HISTIDINE METABOLISM IN LIVER

Thesis by

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ABSTRACT

This is the report of an investigation of histidine metabolism in mammalian liver. Formiminoglutamic acid, reported to be the end-product of histidine metabolism *in vitro*, has been found to be degraded enzymatically to glutamic acid by a rat liver extract. Homogenates of spleen, kidney, and heart do not have such activity. A product of the metabolism of the formimino group is carbon dioxide. The effects of time, enzyme concentration, substrate concentration, temperature, and pH on the reaction are reported. Sulfhydryl groups are essential for activity. Inorganic phosphate is stimulatory, and arsenolysis occurs. On the basis of these findings and pertinent published information a scheme for the degradation of histidine to glutamic acid is proposed.

TABLE OF CONTENTS

I. INTRODUCTION

A. General Aspects

Histidine is one of the amino acids occurring universally in proteins. It is also found in vertebrate muscle, either free (fish), or as a constituent of the dipeptides carnosine and anserine (birds, mammals). A derivative, ergothioneine (or thioneine), the betaine of thiolhistidine, is found in grains and in animal erythrocytes. Another derivative, histamine, the decarboxylation product of histidine, is found in plants and is physiologically important in animals. The important jaborandi alkaloid, pilocarpine, may also be derived from histidine.

It was realized early in this century that amino acids could be metabolized, and much effort has been spent attempting to define the metabolic pathways of the individual amino acids. In the case of histidine metabolism each reaction encountered had no precedent at the time of its discovery, so little wonder it has been a difficult problem! The discovery in recent years that histidine may be implicated in monocarbon metabolism has created renewed interest in its metabolism.

Consideration will be given first to the metabolic pathway from histidine to glutamic acid, which may be considered in three steps, as follows:

(1) Histidine ⟶ urocanic acid + ammonia

(2) Urocanic acid + water ⟶ N-formimino-L-glutamic acid

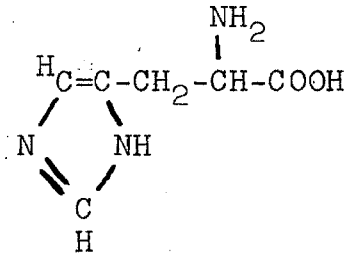
(3) N-formimino-L-glutamic acid + (acceptor) ⟶ L-glutamic acid + formimino-(acceptor)

Then the relation of histidine to monocarbon metabolism will be described, with a discussion of the role of folic acid. It is hoped that this introduction will lead logically to the statement of the present status of the problem.

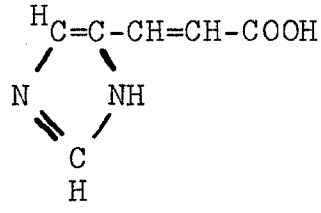
B. The Metabolic Pathway from Histidine to Glutamic Acid

1. Histidine to urocanic acid.

Engeland (1) reported in 1908 that histidine injected into cats was not recovered in the urine and surmised that it was metabolized. Abderhalden, Einbeck, and Schmid (2) found that dogs fed histidine excrete excess urea and ammonia in their urine. The first intermediate in histidine metabolism was discovered in 1917 by Raistrick (3) who found that several enteric bacteria deaminated histidine non-oxidatively to give urocanic acid. The latter had received its name before the discovery of histidine, when Jaffe (4) found it in the urine of a dog. Kotake and Konishi (5) recovered it in dog urine after feeding large amounts of histidine, but it accounted for only a small part of the histidine fed.



Histidine



Urocanic acid

A great step forward in the study of histidine metabolism came with the *in vitro* studies of Edlbacher and co-workers (6-9). The found that L-histidine, but not D-histidine, was metabolized by vertebrate liver preparations to equimolar quantities of ammonia (liberated by sodium carbonate) and an unidentified intermediate. The latter intermediate was not urocanic acid, however, and Edlbacher supposed that there were two separate metabolic pathways for histidine. However, Takeuchi (10) found that the protein precipitated from guinea pig liver extract at pH 5 contained an enzyme which converted histidine to urocanic acid. Edlbacher and co-workers (11, 12) then showed that urocanic acid is converted by liver preparations to the unidentified intermediate noted above.

Proof that histidine is metabolized via urocanic acid came from the studies of Tabor and co-workers (13-15). Tabor and Hayaishi (13) found that histidine-adapted *Pseudomonas fluorescens* converted histidine to glutamic acid. Tabor, Mehler, Hayaishi, and White (14) studied the metabolism of amidine-C^{14}-histidine by cell-free extracts. At the same

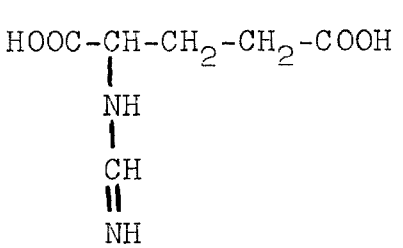
time that glutamic acid and radioactive formic acid were being produced, radioactive urocanic acid was also formed. Mehler and Tabor (15) then studied the metabolism of amidine-C^{14}-histidine in guinea pig liver homogenates, with the addition of unlabeled urocanic acid. Radioactivity appeared in the latter fraction, and it was calculated that at least 84% of the metabolized histidine had been converted to urocanic acid. Urocanic acid, with three unsaturated bonds, has a strong ultraviolet absorption. This absorption was used to study the appearance and disappearance of urocanic acid in liver preparations, further verifying its position in the metabolic pathway.

The first step in the degradation of histidine to urocanic acid is thus a deamination without net change in oxidation state. It appears likely that the α-amino nitrogen of histidine is excreted principally as ammonia and urea.

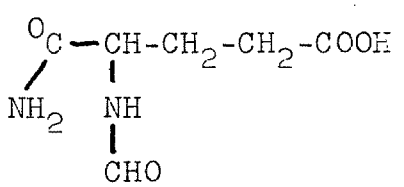
2. Urocanic acid to N-formimino-L-glutamic acid.

As noted previously, Edlbacher and co-workers (6-9) had noted the accumulation of an unidentified intermediate when L-histidine was incubated with vertebrate liver preparations. Equimolar quantities of ammonia (liberated by sodium carbonate) and of the intermediate were produced. In the presence of sodium hydroxide the intermediate produced equimolar quantities of L-glutamic acid, ammonia, and a volatile reducing acid thought to be formic acid. On the other hand, the supernatant liver fraction of Takeuchi (10) degraded urocanic acid

to D,L-isoglutamine, although only after incubation for 4 days. Oyamada (16) identified the reaction product as N-formyl-D,L-isoglutamine and noted that it was then degraded to D,L-isoglutamine. However, this could not be the same product Edlbacher obtained, as the latter had recovered optically active glutamic acid on hydrolysis. A more compatible intermediate, isomeric with formylisoglutamine, was suggested by Walker and Schmidt (17). By electrometric titration during the incubation of histidine with a liver preparation, they found a new reactive group with $pK' = 4.2$ and suggested α-formamidino-glutaric acid as the intermediate. (A better name is N-formimino-L-glutamic acid, hereafter abbreviated FIGA, since this name definitely establishes it as a derivative of optically active L-glutamic acid.)



N-formiminoglutamic acid

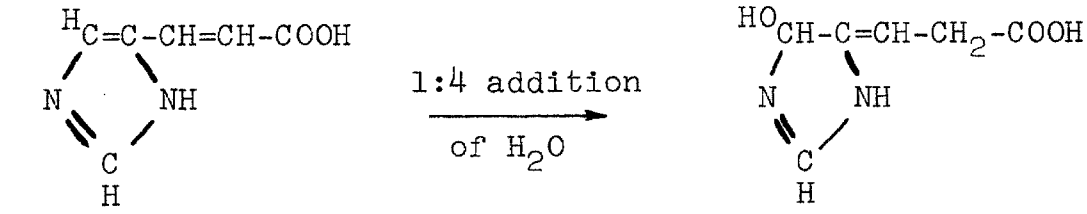


N-formylisoglutamine

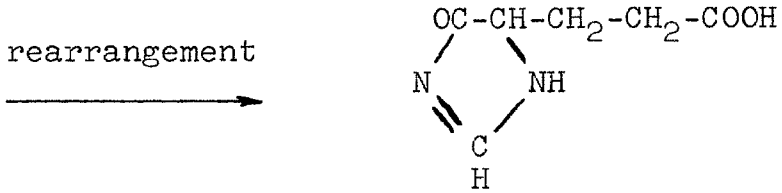
The matter has since been settled in favor of the latter compound. Borek and Waelsch (18) isolated the intermediate from cat liver homogenates incubated with L-histidine.

It was optically active, and it gave rise on hydrolysis to equimolar quantities of L-glutamic acid, formic acid, and ammonia. Titration revealed three groups, with $pK_1' = 2.4$, $pK_2' = 4.7$, and $pK_3' = 11.1$. These findings rule out N-formyl-D,L-isoglutamine as an intermediate. Since then Miller and Waelsch (19) and Seegmiller and co-workers (20) have independently synthesized FIGA and shown it to be identical with the metabolic intermediate.

The second step in histidine degradation to glutamic acid, then, is the addition of water to urocanic acid, but it is not a simple hydrolysis. It has been proposed (18) that water is added in a 1:4 addition, with rearrangement of the product to give imidazolonepropionic acid, which in turn hydrolyzes non-enzymatically to give FIGA. There has never been any experimental evidence for an intermediate between urocanic acid and FIGA.

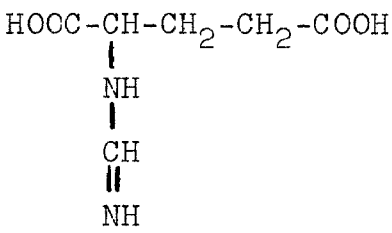


Urocanic acid



Imidazolonepropionic acid

non-enzymatic
hydrolysis



FIGA

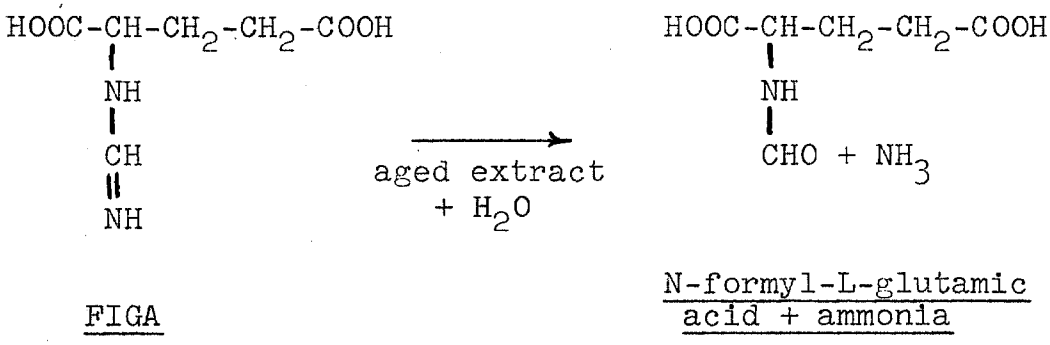
3. N-formimino-L-glutamic acid to glutamic acid.

It is apparent that FIGA is not the end-product of histidine metabolism in the intact animal. Featherstone and Berg (21) showed that L-histidine given to fasting rats was as good a former of liver glycogen as was L-glutamic acid. Tesar and Rittenberg (22) fed γ-N^{15}-histidine to rats and recovered about half of the isotope in tissue proteins and about half in the urine. The urinary amino acids, including glutamic acid, were high in α-N^{15}.

The first direct evidence for conversion to glutamic acid by animals was provided by Abrams and Borsook (23), who studied the metabolism of $C^{14}OOH$-L-histidine *in vivo* in rabbits and *in vitro* in guinea pigs. In the former experiment radioactive L-glutamic acid was isolated from the non-protein liver fraction. Decarboxylation by ninhydrin liberated only 10% of the radioactivity, indicating that the γ-carboxyl group of glutamic acid was derived from the carboxyl group of histidine. This is consistent with the data of Tesar and Rittenberg. The *in vitro* experiment

probably did not result from non-enzymatic degradation during isolation, since mild methods were applied. However, there was no control for non-enzymatic conversion. The same criticism applies to the in vitro study of Fournier and Bouthillier (24), who recovered L-glutamic acid, starting with L-histidine. Wolf (25) verified the finding of radioactive glutamic acid in visceral proteins of animals given radioactive histidine. He used α-C^{14}-D,L-histidine and also recovered radioactivity in respiratory carbon dioxide. The latter could not have arisen by simple decarboxylation of histidine, but presumably arose by total oxidation of glutamic acid.

As noted previously, Tabor and Hayaishi (13) found that extracts of histidine-adapted Pseudomonas fluorescens converted L-histidine to L-glutamic acid. Tabor and Mehler (26) subsequently reported that urocanic acid and FIGA were also converted to glutamic acid by such extracts. By the use of aged extracts, they were able to demonstrate two new enzymatic reactions. Aged extract produced N-formyl-L-glutamic acid from all substrates, while aged extract reactivated by ferrous ion converted N-formyl-L-glutamic acid to L-glutamic acid:

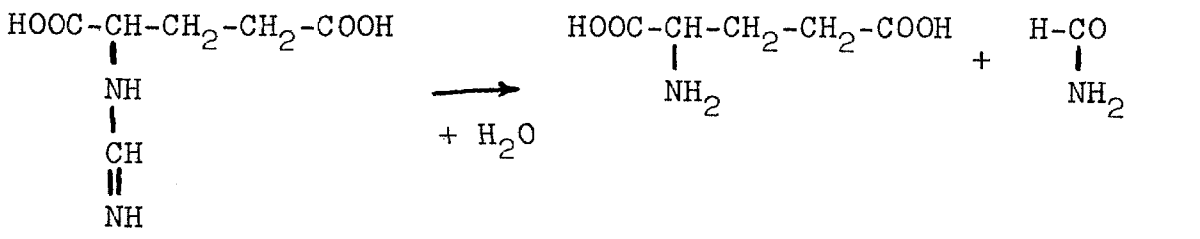


FIGA

N-formyl-L-glutamic acid + ammonia

Glutamic acid + formic acid

The significance of this finding has been particularly difficult to evaluate in view of the finding of Magasanik and Bowser (27) that Aerobacter aerogenes behaves differently. A histidine-requiring mutant of this organism in the absence of glucose elaborates adaptive enzymes which degrade histidine to glutamic acid and formamide. Urocanic acid and FIGA are intermediates. N-formyl-L-glutamic acid is not metabolized. Wachsman and Barker (28) discovered the same pathway in Clostridium tetanomorphum. The question arises whether there are distinctly different pathways in different forms or whether they are variations of a basically single pathway. More will be mentioned on this subject in the next section.



