was 4 µm. In general, rats fall into two classes with regard to their enzyme activity. One class gives uniform results of from 3.9 - 4.5 µm./hour, while the other group under identical conditions are somewhat more variable, but give yields in the range 0.8 - 2.0 µm./hour. The effects of varying the magnesium concentration and the addition of cytochrome C were studied as possible explanations of this effect. Magnesium is known to be required for both the conversion of ornithine to citrulline and citrulline to arginine (28,49). Experiments in this connection showed, however,

that there is no decrease in yield if the magnesium concentration were cut from .0033 M. to .001 M., so that magnesium did not appear to be limiting. In several experiments, cytochrome C was added at concentrations up to 3×10^{-5} M., but no effect on the yield was observed.

No correlations between age of rats (known only approximately) or weight and activity were observed. However, it is possible that the difference noted between groups may have a genetic background. One batch of male rats from an inbred line fell in almost all cases into the 3.8 - 4.5 group. Rats from this source became unavailable, and the new group tended almost exclusively to fall into the 0.8 - 2.0 range.

The results of complete yield controls are shown in Table 3. The only value shown which varies from experiment to experiment is the increase in urea production caused by the presence of substrates. This is the value which is referred to throughout as yield, and all specific activities have been calculated on this basis.

In short, the table shows that about 0.4 μm. of urea are present at t = 0 in 0.4 mls. of homogenate, and another 0.3 μm. are formed during the incubation in the absence of added substrates. The lowest yield (above the value in the absence of substrate) which has been observed was 0.8 μm. or an increase of 270 per cent. The highest yield ever observed was 8.2 μm.; however, yield of this order have only occurred twice.

Figure 6. shows the results of one experiment in which the specific activity of the bicarbonate of the medium was studied

as a function of time. It is important to keep the shape of this curve in mind, as the specific activity of the urea formed from CO_2 will reflect the change. It appears that after 60 minutes, the bicarbonate specific activity has dropped to 80 per cent of its initial value. As is noted in the figure, 40 μm. of bicarbonate were used in place of the customary 24, so that in routine experiments, the bicarbonate specific activity will have dropped still lower by the end of the first hour.

TABLE 3.

YIELD CONTROLS

Conditions	Urea Measured	-Blank
	(μm.)	(μm.)
Substrates - no enzyme	0.26	
Enzyme + substrates, stopped at t = 0	0.64	
Enzyme - no substrates (blank)	0.97	
Enzyme - no substrates 4.45 μm. urea added t=0	5.46	4.49
Enzyme plus substrates	2.63	1.66

B. Krebs cycle compounds, Pyruvate and Acetate

The tricarboxylic acid cycle contains three decarboxylations, one of which is known to be reversible (α-ketoglutaric + $CO_2 \rightleftharpoons$

oxalosuccinic), while oxalacetic and malic acids are known to be products of the addition of CO_2 to pyruvate, which is also a reversible reaction. Data obtained in testing members of the cycle are shown in Table 4.

TABLE 4.

KREBS CYCLE AND RELATED COMPOUNDS

Exp. No.	Compound Tested	Urea Formed	Urea Spec. Activity	Per Cent Control
		(μm.)	(cts./min./μm.)	
A	Control	4.54	152	
	Pyruvate	4.20	173	114
B	Control	1.63	147	
	Pyruvate	3.24	129	88
	α-ketoglutarate	3.61	138	94
	Succinate	2.98	156	106
C	Control	1.66	72	
	Pyruvate	2.69	63	88
	Fumarate	2.33	87	121
D	Control	3.96	142	
	Citrate	2.54	138	97
	Acetate	3.52	178	125

In all experiments, bicarbonate specific activity was 252 cts./min./μm. All compounds were added at a level of 20 μm./vessel.

The results indicate quite clearly that none of the substances tested behaves like an intermediate in the process of fixing carbon dioxide into urea. Since, under the conditions of the experiments,

(Mg^{++}, phosphate, and adenylic acid) the Krebs tricarboxylic acid cycle would be expected to be operating, we may conclude that no member of the cycle, nor pyruvate or acetate is concerned with the particular CO_2 fixation under study.

As can be seen from the data, some members of the cycle tend to stimulate the reaction, especially with enzyme preparations in the 0.8 - 2.0 class. After this fact was established, fumarate was added routinely to some experiments in an attempt to raise the activity of the enzyme. However, activities in the 0.8 - 2.0 class continued to occur despite fumarate addition at a level of 0.03 M.

C. Amino Acids and Related Compounds

1. Histidine - Edlbacher and Kraus (72) have demonstrated an enzyme in the livers of rats, rabbits, and guinea pigs, histidase, which breaks histidine down to glutamic acid + formic acid + 2 ammonia molecules. They postulate the formation of formylglutamine as an intermediate in this process. Edlbacher and Neber (73) have also shown that if histidine is added to liver slices, urea, rather than ammonia, is the end product. As an explanation, they hypothesize that ammonia liberated during histidine breakdown reacts with the formylglutamine formed to produce citrulline. This citrulline would now be available for urea synthesis.

Tesar and Rittenberg (74), in experiments feeding rats histidine labelled with N^{15} in the γ-N, found that excreted urea had twice the N^{15} excess of ammonia. If ammonia labelled with N^{15} is fed, the opposite result is obtained, suggesting that histidine

plays a specific role in urea formation.

Histidine was therefore tested as a possible intermediate, with seven enzyme preparations, using histidine from two different sources. In no case has a significant depression of the urea specific activity been obtained. A sample of the data is shown in Table 5.

TABLE 5.

HISTIDINE

Exp. No.	Compound Tested	Urea Formed	Urea Spec. Activity	Per Cent Control
		(µm.)	(cts./min./µm.)	
A (Prelim)	Control	1.08	161	
	L-histidine (10)	2.12	154	96
B	Control	1.70	30	
	L-histidine (6)	1.68	49	163
C	Control	1.63	180	
	L-histidine (20)	1.56	164	91

Bicarbonate specific activity was 252 cts./min./µm. in all cases. Figures in parenthesis represent µm. histidine added.

One figure in Table 5. warrants further comment. It is the increase in the urea specific activity resulting from the addition of histidine in Experiment B. This increase has been noted with other amino acids, but in nocase has an α-amino acid been found which would give this result consistently. When an increase resulted from the addition of an amino acid, it was generally 30 - 40 percent above

that of the control. This point will be discussed in more detail at the end of the section on amino acids.

2. Methionine, Lysine, and Phenylalanine - Bernheim and Bernheim (59) have compared urea formation from ammonia and from amino acids in rat liver slices. They find that urea production is strongly inhibited by a variety of amino acids; that this inhibition can be overcome by increasing the ornithine concentration; and that the inhibition does not occur when alanine or glutamine are used as nitrogen sources in place of ammonia.

Among the amino acids which they tested and found to inhibit, were methionine, lysine, and phenylalanine. In the cases of methionine and phenylalanine, they tested the D-isomer which was found to be as inhibitory as the L-compound.

Results obtained in testing these substances have confirmed the existence of the inhibition in the homogenate system used. In this test system, both ammonia (.0067 M.) and L-glutamate (.05 M.) are present. These data, in addition to the radioactivity measurements are given in Table 6.

It is apparent from the results that none of these compounds is an intermediate. These results again demonstrate the role amino acids may have in raising urea specific activity.

TABLE 6.

