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Factors concerned in tyrosine metabolism

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FACTORS CONCERNED IN TYROSINE METABOLISM

by

Richard S. Schweet

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ACKNOWLEDGMENT

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I. INTRODUCTION

Studies of the intermediary metabolism of amino acids have assumed increasing importance in recent years. These studies have been stimulated not only by the importance of amino acids in nutrition but also by recent findings implicating various vitamins as components of enzyme systems involved in the metabolism of amino acids. Besides providing new concepts of the functioning of vitamins in the body, our understanding of nutritional deficiency diseases has been clarified by the investigations of vitamin-amino acid interrelationships.

The metabolism of the aromatic amino acids, phenylalanine and tyrosine, has been studied extensively. Some of the findings which have been responsible for the widespread interest in these two compounds are: phenylalanine is an essential amino acid and is readily converted to tyrosine in the body, thyroxine and adrenaline may be derived from tyrosine, melanin pigments are formed from tyrosine, and finally, certain diseases are characterized or accompanied by an abnormal utilization of the aromatic amino acids so that partial breakdown products appear in the urine. These disorders include some of the "inborn errors" of metabolism, hydroxyphenyluria in premature infants, pernicious anemia

in relapse, certain cyanotic conditions, and rheumatic fever.

Many of the investigations, however, have been of a clinical nature or have been concerned with the intermediates produced in tyrosine catabolism. With the finding that the vitamin C-deficient guinea pig fails to metabolize tyrosine completely, a new approach to the problem under controlled experimental conditions was made possible. This approach has now shown that pteroylglutamic acid and a factor found in purified liver extracts, in addition to vitamin C, are components concerned in tyrosine metabolism.

The present investigation is an attempt to investigate further the mechanism by which these factors act on tyrosine metabolism in the vitamin C-deficient guinea pig. Liver extracts used for the treatment of pernicious anemia were studied extensively in order to analyze the testing procedure, study the relationship between their antipernicious anemia unitage and their activity in the tyrosine metabolic system, investigate the nature of the substance in the extracts responsible for their action and finally, examine their action on blood constituents under varied experimental conditions.

The advent of isotopes as tracers provided a new, powerful tool for metabolic research. With the tyrosine molecule marked in various positions, the utilization of the amino acid under the influence of the three factors under

investigation could be followed. Initial studies of the first step, with N^{15} in the amino group of the molecule, are reported here. Further work along these lines may eventually result in the assignment of specific chemical functions in the tyrosine metabolic scheme to these factors.

II. HISTORICAL

A. Tyrosine Metabolism in General

The initial impetus to the study of tyrosine metabolism came from the work of Bödeker (1). In 1859, this author isolated a reducing compound from the urine of diabetic patients. He was unable to identify the substance, but called it "alkapton." Later, Wolkow and Baumann (2) established the structure of the compound as hydroquinone acetic acid on the basis of its empirical formula and functional groups. They called the compound homogentisic acid, the next higher homologue of gentisic acid.

They further discovered that tyrosine was the precursor of the homogentisic acid excreted by feeding extra amounts of the amino acid to persons with alcaptonuria. Falta and Langstein (3) later showed that phenylalanine also was converted to homogentisic acid by the alcaptonuric.

In 1904, Neubauer and Falta (4) investigated the metabolism of a number of aromatic compounds by the alcaptonuric. The only compounds, in addition to the two amino acids, which yielded homogentisic acid were phenylpyruvic and phenyllactic acids. Later, p-hydroxyphenylpyruvic, but not p-hydroxyphenyllactic acid, was also found to yield homogentisic acid in the alcaptonuric (5).

In 1906, Embden, Salomon and Schmidt (6) applied per-
fusion techniques to the study of alcaptonuria. They found
that when tyrosine, phenylalanine and homogentisic acid were
perfused through surviving liver, acetone bodies appeared
in the effluent. The ketogenic nature of the aromatic amino
acids was confirmed by Blum (7) when he showed that feeding
these compounds to diabetics produced an increased excretion
of ketone bodies. Edson (8) has demonstrated the ketogenic
nature of tyrosine and phenylalanine using the Warburg
technique and rat liver slices.

The mechanisms involved in the conversion of tyrosine
to ketone bodies were also discussed by Blum (7). Since
p-hydroxyphenylacetic acid did not give homogentisic acid
in the alcaptonuric patient, he felt that oxidation of the
tyrosine aromatic nucleus occurred prior to sidechain
degradation. In addition, Blum thought that homogentisic
acid was a normal intermediate in tyrosine oxidation although
he was unable to isolate the compound from normal individuals.

Neubauer (5) combined all of these results into a scheme
for the normal oxidation of tyrosine and phenylalanine. This
scheme postulated a "quinol-type" intermediate. Quinols had
previously been prepared by several authors, particularly
Bamberger (9). Neubauer believed that the first step in
amino acid catabolism was oxidative de-amination yielding
the corresponding keto acid. For the aromatic amino acids,

he proposed the following scheme:

$$\underset{\text{phenylalanine}}{\overset{\displaystyle\bigcirc}{\underset{\displaystyle\underset{\text{COOH}}{\underset{|}{\overset{|}{\text{H-C-NH}_2}}}}{\overset{|}{\text{CH}_2}}}} \longrightarrow \underset{\text{COOH}}{\overset{\displaystyle\bigcirc}{\underset{\underset{\text{COOH}}{\underset{|}{\overset{|}{\text{C=O}}}}}{\overset{|}{\text{CH}_2}}}}$$

A.   B.

$$\underset{\text{COOH}}{\overset{\text{OH}-\bigcirc}{\underset{\underset{\text{COOH}}{\underset{|}{\text{H-C-NH}_2}}}{\text{CH}_2}}} \longrightarrow \underset{\text{COOH}}{\overset{\text{OH}-\bigcirc}{\underset{\underset{\text{COOH}}{\text{C=O}}}{\text{CH}_2}}} \xrightarrow{\text{(O)}} \Big[\; \overset{\text{O}=\bigcirc}{\underset{\text{HO}\;\;\text{CH}_2\text{-CO-COOH}}{}} \;\Big] \longrightarrow$$

OH–⬡(OH)–CH₂-CO-COOH → OH–⬡(OH)–CH₂-COOH $\xrightarrow[\text{///}]{1}$ Acetone bodies ⟶ $H_2O + CO_2$

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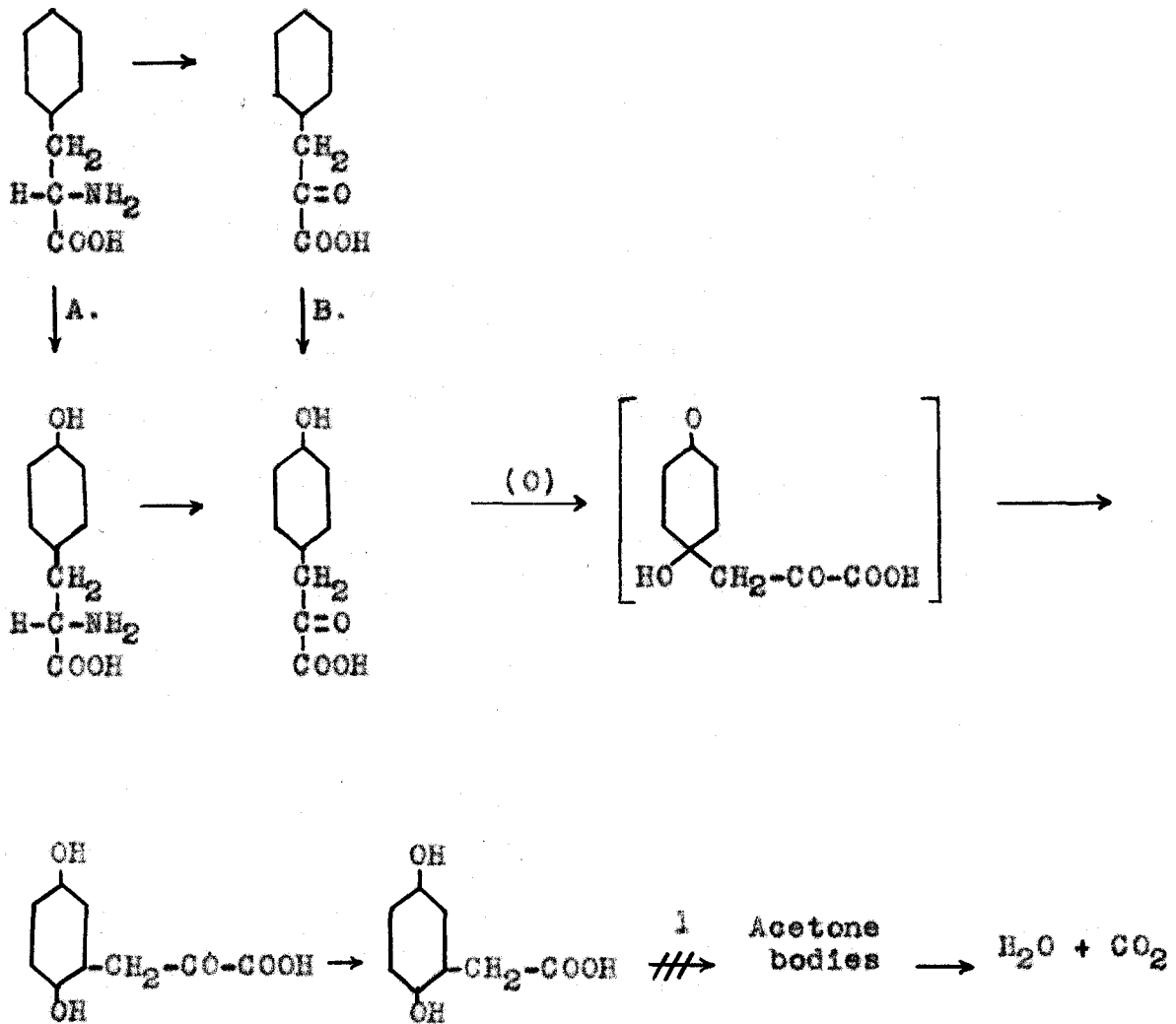
[1] Alcaptonuric unable to proceed past this step.

Evidence for the conversion of phenylalanine to tyrosine
had previously been given by the perfusion experiments of
Embden and Baldes (10). However, these authors noted that

phenylpyruvic acid when perfused through surviving liver, in
contrast to the p-hydroxy compound, did not yield acetone
bodies. They favored, therefore, Reaction A over Reaction B
as part of the normal pathway of metabolism. Moss and
Schoenheimer (11) have recently confirmed the conversion of
phenylalanine to tyrosine by the use of deuterium-labelled
phenylalanine.

In 1910, Dakin (12) was able to point out some flaws in
Neubauer's hypotheses. He showed that compounds such as
p-methoxyphenylalanine and p-methylphenylalanine (13), which
supposedly could not yield the quinol postulated by Neubauer,
were still metabolized by normal individuals. These com-
pounds yielded acetone bodies when perfused through liver and
were metabolized by alcaptonurics without production of
homogentisic acid. Dakin (14) concluded that homogentisic
acid need not be a normal intermediate in tyrosine oxidation.
Since Jaffe (15) had shown benzene was oxidized to muconic
acid by dogs, Dakin (13) proposed a scheme which did not
involve the introduction of hydroxyl groups into the tyrosine
nucleus:
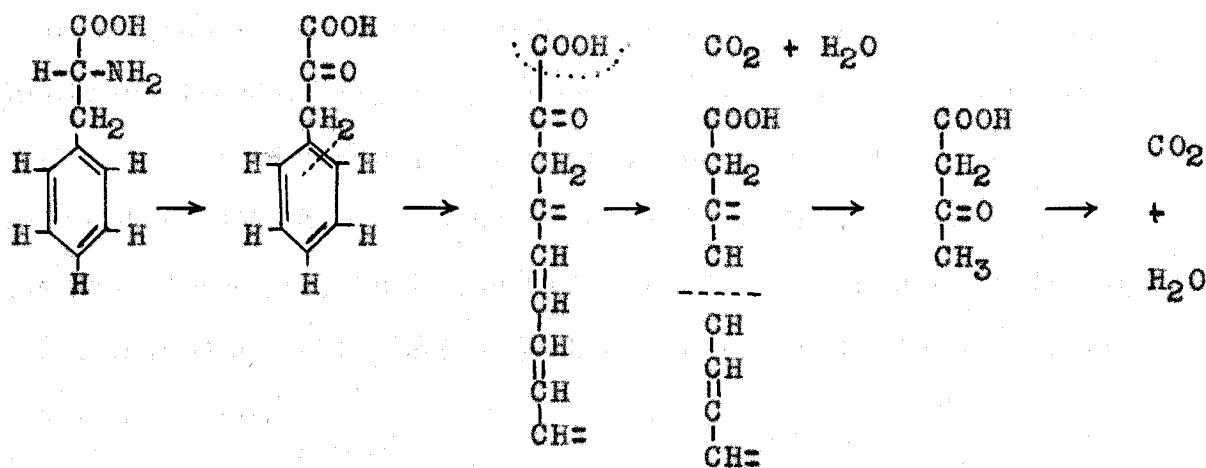
Dakin's views have received support from the work of Fromherz and Hermanns (16, 17). These authors found that p-methyltyrosine and m-methyltyrosine were metabolized by both normal and alcaptonuric individuals without the production of homogentisic acid derivatives, thus confirming Dakin's work.

The most recent investigations of alcaptonuria have been made by Neuberger and colleagues. In 1947, Neuberger, Rimington and Wilson (18) reported that 80-100% of ingested tyrosine or phenylalanine appeared in the urine as homogentisic acid. The low blood values of homogentisic acid led them to suggest that this compound was either formed in the kidney or excreted at an extremely rapid rate. Later, Neuberger (19, 20) fed L-2,5-dihydroxyphenylalanine and noted its efficient conversion to homogentisic acid. He considered this compound, therefore, a normal intermediate in the

oxidation of tyrosine. The mechanism involved in the normal
metabolism of tyrosine as suggested by Neuberger (21)
utilizes some of the more modern concepts of organic chemistry
to explain the postulated scheme. The phenoxide ion (I) is
attacked by an electrophilic reagent. Preferential attack
in the para position might be facilitated by the side chain
constitution or the enzyme. The removal of two electrons
produces a carbonium ion (II). Attack by hydroxyl ion and
rearrangement of the p-quinol (III) follows, yielding
2,5-dihydroxyphenylalanine as a final product. Similar
rearrangements have been shown by Arnold (22).
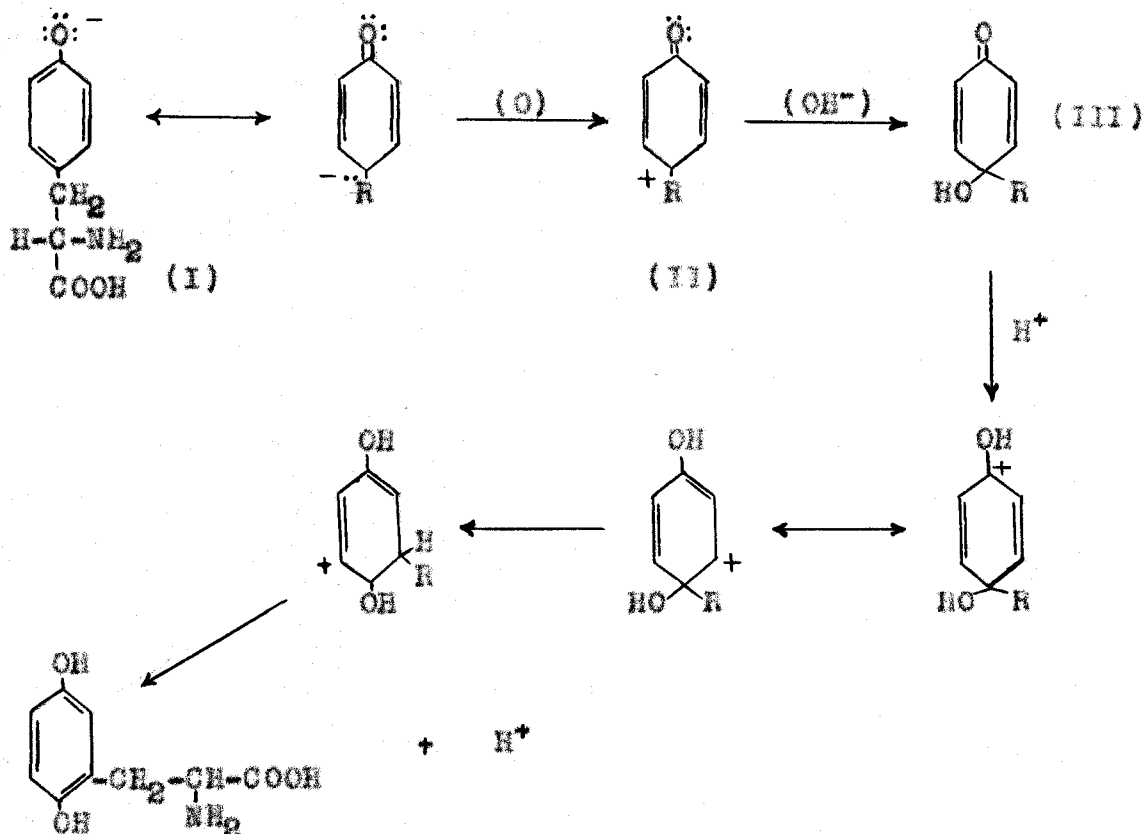
In the light of these recent studies, it would seem that homogentisic acid was an intermediate in the main pathway of tyrosine catabolism, as Neubauer had postulated in 1909. However, the evidence has been indirect and based to a large extent on the study of alcaptonuria. Alternate pathways of oxidation certainly exist and the possibility that they play an important role in tyrosine metabolism has not been excluded. In addition, it should be noted that the mechanisms postulated are even more obscure. None of the important intermediates have been isolated in normal individuals. Finally, if homogentisic acid is a normal intermediate, little is known of the route by which it is converted to acetoacetic acid.

In 1932, Medes (23) reported another "inborn error" of metabolism. This patient excreted p-hydroxyphenylpyruvic acid in the urine. The feeding of phenylalanine or tyrosine increased the excretion of this compound. Homogentisic acid was metabolized completely. This tyrosinosis, as Medes called the defect, resulted also in the excretion of 3,4-dihydroxyphenylalanine when large amounts of tyrosine were fed. Thus, further proof for the conversion of phenylalanine to tyrosine was obtained and also evidence of the oxidation of tyrosine in the meta position. The latter finding implicated tyrosine as a possible melanin and adrenaline precursor. Gurin and Delluva (24) have shown the oxidation of

tritium-labelled phenylalanine to adrenaline, presumably
through the prior formation of a 3,4-dihydroxy compound. The
conversion of 3,4-dihydroxyphenylalanine to melanin has been
well established by the work of Raper (25) and more recently
by Mason (26).

In addition to the utilization of phenylalanine by way
of conversion to tyrosine, other metabolic pathways are known.
Womack and Rose (27) have shown that phenylalanine is an
essential amino acid while tyrosine is not. That this differ-
ence can be reflected in the metabolic pathway has been
demonstrated by Butts, Dunn and Hallman (28). In their first
experiments racemic tyrosine and phenylalanine were fed to
rats and the liver glycogen content and urinary ketone bodies
(after sodium butyrate feeding) determined. Phenylalanine
feeding caused increased liver glycogen, slightly decreased
acetonuria and increased urinary nitrogen. Alanine produced
effects similar to those of phenylalanine, but in greater
amount. Tyrosine caused no change in any of these values.
Later (29) it was found that fasted rats, when fed L-tyrosine,
showed an increase in liver glycogen, in contrast to the
negative findings when DL-tyrosine was fed. The glycogenic
effect of tyrosine has been recently confirmed. This work
will be discussed in Section F.

Defects in tyrosine metabolism have been reported in two
other diseases. Fishberg (30) noted that cyanotic patients

excreted a tyrosine metabolite in the urine. This was
identified as the quinone of homogentisic acid. The author
hypothesized that the oxidizing action of this compound
produced the methemoglobin characteristic of the disease.
Some interruption of tyrosine catabolism, possibly related to
the vitamin C intake, was believed responsible for the
accumulation of the metabolite. Kemmerer (31) has noted the
appearance of tyrosine derivatives in the urine of patients
with rheumatic arthritis. These patients had the same food
intake as controls who did not excrete these compounds.

The early in vitro studies of tyrosine metabolism by
Neubauer and Embden, using perfusion methods were followed by
the adaptation of Warburg manometric techniques to the
problem. In 1934, Bernheim and Bernheim (32) studied the
oxidation of tyrosine and phenylalanine with "broken cell
suspensions." Tyrosine was oxidized best by liver tissues
with the uptake of four atoms of oxygen per mole of tyrosine
under optimum conditions. No ammonia was produced. In con-
trast, phenylalanine was oxidized by both liver and kidney,
ammonia was produced, and the oxygen uptake was one atom per
mole. Cyanide inhibited the tyrosine system but not that of
phenylalanine. A year later, Bernheim (33) showed that not
only were the natural isomers of phenylalanine and tyrosine
oxidized by different enzyme systems, but that the oxidation
of D-tyrosine differed from that of the L-isomer, since
kidney, as well as liver tissue, attacked the unnatural

isomer. The oxidation of D-tyrosine by liver tissue, in contrast to the natural isomer, was accompanied by ammonia production and was not inhibited by potassium cyanide.

Felix and coworkers (34) were able to separate tyrosine oxidation by hog liver into four steps by means of pH differences in the brei. The end products of the reaction were acetone and carbon dioxide. Homogentisic acid and p-hydroxyphenylpyruvic acid were also oxidized. Felix and Schaefer (35) have reported the extraction of an enzyme system from rat liver which oxidized p-hydroxyphenylpyruvic acid with the uptake of 3 atoms of oxygen per mole. These findings do not necessarily indicate that this keto acid is a normal intermediate in tyrosine oxidation, since neither ammonia nor p-hydroxyphenylpyruvic acid have been reported as products of the in vitro oxidation of tyrosine. Furthermore, the work of Neuberger (19), which will be discussed further in Section E, has indicated that 2,5-dihydroxyphenylalanine is an important intermediate in tyrosine metabolism.

In a later paper, Felix and associates (36) detected alanine as one of the products of tyrosine breakdown. Their results agreed with the Neubauer scheme, except for the alanine cleavage. They believed that the tyrosine nucleus only was oxidized to the acetone bodies formed during the incubation. Recent work, which will be discussed in Section F, has shown that side chain cleavage of tyrosine

during its oxidation to acetoacetic acid cannot take place to any large extent. Their isolation of alanine is thus difficult to explain.