FIGA Glutamic acid Formamide

C. The Role of Histidine in Monocarbon Metabolism

1. The relation of histidine to formate metabolism.

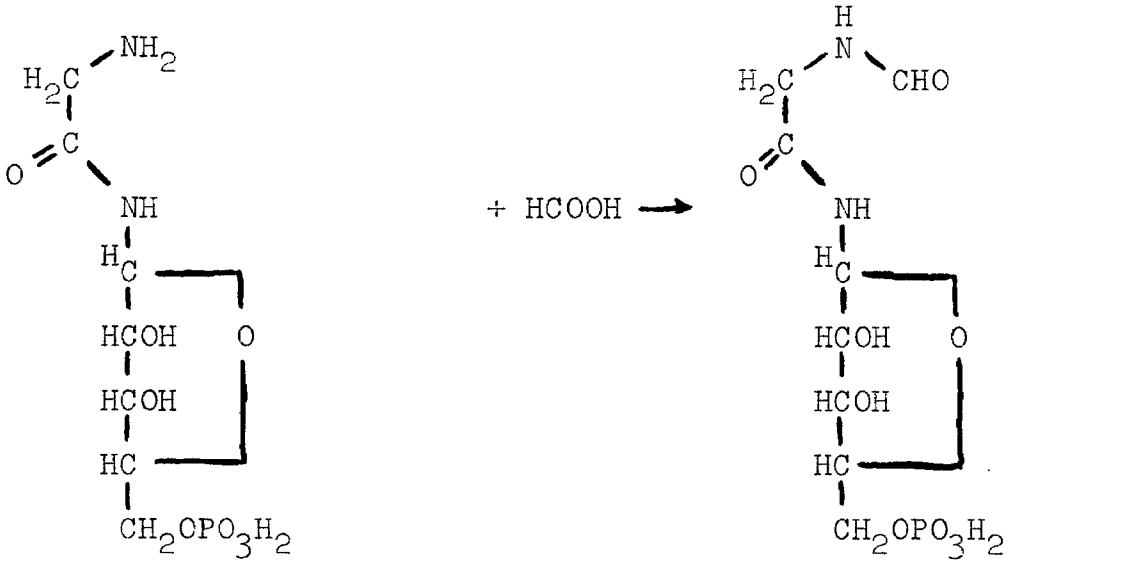
Ackroyd and Hopkins (29) reported in 1916 that a dietary deficiency of histidine or arginine in growing rats resulted in the impairment of growth and a decrease in urinary excretion of allantoin, the end-product of purine metabolism in the rat. Restoration of either amino acid to the diet restored both growth and allantoin excretion to normal levels. It was concluded that these amino acids were interchangeable. However, Rose and Cox (30) showed that histidine is indispensable for the growing rat and that the requirement is not satisfied by arginine. Rose and Cook (31) further found that allantoin excretion is specifically related only to histidine among the dietary amino acids. Direct evidence for this was provided by Popel (32), who injected dogs with histidine via portal vein angiostomy and found the hepatic allantoin content increased many times after ten minutes. No other amino acid had this effect. There was no significant

change in hepatic uric acid, creatine, or creatinine. He concluded that the liver can use histidine for the synthesis of purines.

Isotope techniques were brought to bear on this problem by Barnes and Schoenheimer (33), who fed N^{15}-ammonia to rats and pigeons. Urinary uric acid and allantoin were found to be much richer in N^{15} than were tissue histidine or arginine. So it was concluded the latter were not precursors of purine nitrogen. However, the fact that histidine can be a precursor of purine carbon was dramatically shown by Reid and Landefeld (34, 35). These workers fed amidine-C^{14}-histidine to rats and analyzed urinary allantoin, uric acid, creatine, creatinine, and urea for radioactivity. They showed conclusively that allantoin and uric acid had far more radioactivity than the other nitrogenous compounds and that this radioactivity was derived almost exclusively from purine carbons 2 and 8. These studies, corroborated by Sprinson and Rittenberg (36) in the pigeon and rat, indicated that the amidine carbon of histidine behaved very much like formate in the synthesis of purines.

In 1948 Sonne, Buchanan, and Delluva (37, 38) had studied the problem of purine synthesis by feeding C^{13}-labeled metabolites to fasted pigeons. Urinary uric acid was then degraded to find the isotope concentration in individual carbon atoms. They had found C^{13} in carbons 2 and 8 when C^{13}-formate was fed. $C^{13}O_2$ led to C^{13} in carbon 6, and C^{13}OOH-glycine

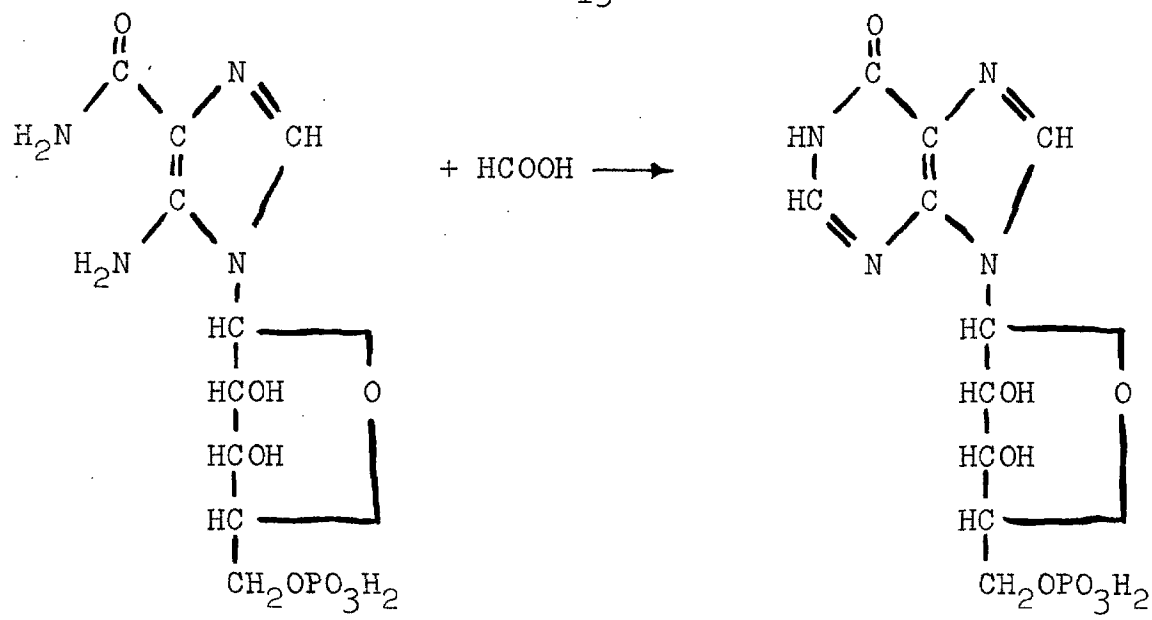
led to C^{13} in carbon 4. It was therefore clear that purines were synthesized from several small metabolites. These findings were substantiated by Heinrich and Wilson (39) by the use of radioactive (C^{14}) metabolites in rats. Formate is now thought to enter into purine synthesis in two different steps. The first is the reaction of glycinamide ribotide with formate to give α-N-formylglycinamide ribotide (40):



Glycinamide ribotide

α-N-formylglycinamide ribotide

The second is the reaction of 4-amino-5-imidazolecarboxamide ribotide with formate to give inosinic acid (41):



4-amino-5-imidazole-carboxamide ribotide

Inosinic acid

Isotope studies revealed that the amidine carbon of histidine appeared in other compounds than purines. Borsook and co-workers (42) injected amidine-C^{14}-L-histidine intravenously into mice and found that about half of the radioactivity was incorporated into proteins while nearly half appeared in respiratory carbon dioxide within four hours. Soucy and Bouthillier (43) injected the same substance into rats and found radioactivity in respiratory carbon dioxide and urinary urea (a reflection of CO_2 radioactivity, presumably). In addition, radioactive serine was recovered from hydrolysates of tissue proteins. The experiments of Sprinson and Rittenberg (36) revealed the same thing. Reid and Landefeld (34,35) had also demonstrated that liver

choline had become radioactive during their study on purine labeling. About two-thirds of the radioactivity was in the methyl groups. Sprinson and Rittenberg (36) found the rest of the radioactivity in the β -carbon. Toporek, Miller, and Bale (44) found that this neogenesis of methyl groups was enhanced by choline deficiency.

These experiments showing radioactivity in CO_2, serine (and β -carbon of choline), and methyl groups are exactly paralleled by studies utilizing radioactive formate. Sakami (45) injected C^{14}-formate into rats and simultaneously fed $C^{13}OOH$-glycine. Liver serine showed C^{13} in the carboxyl group and C^{14} in the β -carbon. Sakami and Welch (46) subsequently demonstrated radioactivity in the methyl groups of methionine and choline after administering C^{14}-formate to rats. Apparently, then, the amidine carbon of histidine, as judged from in vivo studies, is converted to formate or has in common with the latter some intermediate metabolite.

Studies in microorganisms have already been mentioned which indicated that, under certain conditions at least, the amidine carbon could appear as formate (Pseudomonas) or as formamide (Aerobacter and Clostridium). The latter could be explained in keeping with the animal studies by the hypothesis that a formimino-acceptor complex is formed, but that it can dissociate to give formamide (Aerobacter and Clostridium) or be metabolized further (animals). Since the Pseudomonas case must be different in some way from

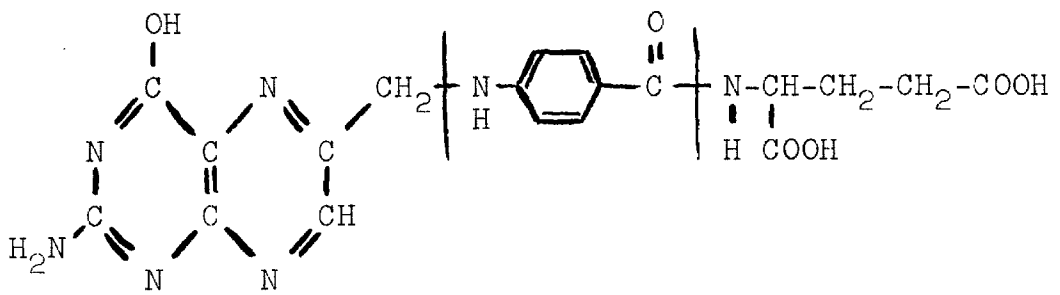
Aerobacter and Clostridium, the difference may lie in the ability to transfer formate, but not the formimino group, to an acceptor.

2. The role of folic acid in histidine metabolism.

In 1951 Bakerman, Silverman, and Daft (47,48) reported that folic acid-deficient rats excrete an alkali-labile glutamic acid derivative in the urine. This derivative was found to increase after feeding histidine (49). Subsequently, Tabor and co-workers (50) showed this derivative to be identical with the one first reported by Edlbacher. When γ-N^{15}-histidine was fed to the deficient animals, 55% of the isotope was recovered in the α-amino group of the glutamic acid derivative.

Prior to this it had already been shown that folic acid was involved in monocarbon metabolism. It was originally discovered as a growth factor for certain bacteria of the lactic acid group. Since these bacteria could grow without it if enough purines and pyrimidines were supplied, it was proper to suppose that folic acid might be involved in purine synthesis (51-53). Another factor implicated in purine synthesis was p-aminobenzoic acid, since sulfanilamide-treated cultures of E. coli accumulated 4-amino-5-imidazolecarboxamide,

which was noted to need but one carbon to be a purine (54). The relationship of these findings became apparent when the structure of folic acid was discovered to be pteroylglutamic acid, containing one molecule of p-aminobenzoic acid per molecule (55).

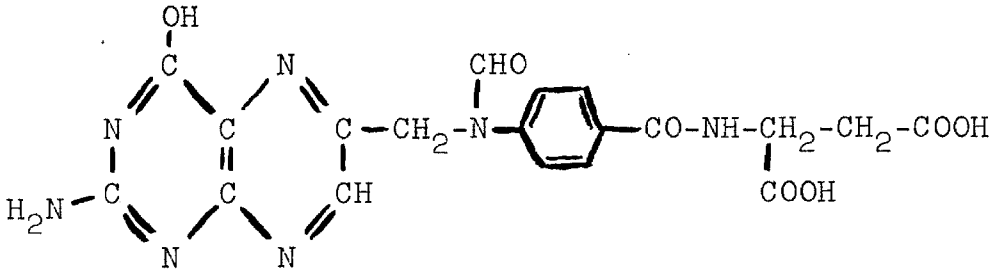


Xanthopterin derivative | p-amino-benzoic acid | Glutamic acid

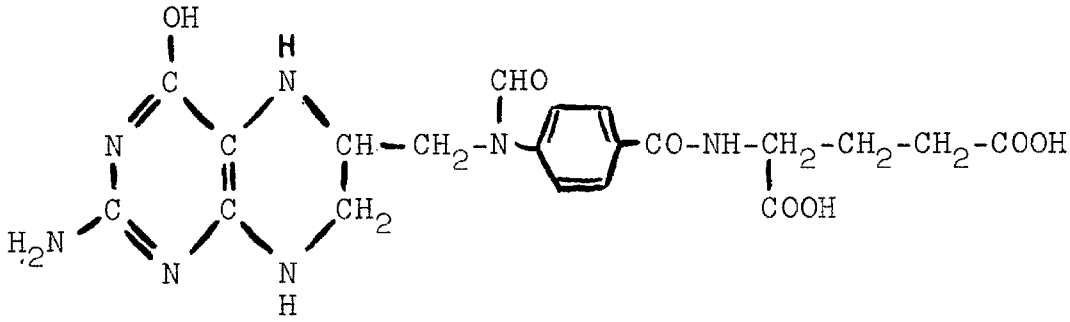
Conclusive evidence that folic acid was involved in monocarbon metabolism came from isotopic studies by Plaut, Betheil, and Lardy (56). Normal rats injected with C^{14}-formate fixed about ten times as much radioactivity into liver proteins as did folic acid deficient rats. The effect on the β-carbon of serine was particularly striking. Oxidation to $C^{14}O_2$ was also considerably reduced in rate. Subsequently, Drysdale, Plaut, and Lardy (57) found that deficient rats also fixed much less formate into carbons 2 and 8 of purines.