METHIONINE, LYSINE, PHENYLALANINE

Exp. No.	Compound Tested	Urea Formed	Per Cent Inhibition	Urea Spec. Activity	Per Cent Control
		(μm.)		(cts./min./μm.)	
A	Control	2.30		150	
	L-methionine (15)	1.92	17	172	114
	L-lysine (15)	1.36	61	194	129
	DL-phenylalanine (30)	0.39	83	---	---
B	Control	3.96		142	
	DL-phenylalanine (40)	2.02	49	193	136
C	Control	4.24		134	
	L-methionine (20)	3.32	22	188	140

Bicarbonate specific activity was 252 cts./min./μm. in all cases. Concentration of glutamate in Exp. C. was .03 M. Figures in parenthesis represent μm. test compound added.

3. Serine and Glycine - These two amino acids are metabolically related (75) and donate carbon atoms for uric acid and heme synthesis (76,77). They have been found, however, not to donate carbon atoms to urea synthesis. (See Table 7.)

The Bernheims (59) have reported that glycine and serine are also inhibitory to urea synthesis from ammonia in rat liver slices, glycine being reported as the most inhibitory amino acid. In repeated experiments with homogenates, glycine inhibition averaged about

50 per cent, while serine inhibits only very slightly.

TABLE 7.

SERINE AND GLYCINE

Exp. No.	Compound Tested	Urea Formed	Urea Spec. Activity	Per Cent Control
		(µm.)	(cts./min./µm.)	
A	Control	2.30	150	
	DL-serine (30)	1.98	200	133
	Glycine (15)	1.73	201	134
B	Control	4.54	152	
	DL-serine (40)	4.40	184	121
C	Control	0.89	72	
	Glycine (20)	0.96	79	110

Bicarbonate specific activity was 252 cts./min./µm. in all experiments. Glutamate concentration in Exp. C. was .03 M. Figures in parenthesis represent µm. test substances added.

4. Proline, Glutamine, and Glutathione - An interesting feature of urea synthesis in homogenates is the high (.05 M.) glutamate concentration required for maximum activity. This fact suggests that there may be some metabolic product of glutamate which is concerned with urea formation, and not glutamate itself. Proline, glutamine, and glutathione are products of glutamic acid metabolism, and for that reason are grouped together.

Bonner (21) has described a series of "arginineless" mutants of Penicillium. The growth requirements of one mutant are satisfied only

by arginine; in another by arginine or citrulline; in a third by arginine, citrulline, or ornithine; a fourth will grow on any member of the ornithine cycle or proline; and a fifth on any member of the cycle, proline or glutamic acid. This raises the possibility that proline might have some function in urea synthesis. Past work on glutamine has been summarized in the introduction.

Results obtained from testing these compounds are shown in Table 8. As can be seen from the data, none act as intermediates; moreover, glutamine does not stimulate the reaction. It would appear then that at least under the experimental conditions employed, glutamine plays no significant role in the process of urea synthesis.

TABLE 8.

PROLINE, GLUTAMINE, GLUTATHIONE

Exp. No.	Compound Tested	Urea Formed	Urea Spec. Activity	Per Cent Control
		(μm.)	(cts./min./μm.)	
A	Control	3.96	142	
	L-Proline (20)	3.58	192	135
	L-glutamine (20)	3.20	197	139
B	Control	4.54	152	
	L-glutamine (20)	4.06	122	80
C	Control	1.63	147	
	Glutathione (20)	2.75	140	95
D	Control	1.66	72	
	Glutathione (20)	1.46	75	104

Bicarbonate specific activity was 252 cts./min./μm. in all experiments. Figures in parenthesis represent μm. of test compound added.

5. Aspartic Acid and Alanine - Results of testing these compounds are given in Table 9. The combination of fumarate and aspartate was also tested in connection with some experiments on the role of glutamic acid, and a sample of these results are given.

TABLE 9.

ASPARTIC ACID AND ALANINE

Exp. No.	Compound Tested	Urea Formed	Urea Spec. Activity	Per Cent Control
		(μm.)	(cts./min./μm.)	
A	Control	1.66	72	
	L-aspartate (20)	1.82	80	111
B	Control	0.79	96	
	L-aspartate (40) + Fumarate (120)	1.50	86	90
C	Control	0.89	72	
	L-alanine (20)	0.74	76	106

Bicarbonate specific activity was 252 cts./min./μm. in all experiments. Glutamate was .03 M. in Exp. B. Figures in parenthesis represent μm. of test compound added.

6. Other Amino Acids - Valine (15 μm.), leucine (15 μm.), hydroxyproline (20 μm.), and threonine (20 μm.) were tested in single experiments and were not found to be active.

7. Discussion of Amino Acids - In general, the amino acids had two effects: an inhibition of urea synthesis, and a tendency to raise the urea specific activty. These effects are apparently unrelated since they have occurred in all possible combinations: inhibition with no increase in specific activity, no inhibition accompanied by a rise in specific activity, both occurring together, and both absent. Any explanation then of the role of amino acids in raising the specific activity of urea, must be independent of the observed inhibition by some amino acids.

There are two possible mechanisms by which amino acids could raise the specific activity of urea:

(a) By preventing the formation of urea, or urea intermediates from non-radioactive sources.

(b) By increasing the rate at which intermediates are removed by side reactions, hence lessening the dilution caused by intermediates present at the start of the reaction.

Case (a) can be excluded since there is quantitative evidence to show that all of the urea carbon must come from CO_2. MacKenzie and du Vigneaud (37) in experiments on methionine metabolism, fed rats methionine labelled with C^{14} in the methyl group. The urea and CO_2 excreted on the first day had identical specific activities. The specific activities were also identical on the second day although total urea excretion rose 35 per cent and CO_2 excretion dropped 13 per cent. It has been shown in this paper that methionine has no action as an intermediate in urea synthesis. It would seem improbable, therefore, that any urea carbon were to come from sources

other than CO_2.

We are left then with a mechanism in which amino acids remove intermediates by side reactions. Why the removal of intermediates should result in a higher specific activity for urea can be best understood by considering a pool of intermediate present at the start of the reaction. The rate at which this pool will increase its specific activity will depend on three factors: the size of the pool, the rate at which the intermediate is removed from the pool, and the rate at which radioactive intermediate is introduced.

If the rate of removal of intermediates is increased, the pool, at any given time during the reaction period, will have a higher specific activity than if the rate of removal had not been increased. Further, if the increased removal is to form a product which is not urea, since the urea specific activity will reflect that of the pool, the urea specific activity will increase at a faster rate than if no increased removal of intermediate by side reactions had occurred.

Amino acids could affect this removal either by reacting with an intermediate or by affecting an enzyme system functioning in its removal. The wide range of amino acids which give this effect (histidine, methionine, glycine, serine, lycine, phenylalanine, proline, etc.) would suggest the second mechanism rather than the first, in view of the well known tendency of enzymes to be highly specific.

Amino acids as a class are known to influence enzyme systems (78-80). Among the enzymes affected by amino acids are phosphatases. Bodansky et al. (81) have shown that amino acids as a group are phosphatase inhibitors. This fact seems suggestive in view of the

peculiar phosphate relationship in the conversion of ornithine to citrulline in whole homogenate. (43). The reaction requires both inorganic phosphate and either adenylic acid or ATP, adenylic acid being twice as effective as ATP.

In Figure 7, a scheme is set up which would explain the observed effect of amino acids in increasing the urea specific activity, based on their known inhibition of phosphatases.

Biotin has been shown on the scheme at the point of initial CO_2 fixation since biotin is known to be concerned with the process of CO_2 fixation (39); has been shown to be involved in the ability of rats to convert ornithine to citrulline (41); and in turkeys to be concerned with the Wood-Werkman reaction and the "malic" enzyme of Ochoa (82).