As some of the questions concerned with tyrosine metabolism were clarified it became apparent that studies of the rare alcaptonuric individual and normal tissues in vitro would have to be supplemented by controlled laboratory experiments. Experimental alcaptonuria and other defects in tyrosine utilization were soon produced by appropriate changes in dietary factors. A discussion of each of these factors and its relation to the tyrosine problem will be given in the following sections.

B. Vitamin C and Tyrosine Metabolism

As early as 1930, prior to its actual identification as vitamin C, Szent-Györgyi (37) noted that "hexuronic acid" inhibited the formation of melanin pigments. The pigmentation (38) associated with Addison's disease was decreased by vitamin C supplementation.

In 1939, Sealock and associates (39) investigated further the vitamin C-melanin relationship. They fed the melanin precursors, tyrosine and 3,4-dihydroxyphenylalanine, to scorbutic and normal guinea pigs. It was noted that the vitamin C requirement was increased when these amino acids were fed. In addition, the urines of the vitamin-deficient

animals receiving extra tyrosine darkened on standing. Examination revealed the presence of homogentisic acid in these urines. This compound was isolated and its identity definitely established.

The production of experimental alcaptonuria had also been reported the previous year, first by Papageorge and Lewis (40) and then by Butts, Dunn and Hallman (28). Homogentisic acid excretion in these cases was the result of the feeding of large amounts of phenylalanine to white rats. However, the guinea pig picture was due to a definite vitamin deficiency, the levels of tyrosine fed were smaller, and the conversion of the amino acid to excreted metabolites was nearly complete when the vitamin deficiency was acute.

Sealock and Silberstein (41) then showed that administration of vitamin C caused the disappearance of the urinary metabolites. When 0.5 gm. of tyrosine was fed, administration of 5.0 mg. of the vitamin resulted in removal of the metabolites from the urine. Similar tests on normal human subjects also resulted in homogentisic acid excretion. The difference between this type of alcaptonuria and the hereditary type was emphasized when it was shown by Sealock, Galdston and Steele (42) that vitamin C did not affect the homogentisic acid excretion of the hereditary alcaptonuric. Further work by Sealock and Silberstein (43) revealed the presence of p-hydroxyphenylpyruvic and p-hydroxyphenyllactic

acids among the excretory products, in addition to homo-
gentisic acid. The feeding of L-phenylalanine to the
vitamin C-deficient guinea pig also resulted in the produc-
tion of these same excretory products. Vitamin C produced
its curative effect here also. The specificity of the
vitamin in this connection was demonstrated by the fact that
10 mg. of D-isoascorbic acid was ineffective in removing the
metabolites. However, 200 mg., an amount equal in anti-
scorbutic activity to 10 mg. of L-ascorbic acid, was able to
prevent the metabolite excretion. Phenylpyruvic acid
metabolism (44), but not that of the p-hydroxy derivative,
was also influenced by vitamin C administration.

The clinical application of these findings appeared
shortly when Levine, Marples and Gordon (45) demonstrated
that the hydroxyphenyluria noted in premature infants on a
cow's milk formula (high protein level) was prevented by
vitamin C. The defect could also be demonstrated (46, 47)
in full-term infants by the feeding of a single dose of 1.0
gm. of tyrosine in the absence of vitamin C. Both the
artificially induced and natural hydroxyphenyluria were
corrected by the proper vitamin C intake and the authors
emphasize the importance of the vitamin in the diet of
infants.

In 1942, Sealock (48) found that the administration of
dicarboxylic acids resulted in removal of the urinary

metabolites of guinea pigs receiving extra tyrosine on a
vitamin C-free diet. The effect, however, was temporary, and
repeated doses gradually lost their effect. The action was
shown to be the result of the acidity changes which mobilized
tissue vitamin C and could be duplicated by the use of
ammonium chloride. The acid-base balance, therefore, must be
considered when experiments of this type are undertaken.

In _vitro_ studies by Lan and Sealock (49) with liver and
kidney slices of normal guinea pigs demonstrated an increased
oxygen uptake in the presence of tyrosine substrate. Slices
of these tissues from scorbutic animals did not exhibit this
increased oxygen consumption, but the addition of crystalline
ascorbic acid to the Warburg flasks restored their ability to
oxidize tyrosine. These same authors (50) extended their
studies to 3,4-dihydroxyphenylalanine and demonstrated a
similar dependence on an adequate vitamin C supply. In the
case of this amino acid the most active oxidation took place
with kidney slices, while liver slices were most important
with the former.

Sealock and Goodland (51) have prepared cell-free homoge-
nates of guinea pig liver which oxidized tyrosine with the
uptake of 4 atoms of oxygen per mole of tyrosine oxidized.
The system was shown to contain thermostable, dialyzable
components. Similarly, Sealock and Clegg (52) have shown
that cell-free preparations from kidney tissue will oxidize

3,4-dihydroxyphenylalanine.

We may conclude that the dependence of tyrosine utilization upon adequate vitamin C intake has been established. The demonstration of the in vitro vitamin function in tyrosine oxidation, and the characterization of the crude enzyme systems responsible, represent further progress in clarifying the problem. These studies point to the action of vitamin C at an early stage in tyrosine breakdown. However, as yet no specific location of the vitamin action in the postulated oxidative scheme involving homogentisic acid has been reported. Furthermore, the function of the vitamin in tyrosine metabolism in vivo may be more complex than in vitro studies seem to indicate. These questions, and the more difficult one of the interrelationship of vitamin C with other factors regulating tyrosine metabolism, constitute the subject matter of continuing research.

C. Liver Extracts and Tyrosine Metabolism

1. Origin of tyrosine-liver extract relationship

In 1941, in connection with their work on the hydroxyphenyluria noted in premature infants, Levine and coworkers (47) had tried liver extract as a curative agent. This extract was a crude liver extract, free from vitamin C. Administration of this preparation reduced the metabolite

excretion of one premature infant and one full-term infant
that had been receiving extra tyrosine, but had no effect on
three other premature infants.

In 1943, Swendseid and associates (53) reported an
increased urinary excretion of keto acids by patients with un-
treated pernicious anemia. After treatment by intramuscular
injection of liver extract, the keto acid levels returned to
normal. Correlation of keto acid with hydroxyphenyl values,
as shown in Table I, suggested a disturbance in tyrosine
metabolism. Ten normal patients on the same hospital diet
showed average values of 82 and 195 mg. per 24 hours for
urinary keto acid and hydroxyphenyl compounds, respectively.

Table I

Daily Urinary Excretion Values for Keto Acids and
Hydroxyphenyl Compounds in Pernicious Anemia

Keto acids expressed as pyruvic acid in mg. per 24 hr.				Hydroxyphenyl compounds expressed as tyrosine in mg. per 24 hr.			
Relapse		Remission		Relapse		Remission	
Range	Avg.	Range	Avg.	Range	Avg.	Range	Avg.
228-177	202	107-94	99	714-444	584	292-242	278
158-107	135	63-56	60	799-427	513	290-270	280
153-103	138	97-68	76	428-308	400	260-201	219
135-124	127	93-74	82	682-362	442	287-240	256

Further work in 1947 by this group (54) confirmed these results. Fractionation of the urine revealed that the portion containing the hydroxyphenyl acids showed the greatest decrease when liver extract was administered. This decrease was the first effect of therapy and preceded any blood changes. Vitamin C administration had no effect on patients with normal plasma levels of the vitamin. The authors suggested a possible function of tyrosine in blood formation, and thus linked the derangement of tyrosine metabolism observed directly to the anemia.

On the basis of these facts, Sealock and Lepow (55) investigated the effect of antipernicious anemia preparations on the metabolite excretion of scorbutic guinea pigs receiving extra tyrosine. These preparations contained an insignificant amount of vitamin C and pteroylglutamic acid. The prompt decrease in keto acid excretion (paralleled by total phenolic values) observed is shown in Table II. It is evident that with a given preparation the effect is roughly correlated with the number of units injected. Other liver extracts yielded similar results, although correlation between different extracts was poor. These extracts did not increase the in vitro oxidation of tyrosine by liver tissue, as has been reported for ascorbic acid and pteroylglutamic acid (to be discussed in Section D).

Table II

Keto Acid Excretion Before and After
Liver Extract Injection

Liver extract injected	Keto acid excretion		
	24 hr. before	24 hr. after	% decrease per unit
units	mg.	mg.	
15	202	159	1.42
20	237	172	1.37
20	195	135	1.54
20	215	186	0.67
Average			1.25 ± 0.29
7.5	244	199	2.46
7.5	314	265	2.08
15	253	208	1.19
22.5	325	156	2.31
Average			2.01 ± 0.41

Three reports which implicated tyrosine in hematopoiesis further strengthened the idea that antipernicious anemia liver extracts contained some factor(s) concerned in tyrosine utilization. In 1938, Subbarow and associates (56) concluded a series of investigations on the nature of the factors in liver extracts effective in curing pernicious anemia. They suggested that the interaction of a primary and three accessory factors was necessary. The primary factor had properties which suggested a complex pyridine derivative, while one of the accessory factors was L-tyrosine. Jacobsen and Plum (57) reported that tyrosine was essential for the maturation of reticulocytes. This has been confirmed by Christensen and Plum (58), who noted that the main action of tyrosine was to enhance the action of a heat-labile factor. These reports have not, as yet, been confirmed by other investigators.

With the tyrosine-liver extract relationship revealed, it would seem pertinent to review studies concerning the nature of the factors in liver extract active in alleviation of pernicious anemia, since these may be important in tyrosine metabolism as well.

2. Multiple nature of liver factors

Pernicious anemia, according to Minot and Strauss (59), is a complicated deficiency disorder, the cause of which is

unknown, but may be due to a lack in the diet, or faulty
absorbtion or utilization of one or more dietary factors.
The disease is characterized by a macrocytic, hyperchromic
anemia with the red blood cell count usually below 2,500,000
per cu. mm. As Wintrobe (60) has pointed out, this red blood
cell count is not due to a failure of the cells to mature, as
is the case in certain nutritional anemias, but rather is due
to abnormal maturation which produces the megaloblasts found
in the bone marrow in such large numbers. These megaloblast
cells do not function, however, in oxygen transport. A
gastro-intestinal disturbance, characterized by achlorhydria,
and neural lesions later usually accompany the blood symptoms.

The classical treatment with whole or refined liver
extracts produces remission of all symptoms, but must be con-
tinued or the symptoms reappear. Numerous studies have been
made to determine the nature of the active material present
in these liver extracts. The work of Castle and associates
(61), which showed the presence of an extrinsic factor in
foods, which is acted upon by an intrinsic factor, probably
an enzyme found in normal gastric juice, is now classic.
Cohn and associates (62), and Dakin and West (63), and others
have attempted to isolate the active constituents from liver.
This subject has been reviewed by Subbarow (64).

Progress in this respect, however, was limited by the
difficulty of the assay procedure. This involved the

observation of the increase in the red blood cell count of pernicious anemia patients on administration of the various fractions. Obviously, individual variation and the difficulty in securing patients suitable for assay purposes contributed to the lack of progress.

This impasse was surmounted in 1947 by Shorb (65, 66), who found that Lactobacillus lactis Dorner showed growth response to refined liver extracts. An assay method was developed for the growth factor. The crystalline factor was isolated by Rickes and coworkers (67) in 1948 and the activity of the crystalline substance for growth of Lactobacillus lactis was discussed by Shorb (68). The compound crystallized as red needles which melted at $300^{\circ}C$. As little as 3-6 μg. produced a hematopoietic response in pernicious anemia patients. This growth factor was temporarily named vitamin B_{12}, pending identification of the compound.

Simultaneously, a group in England working under Smith (69) announced the isolation of the same or similar compound by the use of chromatographic adsorbtion techniques. The presence of cobalt, phosphorus and nitrogen in the molecule, the approximate molecular weight of 1500 and some of the physical properties were soon reported independently by both groups (69-71). Numerous reports have appeared this year giving further details of the structure and chemical properties of vitamin B_{12}. The group at Merck, which originally

isolated the compound, has shown the presence of basic groups in the molecule, reported optical activity and degraded the molecule with the production of 5,6-dimethylbenzimidazole as one fragment (72-74). In England, Smith and Parker (75), and Ellis and coworkers (76, 77) have reported analytical and purification details.

The clinical studies with the vitamin have established unequivocally its ability to remove all the symptoms of pernicious anemia. The original reports have been extended by Reisner and West (78), who injected pure vitamin B_{12} into 11 patients with pernicious anemia. All showed complete hematologic remission and the five with neural lesions reported improvement. This alleviation of the nervous symptoms differed sharply from the action of pteroylglutamic acid (to be reviewed in Section D) which produced only temporary increases in red blood cells (79, 80). Similar results confirming the effectiveness of the vitamin have been reported by Castle and associates (81, 82) and Spies and coworkers (83). The usual dosages of vitamin B_{12} have ranged from 5 to 10 μg. per day by injection and somewhat higher when given orally. It has been noted (82) that the oral effectiveness is increased if gastric juice from normal patients is also administered. Vitamin B_{12} has also been reported (83) as effective in the treatment of sprue.

These results, establishing the vital function of

vitamin B_{12} in blood formation, were probably only one facet
of the vitamin action. Work along other lines demonstrated
the importance of this vitamin in growth processes throughout
the body. Thus, the probable tyrosine-vitamin relationship
in blood formation may be extended in the future to the
involvement of vitamin B_{12} in the general anabolic metabolism
of tyrosine. The importance of this possibility, and the
new fields for research opened by it, merits investigation.

Investigation of various diets for poultry had revealed
the absence of important growth factors from vegetable protein
diets. Byerly, Titus and Ellis (84), as far back as 1933,
had reported poor hatchability and decreased chick survival on
these diets. The search for the missing factor(s) was
intensified by the wartime shortage of animal protein.
Animal protein factor (as this was called) activity was re-
ported in fish solubles, liver extracts, cow manure, numerous
animal proteins and fermentation residues. Active work in
purifying the crude factor and developing chick assay methods
was carried on by Bird and coworkers (85, 86), a group headed
by Elvehjem (87), a group under Hill (88), McGinnis and
coworkers (89), and others.

Shortly after the isolation of vitamin B_{12} in 1948, it
was reported by the Merck group (90, 91) that animal protein
factor activity was associated with the compound. The sug-
gestion was made that animal protein factor and B_{12} might be

identical on the basis of the high activity shown by the vitamin in promoting chick growth. Bird and coworkers (92) reported that vitamin B_{12} supported maximum growth of chicks on diets deficient in animal protein factor. The factor was also shown to be essential for mammalian growth. Cary and Hartman (93) reported the ability of the vitamin to substitute for their "Factor X," and it probably is related to the "Zoopherin" of Zucker and Zucker (94). Both of these factors were essential for rat growth on purified diets. The low levels of the factor required for growth made the early results equivocal in some cases, but when the dams of the experimental animals were also depleted by the use of vegetable protein or purified casein diets, the factor was found to be essential for rats by Emerson (95), for pigs by Johnson and coworkers (96), Hogan and coworkers (97) and Luecke and coworkers (98), and for mice by Bosshardt and associates (99, 100). Vitamin B_{12} promoted increased growth in all of these reports.

A large number of bacterial assays were developed. Chick and mammalian assays were complicated by the problem of depleting the assay animals. It was found by Evans and Nelson (101) that, in addition to growth, lactation required an increased amount of animal protein factor. Several authors noted that feeding of desiccated thyroid or iodinated casein also increased the requirement for the factor. This latter

finding was made the basis for new methods of assay, using rats (102, 103), chicks (104, 105), and mice (100, 106).

In 1949, when certain natural sources of these various factors were compared on the basis of their antipernicious anemia, microbiological and animal protein factor activity, certain discrepancies became apparent. Some of these natural products had, for example, more animal protein factor activity than would be predicted from their B_{12} content (based on microbiological assay). Elvehjem and coworkers (107) confirmed this and showed that the ethyl alcohol extract of liver, discarded in the preparation of antipernicious anemia extracts, had an extremely high chick growth potency. Stokstad and coworkers (108) reported that the maximum growth obtained by chicks on vitamin B_{12}-supplemented diets could be increased by the addition of other liver fractions. Cunha and coworkers (109) reported that the growth of pigs on corn-peanut meal rations was increased by a Fuller's earth concentrate of vitamin B_{12}, but that greater maximum growth was obtained when animal protein factor from fermentation residues was used as supplement. A number of authors reported the separation of highly refined antipernicious anemia extracts by paper chromatography and electrolytic methods into as many as six components. Snook and associates (77) discussed the analyses of vitamin B_{12}. The Merck group (73) had reported a cobalt:phosphorus ratio of 1:1, while the

English group's compound (71) had a 1:3 ratio. These dis-
crepancies seemed to indicate that different forms of the
vitamin, possibly produced by the isolation processes,
existed and had biological activity.

Stokstad and coworkers (110) have reported that chroma-
tography of Streptomyces aureofaciens fermentation residues
yielded two crystalline fractions. Both showed biological
activity by chick and microbiological assay. The absorbtion
spectra of these two compounds differed, and the spectrum of
one was similar to that of vitamin B_{12}. The authors named
the other compound vitamin B_{12b}. Vitamin B_{12a} had previously
been described (72) as a hydrogenation product of the vitamin.

Finally, two English reports by Cuthbertson and associ-
ates (111) and Shaw (112) revealed discrepancies between
clinical antipernicious anemia and vitamin B_{12} (by micro-
biological assay) activity of liver extracts. They inferred
that the form or forms of the vitamin present in liver
extracts were more active clinically than would be expected
on the basis of their vitamin B_{12} content.

At the present time, therefore, it seems probable that
more than one chemical compound exists with vitamin B_{12}
activity. The conjugation of the vitamin with unknown sub-
stances probably differs depending on the source. The
activity of these various forms then would depend on the
species used for assay and the criterion used, as growth,

blood formation, and so forth. The elucidation of the structure of the vitamin should clarify many of these questions.

Several attempts have been made to assign these B_{12} compounds a specific function in growth. One of the earliest ideas was advanced by Shive and coworkers (113, 114) and by Wright and associates (115). Inhibition studies on <u>Lacto-bacillus lactis</u> had shown that the nucleoside, thymidine, could replace the requirement of this organism for vitamin B_{12}. The data supported the conception of vitamin B_{12} as functioning in the biosynthesis of the nucleoside. However, more recently it has been shown by numerous authors that purines and other nucleosides essential for bacterial growth are spared by the vitamin and that the relationship depends on the bacterial species being tested.

A relationship between transmethylation and the vitamin has been reported by Salmon (116), who showed that the presence of vitamin B_{12} in the diet of chicks and rats reduced the requirement for choline. Similar data have been presented by Norris and coworkers (117), while Cunha and associates (109) have reported that the enhanced growth of pigs on corn-peanut meal rations, which was produced by methionine supplementation, could also be produced by animal protein factor. When the animal protein factor was present, extra methionine had no effect on growth.

Cary and coworkers (118) concluded that vitamin B_{12}

played a fundamental role in the capacity of mammals to
utilize protein. Their data was concerned with the growth
increases of vitamin B_{12}-supplemented rats over control ani-
mals, as the protein in the diet was increased from 25 to 65%.
As the protein content of the diet was increased, the growth-
promoting effect of the vitamin supplementation increased.
This fact was cited as evidence for their conclusions.

The large number of reports cited in this section, all
appearing since the isolation of vitamin B_{12} in 1948, are
evidence of the importance attached to this factor. The eluci-
dation of the structure of the vitamin, and of the various
forms which occur naturally, has not yet been accomplished.
However, the almost universal participation of these factors
in growth processes and the specific function of the vitamin
in blood formation seem to have been shown conclusively. The
activity of antipernicious anemia liver extracts in tyrosine
metabolism, which has been demonstrated in the guinea pig,
may possibly be related to some function of tyrosine in blood
formation. However, the combined action of pteroylglutamic
acid and liver extracts in blood formation has now been
extended to other growth processes, and it seems probable
that the tyrosine-liver extract relationship as well is not
confined to hematopoiesis. The action of pteroylglutamic
acid in tyrosine metabolism and the more complex interrela-
tionships which have been reported between this vitamin and

other accessory factors will be discussed in the following
section.

D. Folic Acid and Tyrosine Metabolism

The isolation of pteroylglutamic acid, also called folic
acid, and the various conjugates of the vitamin several years
ago resulted in an intensified study of the various functions
of these compounds. This situation is comparable to the
widespread interest today in vitamin B_{12}. The importance of
folic acid in hematopoiesis and its effectiveness in curing
certain anemias was soon demonstrated. The close relation-
ship of pteroylglutamic acid and the liver principle in blood
formation and their apparently synergistic action in curing
certain anemias was demonstrated. Folic acid was found to
improve the blood picture in pernicious anemia. Stokstad and
Jukes (119) reviewed this subject in 1948.

These facts influenced Rodney, Swendseid and Swanson
(120) to investigate the influence of pteroylglutamic acid on
tyrosine metabolism. Rats were made deficient in the vitamin
by feeding sulfasuxidine, and the ability of liver homogenates
from these animals to oxidize tyrosine was investigated in
the Warburg apparatus. Oxygen uptake by the deficient livers
was lower than normal controls and the addition of pteroyl-
glutamic acid resulted in an increased oxidation. In 1949,
the same authors (121) repeated these experiments on folic

acid-deficient rats with some modifications. The in vitro addition of crystalline folic acid partially restored the oxygen uptake of deficient liver brei, as had previously been reported. Vitamin C and liver extract had no effect. The addition of a folic acid antagonist, 4-aminopteroylglutamic acid, in vitro had no effect on tyrosine oxidation. Livers from animals fed this antagonist, however, did show a decreased oxidation of tyrosine. Contrary to the expected result, the addition of folic acid to the system in this instance had no effect. However, when folic acid or liver extracts were included in the diet along with the antagonist, the tyrosine oxidation in vitro was normal. The function of pteroylglutamic acid in tyrosine oxidation seems to be com-plex, therefore, and closely connected with the action of factors in liver extract.

The earlier experiments of Swendseid and coworkers led Woodruff and Darby (122, 123) to investigate the effect of pteroylglutamic acid on the excretion of tyrosine metabolites by the vitamin C-deficient guinea pig. They found that this vitamin abolished the excretion of these metabolites as effectively as did vitamin C.

In the first series of experiments the vitamin was administered orally. In this case, 15 mg. per day for three days was required to reduce the tyrosyl (phenolic) value to 5% of the administered tyrosine. Later it was shown that

lowered excretory values could be maintained for as long as 20 days when the animals were fed a scorbutigenic diet plus 5% L-tyrosine and daily injections of 5 mg. of folic acid were administered. The four groups analyzed consisted of two animals each on the following diets: experimental, experimental plus folic acid, experimental plus vitamin C, and experimental plus both vitamins. The average daily tyrosyl values over the 20-day period for these groups were 37.3, 6.6, 5.2, and 4.6%, respectively.