Hypothesizing that a one-carbon fragment might be

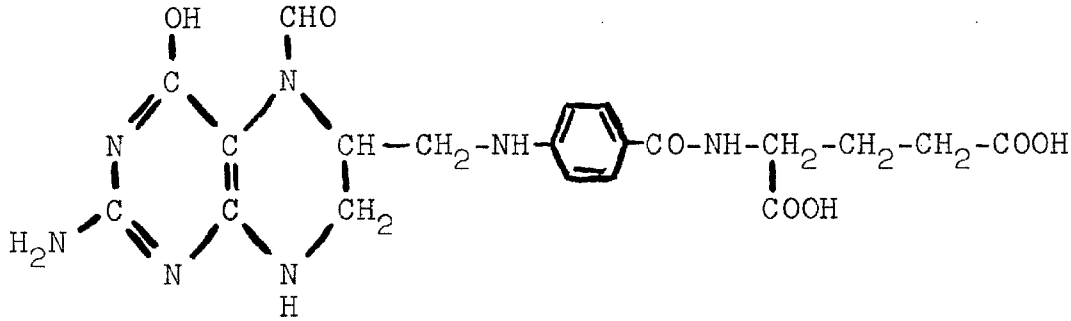
"carried" by folic acid, Gordon and co-workers (58) synthesized N^{10}-formylfolic acid and found it to be an active bacterial growth factor. The naturally occurring "citrovorum" factor, so named because it supports the growth of Leuconostoc citrovorum (59), appeared to be identical with another synthetic derivative, folinic acid-SF, which was shown to have the structure N^5-formyltetrahydrofolic acid (60). Nichol and Welch (61) found that folic acid was converted to the citrovorum factor by rat liver homogenate. Ascorbic acid enhanced the reaction. Aminopterin, a known folic acid inhibitor-analogue, inhibited the reaction (62). A still more active factor is N^{10}-formyltetrahydrofolic acid, first described by Greenberg (63). Jaenicke (64) has found that pig liver extracts formylate tetrahydrofolic acid in the presence of ATP to give the N^{10} derivative. Serine can also be a donor, giving rise to N^{10}-hydroxymethyltetrahydrofolic acid, which can be oxidized by DPN or TPN to the N^{10}-formyl compound. Using β-C^{14}-serine as a one carbon donor Jaenicke was able to show that the N^{10}-formyl compound appears quickly and that the N^5-formyl compound (citrovorum factor) appears after a delay, suggesting it is a further metabolite. Greenberg, Jaenicke, and Silverman (65) found that the N^{10} compound formylated 4-amino-5-imidazolecarboxamide to give inosinic acid. They also demonstrated the transition compound between the N^{10} and N^5 compounds, viz., the N^5-N^{10}-imidazolinium derivative of formyltetrahydrofolic acid.



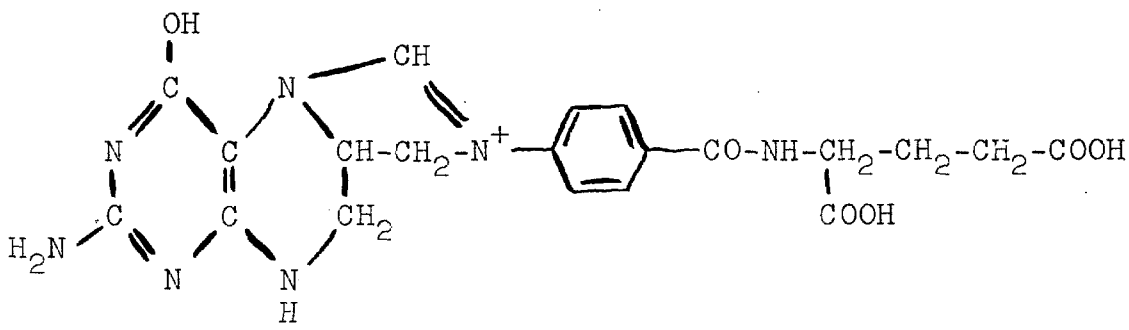
N^{10}-formylfolic acid



N^{10}-formyltetrahydrofolic acid



N^{5}-formyltetrahydrofolic acid



N^5-N^{10}-imidazolinium-formyltetrahydrofolic acid

The only in vitro evidence so far that histidine contributes to "active formate" is the recent publication of *Miller and Waelsch (66) of chromatographic evidence for the appearance of* N^{10}-*formyfolic acid when folic acid is incubated in the dark with liver homogenate in the presence of FIGA. The enzyme was inactivated by Dowex-2-chloride and reactivated by reduced folic acid. Interestingly, N-formyl-L-glutamic acid did not have such activity. They also demonstrated that pigeon liver extracts which incorporate* C^{14}-formate into N-formylglycinamide ribotide incorporate less radioactivity in the presence of FIGA, supporting the notion that FIGA is contributing formate via an active folic derivative rather than by forming free formate first.

The evidence so far accumulated is in favor of the acceptor of formate being tetrahydrofolic acid. There is good evidence that the acceptance of formate leads to N^{10}-formyltetrahydrofolic acid, while the acceptance of the

hydroxymethyl group leads to N^{10}-hydroxymethyltetrahydrofolic acid. There is no direct evidence for the nature of the complex formed when FIGA is the monocarbon donor but the possibility of N^{10}-formimino-tetrahydrofolic acid should be entertained. Presumably this could be transferred as such, hydrolyzed to give N^{10}-formyltetrahydrofolic acid, or, in the cases of Aerobacter and Clostridium at least, hydrolyzed to formamide and tetrahydrofolic acid.

Evidence for a formimino-folic complex has been provided recently by Sagers and co-workers (67). They studied the metabolism by crude extracts of Clostridium acidi-urici of another formimino compound, N-formimino-glycine, shown by Rabinowitz and Pricer (68) to be an intermediate in the degradation of xanthine by Clostridium cylindrosporum. Extracts degrade this compound to glycine, ammonia, and, presumably, formic acid. Extracts treated with Dowex-1-chloride are no longer active, but the addition of tetrahydrofolic acid restores activity. Furthermore, incubation with C^{14}-glycine in the presence of tetrahydrofolic acid resulted in equilibration of radioactivity between glycine and formiminoglycine. Therefore, an intermediate formimino-folic complex was suggested.

D. Present Status of the Problem

The present problem in histidine metabolism then is the conversion of FIGA to glutamic acid in animals, where it is known to occur *in vivo*. The reaction has not been demonstrated *in vitro* in animal tissues. A derivative of folic acid is presumed to be involved in the reaction, but nothing is known of the mechanism.

The present work was concerned with the degradation of histidine *in vitro*. The intermediate, FIGA, was obtained from histidine enzymatically and its degradation to glutamic acid has been demonstrated *in vitro* in a liver extract. The one carbon fragment is metabolized partly to carbon dioxide. The activity of the enzyme depends upon time of incubation, enzyme concentration, substrate concentration, temperature, and pH. Sulfhydryl groups and inorganic phosphate are involved in the reaction. A discussion of the pathway of the degradation of histidine to glutamic acid in organisms studied is presented with a view to a unified picture of histidine degradation.

II. EXPERIMENTAL DATA

A. The Degradation of Histidine by Mammalian Liver

1. The disappearance of diazo-reacting substances.

In a sample experiment the liver from a freshly killed guinea pig was homogenized in 20 ml. of 0.05 M potassium phosphate buffer, pH 8.0. The homogenate was centrifuged in the cold at 100,000 x g. The clear supernatant so obtained was incubated (37°C, with shaking) with an equal volume of a solution of L-histidine in the same buffer, with the addition of 2 or 3 drops of toluene. The final histidine concentration was usually 5.0 mg per ml, or 0.032 M. At intervals an aliquot was taken for reaction with diazotized sulfanilic acid, according to the method of Jorpes (69). Diazo absorption was measured spectrophotometrically at λ = 480 mμ. Fig. 1 shows the disappearance of diazo color with time. The average rate of degradation of histidine to a non-diazo reacting compound is about 1 micromole per ml per hour.

The degradation of histidine and that of urocanic acid by such a liver preparation were compared at pH 8.5. The diazo color disappeared more rapidly when urocanic acid was the substrate. This finding is consistent with the initial degradation of histidine to urocanic acid.

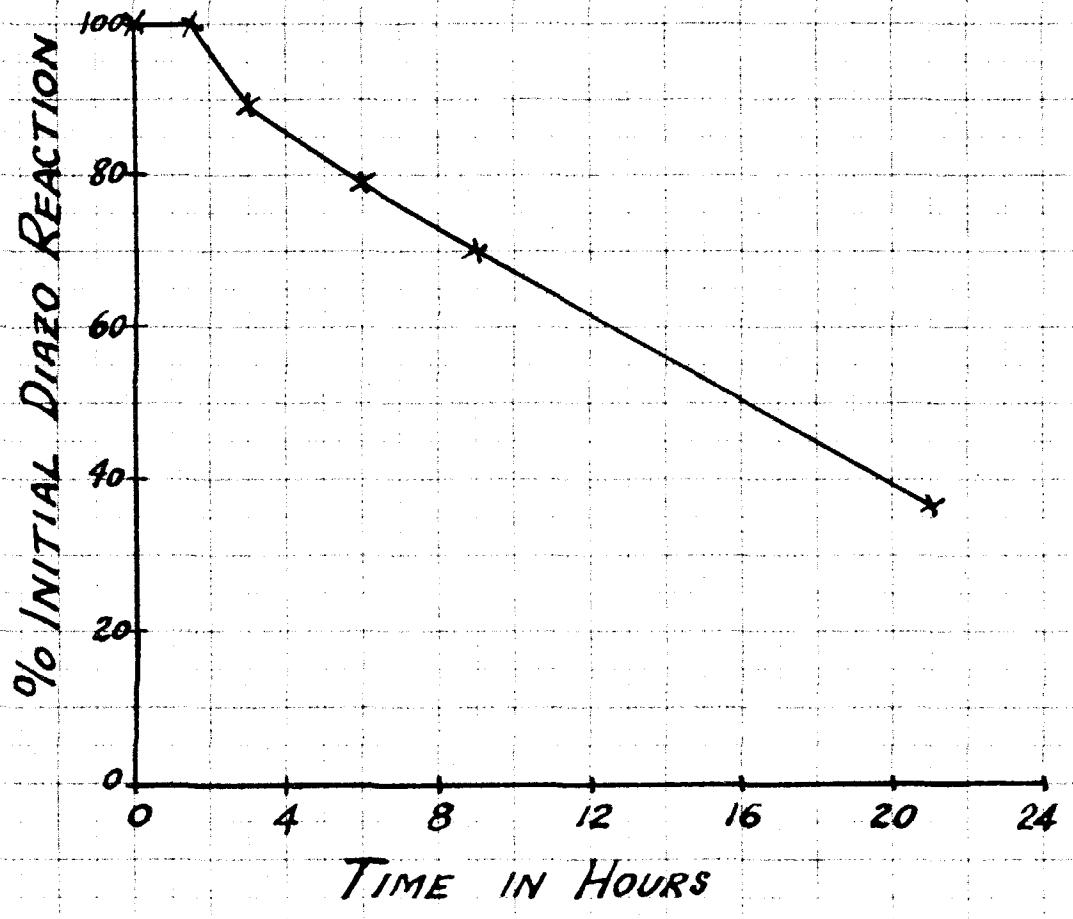


Fig. 1. Incubation of Histidine with Guinea Pig Liver Extract at pH 8.0. Disappearance of Diazo Reaction.

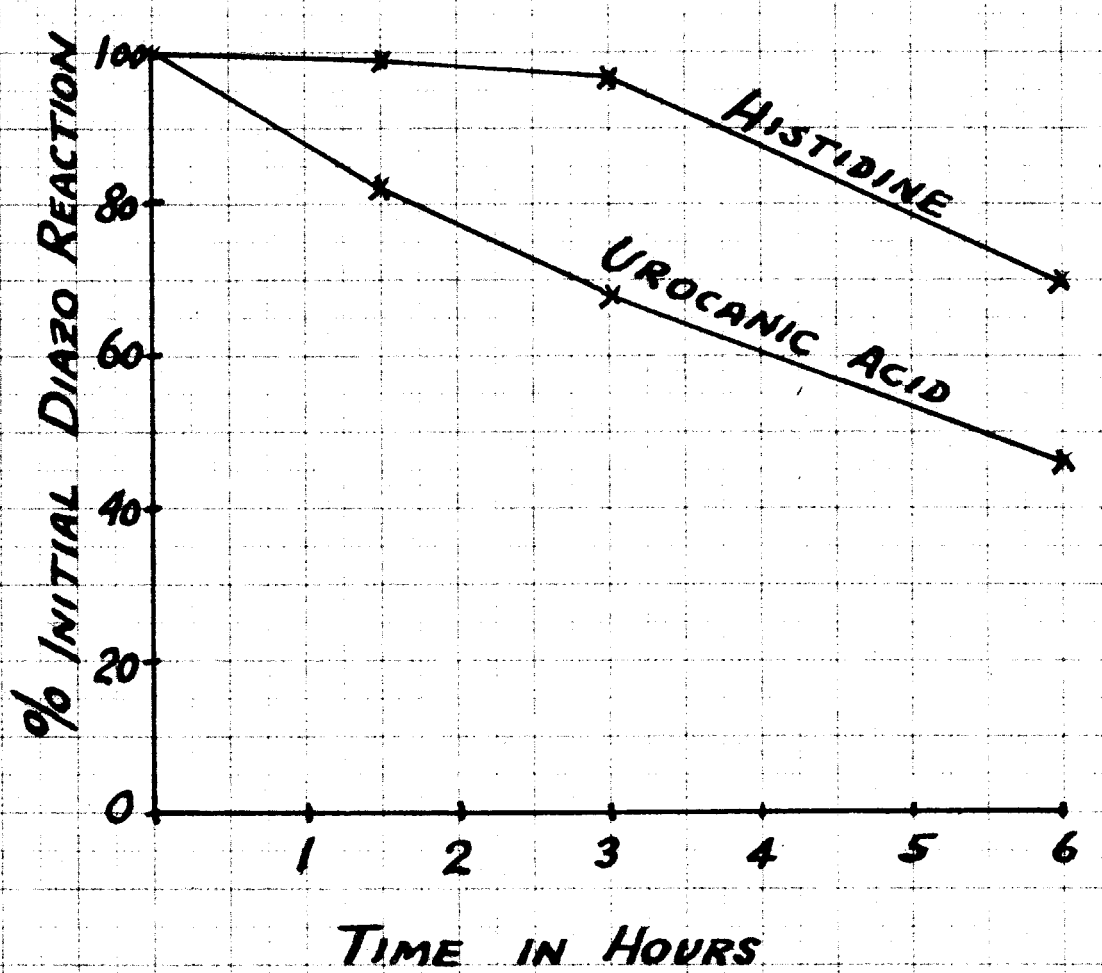


Fig 2. Incubation of Histidine and Urocanic Acid with Guinea Pig Liver Extract at pH 8.5.