One point warrants further discussion. This is the question of whether any amino acids by raising the specific activity of urea might not mask their own action as a source of urea carbon. This effect is theoretically impossible since not enough of the amino acid could be manufactured using all the radioactive CO_2 present to raise the specific activity to the levels observed.

D. Carbamyl Glutamic Acid and Carbamyl Aspartic Acid

The literature on these compounds has been reviewed in detail in the introduction. Carbamyl L-glutamate, or a closely related compound, has been postulated as an intermediate (29) in the conversion of ornithine to citrulline, since it is more active in the semi-isolated "washed residue" preparation than is glutamic acid. In addition, its activity relative to glutamic acid is increased if CO_2 is

omitted from the system.

Tests of this compound in the homogenate system used in this study, however, have led to the conclusion that, while carbamyl glutamic acid appears to play a role in urea formation, it does not function as a source of urea carbon.

First tests of carbamyl glutamic and carbamyl aspartic acids* as intermediates in urea synthesis showed no tendency on the part of either compound to depress the specific activity of the urea formed. Carbamyl aspartic acid has been tested using five enzyme preparations, and carbamyl glutamic acid on a total of eleven. In no case has there been any depression of urea specific activity by carbamyl aspartic acid and in only one case with carbamyl glutamic acid. A sample of the data is shown in Table 10.

These results clearly rule out carbamyl glutamic acid and carbamyl aspartic acid as sources of urea carbon. The results also show that carbamyl glutamic and carbamyl aspartic acids, like the amino acids, have an erratic tendency to significantly raise the urea specific activity. It would seem reasonable to assume that the mechanism suggested to explain this action by amino acids would apply to carbamyl glutamic and carbamyl aspartic acids.

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* Three samples of carbamy L-glutamic acid and one of carbamyl L-aspartic acid were used. The sample of carbamyl L-aspartic acid and one of the samples of carbamyl L-glutamic acid weregenerously supplied by Dr. H. K. Mitchell. Another sample of carbamyl L-glutamic was synthesized according to the procedure of Nyc and Mitchell (83). The third sample was a gift of Dr. P.P. Cohen. All three samples of carbamyl L-glutamic acid had the same melting point, 158-9°C (corr.) and mixed melting points showed no depression. The three samples of carbamyl L-glutamic acid have been used interchangeably, and no difference in behavior has been observed.

TABLE 10.

CARBAMYL L-GLUTAMIC ACID AND CARBAMYL L-ASPARTIC ACID

Exp. No.	Compound Tested	Urea Formed	Urea Spec. Activity	Per Cent Control
		(μm.)	(cts./min./μm.)	
A	Control	0.70	118	
(Prelim)	Carb. L-asp. (10)	0.62	156	132
	Carb. L-glut. (10)	0.62	140	118
B	Control	0.93	64	
	Carb. L-asp. (6)	0.96	91	142
	Carb. L-glut. (6)	1.04	78	122
C	Control	4.54	152	
	Carb. L-asp. (20)	4.32	162	107
	Carb. L-glut. (20)	4.02	192	126
D	Control	1.63	147	
	Carb. L-glut. (20)	2.54	154	105
E	Control	1.66	72	
	Carb. L-glut. (20)	1.88	76	106
	Aspartic Acid (20)	1.82	80	111
	Carb. L-glut. (20) + Aspartic acid (20)	1.88	78	108

In Experiment A., the concentration of bicarbonate was .0075 M. with 224 cts./min./μm. In all other experiments, the bicarbonate concentration was .006 with 252 cts./min./μm. Figures in parenthesis represent μm. test compound added.

In view, however, of the importance of carbamyl glutamic acid, further tests on the nature of its action were carried out. Among the first experiments was an attempt to confirm the work on carbamyl glutamic acid using the semi-isolated "washed residue" preparation.

The incubation was performed in the usual manner, using washed residue* as the enzyme. The citrulline formed was converted to urea by adding aspartic acid (.01 M.) and a combination of washed residue supernatent and rat heart homogenate. The results, given in Table 11, confirm that carbamyl glutamic acid is more effective than glutamic acid in the washed residue preparation.

TABLE 11.

USE OF WASHED RESIDUE

Conditions	Urea Formed (μm.)
.03 M. L-glutamate	0.14
.03 M. Carb. L-glutamate	0.50
L-citrulline (2 μm.)	0.22
L-citrulline (4 μm.)	0.44

Concentration of adenylic acid, ornithine, ammonia, and buffer as usual.

Following this test, glutamic acid and carbamyl glutamic acid were compared in whole homogenate. In most cases, carbamyl glutamic acid alone caused a small, but distinct, quantity of urea to be formed,

* Washed residue was prepared by centrifuging a 20 per cent homogenate at 3000 g. 10 minutes in a refrigerated centrifuge. The precipitate was washed once with an equal volume of cold isotonic KCl and resuspended in isotonic KCl for use.

which was non-radioactive. This level of synthesis was never more than about half that obtained with an equimolar quantity of glutamic acid. When both compounds were tested together, a synergistic effect was noted. The data pertaining to these effects are shown in Table 12.

TABLE 12.

CARBAMYL L-GLUTAMIC ACID AND L-GLUTAMIC ACID

Exp. No.	Substrate*	Urea Formed	Urea Spec. Activity	Per Cent Control
		(µm.)	(cts./min./µm.)	
A	.03 M. L-glutamate	4.24	134	
	.01 M. L-glutamate	2.67	154	115
	.03 M. Carb. L-glut.	0.46	0	0
	.01 M. Carb. L-glut.	0.04	0	0
	.01 M. Carb. L-glut. + .01 M. L-glut.	4.35	217	162
B	.03 M. L-glutamate	0.89	72	
	.01 M. L-glutamate	0.59	66	92
	.03 M. Carb. L-glut.	0.47	0	0
	.01 M. Carb. L-glut.	0.12	0	0
	.03 M. Carb. L-glut. + .03 M. L-glut.	1.85	102	142

*All experiments include ornithine, ammonia, and adenylic acid as additional substrates.
In Experiment B., .03 M. fumarate was present in all vessels. Bicarbonate specific activity was 252 cts./min./µm. in both experiments.

To clarify the situation regarding the role of carbamyl glutamic acid may play in the process of urea formation, some of the facts known about the action of this compound are summarized below.

1. Carbamyl glutamic acid, when tested in homogenate does not donate its carbon atoms to urea synthesis.(This paper).
2. It exhibits the typical behavior of an amino acid in raising the specific activity of the urea formed. (This paper).
3. When both carbamyl glutamic acid and glutamic acid are added together in equimolar quantities, a synergistic effect on the level of urea synthesis is noted. (This paper).
4. Carbamyl glutamic acid is less effective than glutamic acid in homogenates, but is more effective than glutamic acid when washed residue is used as an enzyme system, the relative effectiveness of carbamyl glutamic acid being increased if CO_2 is omitted from the reaction mixture. (44, this paper).
5. When washed residue is used as the enzyme, a balance sheet of ammonia shows onemole of ammonia used when citrulline is formed from ornithine and carbamyl glutamic acid, while two moles are used when citrulline is formed from ornithine and glutamic acid (29).

Point 1. suggests that carbamyl glutamic acid is not concerned with the reactions involved in the formation of the first substance containing radioactive CO_2. Point 3. suggests that carbamyl glutamic acid is concerned with the further reactions of this substance to form a product which will react with ornithine to form citrulline. Point 5. raises the possibility that this function of carbamyl glutamic acid may be to introduce a nitrogen atom into the reaction sequence. This interpretation does not, however, explain why the effectiveness of carbamyl glutamic acid relative to glutamic acid should be increased in the absence of CO_2 (Point 4.)