Some criticism may be made of this long-time experiment, since the food intake (and consequently the tyrosine intake) of the one animal reported in detail (folic acid group) fell to zero several times during the experiment. In addition to pteroylglutamic acid, the triglutamate, but not the diglutamate, caused a decreased tyrosine metabolite excretion.

These authors also reported that daily intramuscular injections of 5 U. S. P. units of liver extract produced no change in metabolite excretion. This report is contrary to that of Sealock and Lepow (55). However, the conditions of the two experiments differed considerably. The basal diet was different, the pre-injection level of metabolite excretion in Woodruff and Darby's experiments was considerably lower, their dosage was at the extreme low end of the effective range as reported by Sealock, and they tabulated the average excretion level over a four-day period, while the excretion

during the 24-hour period following the injection has been
used by Sealock as the criterion of effectiveness. This
criterion was used because the effect of the injection of
liver extracts disappeared after the first 24-hour period
(124).

Recently, Govan and Gordon (125) have reported that
the administration of 10-30 mg. of folic acid removed the ab-
normal tyrosyl metabolites excreted by premature infants on
cow's milk diets. However, the variability of response,
which had previously been noted when liver extract was used
(47), was again reported. Data on 10 infants was given. Four
of these responded to folic acid treatment, while the others
did not. Five of these others, however, did respond to
vitamin C therapy.

The results cited in this section definitely implicate
folic acid in tyrosine metabolism. Its action resembles that
of vitamin C in the amounts used, the duration and intensity
of the tyrosine metabolite decreases produced, and the
demonstration of an in vitro effect on tyrosine oxidation.
On the other hand, reports in fields other than tyrosine
metabolism, stress the close relationship of folic acid to
liver extracts in blood formation and growth processes. Some
of the more pertinent relationships among these three factors
will be cited in the following paragraphs. No information is
available, however, as to what connection these factors have

with one another in tyrosine metabolism. If folic acid and vitamin C are concerned with a catabolic pathway of the amino acid, do they act at different stages of the same one, or do they act on two different pathways? Do the liver factor(s) act in an anabolic capacity? If so, what anabolic pathway of tyrosine is affected? These questions have not yet been answered.

Wintrobe (126) has shown that a folic acid deficiency in pigs resulted in a macrocytic anemia which closely resembled that found in pernicious anemia. Even the megaloblastic bone marrow was evident. The primary cure for this nutritional anemia was the administration of folic acid, although liver extracts and vitamin B_{12} had some curative effect. On the other hand, liver extracts seem to be the primary agents in the cure of pernicious anemia, though folic acid may alleviate the anemia. Bethell (127) has reported one case of pernicious anemia where a folic acid antagonist, 4-aminopteroylglutamic acid, blocked the usual response to the administration of vitamin B_{12}. This relationship appears to be similar to that found by Swendseid (121).

Very recently, Bennett (128) has reported experiments with rats on synthetic diets which she has interpreted to indicate a vitamin B_{12}-folic acid function in the utilization of homocystine. These rats showed normal rates of growth on a synthetic diet (amino acid mixture) containing no known

methyl donors, and homocystine as the only sulphur-containing amino acid. However, the addition of sulfasuxidine to the diet resulted in poor growth. Addition of biotin improved growth slightly, and high levels of folic acid, in addition to biotin, restored growth to normal in some animals. Animals which did not respond to these two vitamins, did show good growth when "Liver Extract Lilly" was added also. Growth rates reported for these various groups in gm. per day were: basal, 1.5; basal plus 2% sulfasuxidine, 0.3; basal plus biotin and folic acid, 1.3; basal plus biotin, folic acid and liver extract (growth of this group without liver extract was 0.0), 1.3. The author interpreted these results to indicate that folic acid and a factor found in liver extracts were required for homocystine utilization under the experimental conditions. Differences in storage levels of other B vitamins, of which vitamin B_{12} was probably the most important, accounted for the variability in the response to folic acid alone.

Elvehjem and coworkers (129) have studied the action of different factors on niacin-deficient dogs. These dogs lost weight and developed a macrocytic anemia. Finally, they ceased to respond to niacin supplementation. At this stage, folic acid produced hematopoietic responses, but the greatest gains in blood formation and weight were produced by administration of purified liver extracts in addition to these other

factors. The same group (130) has studied phenylhydrazine-induced anemia in folic acid-deficient chicks. Here again, folic acid produced some improvement, but the further addition of antipernicious anemia extracts was required for most rapid return to normal blood levels. Recently, the activity of various enzymes (131) from these folic acid-deficient chicks was studied. The effect of folic acid and vitamin B_{12} additions in vitro to these systems was reported, but no definite conclusions were drawn.

Day and coworkers (132) have reported that the growth depression produced by addition of 10% glycine to the diet of rats is prevented by inclusion of folic acid in the diet, though vitamin C and liver extract also had some effect.

Johnson and Dana (133) fed rats a folic acid-deficient diet, plus sulfasuxidine. The addition of vitamin C to this diet caused a significant weight gain and disappearance of hemorrhages. The further addition of folic acid produced a significant increase in reticulocytes. Elvehjem and coworkers (134) noted that vitamin C aided chicks on a folic acid-deficient diet. The hemoglobin levels were increased in this case and growth was affected by folic acid only.

E. Other Dietary Factors

1. Amino acids and other vitamins

A number of papers have appeared in which defects in tyrosine metabolism have been noted when dietary factors other than those previously discussed were omitted. Experimental alcaptonuria has been produced in rats by feeding large amounts of tyrosine or phenylalanine by Butts and co-workers (28), Papageorge and Lewis (40), Abbott and Salmon (135), and Lanyar (136); and in mice by Lanyar (137).

In 1945, Neuberger and associates (138) showed that the excretion of homogentisic acid by the rat receiving extra tyrosine was dependent on the cystine and methionine intake. Small amounts of tyrosine could produce the metabolic defect when these amino acids were absent from the diet. It was later shown (139) that albino rats developed the defect more easily than pigmented strains, but that when the protein intake was low, or essential amino acids were missing from the diet, both strains failed to metabolize tyrosine completely. Thus the primary defect in this experimental alcaptonuria, it was suggested, was an overloading of the catabolic pathways by extra tyrosine, which was accentuated when the anabolic pathways were blocked by lack of essential amino acids. As a result, the intermediate metabolic product of tyrosine oxidation, homogentisic acid, appeared in the urine.

Several authors have reported toxic effects when large doses of tyrosine were fed to very young rats. Martin and coworkers (140, 141) noted skin lesions and hyperglycemia under these conditions. The primary defect seemed to be a riboflavin deficiency, but the addition of cystine or glycine to the diet decreased the symptoms. Marnay (142) has reported similar effects when tyrosine was fed at a level of 5% in the diet, in addition to that found in the 19% casein which was the protein source. He also reported that cystine had a curative action. Thus it is possible that cystine has a more specific relationship to tyrosine metabolism than the work of Neuberger would indicate.

2. Purified diets for guinea pigs

The multiplicity of factors which had been reported to be of importance in tyrosine metabolism indicated the use of a purified diet, the composition of which was known, for any experimental work concerned with tyrosine utilization. Attempts to produce such a diet had been initiated soon after the guinea pig was introduced as a laboratory animal. However, it was not until a few months ago that a diet was devised which would permit growth equal to that produced on natural diets. Woolley and coworkers (143, 144) found that when three factors were added to their casein-sucrose ration, a growth rate of 4 gm. per day was obtained. These factors were identified as folic acid, some constituent of liver

extracts, and a bulk factor which was supplied by cellulose and protein. King and associates (145) developed a ration which supported daily growth of 4.4 gm. for 100 days. At least two nutrients of vitamin nature, one found in crude casein and the other in rice polishing concentrates, were needed.

Elvehjem and Hart (146) reported results using a sucrose-casein ration, which when supplemented with various crude materials, supported growth equal to that on a commercial ration. These supplements included Cellu flour, beet pulp and gum arabic at the 30% level. The gum arabic was best, but an hydrolyzate prepared from it had no growth-promoting action. Presumably some bulk factor, which previous authors also had noted as being necessary, was furnished. A ration containing sucrose, casein, vitamins, salts and 15% gum arabic produced growth of 5.1 gm. per day. The addition of alfalfa leaf meal improved this diet so that normal growth of 7-8 gm. per day was obtained. Astonishingly, however, the ash of this last supplement proved as effective as the meal. The authors hypothesized that the ash furnished a high level of potassium, which was needed by the guinea pig. The final results (147) confirmed this idea. A diet containing sucrose, 30% casein, 15% gum arabic, vitamins, salts, and soybean oil, and supplemented with 2.5% potassium acetate and 0.5% magnesium oxide supported normal growth during the experimental period.

F. Isotope Studies of Tyrosine Metabolism

In 1939, Schoenheimer and his collaborators (148-151) instituted their studies of protein metabolism which eventually resulted in the present concept of the dynamic state of body constituents. For these studies they used amino acids labelled with isotopic nitrogen. They discussed theoretical considerations involved in the use of N^{15} and methods of analysis and demonstrated that the amino group of amino acids did not exchange with the surrounding nitrogenous constituents unless chemical reaction took place. After these preliminary papers, the authors proceeded to synthesize amino acids with N^{15} in the amino group and feed these to rats.

Their tyrosine experiment (151) involved the addition of small amounts of DL-tyrosine (containing 2.04 atoms % excess N^{15}) to the diet for 10 days. At the end of the 10-day period, when the animal was killed, 1.86 gm. of the isotopic tyrosine and 0.93 gm. of the normal tyrosine in the casein had been consumed. Analyses of urinary constituents, liver, blood and carcass fractions were made.

In general, the isotopic nitrogen of the tissues was found mainly in protein and, apart from tyrosine, was found in four different fractions: 1) in the α - amino group of the dicarboxylic amino acids; 2) in histidine from the liver; 3) in the "potential urea" portion of arginine; 4) in the

protein "amide nitrogen" (liberated as ammonia during prote-
olysis). The only fraction isolated from protein which
contained no isotopic nitrogen was lysine. All of these pro-
tein fractions only accounted for a small portion of the total
isotope in the protein, so other fractions not analyzed
evidently contained considerable amounts of the isotopic
nitrogen.

Table III shows a summary (by the writer) of the analyses
for which the "% yield" could be calculated. No quantitative
recovery was reported for those fractions not listed in the
table, and Schoenheimer's conclusions were drawn from a con-
sideration of the determination of "atoms % N^{15} excess" only
in these cases. The calculated "% yield" in Table III is
based on the isotopic DL-tyrosine consumed.

Despite the fact that the animal remained in nitrogen
equilibrium throughout the experimental period, Schoenheimer
noted that about 48% of the dietary tyrosine nitrogen remained
in the body. This was the first demonstration of the exchange
between food and body constituents, which was later expanded
into the theory of the dynamic state of body constituents.

It should be noted that all these results were calcu-
lated on the basis of the racemic amino acid fed. Undoubtedly,
the metabolism of the D-isomer differs from that of the
natural form in some respects and this obscures more quanti-
tative interpretation of these results. The fate of a large

Table III

Isotope Recovery After Feeding DL-Tyrosine Containing N^{15}

Source	Substance	N^{15} excess atom %	Total N mg.	N^{15} excess mg.	% yield
Diet	Total tyrosine consumed	1.36	213.0	2.90	
Liver	Non-protein N	0.038	16.5	0.0063	0.2
	Total protein	0.039	224.0	0.087	3.0
	Tyrosine	0.316	5.9[1]	0.018	0.6
	Amide N	0.044	10.7	0.0047	0.2
Carcass	Non-protein N	0.015	947.0	0.142	4.9
	Total protein	0.012	8900.0	1.06	40.0
	Tyrosine	0.100	213.0[1]	0.21	5.6
	Amide N	0.012	383.0	0.046	1.6
Blood	Protein from plasma	0.029	47.5	0.014	0.5
	Non-protein N from plasma	0.066	1.8	0.001	0.03
Urine	Total N first 9 days	0.077	1950.0	1.50	51.8
	Total N last day	0.067	167.5	0.11	3.8
	Urea last day	0.067	140.0	0.094	3.2
	Ammonia last day	0.197	3.0	0.006	0.2
	Total recovery				104.2

[1]Calculated from analysis on another animal.

portion of the isotopic nitrogen in the protein has not been determined, and further analyses should yield additional information concerning the metabolism of this amino acid.

The experiment cited above is the only reported one in which isotopic nitrogen was used to mark the tyrosine molecule. Studies with marked tyrosine, in which the various dietary factors implicated in the metabolism of the amino acid are varied for the purpose of determining their site of action and function, should yield important information. The most logical start for such a series of experiments would utilize isotopic nitrogen, since it is quite possible that one of these vitamin factors may function in the removal of the amino group from the molecule. Also, for in vivo experiments isotopic nitrogen is more easily traced than deuterium or isotopic carbon since the dilution factor in the body is many times smaller. No studies such as these have been reported.

Radioactive carbon (C^{14}) and heavy carbon (C^{13}), however, have been incorporated in the tyrosine molecule for the purpose of studying some particular catabolic pathway. These investigations have been metabolic turnover studies in vivo, utilizing racemic tyrosine, thus making interpretation difficult; others have been in vitro investigations of some particular catabolic pathway.

Winnick, Friedberg and Greenberg (152) fed tyrosine

labelled with C^{14} in the β-position of the sidechain to a normal and a tumor-bearing rat. The carbon chain of the tyrosine, in contrast to the amino group, did not contribute significantly to the formation of other amino acids. Approximately 30% of the administered dose was found in protein, all as tyrosine. Intestinal mucosa had the highest C^{14} concentration, followed by kidney and blood plasma. The liver had some C^{14}, but very little was found in adrenals or thyroid. These findings may be related to the short time factor to some extent, since the dose was injected into the jugular vein and the animal killed six hours later.

Of the 1.0 mg. of tyrosine administered, approximately 30% was found in protein and 27% in the urine. The rest has not been accounted for by the analyses. Tyrosine, urea, acetoacetic acid, ketones, hippuric acid and creatinine accounted for 50% of the urinary radioactivity. The authors believed that the following reactions accounted for the radioactivity in hippuric acid and creatinine:

tyrosine $\longrightarrow$ acetoacetic acid $\longrightarrow$ acetic acid $\longrightarrow$ glycine.
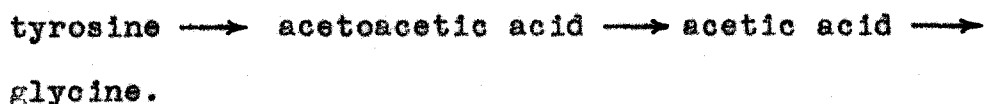
Reid and Jones (153) reported a similar study of the administration of radioactive tyrosine to mice bearing a melanosarcoma. After 72 hours, 30% of the administered dose appeared in the respiratory carbon dioxide, 40% in the urine and feces, and 30% in the carcass. Radioactivity was found

in every tissue of the body, and the highest activities, in general, were found to confirm the findings previously described. However, the adrenals and thyroid also showed high activity.

Weinhouse and Millington (154) incubated L-tyrosine, containing C^{14} in the β-position of the sidechain, with rat liver slices. Acetoacetate was isolated and degradation studies located almost all of the radioactivity in the α-carbon atom. The carboxyl group showed little radioactivity. The authors concluded that their findings were in accord with the scheme:

HO-⟨ ⟩-$\overset{*}{C}H_2$-CH-COOH ⟶ HO-⟨ ⟩-$\overset{*}{C}H_2$-CO-COOH
                    |
                   NH_2

CH$_3$-CO-$\overset{*}{C}H_2$-COOH ⟵ [cyclohexane with OH, OH]-$\overset{*}{C}H_2$-COOH
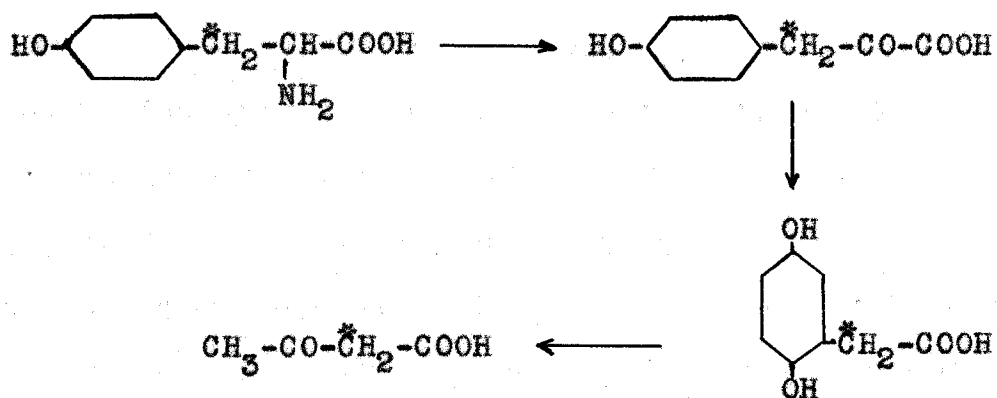
Gurin and coworkers (155, 156) have reported confirmatory experiments using labelled phenylalanine. The DL-form was used with C^{14} in the carboxyl group and the α-position of the sidechain. Incubation with rat liver slices, and isolation of the acetoacetic acid produced, showed the presence of C^{14} in the carboxyl group only. The respiratory carbon

dioxide also contained some isotope, showing that the original carboxyl group of the phenylalanine had been eliminated. The labelled amino acid was also fed to phlorizin-diabetic rats, but the excreted radioactivity was quite low.

In another experiment, phenylalanine ring-labelled in the 1,3 and 5 positions was incubated as previously and acetoacetate was isolated. Most of the radioactivity was found in the methyl carbon atom of the acetoacetate. The only mechanism which would explain this finding would involve a shift in the side chain as shown below:

$$CH_2\text{-}CH\text{-}COOH \longrightarrow \quad CH_2\text{-}CH\text{-}COOH \longrightarrow \quad \overset{*}{C}H_3 \quad + \quad CO_2$$
$$NH_2 \qquad\qquad NH_2 \qquad\qquad CO$$
$$CH_2$$
$$COOH$$
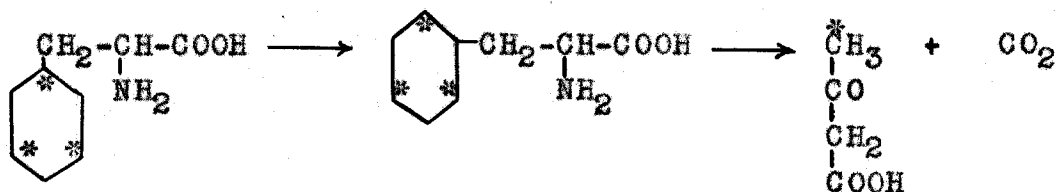
This scheme is in accord with the previously postulated mechanisms of Neuberger (21) and Neubauer (5), which involve quinol intermediates.

In November of 1949, Lerner (157) extended these studies in an effort to determine the fate of the other portions of the tyrosine molecule. For this purpose, L-phenylalanine was synthesized with C^{14} in every position of the ring and C^{13} in the α-position of the sidechain. Also, L-tyrosine with C^{14} in the β-position of the sidechain was used. These compounds were incubated with rat liver slices. The acetoacetic acid

isolated from phenylalanine incubation contained C^{14} in the β-and γ-positions and C^{13} in the carboxyl group. This confirmed the work of Gurin, cited above, and indicated that the acetoacetic acid was formed from two ring carbons and the α- and β-carbons of the phenylalanine side chain split off as an intact molecule.

The author also isolated malic acid with C^{14} in every position. This compound, as well as acetoacetic acid, was a major component of the oxidation, since 14.2% of the starting radioactivity was recovered as malic acid and 7.3% as carbon dioxide derived from malic acid. This compound was formed from the four ring carbon atoms which were not involved in acetoacetic acid formation. The studies with labelled tyrosine confirmed those with phenylalanine. Cleavage of homogentisic acid, as shown below, could yield these two products:
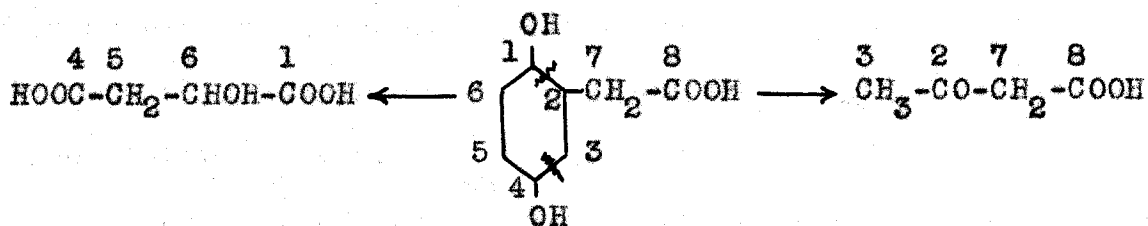
III. EXPERIMENTAL

A. Effects of Concentrated Liver Extracts

1. Methods

The guinea pigs used for these experiments were obtained from commercial dealers and maintained in stock cages on Purina Rabbit Chow, complete ration, supplemented with carrots and cabbage. Later, some animals were kept on Rockland Guinea Pig Ration (vitamin C-fortified). Animals weighing from 250 to 500 gm. were used for experiments. For the metabolic experiments they were housed in individual wire mesh cages supported in large funnels. Feces and food particles were caught in fine mesh screening underneath the cage, while the urine ran into a collecting bottle. This bottle contained 10 ml. of 2.0 N. hydrochloric acid.

Only male animals were used for experiment. These were fed a basal diet which contained insignificant amounts of vitamin C. The basal diet was prepared by grinding Purina Rabbit Chow to a fine powder and spreading in a thin layer. Exposure to the air in this manner for five or more days destroyed the small amounts of the vitamin originally present. After two to three days on this basal diet, the animals were fed daily supplements of 200 mg. per 100 gm. of body weight

of L-tyrosine. L-Tyrosine, purchased from Merck and Company, was fed as a 10% mixture with the basal diet. In addition to the tyrosine supplement, the food pans always contained basal diet, and more was added when the animals had consumed the supplement. A few experiments in which the animals did not eat all of the supplement were discarded. The guinea pigs were weighed daily while on experiment. At the start of each 24-hour period approximately 0.9 gm. of Squibb or Mead Brewers' Yeast was sprinkled on top of the diet mixture. All animals were fed 1.0 ml. of cod liver oil per week by pipette. A supply of fresh water was always available.

At the end of each 24-hour period, the splattered urine was washed from the funnel into the collecting bottles. The urine samples plus washings were filtered through No. 4 Whatman filter paper into 100 ml. graduated cylinders and diluted to that volume with distilled water. Analyses were done immediately on aliquots of this or of a 1:20 dilution.

