A Study of the Ability of Antipyretics to Act as Carboxlyase Models

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A STUDY OF THE ABILITY OF ANTIPYRETICS TO ACT AS CARBOXYLASE MODELS

by

Jay Alan McMahon

A Thesis Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Master of Science

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"No questions are unanswerable.
Whatever curiosity the order of things has awakened in our minds, the order of things can satisfy."

- Ralph Waldo Emerson
Jay Alan Mc Mahon, the last of three children, was born on August 24, 1938 in Chicago, Illinois. In his early years he attended St. Dorothy Parochial School and Mount Carmel High School in Chicago. Following graduation from high school in June of 1956, he attended the University of Michigan and Loyola University, where he completed a pre-dental course of study.

In September of 1959, he began his professional studies at Loyola University School of Dentistry, Chicago, Illinois and received the degree of Doctor of Dental Surgery in June of 1963. The following September he entered the graduate school of Loyola University to begin a two year program toward a Master of Science degree in Oral Biology.
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My deepest appreciation would most certainly go to two of the most devoted parents any individual could ever desire. For their many years of love and sacrifice for me, I owe far more than I will ever be able to repay.

God bless you all.
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CHAPTER I
INTRODUCTION AND STATEMENT OF THE PROBLEM

An understanding of the mechanism of action of therapeutic agents in the body has been and still remains one of the primary goals of biochemical research programs. Such knowledge is an invaluable tool for the improvement in the quality of existing drugs as well as the discovery of new ones. It is important to know whether these agents act in the body in their original state or are perhaps transformed into some other compound which then can produce the desired effect.

The knowledge of the mode of activity of antipyretic-analgetic agents in the body is far from resolved. An antipyretic compound is one that causes a reduction in the body temperature when a fever or abnormally high temperature is present. It has been known for a long time that antipyretics act to increase the heat loss of the body rather than to decrease the body's heat production. This is accomplished by means of peripheral vasodilation, hydremia, polypnea, and especially by an increase in the amount of perspiration. The exact mechanism by which this is accomplished remains somewhat obscure. Because the temperature regulating center is in the hypothalamus, it is likely that they influence this part of the brain in
some manner, directly or indirectly. Yet, it is still not clear whether they affect this temperature reduction prior to a transformation in their chemical nature brought about by the activity or influence of some organ or system of the body.

At the same time, certain of these compounds have exhibited undesirable side effects that have resulted, in some cases, in their use being discontinued. For example, 4-aminoantipyrine, one of the most potent antipyretics, has exhibited the tendency to cause agranulocytosis, the white cell counterpart of aplastic or toxic anemia. In this disease there is an insufficient number of white cells produced by the body. A deeper insight into the mechanism of action of this drug might reveal a cause not directly related to the drug but rather to some by-product, which could possibly be eliminated without damaging the property of antipyresis. This concept could certainly be extended to the other antipyretic compounds showing similar side effects.

It was revealed some time back that this same compound, 4-aminoantipyrine, acted as a catalyst in the decarboxylation of acetoacetic acid into acetone and carbon dioxide. In the human body this same reaction is catalyzed by the biologic enzyme, carboxylase. Thus, a laboratory synthesized compound acted in a manner similar to an enzyme, a product of cellular metabolism. Further studies have revealed that there exists
other similarities between these two types of "catalysts". For this reason, compounds which act in this way are called "enzyme models".

It was found that the mechanism of action of the carboxylase enzyme is very much like the action of 4-aminoantipyrine, and further, that varying the environment as to pH and temperature also had similar effects on each.

Experimental studies on other antipyretic compounds have been negative with regard to their ability to act as enzyme models. Investigation of this problem has revealed that certain molecular arrangements are necessary for the compounds to act as organic catalysts for this decarboxyla.tion reaction. The other antipyretic compounds do not have this prerequisite as aminoantipyrine does.

It remains quite possible, however, that in the human body these compounds are acted upon by an organ or system to bring about a transformation in their chemical and structural nature prior to their action as antipyretics. The antipyretic effect could stem from the fact that in the body they act as enzyme models of one type or another. The investigation carried out and discussed in the following pages represents an attempt to determine if certain antipyretic compounds act as organic catalysts for the decarboxylation of acetoacetic acid. It will also determine if these same compounds are affected by the activity of the liver and also yeast with re-
spect to bringing about a transformation into a particular enzyme model.