From the above results it was concluded that the liver preparations used contained the enzymes histidase and urocanase. Further evidence for this conclusion was obtained by paper and column chromatography of incubation mixtures.

2. Paper chromatography of histidine incubation mixtures.

Incubation mixtures of the type described above were chromatographed on paper at 20°C. The most commonly used solvents were propanol-acetic (3 volumes of n-propanol and 1 volume 1.0 N acetic acid) and propanol-ammonia (3 volumes of n-propanol and 1 volume of 0.2 N ammonium hydroxide). Colors were developed by means of the following spray reagents: (a) diazo reagent (70), (b) ninhydrin reagent (1 mg ninhydrin per ml of pyridine), and (c) FCNP reagent (alkaline ferricyanide-nitroprusside reagent) (71). The last reagent is known to produce a pink, orange, or red color with guanidine, arginine, glycocyamine, and creatine, and a blue color with creatinine.

Unincubated liver extracts contain several substances reacting with ninhydrin, the only one of which compares in intensity to added histidine being one corresponding in R_f to glutamic acid. Histidine incubation mixtures showed the steady disappearance of the diazo and ninhydrin colors of histidine. Incubations at pH 9.0, but not 7.0, 7.5, or 8.0, revealed the appearance of a second diazo spot, ninhydrin negative,

identical in R_f with that of urocanic acid. Histidine and urocanic acid incubations both gave rise, particularly in the pH range 7.0-8.0, to a diazo negative, ninhydrin positive spot above and overlapping the glutamic acid in propanol-acetic chromatograms and superimposed on glutamic acid in propanol-ammonia chromatograms. Its purple ninhydrin color developed slowly over a period of hours, although more quickly if heated. The same area gave a definite red color with FCNP reagent. Its chromatographic behavior was identical to synthetic FIGA kindly supplied by Dr. Alexander Miller (19). (See Table 1 and Fig. 3 for summary of reactions). Unfortunately, this is not a very sensitive reaction, not detecting less than 20 micrograms of FIGA.

The histidine incubation mixture was subjected to paper electrophoresis. The paper was pressed with 0.1 M potassium phosphate buffer at pH 7.0 and suspended in a chlorbenzene bath. With a potential of 500 volts and a current of 3 milliamperes for $1\frac{1}{2}$ hours, histidine moved 11 mm toward the cathode while FIGA and glutamic acid moved 13 mm and 15 mm, respectively, toward the anode.

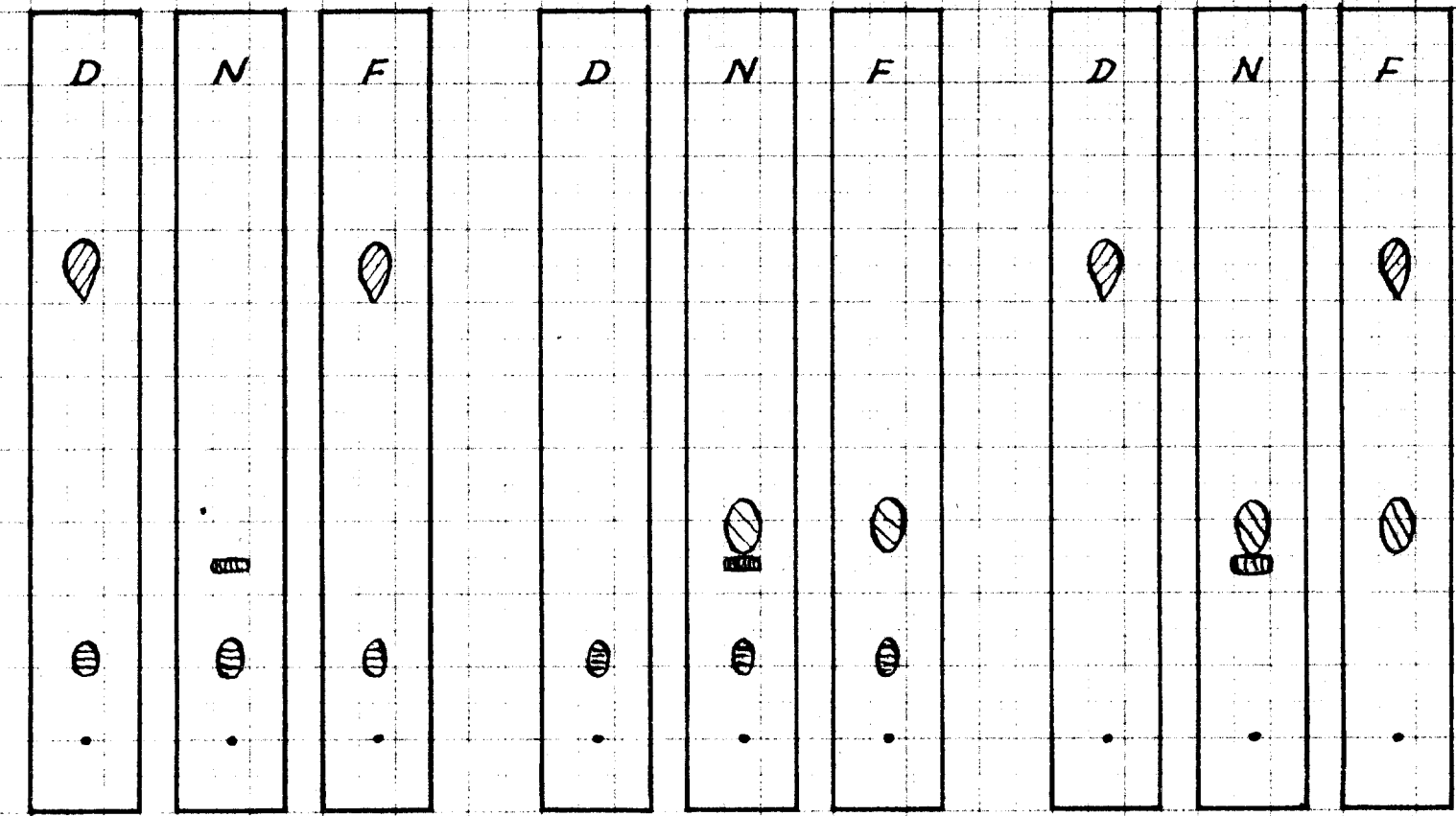
In another sample experiment soluble preparations of guinea pig (or rat) liver were incubated at pH 8.0 with 0.032 M L-histidine labeled in the carboxyl group with C^{14} (72). When such mixtures were chromatographed on paper the only new site of radioactivity coincided with the spot giving

Table 1.

Paper Chromatographic Data for

Histidine Metabolites

Compound	Solvent R_f (20°C)		Color reaction		
	propanol-acetic	propanol-ammonia	Diazo	Ninhydrin	FCNP
Histidine	0.11	0.22	red	brown	violet
Urocanic acid	0.65	0.33	red-orange	--	violet
FIGA	0.28	0.09	--	purple (delayed)	red
Glutamic acid	0.24	0.09	--	purple	--



HISTIDINE-pH 9 HISTIDINE-pH 8 UROCANIC ACID-pH8

FIG 3. PAPER CHROMATOGRAPHY OF HISTIDINE AND UROCANIC ACID INCUBATION MIXTURES IN PROPANOL – ACETIC SOLVENT AT 20°C.

D= DIAZO N= NINHYDRIN F= FCNP

the red FCNP color and delayed ninhydrin reaction.

These findings indicated that soluble guinea pig liver preparations degraded histidine and urocanic acid to the intermediate FIGA, and were therefore suitable for preparation of the latter. For the purpose of separation of FIGA from reaction mixtures column chromatography was used.

3. Column chromatography of histidine incubation mixtures.

In a sample experiment an incubation mixture of the type described above, containing $C^{14}OOH$-L-histidine, was precipitated from 5% trichloracetic acid and filtered. The filtrate was put on a column (0.9 cm diameter, 60 cm high) of the cation exchange resin, Dowex-50 (8% cross-linked, hydrogen form). The column was washed with water until the effluent was no longer acidic. This fraction was designated the front fraction. It contained trichloracetic acid where ether extraction was not performed. The absorbed material was then eluted with 1.5 N HCl, followed by 4.0 N HCl. Fractions of about 5 ml were collected by means of an automatic fraction collector. Aliquots from these tubes were dried on Tygon-coated copper disks under heat lamps and their radioactivity was measured in a Geiger-Müller end-window counter.

Fig. 4 shows the distribution of radioactivity among such fractions. Small amounts of radioactivity were found irregularly in the front fraction. Two radioactive fractions were eluted by 1.5 N HCl, and one eluted by 4.0 N HCl. The

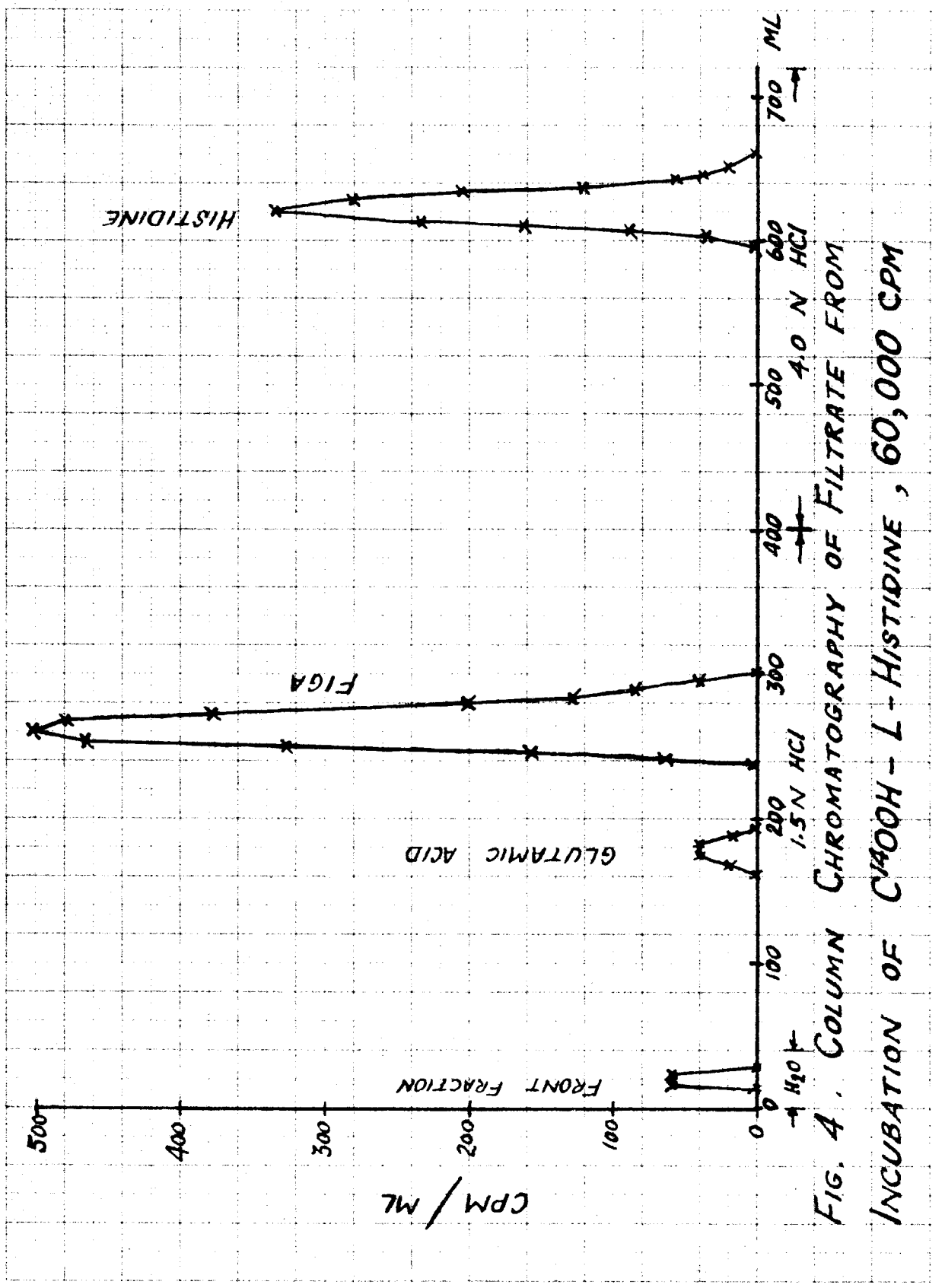


FIG. 4. COLUMN CHROMATOGRAPHY OF FILTRATE FROM INCUBATION OF $C_{14}OOH$ - L-HISTIDINE, 60,000 CPM

last fraction contained a compound with the color reactions and the R_f of histidine in two chromatographic solvents. The first, eluted at approximately 150 ml of 1.5 N HCl, was small. Its radioactive component was identical by paper chromatography with glutamic acid. The second, eluted at approximately 240 ml, was the largest in 18 hr. incubations. The radioactive component was identical by paper chromatography with FIGA. This relationship of radioactive peaks on column chromatography is similar to that described by Abrams and Borsook (23).

The radioactive component in the front fraction has not been identified. It has been obtained in small amounts after incubation with histidine labeled in the carboxyl carbon, the α-carbon, or all carbons. Its R_f in propanol-acetic was 0.60. It was ninhydrin and diazo negative. (N-formylglutamic acid had an R_f of 0.30 and gave a weak ninhydrin reaction). It was extractable into ether. The fraction containing this compound was refluxed for 4 hours in 6 N HCl. The refluxed solution was neutralized and rechromatographed on a Dowex-50 column. All the radioactivity appeared in the front fraction again. None appeared in the glutamic acid fraction (non-radioactive glutamic acid was added to locate this fraction). Similarly, heating on a boiling water bath in 6 N NaOH for $1\frac{1}{2}$ hours gave radioactivity eluted only at the front. It thus appears that the front fraction component was not N-formylglutamic acid.