Analyses for keto acid were done with the 2,4-dinitro-phenylhydrazine reagent according to the method of Penrose and Quastel (158), as modified in this laboratory (124). Total tyrosyl values (phenol) were determined by the method of Folin and Ciocalteu (159), as modified in this laboratory (124). This method depends on the quantitative development of a blue color when phosphotungstic-phosphomolybdic acid is added to a phenol-containing solution. All readings were

made on the Klett-Summerson photoelectric colorimeter with the appropriate filter and standard. Due to the instability of p-hydroxyphenylpyruvic acid, it could not be used as a primary standard for the keto acid determination. Instead, the colorimeter was calibrated with a freshly-prepared solution of p-hydroxyphenylpyruvic acid.

The experimental procedure followed, unless otherwise indicated, was to feed the tyrosine supplement and analyze at the end of each 24-hour period until the keto acid excretion was 30% or more of the administered extra tyrosine. The various vitamin supplements were then given at the start of the 24-hour period and analyses continued usually for several days following vitamin supplementation. Vitamin C was given by mouth as a water suspension, liver extract was injected intraperitoneally and folic acid injected subcutaneously, unless otherwise noted.

2. Analysis of assay procedure

The first series of injections of liver extract were preliminary in nature, and made for the purpose of studying the variation when the same dose was injected into different animals and to follow the proportionality of response to administered dose. Two preparations were tested. These were high-potency experimental extracts identified as Armour 1080 and 1103. The antipernicious anemia potency of these

extracts was approximately 20 U. S. P. units per ml., although they were prepared from different liver batches. A few injections were made subcutaneously, but later the intraperitoneal route was adopted, as it was felt more rapid absorbtion and hence greater effectiveness was obtained.

A typical experiment is shown in Figure 1. The prompt decrease in both keto acid and tyrosyl values upon administration of liver extract can be seen. The level of metabolites is plotted as per cent of theoretical yield, based on the amount of extra tyrosine fed. The changes in both tyrosyl and keto acid values parallel each other approximately, as was usually the case. The rapid increase in metabolite excretion on the second day after injection was also typical.

The results of the complete series of injections are reported in Table IV. Only keto acid values are given, since the tyrosyl changes paralleled these so closely. In some cases, more than one injection was made, but the effect of these later injections was very small (as will be shown). Therefore, only the excretion values in the 24-hour period following the first injection are reported. When a guinea pig was used more than once, it was only after several weeks on the stock diet prior to the second experiment.
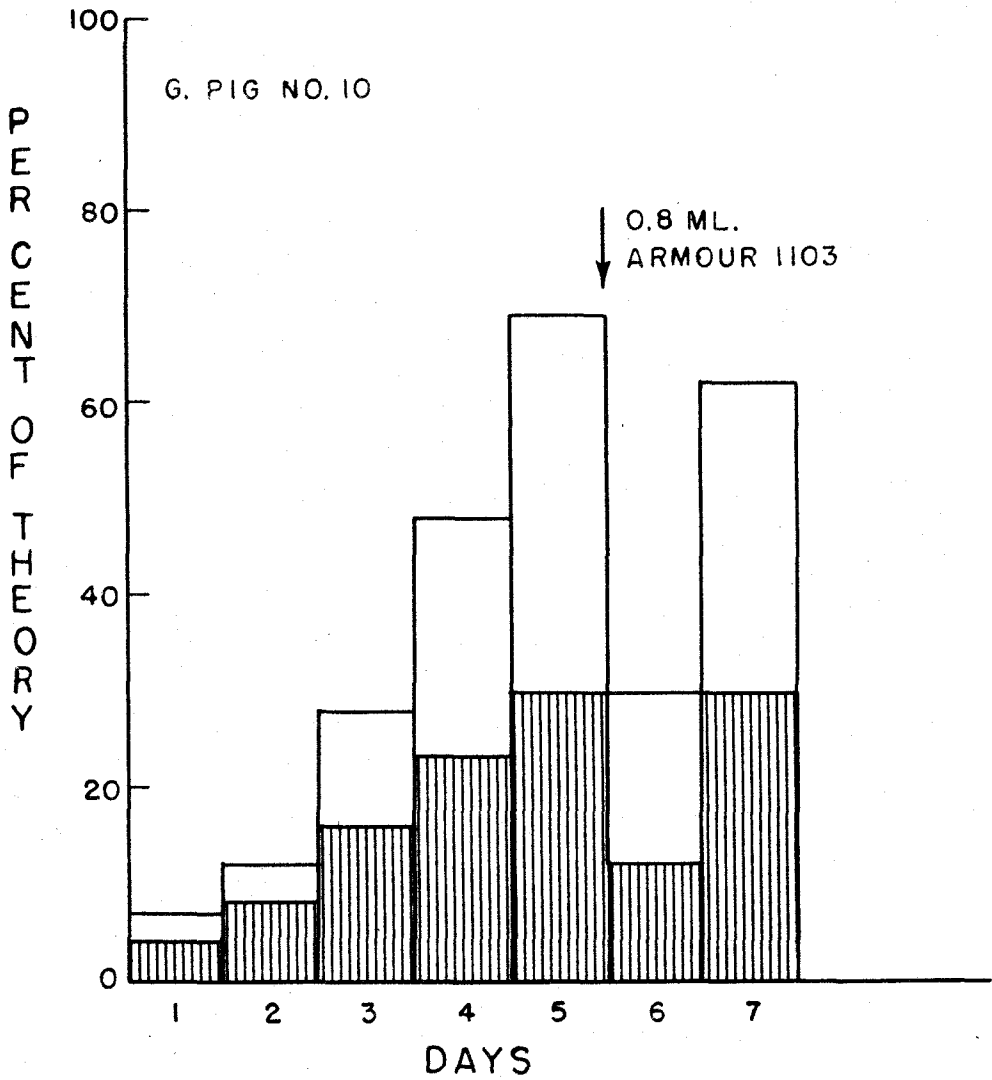
FIG. I   EXCRETION OF KETO ACID (SOLID COLUMNS) AND TYROSYL METABOLITE CALCULATED AS TYROSINE (CLEAR COLUMNS).   ARROW INDICATES LIVER EXTRACT INJECTION.

Various calculations may be made to express the change in the level of metabolite excretion. The "Decrease" in Table IV is the simple difference between the keto acid levels, expressed as per cent of the theoretical yield, before and after injection. This expression will be used in most of the tables and is represented by the figure 5.9 in Column 7 of Table IV. However, the magnitude of this change in relation to the pre-injection level may be expressed by dividing this figure by the keto acid level (%) prior to injection and multiplying by 100. This "Percentage Decrease," in the example cited, would be equal to 5.9 divided by 50.3 multiplied by 100, or 11.7%.

The results given in Table IV show that although the two preparations had similar antipernicious anemia activity, their ability to decrease metabolite excretion was quite different. In addition, the variability within each group was high, particularly in the case of the less effective extract. The proportionality of decrease to dose was poor, since the smaller injections produced a greater response per 0.1 ml. injected. This may have contributed to the variability within the group and accordingly a series of experiments was designed to test the effect of injection volume on response.

Table IV

Keto Acid Excretion Before and After
Liver Extract Injection

G. pig no.	Wt.	Keto acid before		Keto acid after		De- crease	In- jec- ted	De- crease per 0.1 ml.
		Urinary excretion						
	gm.	mg.	%	mg.	%	%	ml.	%
							Armour 1080	
3	380	382	50.3	337	44.4	5.9[1]	0.1	5.9
4	340	404	59.5	350	51.5	8.0	0.2	4.0
8	395	296	37.5	169	21.4	16.1	1.0	1.6
4	350	386	56.8	222	31.7	25.1	1.0	2.5
Average								3.5
							Armour 1103	
8	440	304	34.5	291	32.4	2.1	0.8	0.3
4	400	217	27.2	362	44.2	+17.0[2]	0.8	+2.1
6	460	320	34.8	385	41.8	+ 7.0	0.8	+0.9
7	450	260	28.9	195	21.7	7.2	0.8	0.9
10	450	271	30.2	108	12.0	18.2	0.8	2.3
Average								0.1

---

[1]Difference between keto acid level (%) before and after injection.

[2]Plus signs indicate an increased urinary excretion.

The effect of injection volume on metabolite excretion may be observed in Table V. All of these injections consisted of 0.5 ml. of Lilly LS-1002-20. This preparation was a commercial antipernicious anemia extract containing 20 units per ml. Previous work in this laboratory (124) had shown this extract to have good activity in these guinea pig experiments. Only keto acid values are reported, since tyrosyl values paralleled these closely.

The relative ineffectiveness of this preparation, in general, may be seen. Since the same extract had previously given good results, evidently there was a loss in potency during storage of this highly purified preparation. The age of the extract, even though stored in the cold, is thus a factor which contributes to the variability of results.

The injection volumes seemed to have no definite effect on the response, except that the 5.0 ml. injections gave good decreases in two instances. However, this large injection volume resulted in poor food consumption. It was concluded that 1 to 2 ml. of undiluted 20 unit extract would be best for future work.

Table V

Keto Acid Excretion Before and After Liver Extract Injection

G. pig no.	Wt.	Urinary excretion				De-crease	Injec-tion[1] volume
		Keto acid before		Keto acid after			
	gm.	mg.	%	mg.	%	%	ml.
15	300	258	43.2	280	45.2	+ 2.0[2]	0.5
20	260	236	45.4	244	45.2	0.2	0.5
23	300	218	36.4	233	38.8	+ 2.4	0.5
27	420	252	30.0	459	53.4	+23.4	0.5
17	300	172	28.6	228	39.3	+10.7	1.0
18	330	217	32.9	98.5	15.4	17.5	1.5
16	340	223	36.0	396	58.3	+22.3	2.0
19	360	332	46.2	310	45.6	0.6	5.0
24	430	244	28.4	165	19.2	9.2	5.0
22	390	322	41.3	324	42.7	+ 1.4	5.0
28	390	203	26.1	95.5	12.6	13.5	5.0

[1]All of these injections consisted of 0.5 ml. of Lilly LS-1002-20 diluted to the indicated volume.

[2]Plus sign indicates an increased urinary excretion.

While a considerable difference in response from animal to animal was still observed when more potent extracts were used, some decrease in metabolite excretion was always evident. Table VI lists the results obtained with freshly purchased commercial extracts of similar potency to those previously used. Two preparations, Eli Lilly No. 377 and Reticulogen No. 360, assayed at a minimum of 15 units per ml. were used. The average "Percentage decrease" shown in Table VI represents a drop in keto acid excretion of more than half. Tyrosyl values paralleled these changes closely and are therefore not reported.

It may be pertinent at this point to show the excretory pattern of an animal receiving extra tyrosine but no other supplement. Although numerous reports (see Historical) have appeared indicating that metabolite excretion remains high in the absence of vitamin C or other active supplement, one such control is shown in Figure 2. The excretory values remained high, particularly when compared with those recorded in Table VI after liver extract injection. The average of each two-day period is recorded, rather than individual days, in order to show a longer period on the graph. This animal was maintained on the C-free basal diet plus extra tyrosine for 26 days. While the excretory values fluctuated somewhat toward the end of the experiment, the keto acid values never dropped below 30%. These fluctuations can be definitely

Table VI

Keto Acid Excretion Before and After
Liver Extract Injection

G. pig no.	Wt.	Urinary excretion				Decrease	Percent-age de-crease[1]	In-jected[2]
		Keto acid before		Keto acid after				
	gm.	mg.	%	mg.	%	%		ml.
26	350	270	38.5	131	18.2	20.3	52.9	1.0
22	450	343	38.2	92.5	10.0	28.2	73.9	1.0
35	530	304	28.6	196	18.5	10.1	36.6	1.0
38	330	331	50.2	100	17.3	32.9	65.5	1.0
Average							57.2	

[1]"Decrease" divided by "Keto acid before (%)" x 100.

[2]The first 3 injections are Lilly No. 377 and the last is Reticulogen No. 360.
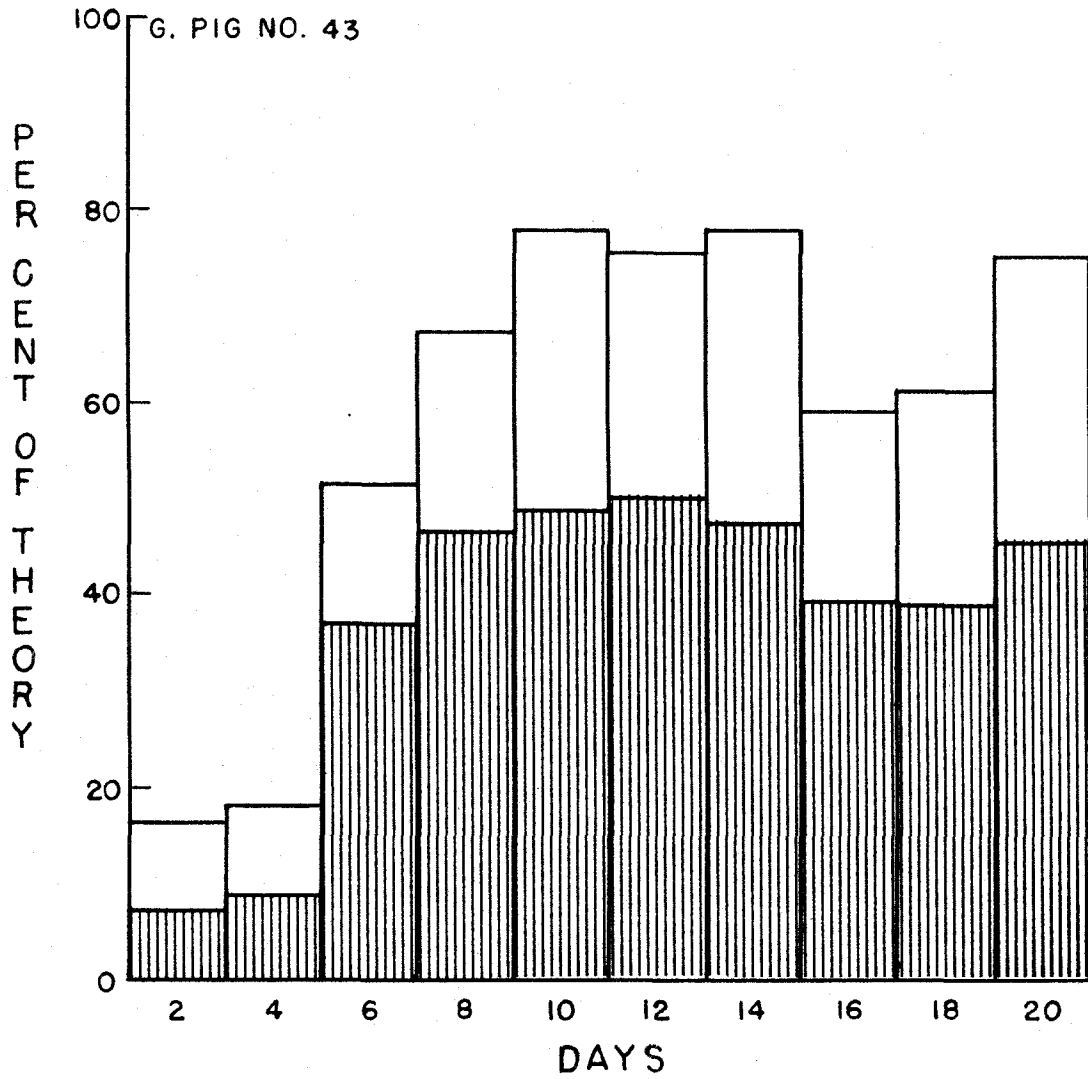
FIG. 2   EXCRETION OF KETO ACID (SOLID COLUMNS) AND TYROSYL METABOLITE CALCULATED AS TYROSINE (CLEAR COLUMNS). CONTROL ANIMAL, AVERAGE OF 2 DAYS IN EACH COLUMN.

attributed to the variation in food consumption which began
to appear when the vitamin C deficiency became acute. The
usual experiment lasted less than 10 days. It should be
noted that, in addition to the factor of poor food consump-
tion, if the room temperature accidentally dropped to
freezing or below for a period of several hours, the metabo-
lite excretion would decrease. Such experiments were
discarded.

Following the rather sharp decreases obtained with the
1.0 ml. injections shown in Table VI, it was felt that even
greater decreases in metabolite excretion could be obtained.
Vitamin C and folic acid completely abolish metabolite
excretion if the dosage is large enough. However, with liver
extracts, as has been shown in Table IV, the larger dosages
seemed to produce a proportionately smaller effect. Further-
more, when more than 1.0 ml. was injected, toxic effects were
noted. These may have been due to the 0.5% phenol included
in these parenteral liver extracts as a preservative.

Therefore, a series of experiments were performed to
test the effect of consecutive injections on the level of
metabolite excretion. Two series of experiments were run:
consecutive daily injections and consecutive injections on
alternate days. These results are tabulated in Table VII.
Both keto acid and tyrosyl metabolite excretion are given so
that comparison of these values may be made.

Table VII

Metabolite Excretion with Consecutive Liver Extract Injections

G. pig no.	Day	Keto acid excreted		Tyrosyl value excreted		In-jected
		mg.	%	mg.	%	ml.
26	1-2	122	17.6	192	26.4	
	3	270	38.5	495	70.7	
	4	131	18.2	222	30.8	1.0[1]
	5	308	44.0	562	80.3	
	6	322	46.0	569	81.3	1.0
	7	292	40.7	527	73.2	
	8	285	40.7	452	64.5	1.0
	9	analysis omitted, poor food consumption				
	10	288	41.2	545	77.9	
	11	217	31.0	366	52.3	1.0
	12	268	38.3	517	73.9	
	13	288	42.4	517	76.0	1.0
	14	302	43.2	531	75.9	
	15	384	54.9	641	91.7	1.0
	16	348	49.8	630	90.0	
31	1-4	187.1	19.3	221	22.5	
	5	325	34.6	411	43.8	
	6	149	15.9	185	19.7	1.0[2]
	7	328	34.9	511	54.4	1.0
	8	348	37.1	478	50.8	1.0
	9	151	16.8	215	23.9	
	10	234	26.0	354	39.4	
	11	228	25.4	360	40.0	
	12	222	26.4	333	39.7	

(continued)

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[1] Lilly No. 377.

[2] Reticulogen No. 360 plus 5 mg. $CoSO_4 \cdot 7H_2O$ (Also see Table XII).

Table VII (continued)

G. pig no.	Day	Keto acid excreted mg.	%	Tyrosyl value excreted mg.	%	In-jected ml.
27	1	24.6	2.8	43.5	5.1	
	2	252	30.0	322	38.4	
	3	459	53.4	852	99.1	0.5[1]
	4	626	71.2	996	113	0.5
	5	442	51.4	720	83.8	2.0
	6	479	55.7	756	88.0	2.0
	7	376	43.7	580	67.5	2.0
28	1	203	26.1	295	37.8	
	2	95.5	12.6	159	20.9	0.5[1]
	3	156	20.5	307	40.4	0.5
	4	388	51.2	612	80.6	0.5
	5	80.0	10.8	143	19.4	2.0
	6	393	51.7	663	87.3	2.0
	7	457	60.2	828	109	2.0
	8	471	62.0	829	109	2.0
17	1-5	23.0	3.0	66.0	8.5	
	6	229	29.4	396	50.8	
	7	analysis omitted, animal ate poorly				0.7[2]
	8	21.6	2.9	58.9	7.9	
	9	237	32.1	370	50.0	
	10	306	41.5	520	70.4	1.0
	11	324	43.8	584	79.0	1.0
	12	248	33.5	521	70.5	1.0
	13	226	30.8	471	63.7	1.0
	14	186	24.8	414	54.5	1.0
	15	274	36.1	480	63.2	1.0
	16	282	36.2	560	72.0	

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[1] Lilly No. LS-1002-20.

[2] Lilly No. 377.

In contrast to the action of folic acid and vitamin C, it was found that the first injection produced the greatest decrease. The second injection generally had no effect, while further administration of these extracts resulted in irregular decreases, none of which were as great as the decrease in the 24-hour period after the first injection. Although no set of experiments was performed to determine the time interval required for a second injection to have as great an effect as the first, a few observations indicated that the refractory period lasted at least four or five days. In some cases, there was a slight decrease after the end of the injection series. In all cases, however, the series of injections failed to produce the steady decline in metabolite levels observed with the administration of vitamin C or folic acid over a period of days.

The problem of completely abolishing metabolite excretion with liver extracts remained unanswered. Furthermore, some new questions were raised. Was the refractory period related to the depletion of some accessory factor by the first injection? Why was there a slight decrease in metabolite levels after a series of injections was concluded? Evidently, the response of the animal to liver extract was a complex phenomenon, possible involving mobilization from body stores of unknown factors.

It became evident at this time that the response to

these liver extracts was quite different from the previously reported type of response to vitamin C and folic acid. It was felt that any attempt to investigate the nature of this difference was complicated by the fact that the liver extracts were complex mixtures, which differed from batch to batch and changed as they aged; and that other factors, which were present to a different extent in different animals, affected the action of a given injection.

For these reasons, two lines of investigation were initiated: the first was a study of the characteristics of the active agent in these liver preparations, and the second involved a study of factors which would enhance the action of liver extracts. An attempt to use a purified diet was part of this second study. It was hoped that the clarification of these two points would permit more control of the experimental factors and thus eliminate some of the discrepancies previously noted. The results of these experiments are given in the next three sections.

However, the main purpose of the investigation was to study the mechanism of action of the factor in these extracts, and the function of this factor in relation to the action of vitamin C and folic acid. Therefore, the work on blood, reported in the last section of the liver extract work, was initiated in conjunction with these other studies.

3. Characteristics of active agent

One of the first attempts to investigate the nature of
the active agent in liver extracts involved the testing of a
liver dialyzate[1]. These preparations reactivated a dialyzed
brei containing an enzyme system which oxidized tyrosine _in_
vitro. Their vitamin C content was quite low. As the
dialyzate was fractionated and the activity for the tyrosine
oxidizing system _in_ _vitro_ concentrated, some of the more
active fractions were tested for their ability to reduce the
tyrosyl metabolite excretion of the vitamin C-deficient
guinea pig. Some of these dialyzate injections, as shown in
Table VIII, produced a considerable drop in excretory levels.
Others had no effect. In many cases, however, the prepara-
tions had an irritating effect and the animal ate poorly.
This might have been responsible for the decreases noted.
Therefore, it was decided to await further purification of
these dialyzates before additional testing was done.