E. Vitamins

1. Biotin and Thiamin - MacLeod et al. (41) have compared the rates of conversion of ornithine to citrulline in livers of normal and biotin deficient rats. Using a semi-isolated enzyme system, they found that the rate in deficient animals was much lower than that in normal rats. MacLeod and Lardy (40) have also found that normal intact rats fix injected $NaHC^{14}O_3$ into the arginine of liver protein six times as fast as biotin deficient animals.

Thiamin pyrophosphate (cocarboxylase) is known to be the co-enzyme for several reactions involving CO_2. Among these are three reactions involving the decarboxylation of pyruvic acid, and another in which succinic semi-aldehyde is formed as a result of the decarboxylation of α-ketoglutaric acid. Leuthardt and Glasson (62,63) have observed that the addition of thiamin to liver slices obtained from B_1 deficient rats, results in an increased yield, if pyruvate is used as the substrate, but not if oxalacetate is used instead.

A sample of the results obtained in testing biotin and thiamin are given in Table 13. As can be seen, neither acts as an intermediate, nor increases the yield. In fact, biotin tends to depress the yield slightly.

TABLE 13.

BIOTIN AND THIAMIN

Exp. No.	Compound Tested	Urea Formed	Urea Spec. Activity	Per Cent Control
		(µm.)	(cts./min./µm.)	
A	Control	1.66	72	
	Biotin (20)	1.40	75	104
B	Control	0.89	72	
	Biotin (20)	0.66	78	108
C	Control	4.57	161	
	Thiamin (20)	4.36	162	101

Bicarbonate specific activity was 252 cts./min./µm. in all experiments. In Experiment B., glutamate was 0.03 M. Figures in parenthesis are µm. of test compound added.

2. Riboflavin - The first experiments carried out on riboflavin indicated that, in the test system used, it behaved like an intermediate in the process of fixing CO_2 into urea. The data obtained are shown in Table 14 (see next page).

Further evidence, however, suggests that the role of riboflavin in urea synthesis is not that of an intermediate. Experiments have shown that the yield in the presence of riboflavin is not dependent on the operation of the ornithine cycle. Experiment A. in Table 15. shows that the presence of the standard substrate mixture (glutamic acid, ornithine, ammonia, and adenylic acid) is not required; and furthermore, as Experiment B. demonstrates, even the presence of

homogenate is not essential. Since the riboflavin reaction is completely independent of the urea synthesis system, it raises the question of whether the substance reacting with the urease preparation to give ammonia is actually urea.

TABLE 14.

RIBOFLAVIN

Exp. No.	Compound Tested	Urea Formed	Total Urea Radioact.	Urea Spec. Activity	Per Cent Control
		(μm.)	(cts./min.)	(cts./min.μm.)	
A	Control	4.24	566	134	
	Riboflavin (20)	1.73	88	50	37
B	Control	1.66	119	72	
	Riboflavin (20)	2.56	8	3	4
C	Control	0.79	76	96	
	Riboflavin (20)	3.99	3	1	1
	Riboflavin (1.0)	0.74	2	2	2
	Riboflavin (0.05)	0.14	0	0	0

Bicarbonate specific activity was 252 cts./min./μm. in all experiments. Glutamate concentration in Experiment C. was 0.03 M. Figures in parenthesis represent μm. test compound added.

Riboflavin itself does not react with the urease solution to give ammonia, indicating that one or more of the treatments prior to the addition of urease renders riboflavin susceptible to attack by the urease preparation.* Of interest in this connection is the apparent high concentration of flavoprotein in the urease used, as evidenced

* For details of the analytical procedure, see Sec. II-C or Figure 4.

by its intense yellow-green color.

TABLE 15.

RIBOFLAVIN BREAKDOWN

Exp. No.	Conditions	Yield "Urea" (μm.)
A	Homogenate + riboflavin	2.68
	Homogenate + riboflavin + substrate mixture	2.24
B	Homogenate alone	0.94
	Riboflavin alone	2.90
	Homogenate + riboflavin	3.74

The substrate mixture was omitted in Experiment B. Otherwise, the experiments were carried out in the usual manner. Riboflavin was added at a level of 20 μm.

The figures on total urea radioactivity given in Table 14. show that the presence of riboflavin strongly inhibits the synthesis of urea from $C^{14}O_2$.* Confirmation of this fact was obtained by testing riboflavin at low concentrations. The addition of 0.05 μm. of riboflavin (a riboflavin concentration of 1.3×10^{-5})caused almost complete inhibition.

In an attempt to elucidate the nature of the riboflavin inhibition, tests were carried out to see if radioactive urea precursors would accumulate in systems inhibited by riboflavin. In one set of

* Experiments have shown that riboflavin does not interfere with urea recoveries, indicating that the inhibition is in the synthetic and not the analytical reactions.

vessels to which 20 μm. of riboflavin had been added, the solution and precipitate (denatured protein and riboflavin) remaining after the removal of urea CO_2, were tested and found to be non-radioactive. This fact suggests that the riboflavin inhibition occurs at an early stage in the process of fixing CO_2 into urea.

F. Formic Acid, Para-amino-benzoic acid, and 4 amino-5 imadazole carboxamide

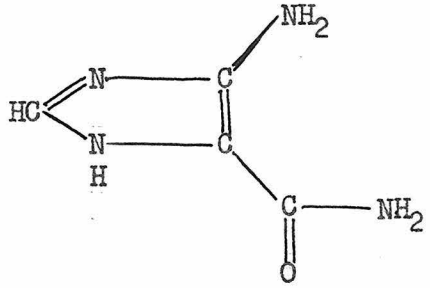
Formic acid has been implicated in urea formation since the work of Bach (15), who showed that urea synthesis in rat liver slices, using a bicarbonate buffer with glutamic acid and NH_4Cl as substrates, was greatly stimulated by the addition of sodium formate. Tests of formate as an intermediate have been consistently negative, however, and indicate no direct role of formate in urea synthesis.

PAB when tested proved to be a very strong inhibitor of urea synthesis. As the data to be presented show, inhibition can be detected at concentrations aslow as 1.3×10^{-5} M. PAB and the other inhibitors which have been reported in this paper (amino acids and riboflavin) have as a common characteristic either a primary or secondary amino group in their molecules.

Subsequent evidence suggested that one of the points of inhibition may be the conversion of ornithine to citrulline. In some experiments carried out using the semi-isolated enzyme system of Cohen, which catalyzes the formation of citrulline from ornithine, ammonia, glutamic acid, adenylic acid, and CO_2; it was observed that increasing ornithine concentrations inhibited the formation of citrul-

line. Data are only available from two experiments, but in these the ornithine concentration required for 50 per cent inhibition was directly proportional to the maximum rate obtained. If the rate (which was varied by changing the concentration of bicarbonate) is a function of the concentration of some intermediate, this would suggest that ornithine(and possibly other inhibitors) compete with the intermediate for an enzyme surface. This interpretation of the inhibition results is in agreement with the evidence suggesting that riboflavin inhibition occurs at an early stage in urea synthesis.

Studies of sulfonamide inhibition in E. Coli and its reversal by PAB and purines (84) have indicated that the substance 4 amino-5imadazole carboxamide



is converted to purines by reactions requiring the presence of PAB and that sulfa antagonizes this reaction. For this reason, 4 amino - 5 imadazole carboxamide was tested in urea synthesis.* The results of these tests and those of formate and PAB are shown in Table 16.

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* A sample of the hydrochloride of this compound was kindly supplied by Dr. E. Shaw of the Rockefeller Institute for Medical Research. The sample had a melting point of 225-230° C (melting point in the literature 255-6°C) and was grey in color. It yielded a faintly purple solution.