The tubes comprising the FIGA fraction were almost completely free of substances reacting with ninhydrin, except the first tube. Therefore, all tubes save the first were pooled and the FIGA taken to dryness either by lyophilization or by vacuum distillation at 30-35°C. Water was added, the final pH being in the range 2-3. It was kept frozen for further use. Under these conditions there was no breakdown to glutamic acid over a period of months. Radioactive FIGA was prepared not only from $C^{14}OOH$-L-histidine, but also from α-C^{14}-D,L-histidine (California Foundation for Biochemical Research) and from uniformly labeled C^{14}-L-histidine (Schwarz Laboratories, Inc.). The latter was used most frequently. It had a specific activity of 72,000 cpm per mg or 11,200 cpm per micromole and, since the liver preparations had no appreciable histidine already present, it was assumed that the specific activity of the FIGA obtained was also 11,200 cpm per micromole.

5. Conclusions.

A soluble mammalian liver preparation in phosphate buffer degrades L-histidine. At pH 9.0, a major product is urocanic acid. At pH 7.0-8.5, the major product is FIGA. The latter is also formed when urocanic acid is the substrate.

FIGA is diazo negative, but gives a delayed ninhydrin reaction and positive FCNP test. It is anionic at neutral

pH, approximately to the same extent as glutamic acid, as judged by paper electrophoresis. It is a stronger cation than glutamic acid, as judged by cation exchange chromatography. It is identical by paper chromatography with synthetic FIGA. FIGA can be obtained relatively free from other ninhydrin-reactive compounds by means of the column chromatographic procedure described.

Glutamic acid is formed from histidine in these same liver preparations, but to a small extent, and possibly non-enzymatically. Another small fraction derived from histidine is recovered at the front by cation exchange chromatography. Its identity is unknown but it appears not to be a precursor of glutamic acid, as FIGA is.

B. The Degradation of FIGA by Mammalian Tissue

1. Enzymatic degradation of FIGA in liver preparations.

A reprint of a publication of evidence for the enzymatic degradation of FIGA (referred to in the reprint as α-formamidinoglutaric acid) to glutamic acid follows. As noted therein, a soluble rat liver preparation degraded radioactive FIGA to a new radioactive compound, identical on column and paper chromatography to glutamic acid. A boiled enzyme preparation had no activity at pH 7.8. FIGA is converted non-enzymatically to glutamic acid in the presence of strong alkali at room temperature.

(*Reprinted from Nature, Vol.* 176, *pp.* 830–831, *Oct.* 29, 1955)

Enzymatic Conversion of Alpha-Formamidinoglutaric Acid to Glutamic Acid by Rat Liver

A PREVIOUS report from this laboratory[1] gave evidence that histidine is converted to glutamic acid *in vivo* and *in vitro*. The *in vitro* experiment, done with guinea pig-liver slices, is subject to the criticism that degradation of α-formamidinoglutaric acid, the principal conversion product, may have occurred non-enzymatically. Direct evidence for such a conversion has been found so far only in micro-organisms adapted to histidine[2]. I have now shown the presence in rat liver of an enzyme which degrades α-formamidinoglutaric acid (N-formiminoglutamic acid) to glutamic acid.

Radioactive α-formamidinoglutaric acid was prepared by incubating homogenized rat liver with L-histidine-^{14}COOH (20,000 counts/min. per mgm. or 3,100 counts/min. per μmole) at 37° C. for 18 hr., precipitating the proteins with trichloracetic acid and separating the radioactive products by elution with hydrochloric acid from a column of 'Dowex-50' resin. The fraction containing α-formamidinoglutaric acid (200–280 ml. of 1·5 *N* hydrochloric acid) was lyophilized and stored in aqueous solution at *p*H 2–3. The radioactive α-formamidinoglutaric acid had an R_F on paper of 0·28 with 3 parts *n*-propanol/1 part 1·0 *N* acetic acid as solvent. It gave a delayed ninhydrin reaction (purple) and an immediately positive alkaline ferricyanide–nitroprusside reaction (pink)[3]. Synthetic α-formamidinoglutaric acid, kindly supplied by Dr. Alexander Miller[4], had identical R_F and colour reactions. α-Formamidinoglutaric acid is unstable in the presence of 1·0 *N* sodium hydroxide and is converted to a compound identical, by the above column and paper chromatographic procedures, with glutamic acid.

α-Formamidinoglutaric acid so prepared was converted to glutamic acid enzymatically by a soluble rat-liver preparation. A rat liver was homogenized at 0–4° C. in 20 ml. of 0·1 *M* potassium phosphate buffer, *p*H 7·8, and centrifuged at 100,000 *g* for 1 hr. The clear supernatant (protein nitrogen = 2·0–3·5 mgm./ml.) was incubated at 37° C. for 2 hr. in varying concentrations in a total volume of 1·0 ml. (0·1 *M*

in potassium phosphate buffer, *p*H 7·8) with 2,000 counts/min. (= 0·65 μmole) α-formamidinoglutaric acid. The reaction was stopped with trichloracetic acid, and the filtrate put on a small column (0·3 cm. × 55 cm.) of 'Dowex-50'. The column was eluted with 1·5 *N* hydrochloric acid, and fractions of 0·5 ml. were collected. These were evaporated to dryness and tested for radioactivity. Unaltered α-formamidinoglutaric acid was recovered in fractions 18–25. A radioactive peak in fractions 11–15 was identical on paper chromatography with glutamic acid, giving an immediate ninhydrin colour (purple) and no colour with alkaline ferricyanide – nitroprusside. No other radioactive peaks were obtained.

Enzyme activity was measured with enzyme (1) unmodified, in varying concentrations, (2) boiled for 10 min., (3) incubated in the presence of 0·01 *M* glutathione, (4) incubated in the presence of 0·0025 *M* *p*-chloromercurobenzoate, (5) pre-incubated 2 hr. at 37° C. with and without glutathione, and (6) incubated in the presence of folic acid (1×10^{-4} to 5×10^{-3} *M*) with glutathione. The percentage conversions to glutamic acid (calculated from amounts formed and α-formamidinoglutaric acid remaining) under various conditions are shown in Table 1.

It is concluded that rat liver contains an enzyme, which can be solubilized, and which catalyses the degradation of α-formamidinoglutaric acid to glutamic acid, without the accumulation of any intermediate. Similar homogenates degrade histidine to α-formamidinoglutaric acid much more rapidly. The enzyme which degrades α-formamidinoglutaric acid is unstable, but less so in the presence of glutathione. Low activity and instability of this enzyme probably account for the accumulation of the acid when histidine is incubated with liver homogenate. Folic

Table 1. Conversion of α-Formamidinoglutaric Acid to Glutamic Acid

Enzyme	Protein nitrogen (mgm.)	Percentage conversion to glutamic acid
None	0	0
Boiled	0	0
Unmodified	1·0	100
Unmodified	0·6	83
Unmodified	0·5	65
Unmodified	0·3	36
Unmodified	0·2	20
Glutathione added	0·3	74
Folic acid + glutathione	0·3	0
p-Chloromercurobenzoate	1·0	22
Preincubated	1·0	33
Preincubated + glutathione	1·0	85

acid inhibits the enzyme, thereby raising the question whether it is acting as an analogue of a coenzyme such as tetrahydrofolic acid.

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Many experiments subsequent to this publication were performed with 1.0 micromole of FIGA in a 1.0 ml incubation mixture, the filtrates being separated by chromatography on larger columns (0.6 cm x 50 cm) of Dowex-50. Fractions of 3 ml were collected and aliquots taken to locate radioactive peaks. The tubes of a peak were then pooled and an aliquot taken to measure the total radioactivity of the peak. Glutamic acid was consistently eluted at 24-30 ml of 1.5 N HCl, while FIGA was eluted at 40-48 ml of 1.5 N HCl.

Since glutamic acid has but 5 carbon atoms, 9.3×10^3 cpm ($\frac{5}{6} \times 11.2 \times 10^3 = 9.3 \times 10^3$) correspond to one micromole of FIGA converted to glutamic acid. The sum of the micromoles of radioactive FIGA and glutamic acid accounted in all short term incubations for the 5 carbon "backbone" of the initial FIGA.

2. Tissue specificity.

In addition to a rat liver preparation, homogenates of the kidneys, the spleen, and the heart of a rat were made in 3.0 ml, 3.0 ml., and 2.0 ml, respectively, of 0.1 M potassium phosphate buffer at pH 7.5. Incubations were carried out at 37°C and pH 7.5 for one hour in the presence of 0.01 M glutathione and with one micromole of radioactive FIGA. The following results were obtained by column chromatography:

Tissue	Micromoles glutamic acid formed
liver	0.70
kidney	0.00
spleen	0.00
heart	0.00

Although only a few organs were tested, the degradation of FIGA to glutamic acid apparently occurs only in liver. This is not surprising in view of the fact that this is the only organ found by Edlbacher (6-9) to degrade histidine to this intermediate. Since the findings of Abrams and Borsook (23) show that FIGA does not accumulate in the liver in vivo, it is probable that other organs are never presented with this substrate. These results are in contrast to those reported by Goriukhina (73), who states that FIGA is not degraded by liver, but is by kidney. The kidney reaction is stated to give an unidentified amino compound. His statement that histidine administration fails to give urinary FIGA because of the degradation of the latter by renal enzymes is not consistent with the finding of Abrams and Borsook (23) that glutamic acid was the only radioactive metabolite of histidine in liver filtrates 20 minutes after histidine injection.

3. Glutamic acid as a product of the reaction.

As noted above, the product of the reaction was identical by paper and column chromatography to glutamic acid.

In addition 8000 cpm (less than 1 mg) of the product obtained by column chromatography were taken to dryness and 500 mg of L-glutamic acid hydrochloride were added. This was crystallized three times from ethanol by the addition of ether. The following specific activities were found:

Sample	Weight (mg)	Specific activity (cpm/mg)
Original	500	16.0 (calculated)
1st crystallization	210	14.6
2nd "	113	14.1
3rd "	51	14.2

Since glutamic acid is the only radioactive product in short term incubations of FIGA labeled in the carboxyl or α-carbon, the conversion appears to occur in one step. Of course, this does not rule out a two-step reaction in which the first is rate-limiting.

4. Fate of the monocarbon fragment.

The only studies on the fate of the monocarbon fragment were done utilizing the uniformly labeled FIGA. In a sample experiment one micromole of FIGA (11,200 cpm) was incubated in 1.0 ml at pH 7.5 with the soluble liver preparation. The incubation was carried out in a Warburg flask with 0.20 ml of 6 N KOH in the center well and 0.14 ml of 35% trichloracetic acid in the sidearm. At the end of 100 minutes at 37°C the acid was tipped in and the mixture incubated 30

minutes to absorb CO_2 in the center well. The contents of the center well were removed, and potassium carbonate was added. Barium chloride was added and the precipitate of barium carbonate was centrifuged, washed with water, alcohol, and ether, dried and measured for radioactivity. The $BaCO_3$ contained 562 cpm (corrected), which represents 0.30 μmole of the one micromole of one carbon fragment present initially ($\frac{562}{\frac{11,200}{6}} = 0.30$). The incubation mixture was fractionated as usual by column chromatography. The glutamic acid fraction contained 9100 cpm, corresponding to 0.98 micromoles of FIGA converted ($\frac{9100}{\frac{11,200 \times 5}{6}} = 0.98$). There was no radioactivity in the fraction usually containing FIGA. The front fraction gave but 160 cpm. The radioactive material did not distill in vacuo, as formic acid would have done. The major part of the one carbon radioactivity, about 0.6 micromole, was not accounted for.

When uniformly labeled glutamic acid, recovered from previous experiments, was so incubated, no radioactive CO_2 was recovered, providing evidence that the above CO_2 radioactivity really was derived from the one carbon fragment. The boiled enzyme control with FIGA produced no radioactive CO_2 either. When 0.01 M sodium formate (non-radioactive) was added, only 0.10 micromole of monocarbon radioactivity was recovered in the front fraction, although 0.96 micromoles of glutamic acid were formed. The addition of 0.01 M formamide had no effect.

5. Conclusions.

FIGA is degraded to glutamic acid by liver, but not by kidney, spleen, or heart. The one carbon unit is metabolized partly to CO_2, but no intermediates have been identified. Inhibition of CO_2 production by added sodium formate but not by formamide, suggests that the former is more closely related to the intermediate than the latter. Difficulty in locating other products of the one carbon fragment might be due to its assimilation into compounds known to incorporate formate.

C. Variables in the Enzymatic Degradation of FIGA

1. Time.

Incubation tubes containing 0.7 ml of liver preparation and 1.0 micromole of FIGA per ml were run for varying times at pH 7.5 and 37°C, with the following results (see also Fig. 5).