At the time this work was being done in 1948, the first
publications on vitamin B_{12} were appearing. Since evidence
was available in this laboratory that the factor in these
antipernicious anemia liver extracts responsible for their
clinical action was the factor responsible for the action
observed in our experiments, these publications were followed
in some detail.

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[1]These dialyzates were prepared by Philip L. White.

Table VIII

Keto Acid Excretion Before and After
Injection of Liver Dialyzates

Urinary excretion

G. pig no.	Wt.	Keto acid before		Keto acid after		De-crease	In-jected
	gm.	mg.	%	mg.	%	%	ml.
12	400	385	48.2	318	39.8	8.4	0.5[1]
13	290	274	47.3	214	36.9	10.4	1.0
14	370	326	44.0	420	58.3	+14.3[3]	0.5
15	400	350	43.8	411	54.2	+10.4	1.0[2]
16	430	310	36.1	60.0	7.0	29.1	1.0
18	410	302	36.8	63.0	7.7	29.1	2.0

[1]Dialyzate No. 2-205B, equivalent to 4 gm. of liver per ml.

[2]Dialyzate No. 2-208B, equivalent to 5 gm. of liver per ml.

[3]Plus sign indicates an increased urinary excretion.

The first information on the chemical characteristics
of vitamin B_{12} was reported by Smith (71). He noted that
boiling this factor with 1.0 N. hydrochloric acid for one
hour did not inactivate the preparation. A sample of liver
extract Lilly No. 377, one of the more active extracts used
in this work, was treated in this manner, neutralized and
filtered. One sample so treated, equivalent to 1.0 ml. of
the original extract, when injected produced a keto acid
decrease from 32.2 to 14.9%; another, equivalent to 2.0 ml.
of the original, produced a decrease from 46.0 to 42.5%.
These results indicated that the acid treatment had not in-
activated the factor to any great extent and strengthened
the hypothesis that vitamin B_{12} would possess activity in
reducing the tyrosine metabolite excretion of the vitamin C-
deficient guinea pig.

The direct testing of this hypothesis became possible
when a vitamin B_{12} concentrate was received. This was a
Merck product, called "Experimental Vitamin B_{12} Mixture,
8R3890," and containing vitamin B_{12} concentrate, charcoal
and soybean flour. The concentration of the vitamin was
10.0 μg. in 2.27 gm. of the mixture. Information received
later stated that this mixture was a source of the pure
vitamin, uncontaminated by other liver factors.

The results of these feeding experiments with the vita-
min are reported in Table IX. At the 20 μg. level, the

decrease in metabolite excretion was greater than any pro-
duced with liver extract injections. Lower levels of the
vitamin had less effect. One experiment in which this con-
centrate was fed at the 10.0 μg. level for three successive
days resulted in the same small decreases observed with
liver extract after the first 24-hour period. The oral
administration of a large dose of Lilly No. 377, an active
liver extract when injected, is included to show the inef-
fectiveness of these extracts by this route. Vitamin B_{12}
has been reported to have some effect when administered
orally to pernicious anemia patients (82), in contrast to
the ineffectiveness of liver extracts by this route. This
difference is shown in these experiments also.

The great drop in metabolite excretion at the 20 μg.
level, as compared with 1.0 ml. of the best liver extracts
(recorded in Table VI), becomes more significant when the
vitamin B_{12} content of these liver extracts is examined.
Elvehjem (103) has reported that 1 U. S. P. unit of Reticulo-
gen is approximately equal to 1.0 μg. of vitamin B_{12} by
microbiological assay. Thus 1.0 ml. of Lilly No. 377 should
contain 15-20 μg. of the vitamin, but the effect of this
amount of liver extract was much smaller than the effect of
that amount of the vitamin itself. The conclusion to be
drawn, that vitamin B_{12} accounted for the activity of these
liver extracts in reducing metabolite excretion, must be

Table IX

Keto Acid Excretion Before and After Feeding of Vitamin B_{12}

G. pig no.	Wt.	Urinary excretion					Percentage decrease[1]	Amount fed[2]
		Keto acid before		Keto acid after		Decrease		
	gm.	mg.	%	mg.	%	%		μ g.
16	480	197	20.5	345	35.2	+14.7	+71.7[4]	5.0
15	440	356	40.4	362	42.1	+ 1.7	+ 4.2	10.0
24	500	250	25.0	209	21.4	3.6	14.4	10.2
18	470	216	23.5	32.4	3.4	20.1	85.6	20.0
25	470	340	36.2	378	40.2	+ 4.0	+11.0	5.0 ml.[3]

[1] "Decrease" divided by "Keto acid before (%)" x 100.

[2] Fed vitamin B_{12} concentrate containing 10.0 g. per 2.27 gm. plus charcoal and soybean flour.

[3] Liver extract Lilly No. 377.

[4] Plus sign indicates an increased urinary excretion.

qualified, since no further experiments with this concentrate were performed. The soybean flour and charcoal introduced unknown factors into the diet, and it was felt that the crystalline vitamin would soon become available. For example, it is quite possible that the protein level of the diet is important in the action of vitamin B_{12} and the soybean flour increased this considerably. Work with crystalline vitamin B_{12} was undertaken by others in this laboratory when it became available, and its activity in the guinea pig was confirmed[1], but its action at the 20 μg. level was not nearly so great as that reported here. The question of whether vitamin B_{12} accounts for all of the activity of liver extract has not yet been answered.

4. Liver extract in conjunction with other factors

The two factors which had been shown to act in tyrosine metabolism, in addition to liver extracts, were folic acid and vitamin C. Small amounts of these in the natural diet used or different storage levels in the experimental animals might account for some of the variability observed in the response to liver extract. It was planned, therefore, to include these at a constant level in the diet, but in small amounts so that the animal excreted the usual high percentage of metabolites. At this point, liver extract would be

[1]Sealock, R. R., and Wu, M. C. Unpublished data.

administered. Two such experiments, in which the animal received 1.0 mg. of vitamin C daily, are reported in Table X and Figure 3. Also included in this table is an experiment in which 1.0 mg. of cobalt chloride was administered with each liver extract injection. This experiment was done after the report appeared that vitamin B_{12} was a cobalt complex (70). It was hoped that the cobalt chloride would result in an increased amount of liver extract activity.

As shown in Table X, the action of the liver extract was enhanced by the addition of 1.0 mg. of vitamin C daily. Not only were the decreases in metabolite excretion high, but there was a response to the second liver extract injection. Despite the continuation of the vitamin C, when the injections were concluded the metabolite excretion rose. Further confirmation of this action of vitamin C in enhancing the effect of liver extract is given in Figure 3. This experiment is reported in detail because of the spectacular decrease to 2.65% keto acid excretion under the influence of daily injections of Reticulogen. Finally, however, the excretion level rose despite further injections. The experiment was continued in order to observe the effect of the vitamin C alone. Sealock and Silberstein (43) have reported that 1.0 mg. of the vitamin may cause some decreases in metabolite levels. This was shown to be the case in this experiment on the 14th and 15th days (Figure 3).

Table X

Effect of Various Accessory Factors
on Liver Extract Activity

G. pig no.	Wt.	Day	Keto acid excretion		In-jected
	gm.		mg.	%	ml.
22	470	1-3	104	11.0[1]	
	450	4	343	38.2	
	460	5	92.5	10.0	1.0[2]
	450	6	450	50.0	
	440	7	159	18.1	1.0
	440	8	211	24.0	1.0
	440	9	142	16.2	1.0
	440	10	411	46.7	
23	445	1-5	74.2	8.2	
	460	6	353	38.4	
	460	7	267	29.0	1.0[3]
	460	8	189	20.6	
	450	9	325	36.2	1.0
	440	10	331	37.7	1.0
	460	11	368	40.1	

[1]Animal fed 1.0 mg. vitamin C daily.

[2]Liver extract Lilly No. 377, 15 units per ml.

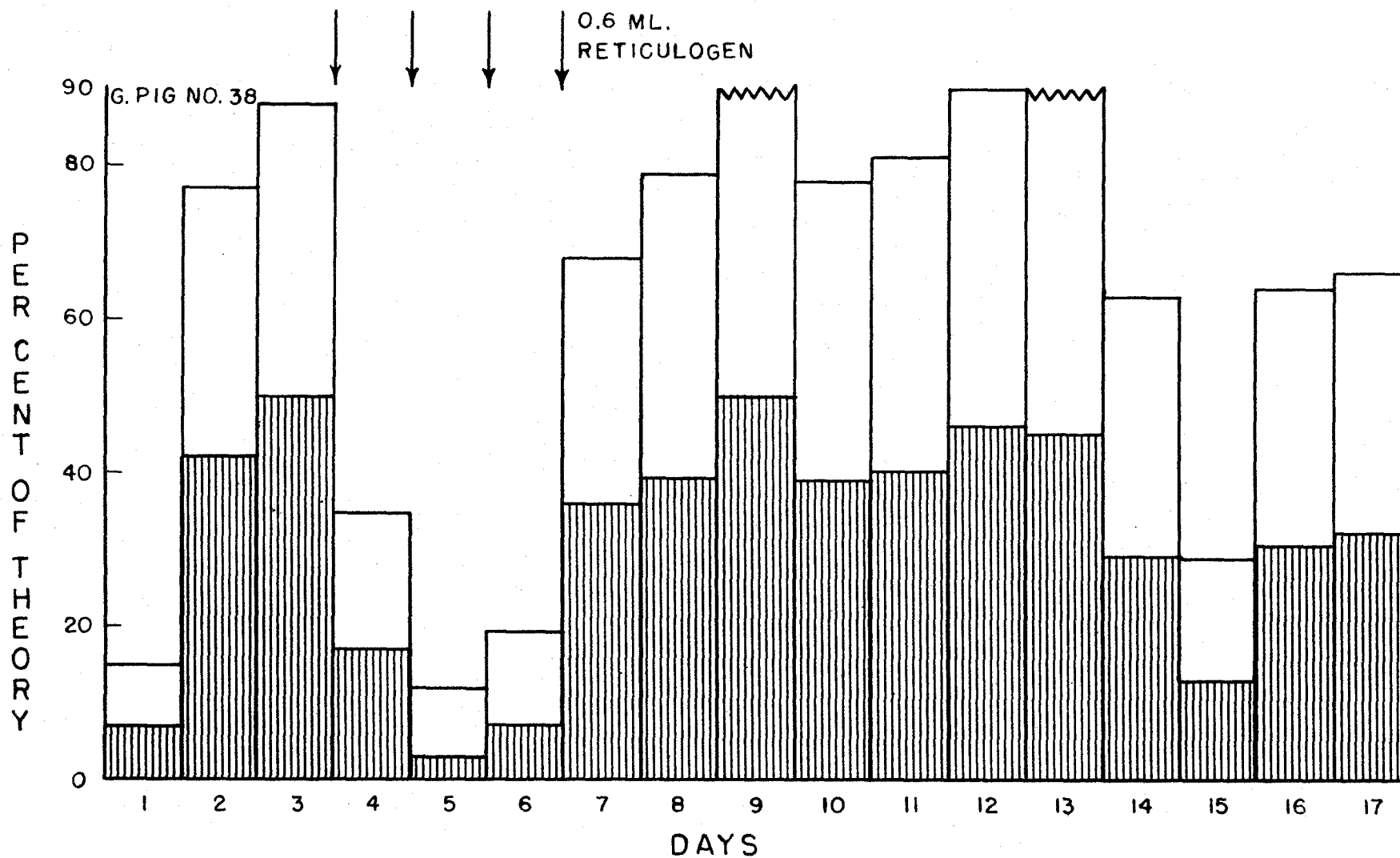[3]Liver extract Lilly No. 377 plus 1 mg. $CoCl_2 \cdot 6H_2O$.

FIG. 3 EXCRETION OF KETO ACID (SOLID COLUMNS) AND TYROSYL METABOLITE CALCULATED AS TYROSINE (CLEAR COLUMNS). ANIMALS FED 1.0 MG. OF ASCORBIC ACID DAILY. ARROWS SHOW LIVER EXTRACT INJECTIONS.

It was therefore concluded that carefully controlled experiments, using smaller amounts of vitamin C, would have to be run before any conclusions regarding a vitamin C-liver extract synergism could be made. It was decided that crystalline vitamin B_{12} ought to be used for this work in order to minimize the number of variable factors. Therefore, no further experiments with vitamin C, and none with folic acid, were run.

The results with cobalt chloride, reported in Table X, show no enhancement of liver extract action when 1.0 mg. of this compound was injected along with the liver preparation.

5. Use of purified diet

The problems involved in the use of a purified diet and the utility of this type of ration have been discussed in the Historical section. It was hoped that the exact control of vitamin and other constituents, which was possible with this diet, would permit more exact investigation of the various factors active in tyrosine metabolism. The basal diet used was a combination of those reported by Elvehjem (146) and Woolley and Sprince (144).

This was composed of 20% vitamin-free casein (Labco), 15% Cellu flour, 54.8% sucrose, 1% sucrose containing B vitamins, 4% corn oil (Mazola) fortified with fat-soluble vitamins, 5% salt mix, and 0.2% choline chloride. All but

one of these constituents were carefully ground together in a mortar. The corn oil was added last and incorporated by mixing in an evaporating dish, since the mortar would absorb some of the oil. After mixing, the diet was stored in the cold in a brown bottle to minimize the action of light on some of the vitamins.

The B vitamin mix was made from 17.23 gm. of sucrose, 2.00 gm. of inositol, 0.40 gm. of nicotinic acid, 0.20 gm. of p-aminobenzoic acid, 0.10 gm. of calcium pantothenate, 0.03 gm. of riboflavin, 0.02 gm. of pyridoxine and 0.02 gm. of thiamine. For short-time experiments it was felt that the lack of biotin would have no harmful effects. Vitamin C, folic acid and vitamin B_{12} were added to the diet only during the experimental periods as will be described. The constituents of the mix were weighed out, transferred to an evaporating dish and folded together, then ground lightly until thoroughly mixed.

The salt mixture was the one used by Elvehjem (146) and was originally prepared by Hart (160) as part of a ration for white rats. The salts used were C. P. or Reagent grade obtained from stock. To prevent caking, the highly hydrated constituents were ground in a mortar and then added to the others which had previously been ground in a ball mill.

The corn oil was made up in 400 gm. batches and each 4 gm. contained 2000 U.S.P. units of vitamin A, 100 U.S.P.

units of vitamin D, 10 mg. of alpha-tocopherol and 0.1 mg. of vitamin K (2-methyl-3-phytyl-1,4-naphthoquinone). This mixture was kept in the cold to prevent rancidity.

The animals on the natural stock diet were transferred to this purified diet, over a period of seven days usually, by feeding a mixture of the two diets and gradually increasing the proportion of purified diet. Vitamin C was administered orally during this period, but not during the experimental period. If this transition period was not observed, the animals showed signs of polyuria and diarrhea. Even at the conclusion of this period, however, and throughout the experiment a small amount of the stock diet (about 5% of the total diet fed) had to be placed on top of the food to induce the animals to eat. The tyrosine was incorporated with the basal diet at the 10% level, and the experimental procedure was the same as that described previously.

The main difficulty with the diet was its lack of palatability. Some animals would eat for a few days and then refuse the diet. These results were comparable to those obtained by Elvehjem (146) using a similar basal ration. The experiments reported in this section and the first two isotope experiments were successful in that the food intake was fairly consistent from day to day. This could not be predicted, however, and several experiments were started and

then discarded when the animals failed to eat for a day.
Therefore, the remainder of the isotope experiments were run
using the usual Purina chow diet.

Attempts were made to improve the palatability of the
purified diet by increasing the roughage from 15 to 25%, by
reducing the choline content from 0.3 to 0.1%, by decreasing
the salt mixture concentration from 5.0 to 4.0%, by using an
Osborne-Mendel salt mix in place of the Hart mixture, by
including folic acid in the basal diet, by substituting glu-
cose for one half of the sucrose, and by adding the ash from
30 gm. of Purina chow to 100 gm. of purified diet. This last
addition had been suggested by Elvehjem (146). None of these
expedients resulted in any detectable improvement in food
consumption and the original diet, as outlined previously,
was finally adopted.

The experiments using this purified diet were designed
to examine the decreases in metabolite excretion upon admin-
istration of the three factors which had been connected with
tyrosine metabolism. The results are shown in Table XI.
Vitamin C and folic acid produced the expected decreases in
metabolite excretion. Only keto acid figures are reported,
since as usual the tyrosyl values paralleled these. However,
liver extract injections failed to produce any decrease in
metabolite levels. This was surprising and the only explana-
tion advanced was that folic acid or ascorbic acid (not

present in this diet) might be necessary for liver extract activity. Further investigations of the action of liver extract using the purified basal diet, both with and without folic acid, are evidently necessary. These were postponed until a more satisfactory purified diet had been evolved.

The adequacy of the diet when it was consumed is shown by the small weight losses, similar to those on the Purina chow diet, observed during the experimental period. The small weight loss was noted even when the experiment lasted 15 days (Table XI, animal 41). The food intake was lower than the 20 to 30 gm. per day for a 350 gm. animal observed in experiments using the natural diet. This was probably due to the better nutritive value of the protein (casein) in the purified diet. This idea is supported by the small amount of fecal matter excreted on this diet and the lower total nitrogen content (to be reported in Section B) of the urine, indicating more efficient utilization of the ingested food.

It will be noted that the oral administration of folic acid produced a drop in metabolite excretion on the third day after administration, while the subcutaneous injection produced its effect in the next 24-hour period (Table XI, animals 49 and 53). A similar difference between these routes of administration was observed when Purina chow was used as the basal diet (to be reported in Section B).

Table XI

Keto Acid Excretion of Guinea Pigs on a Purified Diet Plus Various Supplements

G. pig no.	Wt.	Food intake	Day	Keto acid excreted		Supple-ment	Amount
	gm.	gm.		mg.	%		mg.
41	410	13.4	1-2	171	20.9		
	380	16.6	3	140	18.4		
	380	12.4	4	46.2	6.1	Vita-min C[1]	20.0
	380	17.0	5	29.2	3.9		
	380	21.3	6	254	33.5		
	380	20.8	7-9	175.8	23.1		
	380	22.6	10	lost by error			
	370	22.5	11	401	54.2		
	370	19.8	12	120	16.2	Vita-min C	25.0
	370	24.4	13	49.2	6.7		
	370	21.2	14-15	329	44.5		
42	405	16.7	1-4	120	15.0		
	400	17.5	5	238	29.8		
	400	15.0	6	128	16.0	Vita-min C	20.0
	400	17.4	7	40.0	5.0		
	390	19.6	8-9	41.0	5.3		

(continued)

[1] Administered orally as a suspension in water.

Table XI (continued)

G. pig no.	Wt.	Food intake	Day	Keto acid excreted		Supplement	Amount
	gm.	gm.		mg.	%		mg.
45	340	10.0	1-6	94.9	13.8		
	350	9.5	7	199	28.5		
	350	11.7	8	297	42.5	Reticulogen[1]	1.0 ml.
	330	11.1	9	336	51.0	Reticulogen	1.0 ml.
	330	12.6	10	334	50.6	Reticulogen	1.0 ml.
	330	15.6	11-12	319	48.3		
49	320	9.4	1	149	23.3		
	320	9.7	2	236	36.9		
	320	6.4	3	203	31.7	Folic acid[2] orally	15.0
	320	8.2	4	200	31.3		
	320	9.9	5	41.6	6.5		
53	270	8.9	1-4	83.4	15.5		
	270	12.4	5	246	45.6		
	270	7.9	6	15.4	2.9	Folic acid subcutaneously	15.0
	270	10.0	7	12.3	2.3		

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[1]Liver extract Reticulogen No. 360, injected intraperitoneally.

[2]The vitamin was suspended in 0.3 ml. of H_2O, then 0.4 ml. of 0.1 N. NaOH plus 0.5 ml. of phosphate buffer, pH 7.2, plus 0.4 ml. of 0.1 N. HCl were added in that order.

After the completion of experiments using this diet, Elvehjem (147) described a modification of the diet, which proved satisfactory. It was similar to the basal ration used in these experiments except for the addition of 2.5% potassium acetate and 0.5% magnesium oxide. A preliminary test in this laboratory[1], using two animals, indicated that this diet was eaten by guinea pigs as well as the stock diet and no fluctuations in food consumption were observed.

Since administration of vitamin C and folic acid had produced considerable decreases in metabolite excretion on this diet, two isotope experiments (reported in Section B) were run. However, the variability in food consumption which made some experiments useless precluded the wide use of this purified diet. These problems may disappear with the more recently reported diet and its use, therefore, is indicated for further isotope experiments and for the study of the action of liver extract and accessory factors.

6. Effects on blood constituents

The reported excretion of tyrosine metabolites in pernicious anemia (54), coupled with the fact that antipernicious anemia liver extracts were active in decreasing tyrosine metabolite excretion in the scorbutic guinea pig (55), suggested some connection between blood formation and the

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[1]Sealock, R. R., and Wu, M. C. Unpublished data.

action of liver extract on tyrosine metabolism. The fact that liver extracts did not affect the oxidation of tyrosine in vitro (55) as did folic acid (121) and vitamin C (49), plus the differences between the action of these vitamins and liver extract in vivo reported here, further strengthened the concept of a blood formation-tyrosine relationship.

To test this hypothesis it was proposed to examine blood constituents under a series of experimental conditions which produced decreases in tyrosine metabolite excretion and see whether any correlation existed. The converse experiment, where blood constituents were varied experimentally and the effect on metabolite excretion determined, was also planned. If a relationship between blood formation and the tyrosine-liver extract interaction could be demonstrated, it would be evidence that the tyrosine metabolites, which disappeared from the urine when liver extract was injected, represented the incorporation of tyrosine into some blood constituent.

The red blood cell count, hemoglobin and total nitrogen determinations were selected for study under the various experimental conditions. The problem of obtaining blood samples from the same animal several times without producing harmful effects appeared serious until it was found that the arterioles which ran along the outer edges of the animals' ears could be used. The ears were shaved, blood circulation increased by gentle rubbing and the use of xylene, and the

vessel nicked with a sharp razor. The first few drops were wiped away and discarded, and then three samples were drawn directly into pipettes. The procedure had to be rapid to prevent clotting.

Duplicate 0.02 ml. samples for hemoglobin determinations were drawn into diluting pipettes and then diluted to 5.02 ml. with 0.1 N. hydrochloric acid. After mixing, the samples were transferred to colorimeter tubes, which were matched so that they could be read directly in the Klett-Summerson photoelectric colorimeter, in order to avoid transfers with the small volumes available. The samples were read against distilled water as a blank, using the KS-52 filter after standing for 30 minutes. This modification of the acid hematin method (161) requires standardization of the colorimeter using blood samples whose hemoglobin content has been obtained by some independent method. This was not done, and hence colorimeter readings only were determined. These permitted comparisons to be made, however.