The first stage examines certain selected antipyretic compounds prior to any contact with any tissue or other substance found in the human body. In the second stage, each compound is incubated with yeast prior to testing. Yeast was used because it is a readily available source of fermentative enzymes and afforded an opportunity to see whether these glycolytic enzymes would have any effect on any of the antipyretic substances. It also enabled the operator to run through the incubation and testing procedure prior to the use of liver, which necessitated the sacrifice of an animal.

The third stage was similar to the second except that liver was used in place of yeast. It is a known fact that many drugs are oxidized in the liver microsomes, and a study of foreign compounds has shown that a multitude of drugs are metabolized along a surprisingly few number of chemical pathways. Reduction of azo and nitro compounds, conjugation in the formation of glucuronides and other compounds, hydrolytic cleavage of esters and amides, as well as exchange reactions all take place in the liver. Thus, the liver was chosen as the most logical tissue which might affect this change in these chemical compounds, if this change does indeed occur.
The concept of enzyme models is a relatively recent one. Von Wolfgang Langenbeck must be considered as one of the foremost pioneers in this field. In 1933, he demonstrated that several chemical compounds exhibited activity which had previously been known only to take place with biologic enzymes. Specifically, these compounds showed the ability to catalyze certain reactions which also occur in the body. For this reason they were designated as "enzyme models" or mimics. Further study revealed an even greater similarity to certain enzymes with regard to their activity and the pH of the environment, the effect of chemical competitors on the speed of the reaction, and the specificity of both the substrate and the "enzyme model". He also found that the molecular size influences the potency of the catalyst, the more complex its molecular structure, the more powerful is its catalytic activity.

\[
\begin{align*}
\text{H}_2\text{C}-\text{NH}_2 & < \text{C}-\text{NH}_2 < \text{C}_\text{NH}_2 < \text{COOH} \\
\text{COOH} & < \text{C}_\text{NH}_2 < \text{C}_\text{NH}_2 < \text{COOH}
\end{align*}
\]

Fig. 1. --MOLECULAR STRUCTURE COMPARED TO CATALYTIC ACTIVITY
Widmark and Jeppsson are the only scientists who contributed to the literature with respect to enzyme models prior to the work of Langenbeck. In the early 1920s they made reference to the fact that certain carboxylase active compounds behaved in a manner similar to organic enzymes with respect to hydrogen ion concentration. This result in the light of modern biochemistry is what one might expect. Following their effort, further research was remarkably absent on enzymes like compounds until the work of Langenbeck. It was Langenbeck, however, who seemed to stimulate other research programs, particularly in Japan.

In 1938, Kaneko presented his findings, the result of extensive research with many compounds. He identified the relative catalytic activity of these compounds with their structural arrangement in an attempt to establish a correlation between the two. The results of his experiments are as follows:

1. Aminophenol.
   a. Ortho-aminophenol showed little catalytic activity.
   b. Meta-aminophenol showed some activity.
   c. Para-aminophenol was more active than the other two, but not as active as aniline.

\[\begin{align*}
\text{ortho-} & \quad \text{meta-} \\
& \quad \text{para-}
\end{align*}\]
2. **Phenyldiamine** - the order of activity is the same as aminophenol.

\[
\text{ortho-} < \text{meta-} < \text{para-}
\]

3. **Nitroaniline.**
   a. Meta-nitroaniline is the most active.
   b. Para-nitroaniline is less active than meta-nitroaniline.
   c. Ortho-nitroaniline is the least active of the three.
4. **Aminobenzoic acid** - the order of activity is the same as nitroaniline.

\[
\text{meta-} > \text{para-} > \text{ortho-}
\]

Fig. 6--AMINOBENZOIC ACID ISOMERS - RELATIVE ACTIVITY

5. **Aminobenzine sulfonic acid** - the order of activity was the same as aminobenzoic acid.

\[
\text{meta-} > \text{para-} > \text{ortho-}
\]

Fig. 7--AMINOBENZINE SULFONIC AcID ISOMERS - RELATIVE ACTIVITY

6. **Methylaniline or toluidine.**

   a. Meta-toluidine was 1-1/2 times more active than aniline.

   b. Ortho-toluidine and para-toluidine were less active than aniline.
7. Xylidine.
   a. Ortho and para-xylidine were as potent as aniline.
   b. Meta showed little activity.