Time (minutes)	Micromoles glutamic acid formed
0	0.00
20	0.25
40	0.42
60	0.54
150	0.70
240	0.85
240 (boiled enzyme)	0.00

Enzymatic activity falls off steadily with time, which is to be expected with the progressive decrease in substrate

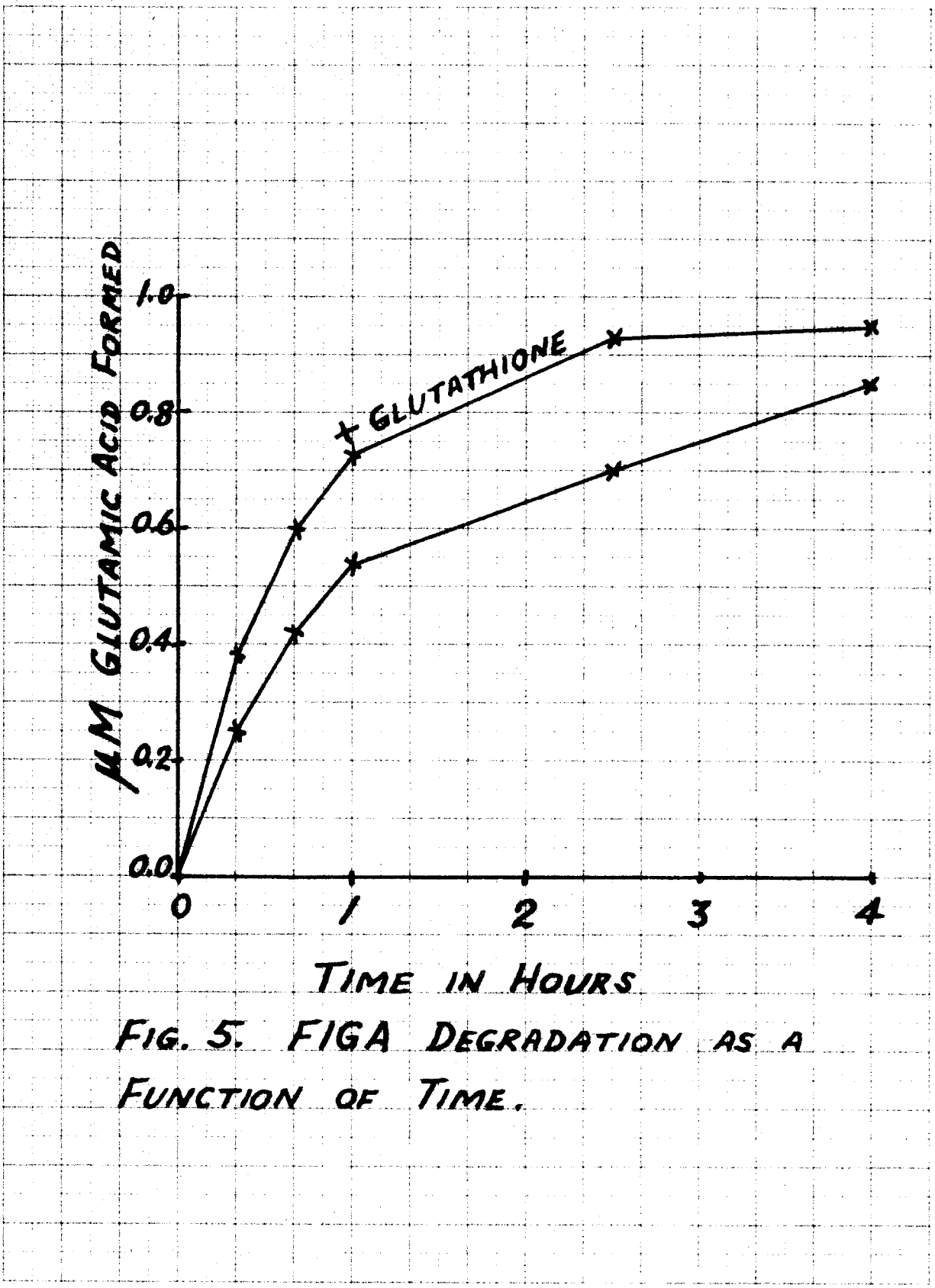


FIG. 5. FIGA DEGRADATION AS A FUNCTION OF TIME.

concentration. However, since preincubation causes a decrease in enzymatic activity, part of the decrease with time may be due to enzyme instability.

2. Enzyme concentration.

As noted in the reprint the rate of reaction depends on enzyme concentration. When one micromole quantities of substrate were incubated with varying amounts of enzyme at pH 7.5 for 1 hour at 37°C in the presence of 0.01 M glutathione, the following results were obtained (see Fig. 6 also):

ml Liver preparation per ml reaction mixture	Micromoles glutamic acid formed
0.00	0.00
0.10	0.08
0.20	0.22
0.30	0.47
0.40	0.57
0.50	0.71
0.70	0.73

There is an obvious dependence upon enzyme concentration, although the curve is not linear. The only significant point is the falling off at higher concentration, which is most likely due to the diminished substrate concentration. Zero time studies were not done because of difficulty in measuring slight reactions.

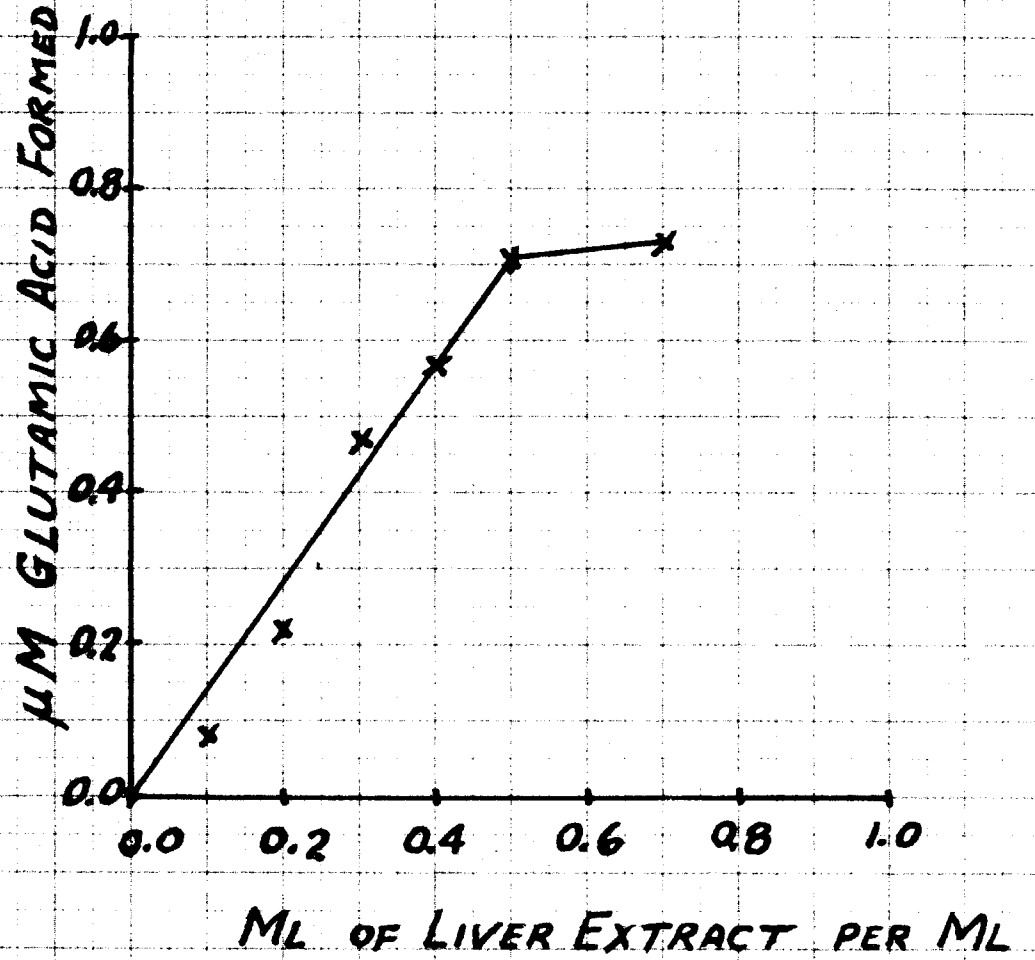


FIG. 6. FIGA DEGRADATION AS A FUNCTION OF ENZYME CONCENTRATION

3. Substrate concentration.

Study of dependence upon substrate concentration was hampered by the difficulty in measuring differences between reactions of small extent. Incubations for 1 hour at 37°C and pH 7.5 in the presence of 0.01 M glutathione gave the following results (see also Fig. 7):

Micromoles FIGA initially present	Micromoles glutamic acid formed
0.10	0.10
0.30	0.28
0.50	0.45
1.00	0.73

These data are most readily explained by the smaller number of free enzyme molecules available at higher substrate concentrations.

4. Temperature.

Incubations were run at different temperatures for 1 hour at pH 7.5, in the presence of 0.01 M glutathione, with 1 micromole FIGA and 0.7 ml enzyme per ml, with the following results (see also Fig. 8):

Temperature	Micromoles glutamic acid formed
4°C	0.03
10	0.29
18	0.63
26	0.67
37	0.71
46	0.21

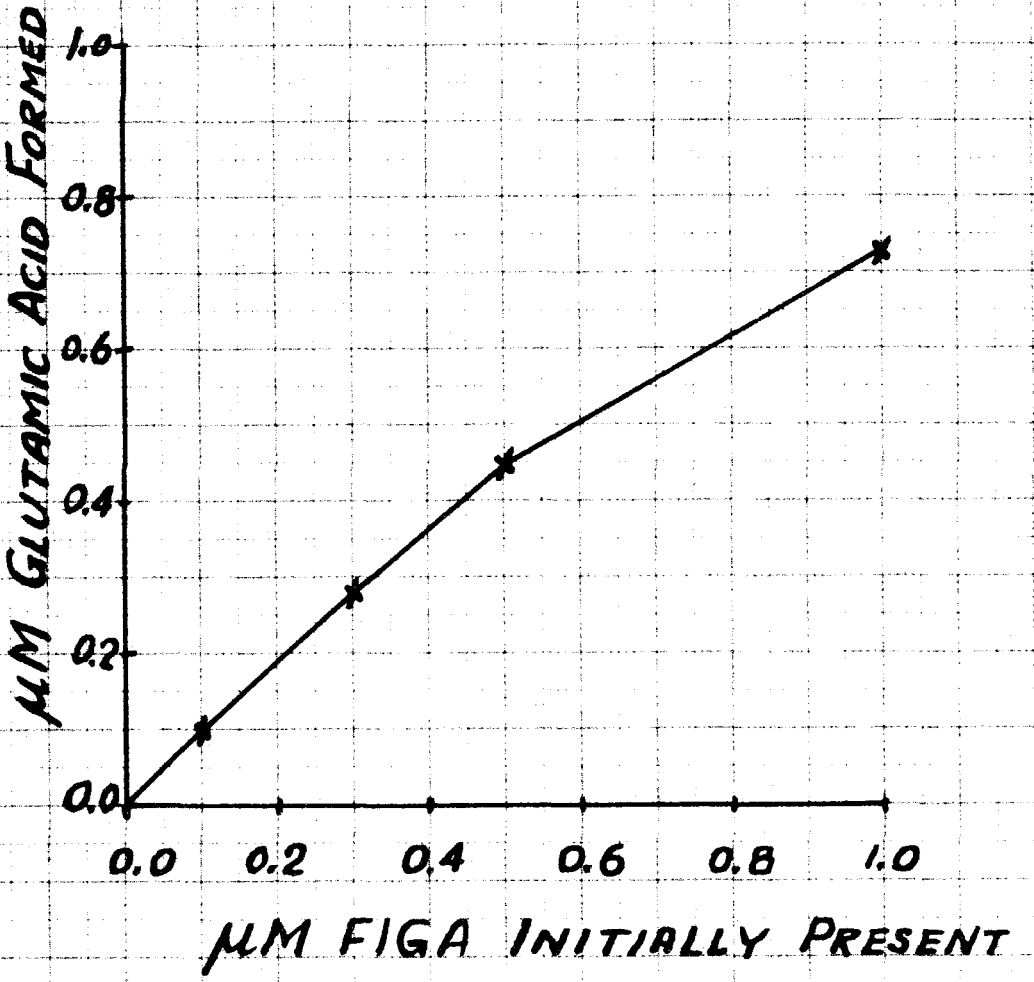


FIG. 7. FIGA DEGRADATION AS A FUNCTION OF SUBSTRATE CONC.

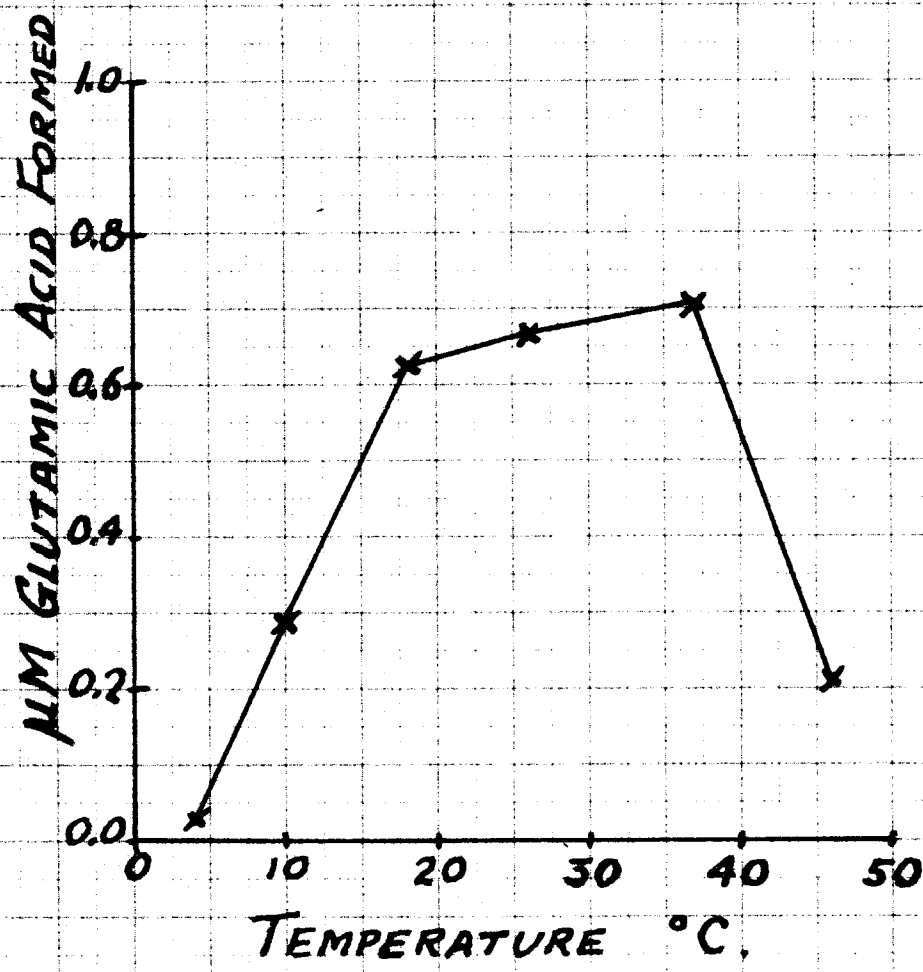


FIG. 8. FIGA DEGRADATION AS A FUNCTION OF TEMPERATURE.

The extent of the reaction in 1 hour increases with temperature in the lower range, reaches a plateau in the middle range, and then falls off sharply over 40°C. The values are not initial rates, so they cannot be used to calculate the temperature optimum. It has already been pointed out that the enzyme(s) has an appreciable rate of inactivation at 37°C, and it may be presumed that this rate is even greater at 46°C. This set of circumstances is reminiscent of catalase inactivation with temperature. Morgulis, Beber, and Rabkin (74) showed that catalase is rapidly inactivated in the presence of H_2O_2 in the middle temperature range. At a concentration of 0.90 N H_2O_2 the following relative catalase activities were found at various temperatures:

Temperature	Activity
0	29.8
8	61.4
19.4	57.7
30	42.7

But when the reaction was studied for very short time intervals at low H_2O_2 concentration, Sizer (75) found that initially the reaction is linear with time at each temperature, and that the temperature of optimal activity is 53°C, above which there is rapid decline in activity.