For determination of total nitrogen, it was convenient to pipette 4.0 ml. of the solution used for the hemoglobin determination into micro-Kjeldahl flasks. The solutions were then digested and distilled, and titrated with 0.01 N. hydrochloric acid.

The red blood cell count was determined on a third blood sample which was drawn into a Trenner pipette and diluted

with Hayem's solution (162). Pipettes and the "bright-line"
counting chamber used were National Bureau of Standards
tested instruments. Red blood cell counts are often inaccu-
rate when done by inexperienced personnel. Results on seven
normal guinea pigs (at the start of various experiments)
showed an average red blood cell count of 6.09 million per
cu. mm. with a range of 5.45 to 6.93 million. One other
animal had a count of 8.19 million, probably the result of
some pathological condition. Wintrobe (163) has reported the
red blood cell count of the guinea pig to be 5.75 million per
cu. mm. with a range of 5.37 to 6.05 million. Our results
were slightly high and more variable. However, it was felt
that these results were good, considering the difficulties
under which the blood samples were drawn. The range repre-
sents individual variation, since repeat determinations on
the same animal showed much closer agreement.

The first group of experiments consisted of a study of
the three blood values described above with five animals.
One of these was placed on the vitamin C-deficient diet plus
extra tyrosine (guinea pig no. 43), another received liver
extract injections but no extra tyrosine (guinea pig no. 37),
while three animals received liver extract injections in
addition to the tyrosine supplement (guinea pigs nos. 29, 35,
and 38). Blood analyses were made at appropriate intervals
during a 20-day period in most cases. The tyrosine

supplement was administered in the usual manner. Each
liver extract injection consisted of 1.0 ml. of Reticulogen.

Figures 4 and 5 show the fluctuations in red blood
cell counts of these animals. The animal receiving tyrosine
only (Figure 5) had a red blood cell count which decreased
steadily. The addition of liver extract to this diet
(Figure 4) seemed to result in a temporary drop in the red
blood cell count, but at the end of the experiment the
values were higher than at the start in all three cases.
The animal receiving liver extract but no tyrosine (Figure 5)
also showed an increase in the red blood cell count. If any
conclusion at all can be drawn from these results, it would
be that tyrosine fed to the vitamin C-deficient guinea pig
decreases the red blood cell count, and the effect of liver
extract would only be to remove this inhibition, since the
increase with liver extract in the absence of tyrosine was
comparable to the increase with liver extract when tyrosine
was present. These results must be considered inconclusive
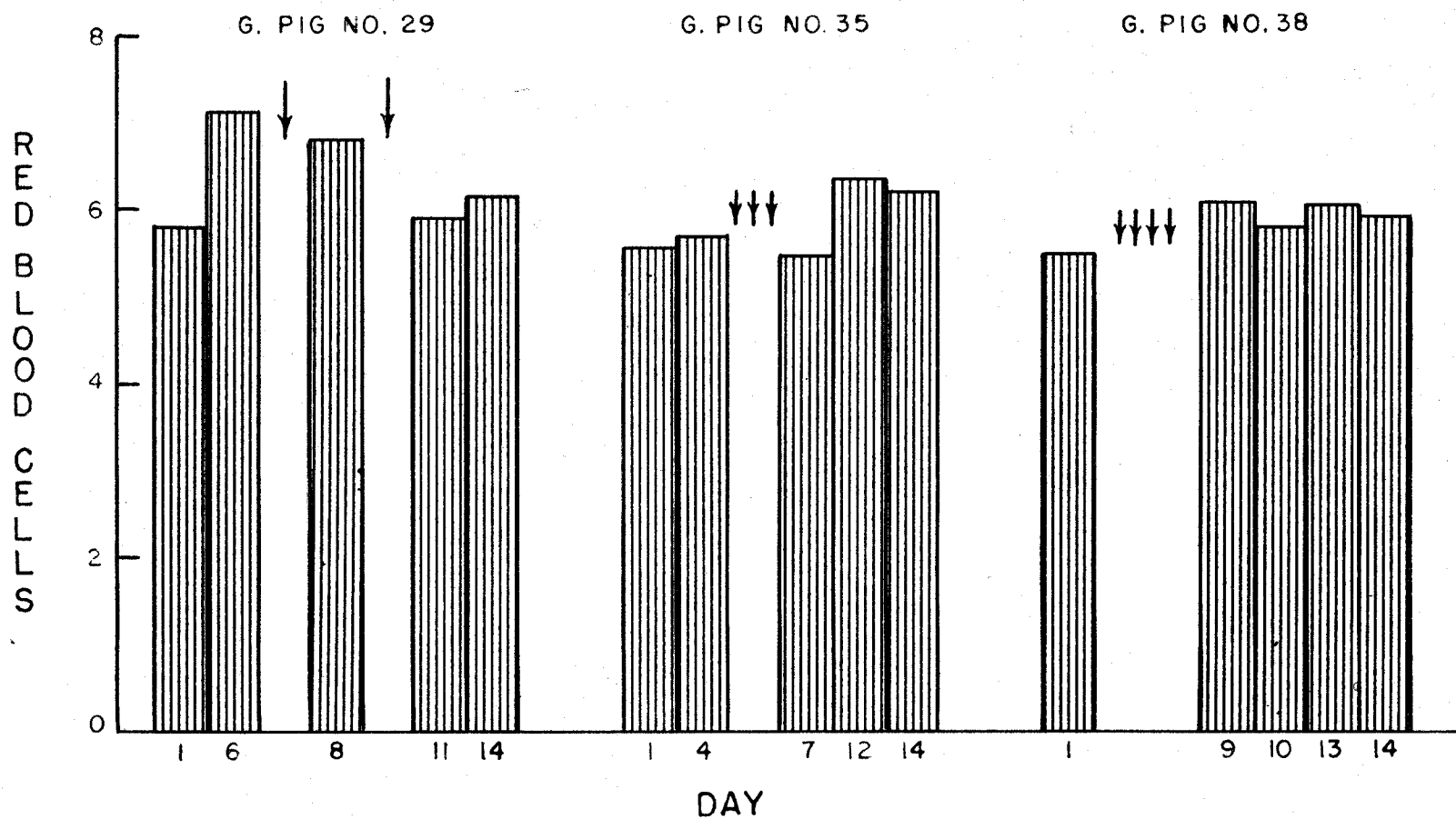in view of the variability observed.

FIG. 4 Red blood cell count (millions per cu. mm.) of three animals receiving tyrosine plus liver extract. Arrows indicate single liver extract injections.
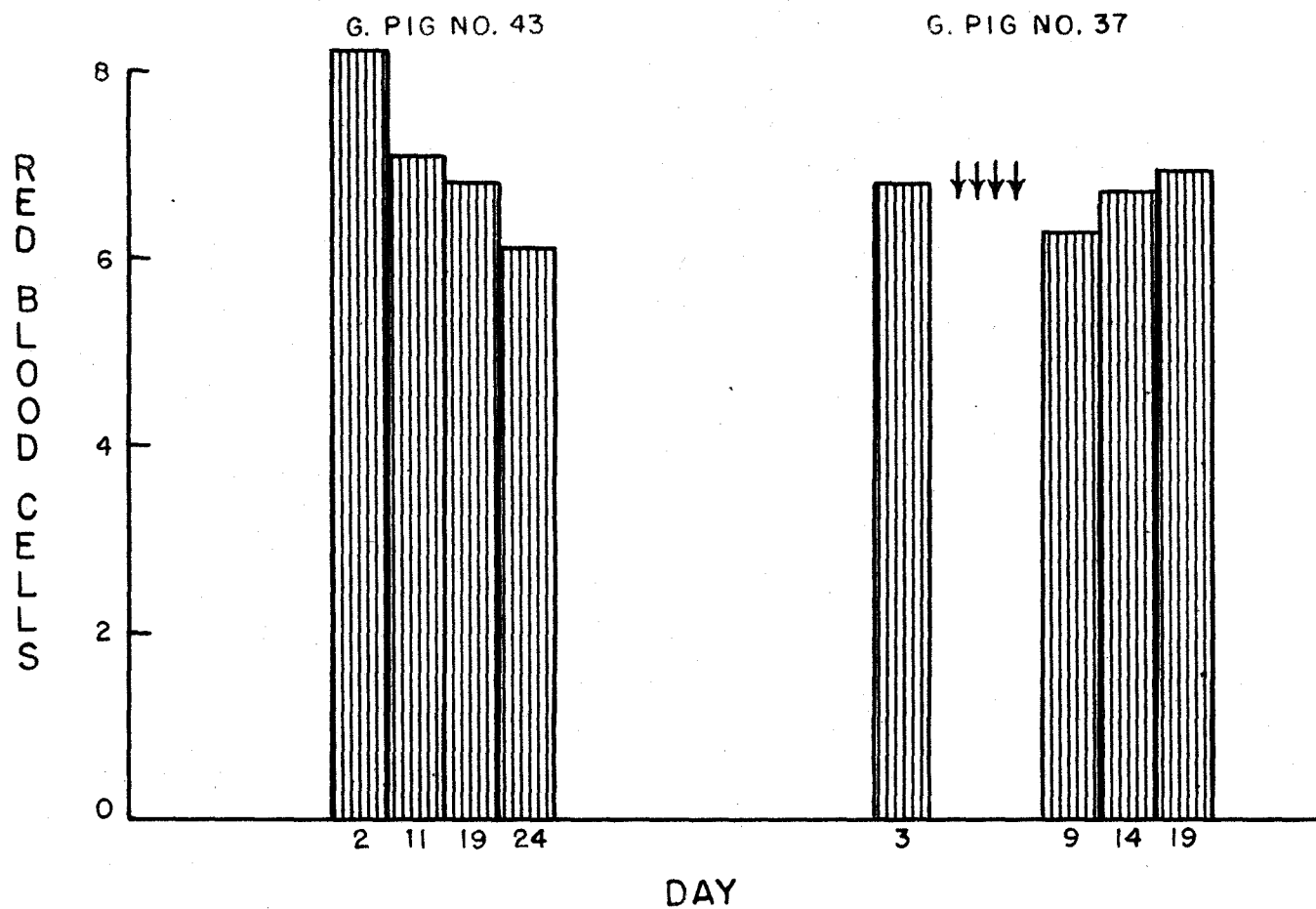
FIG. 5 RED BLOOD CELL COUNT (MILLIONS PER CU. MM.) OF A GUINEA PIG RECEIVING TYROSINE ONLY (LEFT) AND ONE RECEIVING LIVER EXTRACT ONLY (RIGHT). ARROWS INDICATE SINGLE LIVER EXTRACT INJECTIONS.

The total nitrogen determinations on these five animals are shown in Figures 6 and 7. It is evident that no significant changes in the blood nitrogen occurred under these conditions. Hemoglobin values paralleled the total nitrogen values very closely and are not reported.

The metabolite excretion values of these animals were those which would be predicted under the experimental conditions. The animal which received no tyrosine (guinea pig no. 37) excreted practically no tyrosine metabolites. The animal receiving tyrosine but no liver extract (guinea pig no. 43) maintained a high level of excretion throughout the experiment (Figure 2). The changes in excretion levels of the animals on tyrosine with liver extract injection were similar to those previously reported. The data for one of these animals (guinea pig no. 38) are reported in Figure 3.

These experiments seemed to indicate that increases in red blood cells might be correlated with decreases in metabolite excretion. No clear-cut evidence was obtained, however. It was felt that one factor which may have prevented more successful experiments was the initial red blood cell count, which was normal. Any attempts to increase the red cell count would be operating in opposition to the tendency to remain at the normal level.
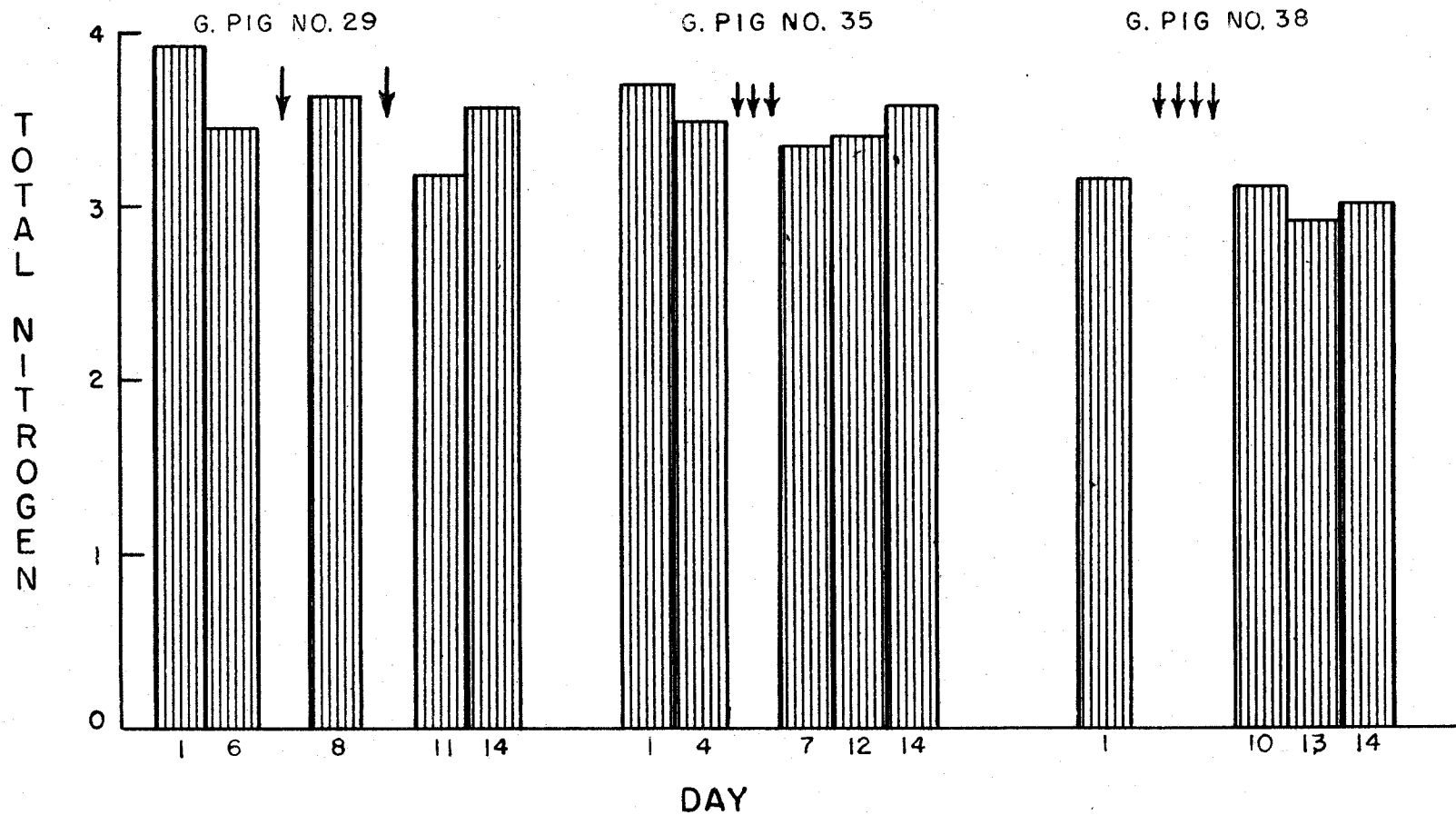
FIG. 6 TOTAL BLOOD NITROGEN (GM. PERCENT) OF THREE ANIMALS RECEIVING TYROSINE PLUS LIVER EXTRACT. ARROWS INDICATE SINGLE LIVER EXTRACT INJECTIONS.
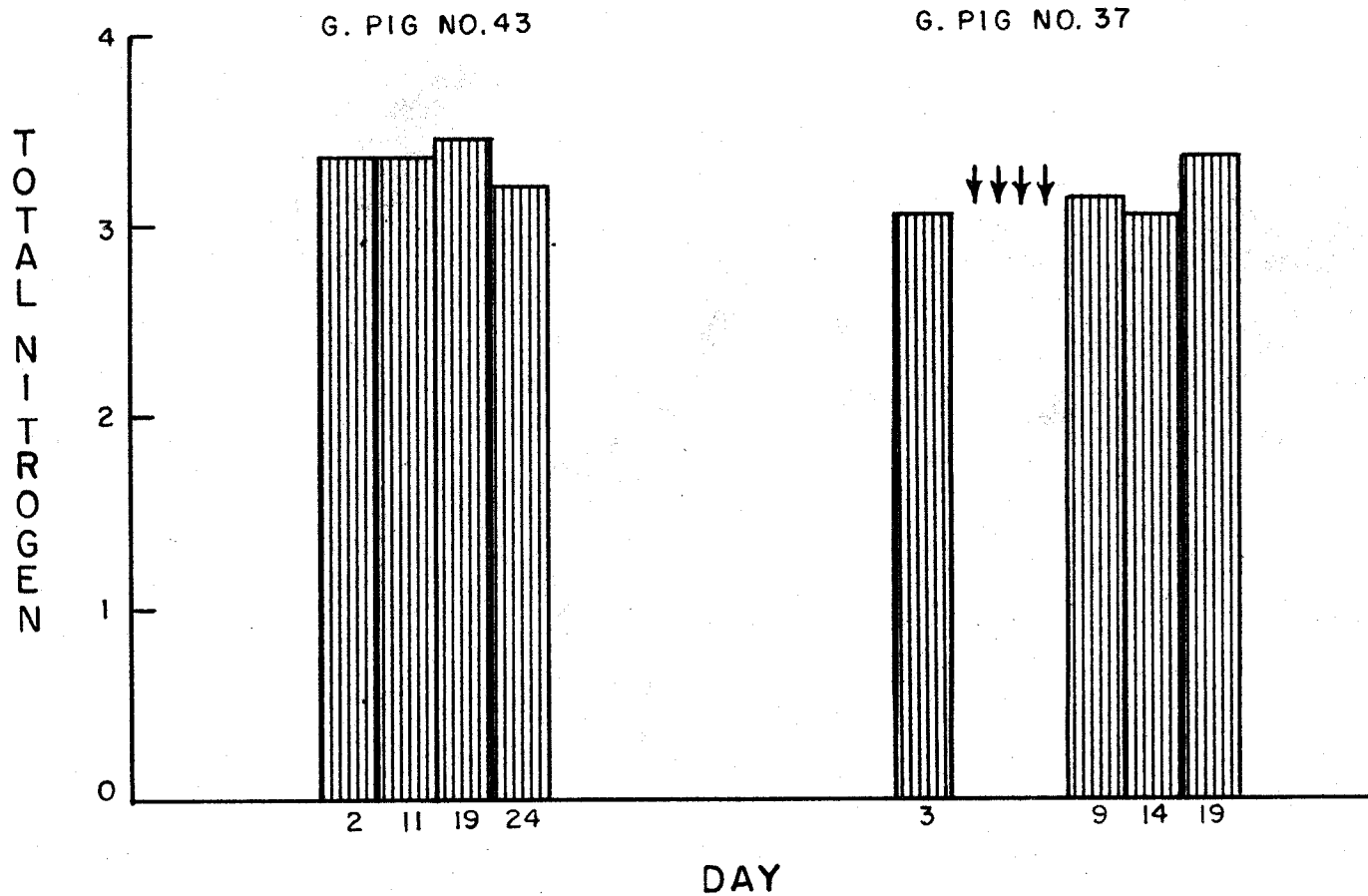
FIG. 7 TOTAL BLOOD NITROGEN (GM. PERCENT) OF A GUINEA PIG RECEIVING TYROSINE ONLY (LEFT) AND ONE RECEIVING LIVER EXTRACT ONLY (RIGHT). ARROWS INDICATE SINGLE LIVER EXTRACT INJECTIONS.

Therefore, it seemed advisable to study tyrosine-liver extract interaction under conditions where rapid red blood cell formation was occurring.

Cobalt polycythemia was one type of blood change which met this requirement. Orten (164) has described this condition as the result of a true stimulus to red blood cell production. Injection of 10 mg. of cobalt sulfate daily to rabbits (165) has been reported to produce an increase in red blood cells of 1.5 to 2.5 million per cu. mm. at the end of 7 days. Hemoglobin values also rose. The mechanism of this cobalt action on red blood cells has not been definitely established. Only one report on cobalt polycythemia in guinea pigs which gave the level of cobalt used was found in the literature. Sutter (166), using a total dose of 2 mg. of cobalt per 10 gm. of body weight (compound not stated), produced increases of 0.5 to 4.0 million red blood cells per cu. mm. in guinea pigs. These amounts of cobalt were close to the toxic level.

For our experiments, two animals were originally started at a level of 2.0 mg. of cobalt sulfate heptahydrate per day. When this failed to produce a definite polycythemia, three animals were started receiving 10 mg. per day for five days and then 5 mg. per day during the experimental period. Only one successful experiment was obtained, since one animal died and the other refused to to eat. Severe weight losses

were observed in all the experiments. The daily dose of
cobalt sulfate heptahydrate was dissolved in water so that,
after neutralization to pH 7.0, it was contained in a volume
of 1.0 ml. Administration was subcutaneous by hypodermic or
oral by pipette, as noted.

The results of the three completed experiments, recorded
in Table XII, indicated that cobalt administration did change
metabolite excretion. Increases in red blood cell counts
took place in two of the animals, but no changes in total
blood nitrogen were noted. Hemoglobin values did not change
and are not reported. The levels of metabolite excretion
fell prior to the injection of liver extracts and failed to
reach the usual levels, even after 17 days of tyrosine feed-
ing in the case of the animal with the greatest degree of
polycythemia (guinea pig no. 31, Table XII). Decreases in
metabolite excretion prior to injection are almost never
observed and usually after injections cease, the metabolite
excretion level rises sharply (Table VII and Figures 1, 2,
and 3). The metabolite excretion pattern reported in
Table XII thus differed sharply from that observed under
these conditions in the absence of cobalt.

The liver extract injections produced smaller decreases
than usual. Therefore, if the decrease in excretory values
resulting from cobalt administration is connected with the
increased blood formation observed, it would appear that the

Table XII

Blood and Urinary Changes of Guinea Pigs
Receiving Extra Cobalt

G. pig no.	Day	Total-N	R. B. C.	Keto acid excretion		In- jected
		gm. %	millions per cu. mm.	mg.	%	ml.
31	0	3.42	6.93			
	7	3.55	7.15[1]	200	18.5	
	8			464	42.9	
	9			388	35.9	
	10			402	36.5	
	11			363	33.0	1.0[2]
	12	3.44	7.13	455	41.4	
	13			345	31.4	
34	0	3.26	6.08			
	7	3.30	6.02[1]	362	32.9	
	8			625	59.0	
	9			530	50.0	
	10			450	41.7	
	11	3.23	5.76	363	33.7	1.0
	12			376	35.5	
	13			367	34.6	

(continued)

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[1] Animal on vitamin C-free basal diet plus 2.0 mg.
$CoSO_4 \cdot 7H_2O$ in 0.1% glucose, orally, each day for 6 days prior
to tyrosine. Cobalt continued during experimental.