8. Toluidine diamine and amidol or diaminophenol were very active.

9. Methyl aniline and methol showed little activity.
10. Dimethyl \(-p\)-phenylendiamine was catalytically active.

\[
\begin{align*}
\text{HN-(CH}_3\text{)}_2
\end{align*}
\]

Fig. 12--DIMETHYL \(-p\)-PHENYLENDIAMINE

11. Aminonaphthalene.

a. alpha aminonaphthalene was stronger than aniline.

b. beta aminonaphthalene was weaker than aniline.

\[
\begin{align*}
\text{alpha} & & \text{beta}
\end{align*}
\]

Fig. 13--AMINONAPHTHALENE ISOMERS

12. Hydrozine - proved to be inactive.

\[
\begin{align*}
\text{HN-NH}_2
\end{align*}
\]

Fig. 14--HYDROZINE

13. Generally speaking, the aromatic hydrocarbons are far better organic catalysts than the alaphatic.

a. Aminocyclohexane is an example of an alaphatic hydrocarbon which is inactive.
14. Ethylene diamine is an aliphatic hydrocarbon which is active as an organic catalyst.

\[ \text{H}_2\text{N-} \text{C-C-NH}_2 \]

15. Pyridine.
   a. Alpha pyridine is slightly active.
   b. Beta pyridine is inactive.

16. Aminopyrine proved to be inactive.
17. Aminoantipyrine is not only an excellent catalyst for the decarboxylation of acetoacetic acid, but also pyruvic acid.

\[ \text{H}_3\text{C}-\text{C}-\text{C}=\text{O} \]

Fig. 19--PYRUVIC ACID.

It was Kuga in 1941 who noted that in addition to the basic molecular structure, the enzyme model must contain certain other groups. The nature of these side groups greatly influences the effectiveness of the catalyst, as one might expect.

\[ \text{H}_2\text{N}-\text{C}-\text{C}-\text{N} \]

Fig. 20--KUGA'S BASIC MOLECULAR STRUCTURE OF AN ENZYME MODEL

Of particular significance is the fact that when this group was associated with a group structurally similar to the keto acids, such as amino derivatives of pyrazalone and amino-methyl-uracil, they showed definite catalytic activity.

He further revealed that the activity of the enzyme models is in direct proportion to the relationship between the dissociation constants of the catalysts and keto acids. Thus, if the dissociation constant of the
enzyme model is relatively high, the constant of the acid must also be high for the effective performance of the catalyst.

Studies by Akamatsu and Fukuda substantiated the work of Kaneko and Kuga. Fukuda indicated the presence of an acetoacetic residue in the aminoantipyrine molecule.

Fig. 21--AMINOANTIPYRINE MOLECULE INDICATING THE ACETOACETIC ACID RESIDUE

It was found that all of the enzyme models contain a free amino group in the alpha carbon position; the beta carbon of the molecule combines with the N of another molecular group. The free amino group appears to be the key to the mechanism, which may be to increase the affinity between the catalyst and substrate. The beta keto catalysts resulting in a substrate with an unstable carboxyl which dissociates; the remaining Shiff's base either dissociates spontaneously to produce the original free catalyst or reacts successively with a second molecule of acetoacetic acid under acetone liberation.

Fig. 22--CATALYTIC DECARBOXYLATION OF ACETOACETIC ACID BY AMINOANTIPYRINE
Beniya had already observed in 1934 that a second amino group in the neighboring position intensifies the decarboxylation activity. The subsequent investigation of Fukuda and Kumiga showed similar results. In these heterocyclic diamino compounds there is no more acetoacetic acid residue, because a methyl has been exchanged with another amino group. It is known, however, that an amino group behaves electrically the same as a methyl group.