5. pH.

There is enzymatic activity over a broad pH range with an optimum around 8.0. The following data (see also Fig. 9) show the extent of reaction with 0.7 ml enzyme and 1 micromole substrate per ml, in the presence of 0.01 M glutathione at 37°C for 1 hour:

pH	Micromoles glutamic acid formed	Micromoles glutamic acid formed enzymatically
6.0	0.03	0.03
6.5	0.05	0.05
7.0	0.55	0.55
7.5	0.71	0.71
8.0	0.89	0.89
8.5	0.64	0.64
8.5 (boiled)	0.00	--
9.0	0.56	0.51
9.0 (boiled)	0.05	--
9.5	0.32	0.17
9.5 (boiled)	0.15	--

An appreciable non-enzymatic reaction occurs in short-term incubations only at a pH greater than 8.5.

6. Conclusions.

The conversion of FIGA to glutamic acid by liver preparations shows the characteristic dependence of enzymes upon time, enzyme concentration, substrate concentration, temperature, and pH. Initially the reaction is of the order of magnitude of one micromole per hour per ml with the same kind of incubation mixture which degrades histidine to FIGA at an average rate of one micromole per hour for a twenty

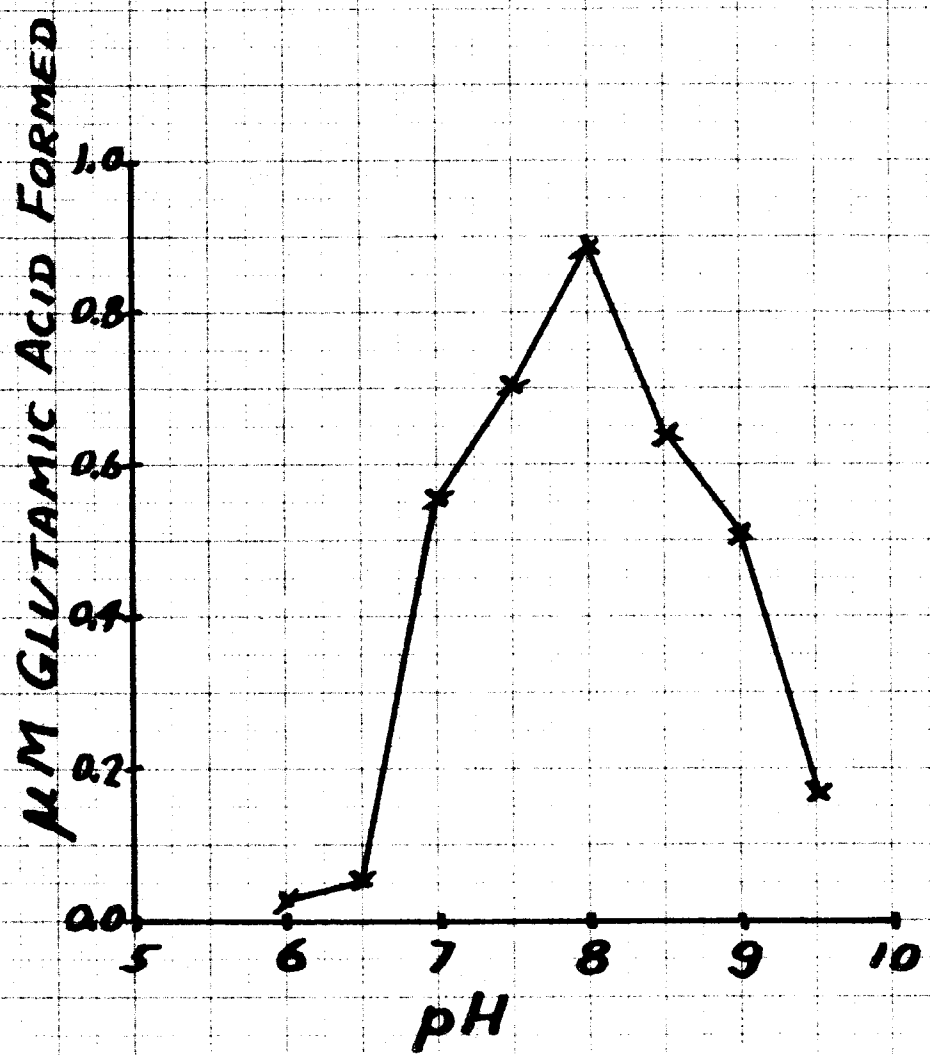


FIG. 9. FIGA DEGRADATION AS A FUNCTION OF pH.

hour period. A loss of activity on preincubation and the accumulation of FIGA in 24 hour histidine incubation mixtures indicate an instability of the enzyme(s) responsible for the degradation of FIGA to glutamic acid.

D. The Effect of Other Agents upon FIGA Degradation

1. Sulfhydryl groups.

Incubations were almost always carried out in an atmosphere of air, but neither a nitrogen atmosphere nor 0.01M potassium cyanide affected the reaction. As noted in the reprint, there was an effect of sulfhydryl groups. The effect of glutathione in a time study was as follows (performed with the previously noted time study - see fig. 5).

	Micromoles of glutamic acid formed	
Time (minutes	No glutathione	0.01 M glutathione
0	0.00	0.00
20	0.25	0.38
40	0.42	0.60
60	0.54	0.73
150	0.70	0.93
240	0.85	0.95

The effect of sulfhydryl groups is also seen in an experiment performed with 0.7 ml liver extract and 0.50 micromole FIGA for 1 hour at 37°C and pH 7.5, with additions as listed:

Addition	Micromoles glutamic acid formed
None	0.42
Glutathione 0.01M	0.50
p-Chloromercurobenzoic acid 0.0025M	0.00

While glutathione had a protective effect, the sulfhydryl inhibitor, p-chloromercurobenzoic acid, completely inhibited the reaction.

To summarize, the stimulatory effect of glutathione shown in the time curve, the conserving effect of glutathione during preincubation, and the inhibitory effect of p-chloromercurobenzoic acid constitute evidence that sulfhydryl groups are essential for enzymatic activity.

2. Phosphate and related compounds.

All enzyme preparations so far mentioned were made in potassium phosphate buffer. In order to test whether the inorganic phosphate itself was having an effect an enzyme preparation was made in 0.1M trihydroxymethylaminomethane ("tris") buffer at pH 7.5. Such a preparation was found to have 151 micrograms of inorganic phosphate per ml (= 5 micromoles per ml) (76). Incubations were made with 0.7 ml "tris" enzyme (about 3.5 micromoles phosphate), 0.01 M glutathione, 1.00 micromole of FIGA, and varying

amounts of added phosphate buffer. After 30 minutes at 37°C and pH 7.5 the following results were obtained (see also Fig. 10).

Micromoles phosphate added	Micromoles glutamic acid formed
0.0	0.19
5.0	0.16
10.0	0.18
20.0	0.30
30.0	0.45
50.0	0.80

The "tris" enzyme is clearly less active than similar phosphate preparations and the addition of orthophosphate is stimulatory. After an initial plateau at low concentrations there is an almost linear effect of further additions of phosphate. The relationship between phosphate addition and substrate degradation is not stoichiometric, although with a crude enzyme preparation this means little. The effect certainly implicates phosphate in the reaction. Phosphorolysis of a labile compound is one possibility, in which case arsenolysis might also occur. Consequently a parallel experiment was run with the same enzyme preparation, with additions of arsenate (sodium arsenate, pH 7.5). The results were (see also Fig. 10):

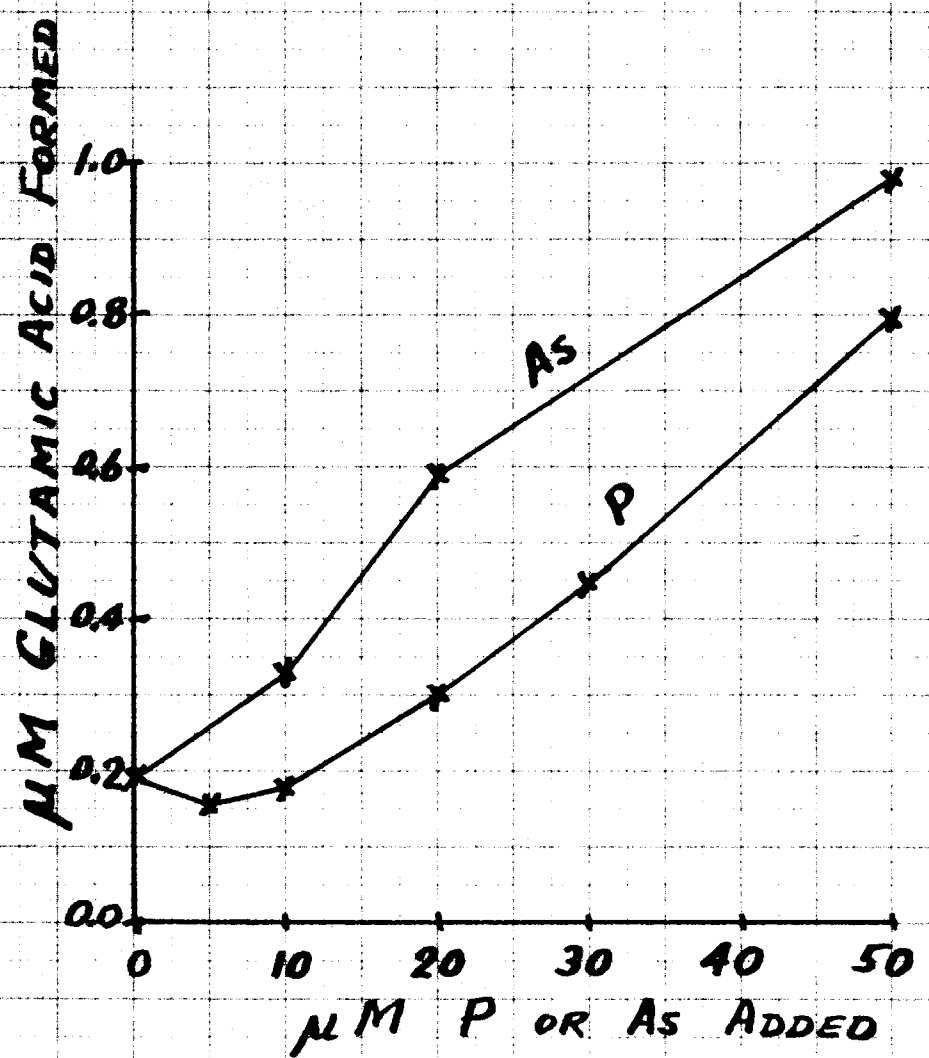


FIG. 10. FIGA DEGRADATION AS A FUNCTION OF ADDED PHOSPHATE OR ARSENATE.

Micromoles arsenate added	Micromoles glutamic acid formed
0.0	0.19
10.0	0.33
30.0	0.59
50.0	0.98

Arsenate then has an even greater effect than phosphate, lending support to the idea that the normal enzymatic degradation of FIGA involves a phosphorolysis which can be substituted by an arsenolysis.

If a phosphorolysis occurs which involves the formation of a high-energy phosphate intermediate, it is possible that adenosinetriphosphate (ATP) is synthesized in a subsequent reaction involving the transfer of a phosphate to adenosinediphosphate (ADP). The following results were obtained on incubating 0.7 ml phosphate enzyme in 1 ml, with 0.01M glutathione and 1.00 micromole FIGA, for 30 minutes at 37°C and pH 7.5.

Addition	Micromoles glutamic acid formed
None	0.42
ADP, 0.01M	0.44
ATP, 0.01M	0.45

The lack of effect of either ADP or ATP does not permit any conclusions about ATP synthesis during the reaction.

Since fluoride ion interferes with some reactions involving phosphate transfer, another experiment was set up designed to demonstrate a differential effect on phosphate and arsenate. "Tris" enzyme, 0.5 ml, was incubated with 50 micromoles of either phosphate or arsenate and with 0.01M glutathione and 1.0 micromole FIGA at 37°C for 1 hour at pH 7.5. The effect of 0.01M sodium fluoride was as follows:

Addition	Micromoles glutamic acid formed
Phosphate	0.90
" + fluoride	0.95
Arsenate	0.95
" + fluoride	0.97

There is no inhibition of either phosphate or arsenate by fluoride.

The above findings then are evidence that a phosphorolysis is involved in the <u>in vitro</u> degradation of FIGA to glutamic acid. Whether ATP is synthesized or not requires direct evidence, either by direct measurement of an ATP increase or by incorporation of P^{32} into ATP during the reaction.

3. Monocarbon acceptors.

Only a few experiments were performed to test the effect of monocarbon acceptors. The inhibitory effect of added folic acid was noted in the reprint. Another preparation of folic acid, at 0.001M, gave a 0.12 micromole conversion while the control gave a 0.38 conversion (from 1.00 micromole FIGA). It is quite possible that an oxidation product of folic acid was a contaminant and inhibited the enzyme. Leucovorin (=folinic acid - SF = N^5-formyltetrahydrofolic acid), 0.001M, gave a 0.38 micromole conversion, showing neither inhibition nor stimulation.

Since the reaction proceeds without any additions, necessary cofactors must be present to some extent. It was thought therefore that the addition of a compound known from in vivo studies to accept the formimino carbon of FIGA might enhance the reaction. As stated in the introduction, 4-amino-5-imidazolecarboxamide ribotide is such an acceptor. This not being available, free 4-amino-5-imidazolecarboxamide was added. This compound had no effect when incubated with 0.58 micromoles of substrate:

Addition		Micromoles glutamic acid formed
None		0.30
4-amino-5-imidazole-carboxamide	0.01M	0.28
4-amino-5-imidazole-carboxamide	0.001M	0.29

In view of the reported bacterial system for transferring the formimino group from N-formimino-glycine to tetrahydrofolic acid (67) glycine was incubated in the present system, again with no effect when 1.0 micromole FIGA was present:

Addition	Micromoles glutamic acid formed
None	0.42
Glycine 0.01M	0.37

4. Attempted enzyme purification.

Dialysis of the liver extract was attempted, but prolonged standing of the enzyme at 4°C alone caused marked loss of activity. Therefore dialysis was performed against large volumes of 0.1M phosphate buffer in the presence of 0.001M glutathione for short periods. In a sample experiment 2 ml of liver extract were dialyzed against 100 ml of buffer twice for one hour periods, with the following results when incubated with 0.58 micromole FIGA in the presence of 0.01M glutathione.