[2] Liver extract Lilly No. 377.

Table XII (continued)

G. pig no.	Day	Total-N gm. %	R. B. C. millions per cu. mm.	Keto acid excretion mg.	%	Injected ml.
31	5	3.62	7.26[1]			
	6	3.76	7.82	84.6	8.6	
	7			228	23.3	
	8			291	29.7	
	9			145	15.4	
	10			325	34.6	
	11			149	15.9	1.0[2]
	12	3.58	9.64	328	34.9	1.0
	13			348	37.1	1.0
	14			151	16.8	
	15			234	26.0	
	16			228	25.4	
	17	3.40	8.27	222	26.4	

[1]Animal on stock diet plus 10 mg. $CoSO_4 \cdot 7H_2O$, subcutaneously, each day for 5 days prior to tyrosine. Received one half this amount of cobalt during experimental.

[2]Liver extract Reticulogen No. 360.

"vitamin factors" necessary for the transformation involved are present in the animal. It would be of interest to perform this type of experiment on an animal which had been depleted of vitamin B_{12}.

The results, then, of these studies on blood constituents, suggested that some relationship existed between tyrosine metabolism and red blood cell formation, and that liver extract played some part in this relationship. The evidence obtained in these few experiments, however, does not warrant any definite conclusions, but serves as a guide to further investigation.

The toxicity observed with cobalt administration suggested that some other means of inducing red blood cell formation be utilized for future work. Phenylhydrazine-induced anemia experiments were planned. It was realized that even if the results obtained were conclusive, the exact mechanism of the relationship between tyrosine and blood formation could not be obtained from these studies. However, if these studies were done with isotopically labelled tyrosine it would be possible to determine whether tyrosine was actually being incorporated into some blood constituent or whether the relationship was indirect. Since isotope studies were being initiated as a part of the comprehensive investigation of all three factors now known to affect tyrosine metabolism, further blood studies were postponed until they could be included in the isotope program.

B. Experiments Using N^{15}-L-Tyrosine

1. Methods

The general plan of these experiments involved the feeding of extra tyrosine to the vitamin C-deficient guinea pig in the usual manner. When metabolite excretion became high, vitamin supplementation was started, and under the influence of the vitamin, the tyrosine metabolites were removed from the urine. During the supplementation period, the isotopic L-tyrosine was fed. After 24 or 48 hours the animal was killed and isotope analyses made.

Supplementation with either vitamin C or folic acid removed tyrosine metabolites from the urine. By marking the tyrosine molecule, it was hoped that information would be obtained concerning the fate of the tyrosine under the influence of each vitamin, what compounds were formed from the tyrosine molecule, and whether the action of these two vitamins differed. Later, when some of the difficulties previously noted had been removed, the action of liver extracts would also be studied.

The L-tyrosine used in these experiments was marked with N^{15} in the amino group. Since evidence had appeared implicating both vitamin C and folic acid in the catabolism of tyrosine, our main interest here was to study the various urinary fractions and determine the distribution of the N^{15}

in control animals, animals receiving vitamin C, and animals receiving folic acid. Feces, intestinal contents and uneaten food were also analyzed so that an accounting could be made of all of the isotopic tyrosine. Blood and carcasses were frozen and saved for future analyses.

The L-tyrosine used was synthesized[1] from Eastman Kodak ammonium nitrate containing 7.12 atoms % excess N^{15}. The synthetic procedure (167) involved the Erlenmeyer azlactone synthesis, ring opening by acetone and water hydrolysis, acid hydrolysis to obtain the p-hydroxyphenylpyruvic acid, and catalytic hydrogenation of the carbonyl group in the presence of isotopic ammonia to obtain the DL-amino acid. The N-acetyl derivative of the racemic amino acid was resolved with D-α-phenylethylamine in order to isolate the isotopic L-tyrosine.

After feeding of the isotopic tyrosine, 24-hour urine samples were collected and the usual analyses for tyrosyl value and keto acid were run. At the conclusion of the experiment, the animal was stunned by a blow on the head, the throat was severed and blood collected in oxalate. The red cells were separated by centrifugation with the addition of 0.9% saline. Red cells and plasma, after separation, were diluted with water plus one drop of concentrated hydrochloric acid to a final volume of 50 ml. and stored in the cold.

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[1]Some of these experiments were performed in cooperation with Dr. Ralph K. Barclay, who synthesized and resolved the N^{15}-L-tyrosine used, and oxidized some ammonia samples to nitrogen for mass spectrometer analysis.

The intestines were removed at once and the contents washed
out. The intestinal contents and uneaten food plus feces
were transferred to separate 500 ml. macro-Kjeldahl flasks
and digested in the usual manner. About 60 ml. of nitrogen-
free, concentrated sulfuric acid was required for each flask.
The digested samples were transferred to 100 ml. volumetric
flasks, diluted to volume, and aliquots were distilled over
in the micro-Kjeldahl still into boric acid. These were
titrated and the ammonia samples then saved for isotope
analysis.

The 24-hour urine samples were diluted to 100 ml. Dupli-
cate 1 ml. aliquots were used for determination of total
nitrogen by the micro-Kjeldahl method. The ammonia samples
obtained were saved for isotope analysis. Duplicate 3 ml.
aliquots were used for the urea analysis by a modification
of the standard urease aeration method (168). The usual five-
tube aeration train with the addition of a small separatory
funnel in tubes 2 and 4 was used for this determination. Ten
ml. of boric acid was placed in tubes 3 and 5. Tube 1 con-
tained 6 N. sulfuric acid to remove any ammonia present in
the air. The 3 ml. urine aliquots were pipetted into tubes
2 and 4. Two drops of bromthymol blue and 1 drop of phenol-
phthalein were added as indicators plus 2 ml. of 0.2 N. phos-
phate buffer, pH 6.5. The system was closed and gentle
suction started. By means of the separatory funnel, one

urease tablet (Urease Dunning, 25 mg. tablet, Hynson,
Westcott and Dunning, Baltimore, Md.) ground up in 5 ml. of
the phosphate buffer was added to each sample. The funnels
were washed down with water so that the final volume in each
sample tube was approximately 20 ml. The enzyme was allowed
to act for two hours at room temperature during which period
the slight aeration provided stirring. The ammonia liberated
by the enzyme action should turn the indicator a yellow-green
color, indicating a pH of 7.0, essential for rapid urease
action.

At the conclusion of the period, the solutions were
made just basic to phenolphthalein with 0.1 N. sodium hydrox-
ide added by means of the separatory funnels. Gentle suction
was applied at the start and more vigorous aeration after the
first hour. Capryl alcohol was added as needed. The aera-
tion period lasted a minimum of four hours. It was found
that if the aeration were too vigorous, ammonia losses occurred.
When aeration was complete, the boric acid in the traps was
titrated with 0.1 N. hydrochloric acid and the solutions
saved for isotope analysis.

It should be noted that this procedure would carry over
any ammonia originally present in the urine. However,
repeated aeration tests with this size urine sample failed to
show any detectable amount of ammonia as such. It is well
known that the urine of herbiverous animals contains a very

low percentage of ammonia. Some ammonia was recovered when
40 to 50 ml. samples of urine were made just alkaline to
phenolphthalein and aerated for five hours. These results
will be reported in the next section.

The residues which remained in the tubes, after the re-
moval of the ammonia derived from urea, were made acid. They
were then transferred quantitatively, in portions, to micro-
Kjeldahl flasks to which one drop of nitrogen-free concentrated
sulfuric acid had been added. Each portion was boiled down
to a small volume over a small flame before the next was
added. The usual micro-Kjeldahl digestion and distillation
procedure was followed when the transfer was completed. The
titrated ammonia samples were saved for isotope analysis.
Blank determinations, using 3 ml. of distilled water, a
urease tablet and the other constituents of the sample tubes,
were run in order to determine the amount of the residual
nitrogen not derived from the urine itself.

The ammonia samples from all of the determinations were
made just acid to Congo red with one drop of concentrated
hydrochloric acid and stored. This acidity was suitable for
conversion of the ammonia samples to nitrogen gas, in which
form these samples would be analyzed in the mass spectrometer.
The oxidation procedure used was the method described by
Rittenberg (169). The ammonium salts obtained from the
Kjeldahl distillation were oxidized with alkaline hypobromite,

according to the following reaction:

$$3OBr^- + 2NH_4^+ + 2OH^- \longrightarrow N_2 + 5H_2O + 3Br^-$$

If the solution is too acid, the following undesirable side reaction may occur:

$$Br^- + OBr^- + 2H^+ \longrightarrow H_2O + Br_2$$

The apparatus used for oxidizing the ammonia samples and collecting the nitrogen gas is shown in Figure 8. "A" is a 50-ml. Erlenmeyer flask with a bent neck attached and a ground-glass joint on the neck. "B" and "C" are fused together and have ground-glass fittings at each end. "B" is a 50-ml. round-bottomed flask, and "C" is a trap. "E" is a ground-glass stopcock which is interposed between the system and the mechanical and mercury diffusion pumps, which are used to evacuate the system. The right side of the apparatus is a Toeppler pump, consisting of a 250-ml. bulb, "J", with 10-mm. glass tubing fused on opposite sides. The bottom tubing extends down into "H", a 500-ml. filter flask containing 350 ml. of redistilled mercury and having a three-way stopcock, "D", fused onto the sidearm. The upper tubing from "J" leads to the three-way stopcock, "F", and a ground-glass joint into which the gas-collecting bulb, "G", fits. This collecting bulb is a 15-ml. bulb fused to a stopcock with a
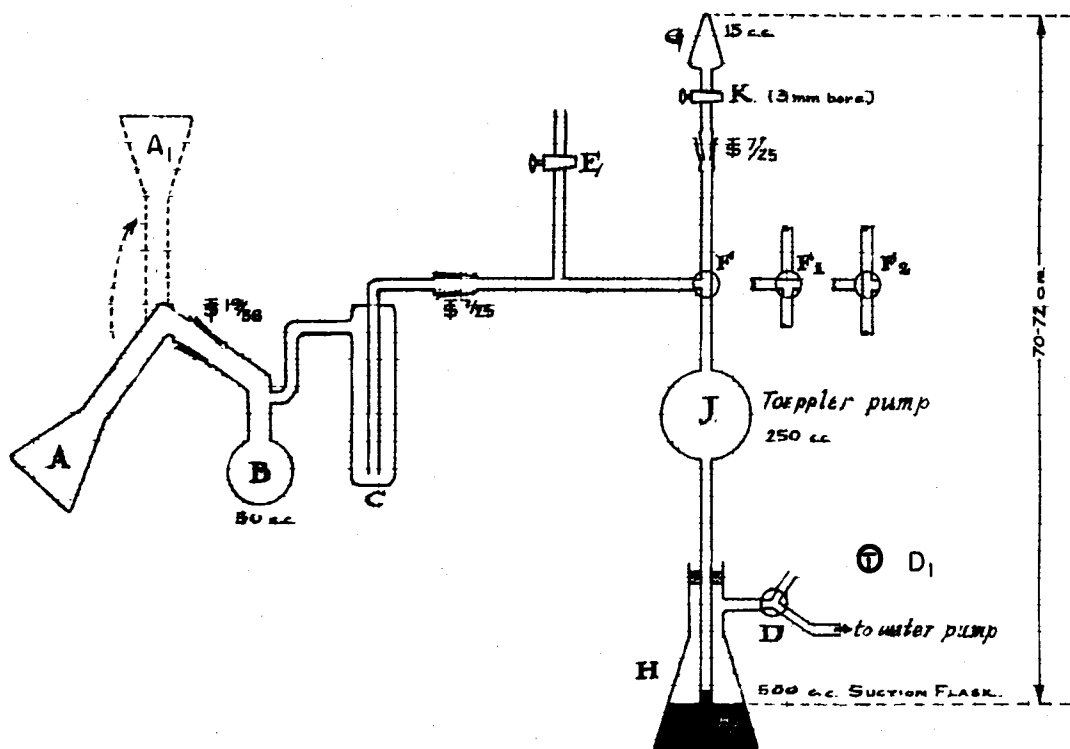
FIG. 8   APPARATUS FOR COLLECTION OF NITROGEN GAS.

ground-glass joint. This joint also fits the inlet system of the mass spectrometer. The distance from the bottom of "H" to the top of "G" is 70 cm., so that when the system is fully evacuated and then air is admitted through "D", the mercury will rise to the top of the gas bulb. A Dewar flask containing alcohol and dry ice is placed around trap "C".

The following procedure was used when operating the apparatus. The gas bulb, "G", was lubricated[1] and placed in position. The Kjeldahl distillate, boiled down to a volume of 3 to 4 ml., was transferred to the flask "B". This transfer need not be quantitative since it is the ratio of N_2^{29} to N_2^{28} which is being determined. The joint at "C" was greased and put in place and the alcohol-dry ice bath raised around it.

Five ml. of diluted sodium hypobromite (stock solution diluted 1:1 with water) was pipetted into flask "A" and this was then greased and placed in the downward position.

The stock solution of hypobromite was made by dissolving 200 gm. of sodium hydroxide pellets in 300 ml. of water. One half of the solution was cooled in ice and 60 ml. of bromine added over a 10-minute period, with stirring. The other one half of the alkali was then added. After two days in the cold, the sodium bromide precipitate which had formed was

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[1] A special lubricating grease which minimized air leakage was used. This grease was Apiezon N, purchased from James G. Biddle Co., 1316 Arch St., Philadelphia, Pa.

filtered off through an asbestos mat. The filtrate was stored in the cold and was stable for months. This stock solution was diluted 1:1 with water just before use.

When the procedure cited above was completed, stopcock "E" was opened and the system evacuated by means of a Cenco Hi-vac mechanical pump and a mercury diffusion pump. The mercury in "H" rises as the system evacuates. This was prevented by starting the water pump with stopcock "D" turned to position "D_1". When the pressure became low, the solutions in "A" and "B" degassed and "B" froze. The vigor with which these changes occurred was controlled by opening or closing stopcock "E".

The degree of evacuation was determined by turning stopcock "F" to position "F_2" and admitting air into "H" by returning the stopcock to position "D". If the system is fully evacuated, the mercury should rise to the top of the gas bulb "G". In practice, the usual vacuum attained left a small disc of air, about the size of a thumb tack, in the gas bulb. This contamination could be measured and easily corrected for in the isotope calculations.

When the system had been satisfactorily evacuated as shown by this test, the mercury was drawn back into "H" by turning its stopcock to position "D_1". Stopcock "F" was left at position "F_2", stopcock "E" was closed and the hypobromite solution poured into "B" by turning the flask to position "A_1".

The mixture in "B" was then heated gently. The reaction in "B" may be considered complete when all the ice has melted and ebullition has ceased. When the reaction was completed, the gas was introduced into "J" by turning the stopcock "F" to position "F_1". Then, by turning to position "F_2" and introducing air into the system at "H" (position "D"), the nitrogen gas was moved up into the gas bulb "G" by the rising mercury. The stopcock at "G" was then closed and the stopcock at "F" opened to that position. The system was then cautiously returned to atmospheric pressure by simultaneously opening "E" and "D".

Care must be taken to admit air to all parts of the apparatus between samples to prevent a carry-over of isotope to the next sample. The various parts were then removed, rinsed with distilled water and the grease removed from the joints with carbon tetrachloride. It was not found necessary to dry the parts thoroughly between samples. The complete oxidation of a sample took about 30 minutes. The samples in the gas bulbs were analyzed in the mass spectrometer as soon as possible. Storage for more than 12 hours should be avoided.

The mass spectrometer used for these analyses was constructed here at the Institute for Atomic Research[1].

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[1] The writer is indebted to Mr. Harry J. Svec of the Institute for Atomic Research, who constructed the mass spectrometer and led him through the intricacies of its operation.

It was the 60-degree, sector-type instrument with two collec-
tors, the currents of which were balanced against each other
in the usual analysis. These features produced a machine
which could detect a 0.3% difference in isotope ratios.
Standard samples of tank nitrogen were run before and after
each series of unknown samples to check the operation of the
apparatus.

Since calculation of the $N_2^{29}:N_2^{28}$ ratio of the unknown
samples involved the balancing of currents from the two
collectors by means of two resistances, the relationship of
these resistances to each other must be known. Direct
experimental determination of this ratio, a tedious procedure,
was avoided by running a standard sample of tank nitrogen and
calculating the ratio of $R_1:R_2$ by means of the equation:

$$(1) \qquad \frac{N_2^{29}}{N_2^{28}} = \frac{Decade}{111,111} \times \frac{R_1}{R_2}$$

The "Decade" reading was obtained experimentally and repre-
sents the adjustment of the two resistances needed to balance
the currents from the two collectors. Since the $N_2^{29}:N_2^{28}$
ratio for normal nitrogen is known, $R_1:R_2$ may be calculated
by equation (1). For this instrument, the ratio of $R_1:R_2$ was
usually close to 0.99 when the nitrogen gas pressure outside
the capillary leak was 5 cm. of mercury. The ratio changed

somewhat when this pressure was lower. The uncorrected $N_2^{29}:N_2^{28}$ ratio of unknown samples was then calculated by means of formula (1) from the "Decade" reading determined experimentally for that sample, using an $R_1:R_2$ ratio calculated as described above. The unknown and tank nitrogen samples were analyzed at the same nitrogen gas pressure outside the capillary leak.

The most widely used $N^{15}:N^{14}$ ratio has been reported as 1:265 ± 8 (170). The "% N^{15}" calculated from this ratio is 0.376. This percentage has been reported as 0.38 by Nier (171). The $N_2^{29}:N_2^{28}$ ratio for normal nitrogen of 0.00760 was then calculated from this figure. These methods of calculation have been modified slightly from those reported by Rittenberg (169). The various steps involve the conversion of an uncorrected $N_2^{29}:N_2^{28}$ value, which has been experimentally determined on the unknown sample, to a "% N^{15}" and then to a "% N^{15} excess" over normal nitrogen. This value is then corrected for the amount of normal nitrogen contaminating the unknown due to air and background nitrogen.

The experimentally determined readings used for these calculations are: $\left[\dfrac{N_2^{29}}{N_2^{28}}\right]_T$, $(N_2^{28})_T$, $(N_2^{28})_B$, and $(O_2^{32})_A$,

where T = total, B = background, and A = air. The last three values are voltage readings. The uncorrected "% N^{15}" can then be calculated from the formula:

This formula may be used for concentrations of N^{15} up to 5%. When the N^{15} concentration is higher than 5%, it is necessary to determine the amount of N^{30} present, as well as the $N_2^{29} : N_2^{28}$ ratio. The "% N^{15} (uncorr.) excess" is then obtained by subtracting 0.38, the value for normal nitrogen, from the "% N^{15} (uncorr.)".

$$(2) \quad \% \; N^{15} \text{(uncorr.)} = \frac{100R}{2}, \quad \text{where } R = \left[\frac{N_2^{29}}{N_2^{28}} \right]_T$$

The "% N^{15} (uncorr.) excess" must be corrected for the presence of normal nitrogen from air, which has contaminated the enriched sample. Any sample containing more than 3% air must be discarded because of the presence of CO^+ ion derived from carbon dioxide. If the sample contains less than 3% air, as determined by the value obtained for $(O_2^{32})_T$, it is possible to obtain the value for the amount of contaminating nitrogen and correct the calculation for this, as shown below.

$$(3) \quad (N_2^{28})_C = (N_2^{28})_B + (N_2^{28})_A,$$

where C = contaminating, B = background, and A = air.

For this mass spectrometer,

$$(4) \quad \left[\frac{N_2^{28}}{O_2^{32}} \right]_A = 10.6$$

This value has been determined experimentally. It differs from the expected ratio of 4 because of the differences in ionization of nitrogen and oxygen in the mass spectrometer. It is then possible to calculate $(N_2^{28})_A$ from the experimentally determined $(O_2^{32})_A$ by means of equation (4) and thus also determine $(N_2^{28})_C$ from equation (3). The percentage which $(N_2^{28})_C$ is of $(N_2^{28})_T$ determines the correction to be applied to "% $N_{(uncorr.)}^{15}$ excess". For example, if $(N_2^{28})_C$ is 2% of $(N_2^{28})_T$ then the "% $N_{(uncorr.)}^{15}$ excess" must be increased by 2%.

A sample calculation, representative of the values obtained, is shown below:

$$\left[\frac{N_2^{29}}{N_2^{28}}\right]_T = 0.00830, \quad (N_2^{28})_T = 9.88, \quad (N_2^{28})_B = 0.068, \quad (O_2^{32})_T = 0.014$$

These values are determined experimentally.

$$\% \ N_{(uncorr.)}^{15} = 100 \ x \ \frac{0.00830}{2} = 0.415$$

$$\% \ N_{(uncorr.)}^{15} \ \text{excess} = 0.415 - 0.380 = 0.035$$

$$(N_2^{28})_A = 10.6 \ x \ 0.014 = 0.148$$

$$(N_2^{28})_C = 0.068 + 0.148 = 0.216$$

$$\frac{(N_2^{28})_C}{(N_2^{28})_T} = \frac{0.216}{9.88} = 0.022$$

$$\% \; N_{(corr.)}^{15} \; excess = 0.035 + (0.035 \times 0.022) = \underline{\underline{0.0358}}$$

2. Results

A number of preliminary experiments designed to check
the experimental procedure and examine the urinary constitu-
ents were run. In addition to the isotope comparisons, it
seemed advisable to study the changes in total and urea
nitrogen of the urine before and after administration of the
same accessory factors which would be used when the isotopic
tyrosine was fed.

The results of experiments in which the nitrogen constitu-
ents were examined before and after vitamin administration are
given in Table XIII. Two of these experiments were isotope
experiments. No change in total nitrogen can be seen,
although considerable variability in these values is evident.
The relationship of urea to total nitrogen is quite uniform,
all the values falling between 73.1 and 83.7%. In all of
these experiments, except the liver extract one, metabolite
excretion on the pre-injection day was high, and showed the
expected low value on the post-injection day reported. There
was no change in metabolite excretion upon administration of

Table XIII

Urinary Nitrogen Constituents Before and After Vitamin Administration

G. pig no.	Pre-injection day			Post-injection day			Supplement
	Urea-N	Total-N	$\frac{\text{Urea-N}}{\text{Total-N}}$	Urea-N	Total-N	$\frac{\text{Urea-N}}{\text{Total-N}}$	
	mg.	mg.	%	mg.	mg.	%	
44[1]	296	361	82.0	323	397	81.4	50 mg. vit. C
47[2]	202	269	75.2	185	232	79.5	75 mg. vit. C
45	188	238	79.0	185	250	74.1	1 ml. Reticulogen
	185	250	74.1	199	272	73.1	1 ml. Reticulogen
49	208	255	81.7	167	223	75.0	15 mg.[4] folic acid
52[3]	285	354	80.5	314	390	80.6	10 mg. folic acid
	314	390	80.6	326	390	83.7	15 mg. folic acid

[1]Guinea pigs nos. 44, 45 and 49 fed on purified diet.