TABLE 16.

FORMATE, PAB, AND 4 AMINO-5 IMADAZOLE CARBOXAMIDE

Exp. No.	Compound Tested	Urea Formed	Total Urea Radioact.	Urea Spec. Activity	Per Cent Control
		(μm.)	(cts./min.)	(cts./min./μm.)	
A Prelim	Control	1.08	170	161	
	+ Formate (100)	1.25	146	116	72
B	Control	1.66	118	72	
	+ PAB (20)	0.00	0		
C	Control	0.79	76	96	
	+ PAB (20)	0.28	0	0	
	+ PAB (1.0)	0.16	0	0	
	+ PAB (0.05)	0.66	48	73	76
D	Control	0.89	64	72	
	+ Formate (20)	0.84	62	74	103
	+ Imad. (20)	1.50	54	36	50
	+ Imad. (20) + Formate (20)	1.30	60	45	62
E	Control	4.57	736	161	
	Imad. alone - substrate mixture - absent.	1.34	0	0	
	+ Imad. (20)	5.50	770	140	87
	+ Imad. (20) + Formate (50)	4.54	646	142	88
	+ Imad. (20) + PAB (0.20)	4.26	618	145	90
	+ PAB (0.20)	3.78	634	168	104

Concentration of glutamate in Experiments D. and E. was 0.03 M. Bicarbonate specific activity was 252 cts./min./μm. in all cases. Figures in parenthesis represent μm. test compound added.

Experiments D. and E. of Table 16. show a depression of the urea specific activity resulting from the addition of 4 amino

5 imadazole carboxamide. That this depression is not due to the action of 4 amino-5 imadazole carboxamide as an intermediate is suggested by two results. First, the substituted imadazole does not depress the total urea radioactivity; it merely increases the yield (this behavior is reminiscent of that of arginine which was reported earlier). Second, this increase in yield is not dependent on the operation of the ornithine cycle.

The urea analysis, then, gives a crude measure of 4 amino-5 imadazole carboxamide by measuring its ability to increase the urea assay over that of the control. Using this method, the data show that both PAB and formate facilitate the conversion of 4 amino - 5 imadazole carboxamide to some substance which will not increase the urea assay.

PAB is concerned with the conversion of 4 amino-5 imadazole carboxamide (or a related compound (85)) to purines. Isotope evidence (76) has shown that formate is the source of the carbon in the number 2 position in purines. This is the carbon which must be added to 4 amino - 5 imadazole carboxamide to form purines. It would, therefore appear probably that a purine is the substance formed which fails to increase the urea assay.

Attempts to secure hypoxanthine synthesis from 4 amino - 5 imadazole carboxamide in homogenates have been reported. Schulman, Buchanan, and Miller (86) have found that incubation of C^{14} labelled 4 amino-5 imadazole carboxamide with pigeon liver homogenates results in the formation of C^{14} labelled hypoxanthine. However, Greenberg (87) has reported that the substituted imadazole is not an intermediate in hypoxanthine synthesis in pigeon liver homogenates.

IV. DISCUSSION

A total of thirty-three substances, including members of the Krebs tricarboxylic acid cycle, a variety of amino acids, vitamins, and some miscellaneous compounds, have been tested as possible intermediates in the process of fixing CO_2 into urea. In testing these substances, several effects have been noticed.

Citrulline and arginine, as predicted by theory, behave as intermediates in urea synthesis, when tested for their ability to depress the specific activity of urea formed from $C^{14}O_2$. The substances 4 amino - 5 imadazole carboxamide and riboflavin also showed the same property of lowering the urea specific activity. However, in both these cases, it was shown that this depression was the result of a mechanism independent of the ornithine cycle.

In the case of riboflavin, it was found that some step in the analytical scheme rendered riboflavin susceptible to attack by the urease preparation used; and that, in addition, riboflavin is a potent inhibitor of urea formation at concentrations as low as 1.3×10^{-5} M. The evidence suggested that the point of inhibition was at an early step in the sequence of reactions studied.

In the case of 4 amino - 5 imadazole carboxamide, it appeared that this substance broke down independently of the ornithine cycle to increase the urea assay value. By the use of this criterion as a measure of the 4 amino - 5 imadazole carboxamide remaining at the end of the reaction period, it appeared that this compound, under the stimulation of PAB and formic acid, was being transformed into

some product which did not affect the urea assay; and that this substance was probably a purine.

The inhibition of urea synthesis by certain amino acids, which had been reported in liver slices, was confirmed for homogenate. In addition, the list of inhibitors was extended to include PAB, ornithine (a substrate for the reaction), and riboflavin (mentioned above). Amino acids and the compounds carbamyl glutamic acid and carbamyl aspartic acid were further shown to raise the specific activity of urea formed under the experimental conditions employed. An explanation of this effect has been offered based on the known inhibition of phosphatases by amino acids.

Tests of the compound carbamyl glutamic acid have indicated that, while it does not donate any of its carbon atoms to form urea, it does play some role in urea formation, probably by aiding the conversion of one intermediate to another.

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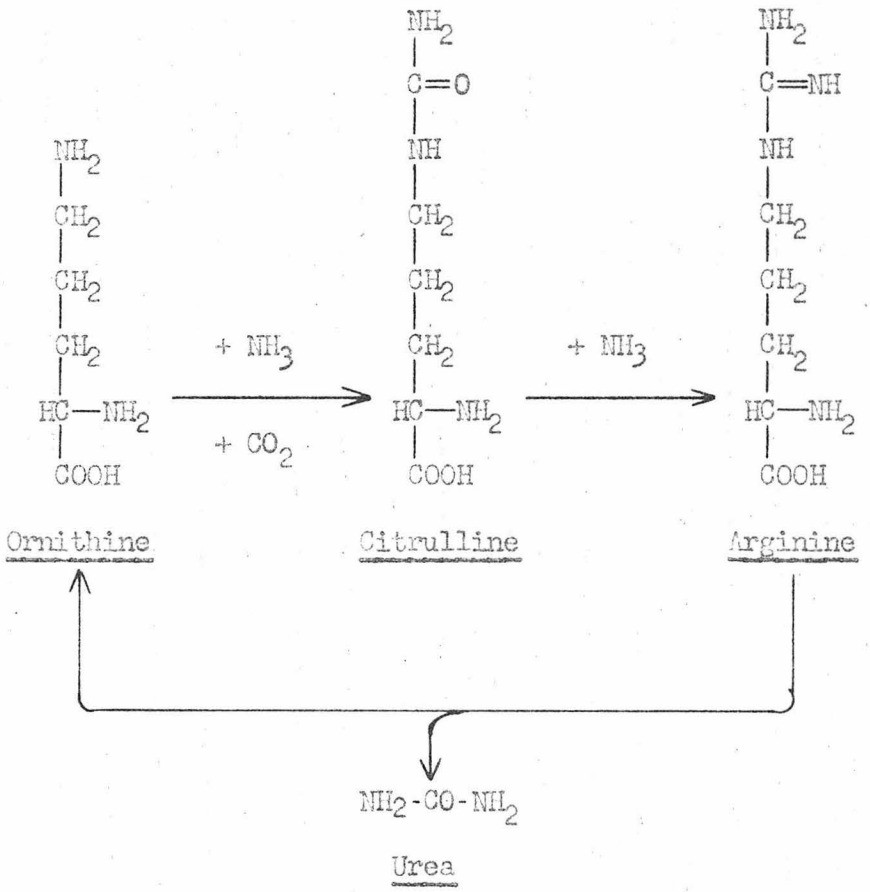
FIGURE 1.