Akamatsu noted that amino-oxindol is an intensive catalyst for alpha keto acid decarboxylation, yet it is scarcely active to acetoacetic acid, probably owing to the absence of beta keto acid residue. The beta carboxylase model, he remarked, must contain a free amino, and moreover a structurally analogous residue for the enhancement of the activity. This activity, on the other hand, is also dependent upon the other part of the catalyst's molecule. He also noted regular variation in catalytic power in different heterocyclic compounds.

In 1952, Schadt, Becker and McLaren of the Department of Chemistry of Polytechnic Institute of Brooklyn studied the thermal catalyzed decarboxylation of phenylglyoxylic acid of 3-amino alpha naphthindole at 70 degrees. They remarked that the kinetics and mechanism of the catalytic action of model substances having enzyme-like activity may be of interest if the information acquired can be applied to the mode of action.
of natural enzymes. The theoretical mechanism of the catalytic decarboxylation of a keto acid by a carboxylase model was proposed as follows:

1. INITIATION.

\[
\text{Cyclohexylamine} + \text{Pyruvic acid} \rightarrow \text{Cyclohexylpyruvate} + H_2O
\]

2. REARRANGEMENT.

\[
\text{Cyclohexylpyruvate}
\]

3. CLEAVAGE.

\[
\text{Cyclohexylpyruvate} \rightarrow \text{Cyclohexylpyruvate} + CO_2
\]

4. FURTHER REACTION OF SHIFF BASE WITH PYRUVIC ACID.

\[
\text{Cyclohexylpyruvate} + \text{Pyruvic acid} \rightarrow \text{Cyclohexylpyruvate} + \text{Pyruvic acid}
\]

Fig. 23--THE THEORETICAL MECHANISM FOR THE CATALYTIC DECARBOXYLATION OF A KETO ACID BY A CARBOXYLASE MODEL

The proximity between the carbonyl oxygen of the 2-carbon and the carboxyl hydrogen, making hydrogen bonding possible was noted at this time. As proposed in the above scheme, hydrogen bonding between the
oxygen and hydrogen atoms probably precedes decarboxylation.

The authors then discussed the kinetics of the decarboxylation of phenylglycyclic acid by 3-amino alpha-naphthoxindole. The mechanism for the reaction is as follows:

1. INITIATION.

\[
\text{phenylglycyclic acid} \ + \ R-\text{NH}_2 \rightarrow \text{3-amino alpha-naphthoxindole} \ + \text{H}_2\text{O}
\]

2. DECARBOXYLATION.

\[
\text{3-amino alpha-naphthoxindole} \rightarrow \text{phenylglycyclic acid} \ + \text{CO}_2
\]

3. REGENERATION.

\[
\text{3-amino alpha-naphthoxindole} \ + \text{phenylglycyclic acid} \rightarrow \text{3-amino alpha-naphthoxindole} \ + \text{phenylglycyclic acid}
\]

4. TERMINATION.

\[
\text{phenylglycyclic acid} \ + \text{ALL ACTIVE FORMS OF THE CATALYST} \rightarrow \text{INACTIVE PRODUCTS}
\]

Fig. 24--THEORETICAL MECHANISM FOR THE DECARBOXYLATION OF PHENYLGLYCYLIC ACID BY 3-AMINO-ALPHA'-NAPHTHOXINDOLE
Pederson proposed the following mechanism for the decarboxylation of beta ketocarboxylic acids: The ordinary form of the undissociated acid (I) is stable. The acetoacetate ion (II) decomposes very slowly. Owing to the weak basic properties of the keto group, it will, to a small extent, take up the hydrogen ions. The concentration of the ampho ion (III), thus formed, is proportional to the concentration of the ordinary undissociated acid. Consequently, it is impossible to decide from the kinetic experiments which of them is unstable. Owing to the attraction of the positive charge, it is reasonable to assume that the ampho ion decomposes much more quickly than the ion (II). By the decomposition we get the enol form of the reaction product (IV).