[2]Animal fed on purified diet, isotope experiment.

[3]Animal fed on Purina chow, isotope experiment.

[4]This dose given orally, while Guinea pig no. 52 received vitamin subcutaneously.

liver extract on the purified diet (Table XI). The decreases
in metabolite excretion which were observed evidently cannot
be correlated with changes in total or urea nitrogen under
these conditions.

A total of six isotope experiments were performed.
Experiments 1 and 2 utilized the purified diet, while Purina
chow was used for the remainder of the experiments. Two of
the animals were controls and no supplementary vitamin was
given, three animals received vitamin C supplementation, and
one animal received folic acid.

In all the experiments, the animals received 0.5 gm. of
isotopic L-tyrosine containing 6.01% N^{15} excess, or a total
of 2.32 mg. of excess N^{15}. Table XIV shows the type of
tabulation used to convert "% N^{15} excess" to "N^{15} excess in
mg." and "% yield" figures. Data on the absorption of the
N^{15} fed are used to illustrate these calculations. In the
case of Experiments 1 through 4 the analyses represent a
24-hour period, while in Experiments 5 and 6 the animals were
killed 48 hours after the isotopic tyrosine was fed. It will
be noted that the absorption and excretion of the tyrosine
fed was essentially complete after the first 24 hours.
Furthermore, the percentage of administered tyrosine nitrogen
which is absorbed is high and appears to be affected only
slightly by the various vitamin supplements on both the
purified and natural diets. These findings illustrate the

Table XIV

Isotope Absorption of Guinea Pigs Receiving N[15]-L-Tyrosine and Various Supplements

Exp. no.	Constit- uent	% N[15] excess	Total-N in sample mg.	N[15] excess[1] mg.	N[15] excess absorbed mg.[2]	%	Supple- ment
	N[15]-L- tyrosine fed	6.01	38.7	2.32			
1	Food-Feces	0.100	46.8	0.0648			vit. C
	Int. Cont.	0.061	204.0	0.124			
	Total			0.189	2.131	91.8	
4	Food-Feces	0.0977	198.6	0.194			vit. C
	Int. Cont.	0.0451	372.0	0.168			
	Total			0.362	1.958	84.3	
5	Food-Feces	0.0388	326.0	0.127			vit. C
	Int. Cont.	0.0255	316.0	0.0806			
	Total			0.208	2.112	91.0	
3	Food-Feces	0.140	276.0	0.386			folic acid
	Int. Cont.	0.048	348.0	0.167			
	Total			0.553	1.767	76.2	
2	Food-Feces	0.193	268.0	0.518			none
	Int. Cont.	0.058	163.8	0.095			
	Total			0.613	1.707	73.7	
6	Food-Feces	0.0402	535.0	0.215			none
	Int. Cont.	0.0256	277.0	0.071			
	Total			0.286	2.034	87.5	

[1] % N[15] excess x Total-N in sample.

[2] Difference between N[15] fed and N[15] remaining in food and feces plus intestinal contents.

essentially physiological conditions under which these experiments were run despite the large amounts of tyrosine fed. The changes in metabolite excretion observed represent true changes in utilization of the amino acid, therefore, unless it is assumed that the amino group of tyrosine is split off prior to absorption. Further details of the experimental procedure for each experiment will be given in connection with the discussion of urinary constituents.

The poor absorption shown in Experiment 2 was due to poor food consumption, since the animal did not even eat all the supplement fed. The high "$\% N^{15}$ excess" in the food and feces was due to uneaten tyrosine in this case.

Table XV shows the results obtained when the urinary constituents were analyzed after vitamin C supplementation. Experiment 1 utilized the purified diet and the usual low total nitrogen excretion can be seen. The animal was fed 75 mg. of vitamin C in three doses at the start and during the 24-hour period of isotope feeding. The keto acid excretion dropped from 35.4 to 10.4%. Tyrosyl values paralleled the keto acid values. In order to assure better removal of metabolites from the urine in Experiments 4 and 5, the animals received daily 25 mg. vitamin C supplements for two and three days, respectively, prior to isotope feeding. The keto acid levels before supplementation were 56.6% in Experiment 4, and 44.5% in Experiment 5. The low values

which prevailed during the isotope feeding period, after supplementation, are shown in Table XV.

The amount of isotope excreted in the urine in 24 hours (Table XV) ranged from 26.7 to 39.1% in these experiments. This range resulted mainly from changes in the isotope content of the residual nitrogen, since the urea nitrogen values were almost the same in all cases, and the rise in total nitrogen values was accounted for by a similar change in the residual nitrogen. One constituent of the residual nitrogen fraction might be unchanged tyrosine. The sum of urea-plus residual-N^{15} agreed closely with the value for total isotope nitrogen, indicating the accuracy of the fractionation procedures.

The yield figures for ammonia nitrogen do not mean much, since the long aeration period used and the small amounts of ammonia recovered make these figures unreliable. A possibility also exists that some ammonia may be lost before the urine reaches the acid in which it is collected. No ammonia could be recovered from the urine of animals fed on a purified basal diet. However, the "% N^{15} excess" figures for ammonia are accurate since they represent a ratio. These indicate that ammonia or some compound which yields ammonia contained a high proportion of N^{15} derived from tyrosine. In every case, the "% N^{15} excess" for ammonia was higher than for the other fractions. Thus, despite the low "Isotope yield" shown

Table XV

Urinary Constituents of Guinea Pigs Receiving
N^{15}-L-Tyrosine and Vitamin C

Exp. no.	Constit- uent	% N^{15} excess	Total-N in sample mg.	N^{15} excess mg.	Isotope[1] yield %	Keto acid[3] %
1	Total-N	0.323	220.0	0.710	33.3	10.4
	Urea-N	0.319	185.7	0.590	27.7	
	Residual-N	0.116	141.3[2]	0.164	7.7	
4	Total-N	0.156	335.0	0.523	26.7	4.63
	Urea-N	0.169	279.3	0.473	24.2	
	Residual-N	0.0358	148.3	0.053	2.7	
	NH_3-N	0.172	0.792	0.0014	0.07	
5 (Day 1)	Total-N	0.171	483.0	0.825	39.1	7.32
	Urea-N	0.164	332.0	0.543	25.7	
	Residual-N	0.111	278.7	0.310	14.7	
	NH_3-N	0.182	4.8	0.0087	0.4	
5 (Day 2)	Total-N	0.0234	451.0	0.106	5.03	5.49
	Urea-N	0.0224	315.7	0.071	3.37	
	Residual-N	0.0133	218.7	0.029	1.38	
	NH_3-N	0.0389	4.0	0.0016	0.08	

---

[1] Based on the N^{15} absorbed (Table XIV).

[2] This value used in calculating N^{15} excess. The blank value of 93.3 mg. must be subtracted to obtain the residual nitrogen of the urine only.

[3] Based on the amount of tyrosine fed.

in Table XV, some ammonia-yielding compound, such as glu-
tamine, may contain significant amounts of the tyrosine
nitrogen.

The nitrogen fractions collected the second day after
isotope feeding (Experiment 5, Table XV) show a total of
5.03% isotope recovery. The completeness of tyrosine utili-
zation in the first 24-hour period is again evident.

The total isotope nitrogen present in the urine of ani-
mals receiving folic acid or no supplement, as shown in
Table XVI, was of the same order of magnitude as the animals
receiving vitamin C. The control animals showed a urinary
keto acid excretion of about 30%, while the animals receiving
vitamins usually showed keto acid values of about 5%; never-
theless, the N^{15} content of the urine was in the same range
for all groups.

The animal receiving folic acid (Experiment 3) received
the vitamin subcutaneously in two daily doses of 10 and 15 mg.
prior to isotope feeding. The keto acid values were 30.0 and
7.26%, respectively, on these two days. This prompt response
to folic acid is in contrast to the response when the vitamin
was given orally. For example guinea pig no. 51 received
15 mg. of folic acid orally. The keto acid value was 40.2%
before supplementation for this animal, and after supplementa-
tion the daily levels were 42.0, 26.9, 5.72 and 5.50%. This
delayed response to orally administered folic acid has been
shown to exist on the purified basal diet also.

Table XVI

Urinary Constituents of Folic Acid-Supplemented and Control Animals Receiving N^{15}-L-Tyrosine

Exp. no.	Constit- uent	% N^{15} excess	Total-N in sample mg.	N^{15} excess mg.	Isotope[1] yield %	Keto acid[2] %
3	Total-N	0.157	390.0	0.612	34.7	3.47[4]
	Urea-N	0.143	325.7	0.467	26.4	
	Residual-N	0.067	155.0[3]	0.104	5.9	
	NH_3-N	0.137	3.3	0.0045	0.25	
2	Total-N	0.212	195.0	0.413	24.2	33.4
	Urea-N	0.212	154.7	0.328	19.1	
	Residual-N	0.058	150.7	0.087	5.1	
6	Total-N	0.153	498.0	0.762	37.5	28.2
(Day 1)	Urea-N	0.158	213.3	0.337	16.6	
	Residual-N	0.115	355.3	0.408	20.1	
	NH_3-N	0.152	2.8	0.0043	0.2	
6	Total-N	0.0214	433.0	0.0926	4.6	3.1
(Day 2)	Urea-N	0.0245	261.7	0.064	3.2	
	Residual-N	0.0220	300.3	0.067	3.3	
	NH_3-N	0.0437	3.7	0.0016	0.1	

[1]Based on amount of N^{15} fed.

[2]Based on amount of tyrosine fed.

[3]This value was used in calculating N^{15} excess. The blank value of 93.3 mg. must be subtracted to obtain the residual nitrogen of the urine only.

[4]Received folic acid supplement.

Of the two control experiments, Experiment 2 was run on the purified diet, the other was not. It is possible that Experiment 6 cannot be compared with the other studies, since this animal had been on the vitamin C-free basal for five days plus 13 days on the tyrosine supplement. All the other experiments lasted less than a week. Furthermore, despite the lack of supplementation, the metabolite excretion of this animal dropped rapidly during this period. The keto acid value was 48.4% prior to the two days reported in Table XVI. This drop in metabolite excretion, of unknown origin, was accompanied by a changed isotope excretion picture. The "Isotope yield" of urea was a smaller fraction of the total "Isotope yield" than that of any other animal. In fact, the residual "Isotope yield" was higher than the urea "Isotope yield", in contrast to the usual 1:5 ratio between these values. The "% N^{15} excess" for urea, however, was quite high for this animal and the low "Isotope yield" for urea was due to a drop in the total amount of urea produced, rather than the failure of the tyrosine nitrogen only to be converted to urea.

The ammonia "% N^{15} excess" values for the vitamin C-supplemented animals were higher than these values for the folic acid or control animals. This difference is seen more clearly when the ammonia "% N^{15} excess" is compared to the urea "% N^{15} excess" of the same experiment. The ratios of

these values for vitamin C-supplemented, folic acid-
supplemented and control animals, respectively, were 1.06,
0.96 and 0.96.

In addition to these differences in ammonia isotope con-
centrations, an altered distribution of the urinary nitroge-
nous constituents was indicated by the urea "Isotope yield"
values of 16.6 and 19.1% for the control animals compared to
the average urea "Isotope yield" of 26.0% for the four
vitamin-supplemented animals (Tables XV and XVI). Thus,
while the values for total isotopic nitrogen in the urine of
all of these animals showed no significant differences, a
difference in the mechanism by which the tyrosine nitrogen
reaches the urine may be inferred from these changes in the
distribution of the isotopic nitrogen of the various frac-
tions when the supplementation was varied. Further control
experiments and analyses of the isotope distribution in the
carcasses must be made to confirm the significance of these
findings.

IV. DISCUSSION

It has been demonstrated that antipernicious anemia liver extracts will decrease the excretion of tyrosine metabolic products by the vitamin C-deficient guinea pig receiving extra tyrosine. Injection of the best extracts resulted in a rather uniform percentage decrease in keto acid excretion of more than one half. However, injection of less active liver preparations produced a more variable response, and large doses of active preparations did not have the proportionally greater effects expected. Furthermore, one experimental extract with a high clinical activity had little action in these studies. The demonstration that vitamin B_{12}, the constituent of these extracts which is responsible for their clinical activity, was active in decreasing metabolite excretion made these results difficult to explain.

A number of similar reports have appeared in the literature concerning the action of liver extracts in growth and blood formation. Elvehjem (107) has reported that the growth increases produced by antipernicious anemia liver extracts in chicks were not proportional to their clinical activity. Vitamin B_{12} produced growth responses here also. Furthermore, certain alcohol extracts which are discarded in the preparation of these extracts were active for chick growth. Shaw

(112) has assayed a number of commercial liver extracts, similar to those used here, for their vitamin B_{12} content by microbiological assay. Preparations of different manufacturers, supposed to have the same clinical potency, varied greatly when assayed. Different batches from the same manufacturer of the same labelled potency had a vitamin B_{12} content of 0.3 to 16.0 μg. per ml. in one case, and 2.5 to 13 μg. per ml. in another. The activity of these liver extracts was greater than would be predicted on the basis of their vitamin B_{12} content. This was determined by running standards of crystalline vitamin B_{12} and a solid liver preparation of known vitamin B_{12} content. Clinical results illustrating this same type of discrepancy have been reported by Cuthbertson and Lloyd (111), who showed that liver extracts which contained less than 10 μg. per ml. of vitamin B_{12} failed to produce the expected blood responses in pernicious anemia patients, while more potent extracts did. The same labelled unitage was injected in all cases.

These results plus the actual isolation of two forms of vitamin B_{12} (110) make it probable that the vitamin exists naturally as different conjugates with other unknown compounds, and also that the processing of the liver extracts results in the formation of compounds having different activities for growth of mammals, bacterial growth and blood formation.

These findings explain some of the discrepancies reported here. The ability of other vitamin factors to enhance vitamin B_{12} activity when administered along with the vitamin has also been reported and coincides with the results found using liver extract with the vitamin C-deficient guinea pig. The isolation of the various forms of the vitamin and the study of the interrelationships which exist between these forms and other factors should eventually provide a more complete explanation of the action of these liver extracts.

All the information reported concerning the function of liver extracts and vitamin B_{12} implicate them in some anabolic scheme concerned with growth or blood formation. The failure of liver extracts to produce continued abolition of metabolite excretion in our experiments, in contrast to folic acid and vitamin C, plus their lack of activity in tyrosine oxidation in vitro, indicate that a similar action may be true here. A number of studies (see Historical section) have postulated a function of vitamin B_{12} in protein utilization. Some of our experiments, where extra soybean protein was included in the diet, showed a great increase in the ability of vitamin B_{12} to decrease metabolite excretion. Thus it is quite possible that liver extract may act catalytically in the incorporation of tyrosine into protein or some other body constituent. The lack of sufficient amino acids or other compounds with which tyrosine can combine may then account

for the inability of liver extract to produce abolition of
metabolite excretion for longer periods. Experiments with a
purified diet, using different protein levels, should be
performed to test this hypothesis. Isotope experiments simi-
lar to those already done with vitamin C and folic acid, with
the tyrosine molecule marked in different positions, are also
planned and would also provide information on this question
by showing the location of the tyrosine molecule when liver
extract was injected.

The isotope experiments reported here have been concerned
with the action of vitamin C and folic acid in tyrosine
metabolism, pending the establishment of experimental condi-
tions under which greater liver extract activity can be
obtained. The L-tyrosine used contained N^{15} in the amino
group.

When the isotopic tyrosine was fed to control and vitamin-
supplemented animals it was found that almost all of the N^{15}
was absorbed in the first 24 hours. If one considers the
possibility of re-secretion into the gut, known to occur with
ammonium salts, it is possible that all of the isotope eaten
was absorbed during this period. Unless deamination of
tyrosine in the gut is postulated, which seems improbable,
these findings indicate the rapid absorption of the whole
tyrosine molecule. Even the large amount of extra tyrosine
consumed, therefore, is capable of being absorbed in a normal

manner when mixed with the food as in these experiments.
The action of vitamin C and folic acid on tyrosine must be
considered to be truly a metabolic relationship, since this
action must occur after absorption of the molecule.

The results reported above are in conflict with those
of Painter and Zilva (172). These authors fed L-tyrosine to
guinea pigs and analyzed for the phenolic value of various
tissues using a modified Millon's test. Their results showed
a more rapid disappearance of phenolic value from the intes-
tine in the presence of vitamin C than in its absence. It
should be noted that in these experiments the tyrosine was
administered in one portion by stomach tube. Poor food con-
sumption and weight losses result from this technique. In
addition, the analytical method used is positive for any
phenolic molecule. In the light of the isotope findings, it
is improbable that these results have physiological
significance.

The analyses of urinary nitrogen constituents revealed
that approximately 30% of the N^{15} absorbed appeared in the
urine in 24 hours, irrespective of the supplementary treat-
ment. The urine of the second 24-hour period contained only
5% more of the isotope, indicating rapid utilization and
elimination of the amino acid. Schoenheimer (151) fed
DL-tyrosine containing N^{15} to a rat for 10 days and found
55% of the isotope in the urine. These results show that a
constant amount of the amino group of tyrosine appears in

the urine when the metabolite excretion is high as well as low. The action of folic acid and vitamin C on the tyrosine molecule does not involve removal of the amino group from the urine, therefore.

One hypothesis which helps to explain these results, postulates that the action of folic acid and vitamin C takes place in the liver and is concerned with tyrosine oxidation. The amino group is excreted as urea. In the absence of vitamin C, the intact tyrosine molecule is not oxidized by the liver, but is transported to the kidney where de-amination occurs and p-hydroxyphenylpyruvic acid appears in the urine. The amino group is also excreted, and if the enzymes of guinea pig kidney act like those of the rat, a large proportion of the amino group should appear as ammonia. Thus, in the control animals the urea N^{15} should be lower and the ammonia N^{15} higher than in the vitamin-supplemented animals. However, in all the animals the total N^{15} would be the same.

Evidence for this last finding has been presented and the urea N^{15} yield for the control animals was slightly less than for the vitamin-supplemented animals, as expected. The results for the amount of ammonia N^{15} were not reliable because of the very small amount of ammonia excreted by this species, but the proportion of ammonia N^{15} was highest in the vitamin C-supplemented animals, contrary to expectations.

Further studies of the N^{15} distribution in the carcasses

of these animals must be made. The isotope concentration in ornithine, citrulline and arginine, as compared to that in glutamine and asparagine, under the different experimental conditions should be particularly informative. The hypothesis mentioned above seems to fit the experimental facts on hand at present.

There are two questions about which little information exists. No data is available on the relationship of vitamin C to folic acid in this scheme. They may both act in the oxidation of tyrosine, but at different steps of the enzymatic pathway. Secondly, it is not known at what stage of the oxidation the amino group of the molecule is removed. The evidence at present (see Historical section) indicates that 2,5-dihydroxyphenylalanine is an intermediate in the oxidation of tyrosine. Therefore, de-animation is not the first oxidative step.

It should not be concluded that these hypotheses exclude other types of relationships between these vitamins and tyrosine. The anabolic functions of vitamin C and folic acid in growth and blood formation are well known. Their relationship to the action of liver extracts on tyrosine metabolism may involve an entirely different metabolic pathway than the oxidative one postulated here. This oxidative pathway is an important one, however, since the amino acid provides an energy source by this mechanism.

V. SUMMARY

The finding that liver extracts, used in the treatment
of pernicious anemia, decreased the excretion of tyrosine
metabolites by the vitamin C-deficient guinea pig has been
confirmed. Suitable extracts reduced the metabolite excre-
tion by approximately half. A vitamin B_{12}-charcoal adsorbate
in a soybean flour carrier, when fed orally, had a similar
action on metabolite excretion. The effect produced was
approximately proportional to the amount of vitamin B_{12}
administered. It has been tentatively concluded that the
activity of liver extracts is due, at least in part, to
their vitamin B_{12} content.

The decreases in metabolite excretion caused by liver
extract were observed primarily in the 24-hour period follow-
ing injections and could not be maintained by continued
injections. The decreases observed were not always exactly
proportional to the antipernicious anemia unitage adminis-
tered. It has been concluded that other factors probably
operate in conjunction with the activity of the liver
extracts to produce the metabolite decreases.

The action of vitamin C or cobalt salts, in conjunction
with these liver extracts, was studied. Daily administration
of 1 mg. of vitamin C resulted in larger decreases in

metabolite excretion after liver extract injection. The cobalt salts used had no effect on the action of the liver extract. Injection of a liver dialyzate resulted in irregular decreases in metabolite excretion.

A purified diet was prepared and the action of these factors on metabolite excretion studied with this basal diet. Vitamin C and folic acid supplementation resulted in the usual cessation of metabolite excretion. In the one experiment with liver extract injections, no change in excretory values occurred.

Studies of blood constituents were made to determine whether the action of liver extracts on tyrosine metabolism was correlated with changes in red blood cell counts, total nitrogen and hemoglobin. A suggestion that decreases in metabolite excretion were correlated with increases in the number of red blood cells was noted.

Further studies of the action of vitamin C and folic acid on tyrosine metabolism were made using isotope-labelled amino acid. L-Tyrosine containing 6.01 atoms % excess N^{15} was fed to control and vitamin-supplemented animals. It was found that 80 to 90% of the isotopic tyrosine nitrogen was absorbed from the digestive tract in the first 24 hours.

Approximately 30% of the isotopic tyrosine nitrogen absorbed by unsupplemented, vitamin C-supplemented and folic acid-supplemented animals appeared in the urine in the

first 24 hours. An additional 5% was excreted in the second 24-hour period. It was concluded that the decreases in metabolite excretion produced by vitamin C and folic acid were not accompanied by changes in the amount of isotopic tyrosine nitrogen excreted.

However, the distribution of isotopic nitrogen in the urinary fractions studied differed when the supplement was varied. The nitrogen derived from the urea fraction contained more isotopic tyrosine nitrogen in the case of vitamin-supplemented animals than controls. The urine fraction which contained the greatest concentration of isotopic tyrosine nitrogen was ammonia in the case of vitamin C-supplemented animals, but this was not true for control or folic acid-supplemented animals.

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