THE ORNITHINE CYCLE



Ornithine Citrulline Arginine

Urea

Figure 2 - Reaction Vessel

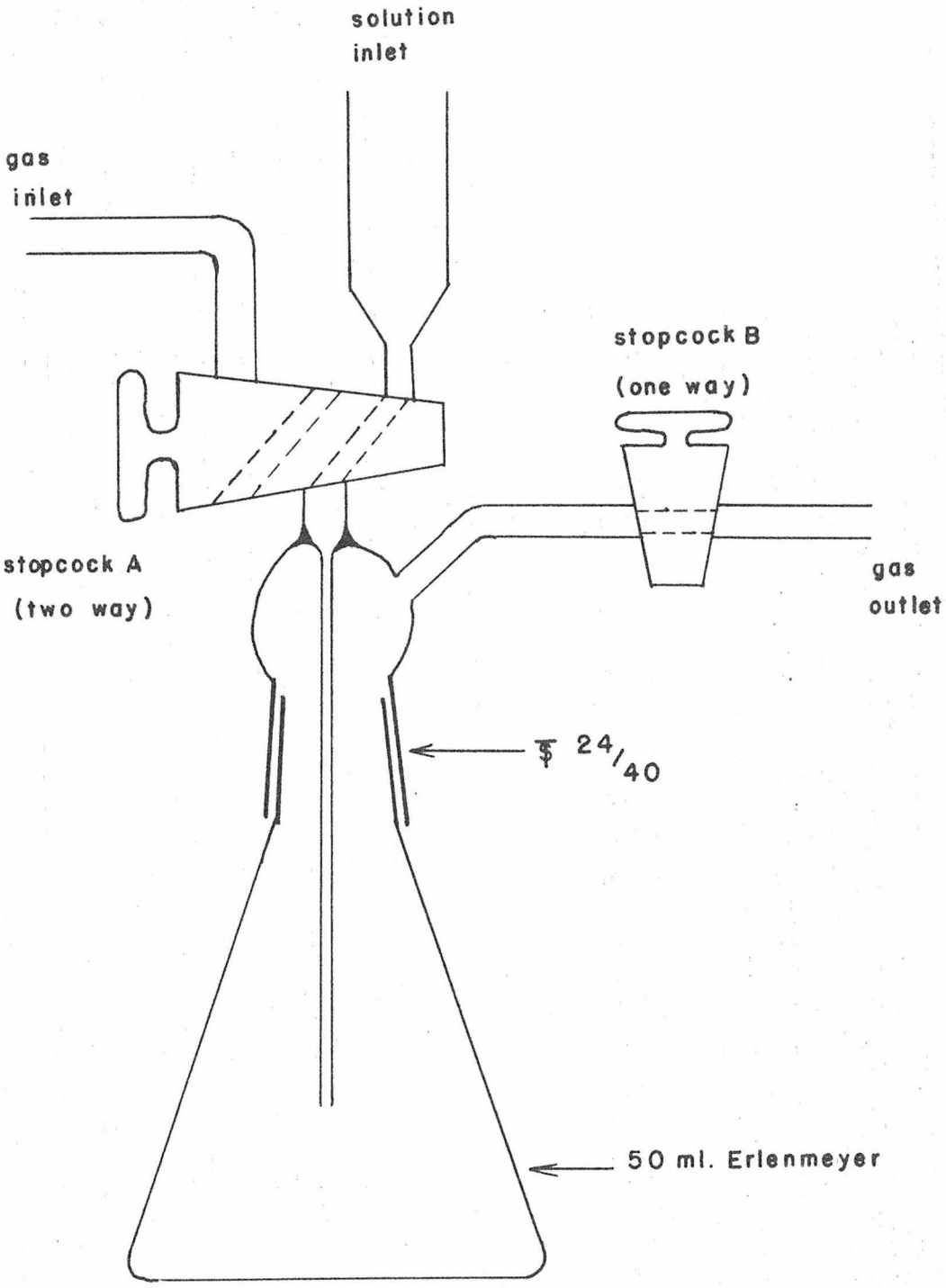


Figure 3

Aeration of CO_2

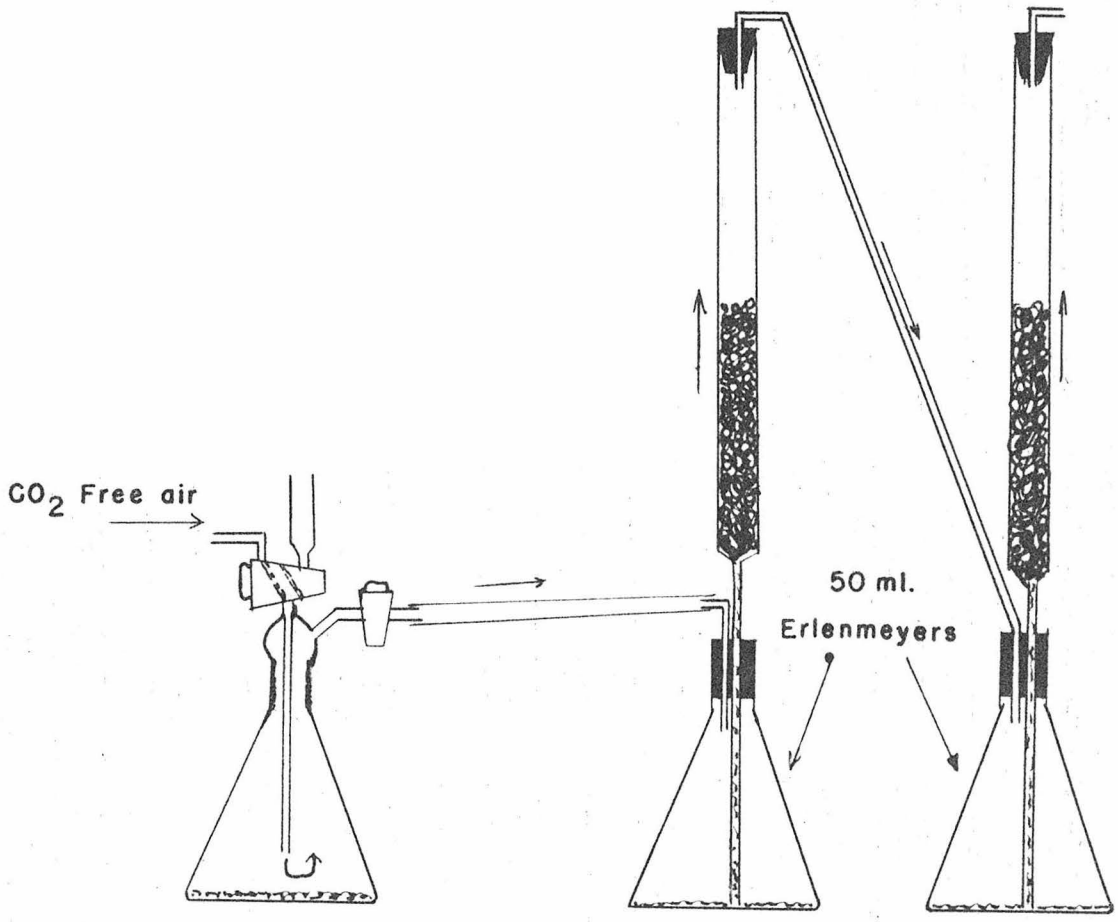
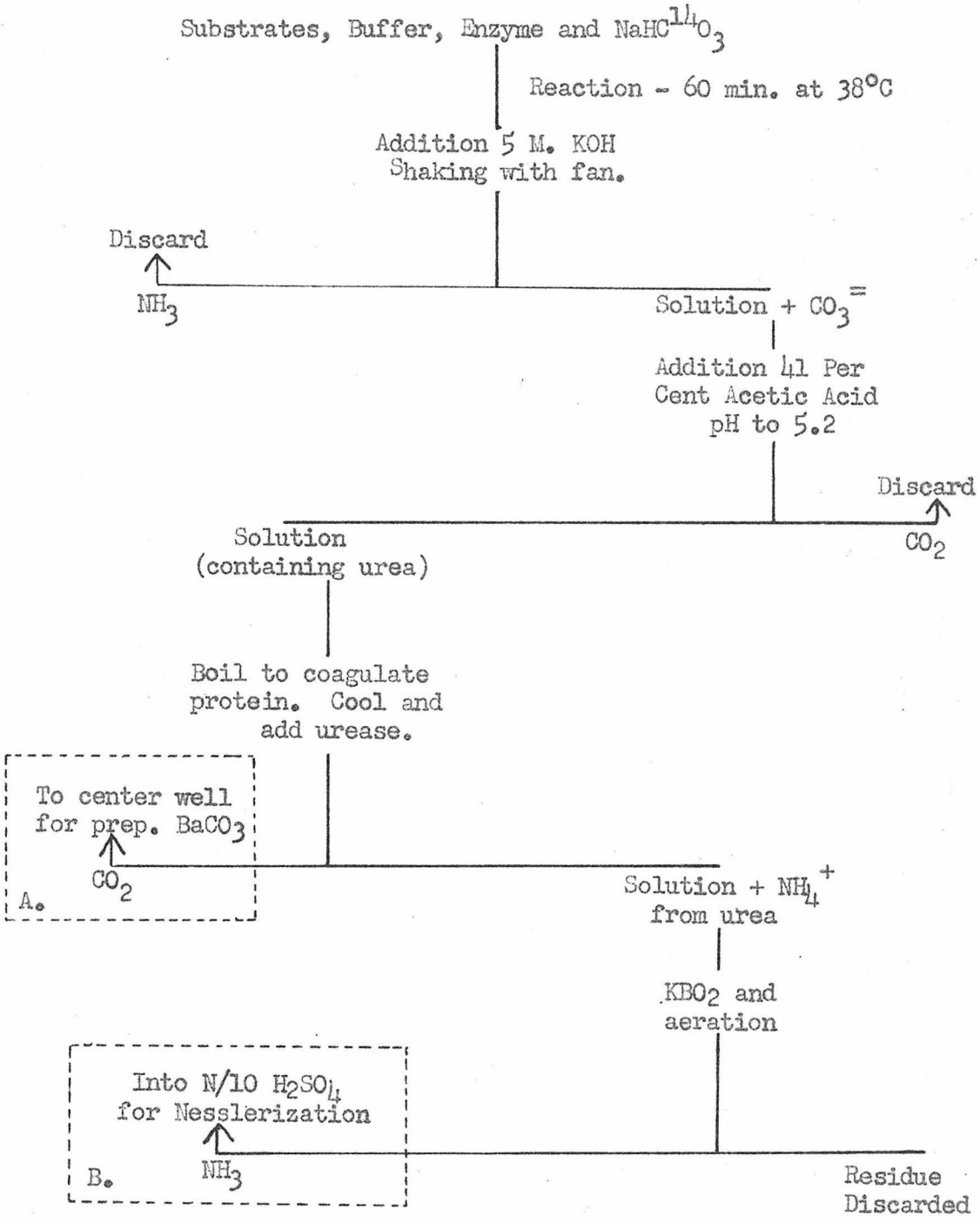