	Micromoles glutamic acid formed
Control	0.46
Dialyzed prep.	0.40

This indicated that no readily dialyzable cofactor was present and it also indicated that dialysis might not be

a limiting factor for ammonium sulfate fractionation.

In a preliminary experiment the protein was precipitated from a phosphate liver extract by saturation with ammonium sulfate. The dialyzed (1 hour x 3) precipitate was redissolved in the original volume and tested for activity with 0.32 micromoles FIGA, with the following result:

	Micromoles glutamic acid formed
Control	0.32
Precipitated	0.07

Loss of activity could have been due to inhibition by any residual ammonium or sulfate ions. Despite the extensive loss of activity a fractionation was performed, giving 3 fractions: (1) 0 -30% saturation--yellowish precipitate, (2) 30-60% fraction--reddish precipitate, and (3) 60-100% fraction--bright red precipitate. The 3 dialyzed precipitates were tested for activity as above with the following results:

	Micromoles glutamic acid formed
Control	0.32
0-30% fraction	0.02
30-60% "	0.06
60-100% "	0.00

This is obviously not satisfactory for purification.

5. Conclusions.

The degradation of FIGA by liver extracts involves sulfhydryl groups and phosphate. A phosphorolytic reaction is suggested by the arsenate effect. No information has been gained about the role of a folic acid derivative as a catalyst. Dialysis against phosphate buffer in the presence of glutathione for short terms does not remove any cofactors.

III. DISCUSSION

The degradation of histidine to glutamic acid has been studied in mammals and microorganisms. The general pathway is described in 3 steps:

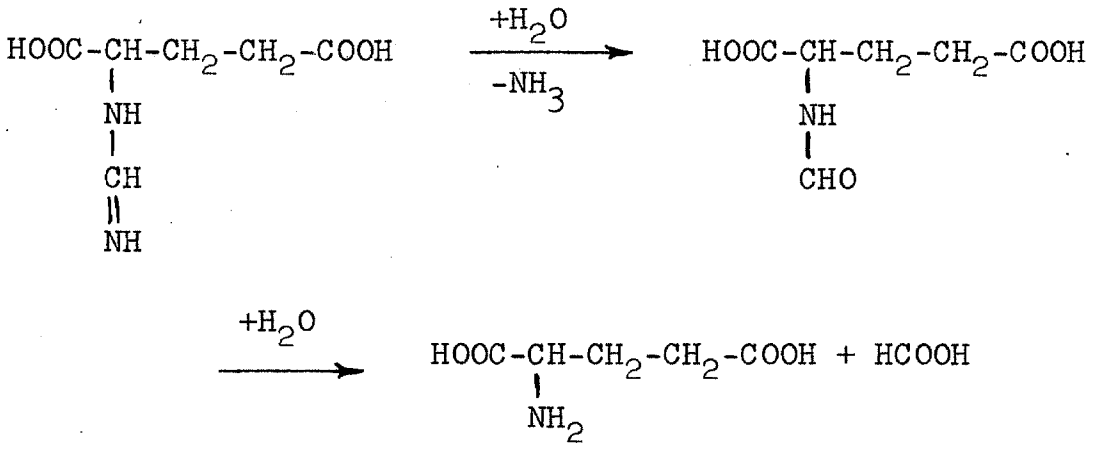
(1) Histidine ⟶ urocanic acid

(2) Urocanic acid ⟶ N-formiminoglutamic acid (FIGA)

(3) N-formiminoglutamic acid (FIGA) ⟶ glutamic acid.

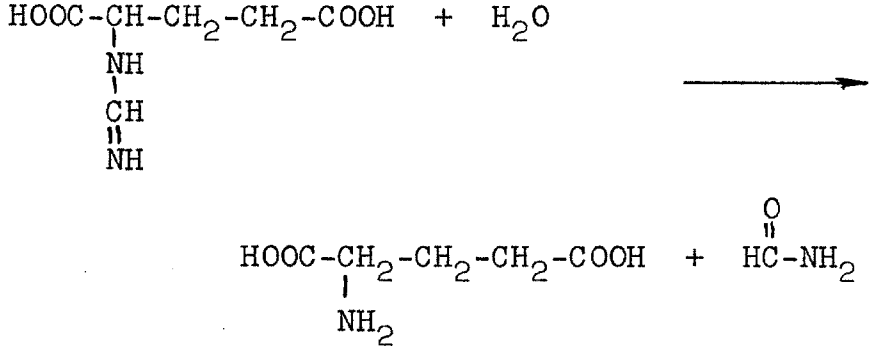
The first two steps each represent a single enzymatic reaction as far as is known, and their product is certainly FIGA in mammals (18-20), Pseudomonas fluorescens (26), and Aerobacter aerogenes (27, 77), and probably FIGA in Clostridium tetanormorphum (28). Both reactions are hydrolytic and one mole of ammonia is formed for each mole of histidine degraded to FIGA.

The third step is a different matter. The first organism for which the pathway was elucidated was histidine-adapted Pseudomonas fluorescens (13, 14, 26), in which the reaction occurs in two steps, with the intermediate formation of N-formyl-L-glutamic acid:



The first enzyme is activated by sulfhydryl groups, while the second is inhibited by them. Ferrous ion activates the second, deformylating enzyme. No other cofactors are known. The extracts were made with phosphate buffer, but the incubations were performed in barbital buffer. It is not known whether a folic acid derivative is involved in the reaction. It has been supposed (26) that each step is hydrolytic.

Another organism that has been studied is histidine-adapted Aerobacter aerogenes (27, 77). This organism degrades FIGA to glutamic acid in one step, with the formation of formamide from the formimino group:



Formamide is not metabolized further. The incubations were performed in phosphate buffer, but there is no study of the effect of phosphate. There is also no information about co-factors, such as a folic acid derivative. The reaction was assumed to be hydrolytic.

A third organism that has been studied is Clostridium tetanormorphum (28), which degrades histidine and urocanic acid (FIGA was not studied) to glutamic acid with the formation of formamide. In this case there is also some formation of formic acid and ammonia. Incubations were performed with washed cell suspensions in phosphate buffer. Again in this case there is no information about cofactors, and the reaction was assumed to be hydrolytic.

As noted in the introduction, in vivo studies demonstrated the conversion of histidine to glutamic acid in mammals (23, 25). The formimino carbon has been found to follow the path of formate to CO_2 (42, 43), purines (34-36), serine (36, 43), and methyl groups (34-36, 44). In vivo studies also demonstrated that FIGA was excreted in the urine by folic acid-deficient rats (47-50). On the other hand, in vitro studies on the degradation of histidine by mammalian liver showed the accumulation of FIGA as the end-product (18-20). Studies on the degradation of the latter have been negative (73, 78, 79).

The present work establishes the fact that a mammalian

(rat) liver extract degrades FIGA enzymatically to glutamic acid, without the accumulation of any intermediate. The fate of the formimino group is uncertain, although CO_2 is one product. Kidney, spleen, and heart extracts are inactive. Miller (66, 80) has obtained chromatographic evidence for the appearance of N^{10}-formylfolic acid when liver extracts are incubated with FIGA and folic acid. Dialyzed and Dowex-2-treated extracts lose this ability but are reactivated by reducing agents (glutathione, ascorbic acid) and dihydrofolic acid. This finding is in keeping with the in vivo studies and suggests that the formimino carbon is metabolized via a formylfolic acid compound. The manner in which the carbon and nitrogen in the formimino group are separated is unknown, although they must be separated in animals in order to account for the in vivo data on the fate of the amidine carbon of histidine. There is no evidence that N-formylglutamic acid is an intermediate, glutamic acid being the only 5-carbon product. If it were, and if folic acid is always concerned with monocarbon transfers, then N-formylglutamic acid, rather than FIGA, should accumulate in folic acid deficiency. This suggests that the amino group is removed, either enzymatically or non-enzymatically, after the formimino group has been transferred to the proper folic acid derivative. This conclusion demands the

existence, at least transiently, of a formimino-folic acid compound. As mentioned before, Sagers and co-workers (67) have shown that N-formimino-glycine is degraded to glycine by extracts of Clostridium cylindrosporum, with tetrahydrofolic acid as apparent cofactor. The equilibration of radioactivity between N-formiminoglycine and glycine suggested to these workers the formation of N-formimino-tetrahydrofolic acid.

The enzyme(s) responsible for converting FIGA to glutamic acid is unstable. The stimulation of the reaction by sulfhydryl groups suggests the possibility that enzyme instability may be related to oxidation of sulfhydryl groups. These groups may be concerned with the transfer, on the enzyme, of the formimino group from FIGA to a firmly bound folic acid cofactor. This function of the sulfhydryl groups would be analogous to their function in acyl transfer reactions.

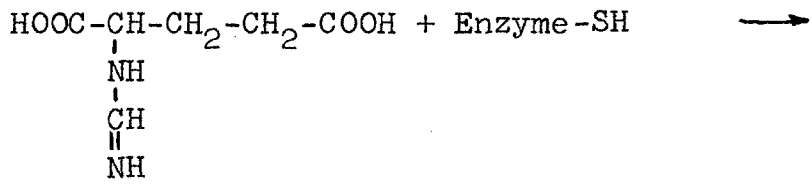
The requirement for phosphate and the effect of arsenate mean that the reaction is not a simple hydrolytic one. It is possible that a formimino linkage, either with sulfur or with a folic acid derivative, on the enzyme surface could be cleaved by phosphorolysis or arsenolysis. The formyl- or formimino-, phosphate so formed would be reactive and could be expected to transfer either the monocarbon unit or the phosphate to an acceptor, in the latter case possibly ADP. It is pertinent that ATP is necessary for the reaction

of formate with tetrahydrofolic acid to give formyltetrahydrofolic acid (81). The formulation of a phosphate transfer is in accord with the very recent finding of Rabinowitz and Pricer (82) that the degradation of N-formiminoglycine to glycine, formic acid, and ammonia by extracts of Clostridium cylindrosporum or C. acidi-urici requires inorganic phosphate and ADP and is accompanied by ATP formation.

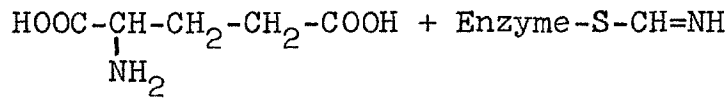
The in vivo data show that to a large extent the formimino carbon is transferred to various acceptors or oxidized to CO_2. The former reaction would be expected to consume the energy of the formyl-folic bond, while the latter could be expected to give ATP, just as with the liberation of formate. Consequently, in an in vitro system, which might not offer the proper conditions for transfer of the carbon fragment to an acceptor, phosphorolysis or arsenolysis presents a major way for regenerating the cofactor. In the case of microorganisms phosphorolysis could represent a major pathway, thereby providing a source of energy.

In conclusion, then, a tentative scheme for the final step in the degradation of histidine to glutamic acid in liver may be presented as follows:

(1) Transfer of formimino group to sulfhydryl group on enzyme.

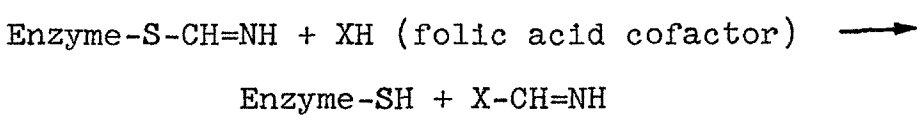


$$HOOC\text{-}\underset{\underset{\underset{\underset{NH}{|}}{CH}}{||}}{\underset{|}{CH}}\text{-}CH_2\text{-}CH_2\text{-}COOH + Enzyme\text{-}SH \longrightarrow$$



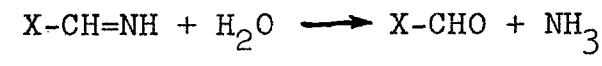
$$HOOC\text{-}\underset{|}{CH}\text{-}CH_2\text{-}CH_2\text{-}COOH + Enzyme\text{-}S\text{-}CH{=}NH$$
$$NH_2$$

(2) Transfer of formimino group to folic acid acceptor.



$$Enzyme\text{-}S\text{-}CH{=}NH + XH \text{ (folic acid cofactor)} \longrightarrow$$
$$Enzyme\text{-}SH + X\text{-}CH{=}NH$$

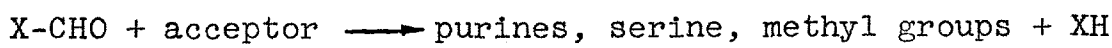
(3) Hydrolysis of formimino group to give ammonia.



$$X\text{-}CH{=}NH + H_2O \longrightarrow X\text{-}CHO + NH_3$$

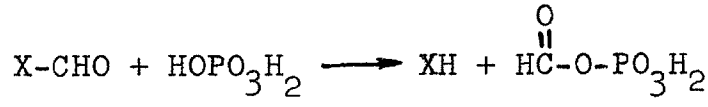
(4) Regeneration of cofactor.

(a) By transfer to acceptor.



$$X\text{-}CHO + \text{acceptor} \longrightarrow \text{purines, serine, methyl groups} + XH$$

(b) By phosphorolysis.



$$X\text{-}CHO + HOPO_3H_2 \longrightarrow XH + \underset{\underset{O}{||}}{HC}\text{-}O\text{-}PO_3H_2$$

(5) Transfer of phosphate to ADP.

(a) Non-oxidatively.

$$HC(=O)\text{-}O\text{-}PO_3H_2 + ADP \longrightarrow HCOOH + ATP$$

(b) Oxidatively.

$$HC(=O)\text{-}O\text{-}PO_3H_2 + ADP + R \text{ (oxidizing agent)} \longrightarrow$$

$$CO_2 + ATP + RH_2 \text{ (reduced form of oxidizing agent)}$$

In the case of formamide-forming organisms, the third reaction could be a phosphorolysis to give formimino-phosphate, a compound which to my knowledge has not been described, but which would represent a step lower in oxidation level than carbamyl phosphate (83). In the case of Pseudomonas the hydrolytic reaction occurs before the monocarbon transfer. If folic acid is involved, the formyl is probably transferred to it directly, in which case phosphorolysis or hydrolysis of the complex could occur, in the former event with energy conservation in the form of ATP.

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