FIGURE 4. ANALYTICAL SCHEME



A. At this point, the measurement of the total number of counts fixed into urea is obtained.

B. At this point, the yield of urea is determined.

Figure 5 - Homogenate conc. vs. yield

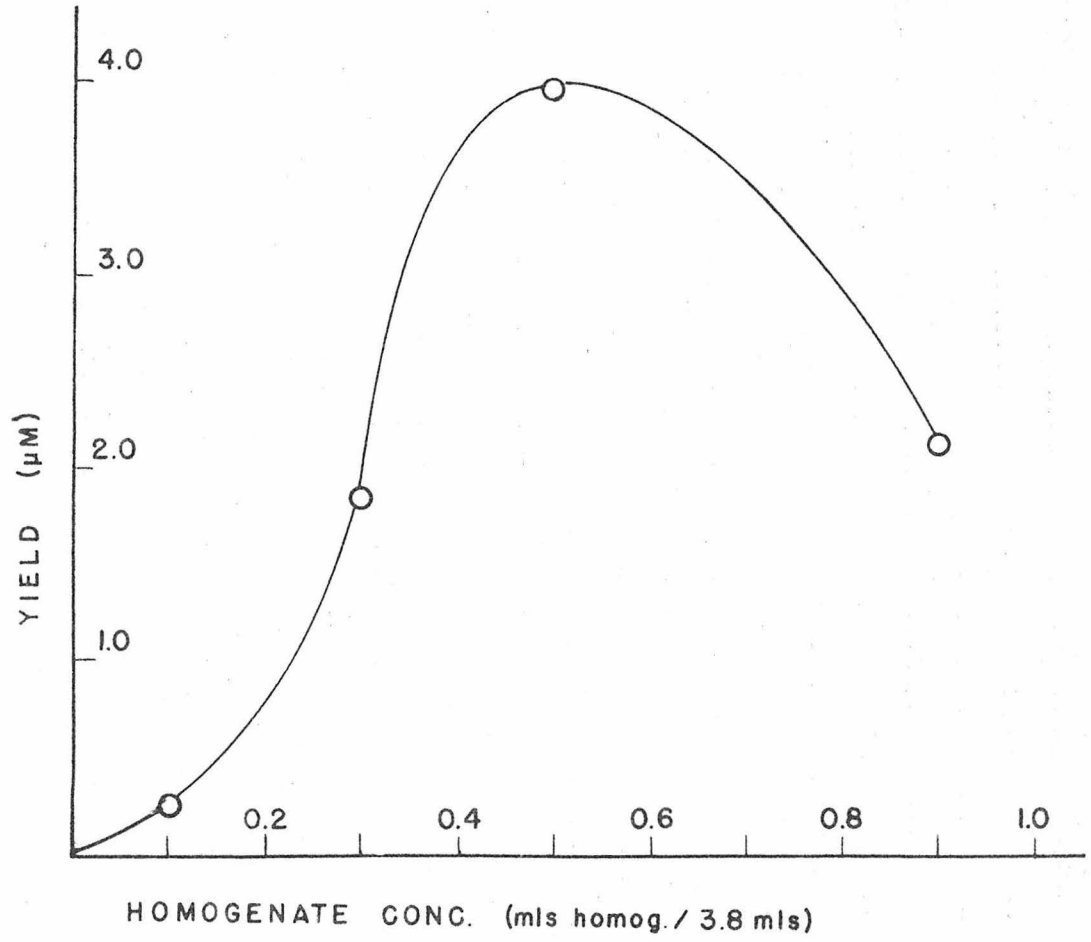


Figure 6

Bicarbonate specific activity vs. time

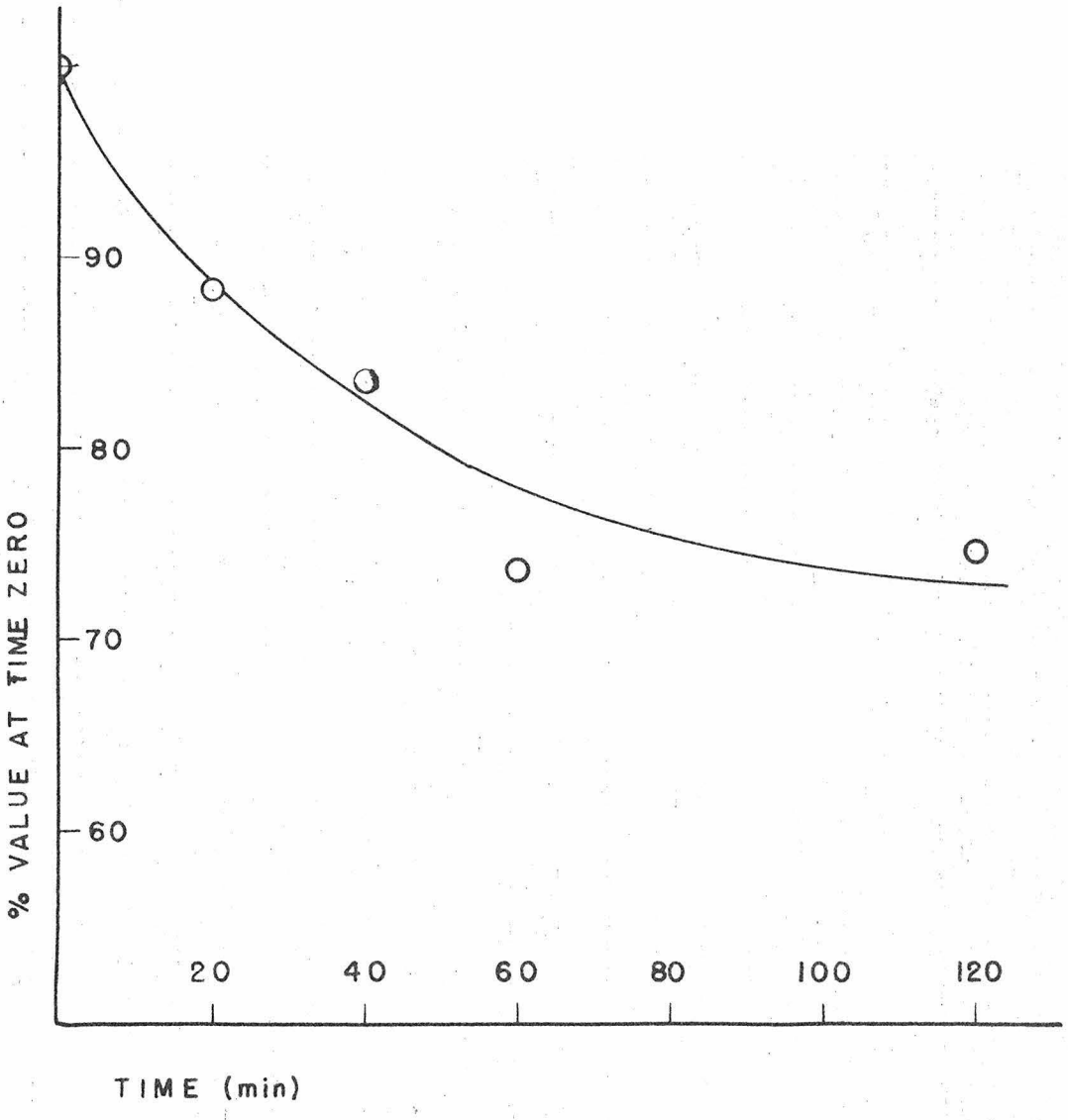
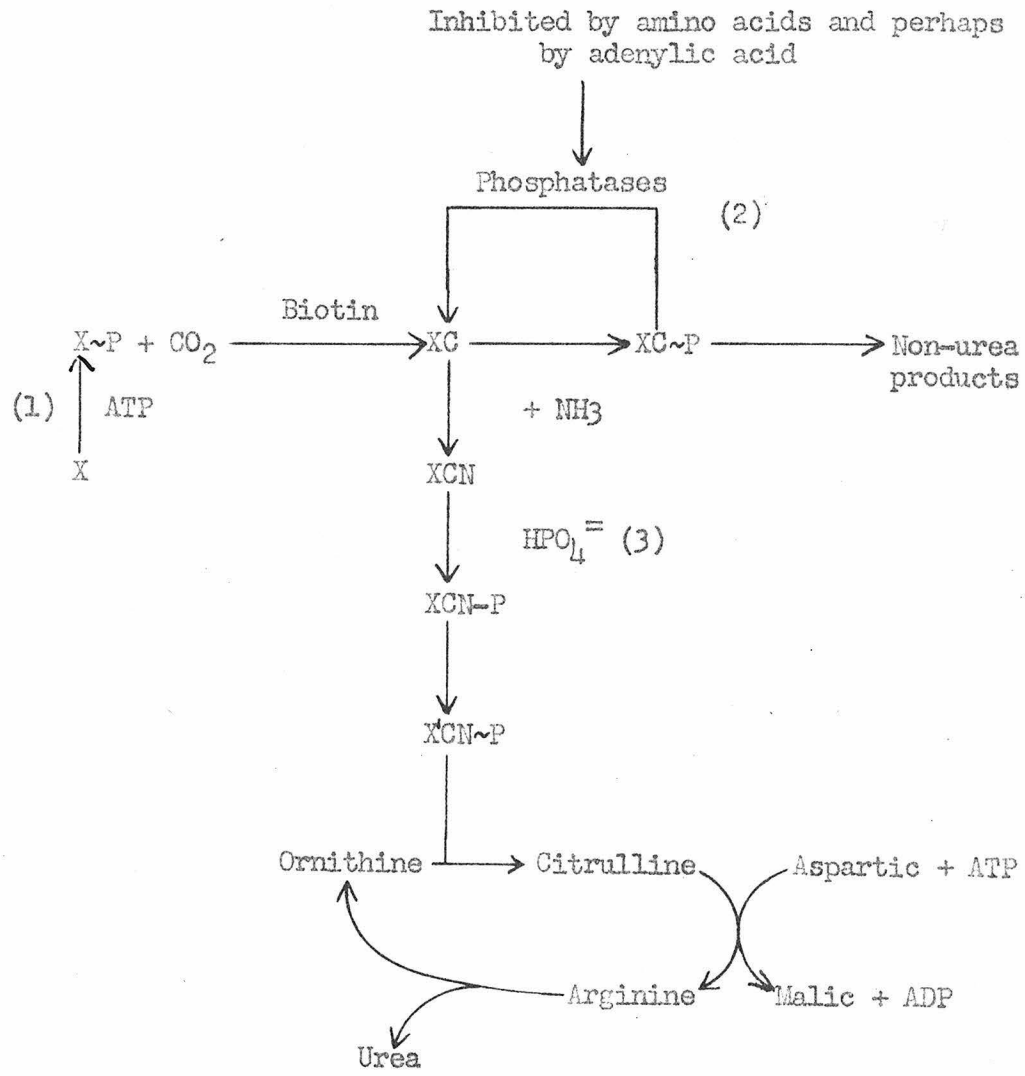


FIGURE 7.

MECHANISM OF AMINO ACID EFFECT



(1) X represents a compound which when phosphorylated, would react with CO_2 to form the primary fixation product XC.

(2) Amino acids by inhibiting the phosphatases breaking down XC~P to XC would stimulate the removal of XC to non-urea products. The greater effectiveness of adenylic acid over ATP might be a consequence of its inhibition of this enzyme.

(3) The requirements for inorganic phosphate would be explained if the system generated its own high energy phosphate for use in the reaction ornithine to citrulline.