\[
\begin{array}{cccc}
I & II & III & IV \\
\text{H}_3\text{C} & \text{H}_3\text{C} & \text{H}_3\text{C} & \text{H}_3\text{C} \\
\text{C}=\text{O} & \text{C}=\text{O} & \text{C}=\text{OH}^+ & \text{C}=\text{OH} + \text{CO}_2 \\
\text{H}_2\text{C} & \text{H}_2\text{C} & \text{H}_2\text{C} & \text{H}_2\text{C} \\
\text{C}=\text{O} & \text{C}=\text{O} & \text{C}=\text{O} & \text{C}=\text{O} \\
\text{OH} & \text{O}^- & \text{O}^- & \\
\end{array}
\]

Fig. 25--REACTION KINETICS FOR THE DECARBOXYLATION OF BETA KETOCARBOXYLIC ACIDS

In order to explain the amine catalysis we assume that an equilibrium of the type

\[ \text{CO} + \text{H}_2\text{NR} \rightleftharpoons \text{CNR} + \text{H}_2\text{O} \]

is quickly established. The group CNR has much stronger basic properties than the keto group. Although the substance CNR may only be formed in a very small concentration by equilibrium (I), to suggest a
greater concentration of ampho ion than before, and a quicker decomposi-

tion.

Westheimer, in 1959, studied the mechanism of the enzymatic
decarboxylation of acetoacetate by examining the oxygen exchange which
accompanies the decarboxylation, labeled in the carbonyl group with $^{18}O$,
in the presence of the crystalline decarboxylase from Clostridium aceto-
butylicum.

His experiments showed that the exchange of $^{18}O$ from the carbonyl
group of acetoacetate is an obligatory part of the enzymatic decarboxylation
process; control experiments establish that the direct exchange of $^{18}O$ from
acetone and acetoacetate, in the presence or absence of enzyme, is incom-
plete. The results are consistent with the hypothesis that the reaction pro-
ceeds by way of Shiff base formation between the ketoacid and the enzyme,
but do not themselves demand this conclusion. This mechanism is very
similar to the one proposed by Pederson for the non-enzymatic amine
catalyzed decarboxylation of acetoacetate.

There are additional scientists, Cramer, Kampe, Mix, Shimoda
and others, who have studied and carried out experiments with enzyme
models. Their work, however valuable it may be, extends beyond the
rather specific scope of this thesis. Yet, I feel in conscience the necessity
to recognize their work before passing on to the next topic.
There have been several valuable clinical studies on the transformations which certain antipyretic compounds have shown to exhibit in the body. The overwhelming amount of work has been carried out by Bernard B. Brodie and his associates. He studied acetanilide, acetophenetidine, antipyrine, and aminopyrine.

The route of acetanilide in man was shown to be as follows: a minor fraction of the drug deacetylates to form aniline: this compound was shown to be a precursor of the substance which oxidizes hemoglobin to methemoglobin; the major fraction of the drug is oxidized to N-acetyl p-aminophenol; this compound is excreted in a conjugated form. The analgesic action of acetanilide is exerted mainly through N-acetyl p-aminophenol which is an active analgesic. The oxidation of acetanilide occurs mainly in the liver.

Fig. 26—TRANSFORMATION OF ACETANILIDE IN MAN

The route of metabolism of acetophenetidin in man is as follows:

The major fraction of the drug is rapidly deethylated to N-acetyl p-aminophenol.
phenol; this compound is excreted in a conjugated form; a minor fraction deacetylates for form p-phenetidin; this compound was shown to be a precursor of the substance which oxidizes hemoglobin to methemoglobin. The analgesic and antipyretic action of acetophenetidin is exerted mainly through N-acetyl p-aminophenol which is an active analgesic and antipyretic.

![Reaction Diagram]

**Fig. 27—TRANSFORMATION OF ACETOPHENETIDIN IN MAN.**

The route of metabolism of antipyrine in man was shown to be as follows: 30 - 40% of the drug is oxidized to 4-hydroxyantipyrine, which is quickly conjugated with glucuroned and possibly sulfuric acid and excreted as such; the remainder of the antipyrine is metabolized through an unknown route.

Pyramidon (dimethylamino pyrine) was discontinued as a therapeutic agent due to an occasional occurrence of agranulocytosis following its use. It does not cause methemoglobinemia or disturbance of RBCs or cause gastric distress sometimes associated with the salicylates. The drug is absorbed almost completely from the gastrointestinal tract and is fairly evenly distributed throughout the body water. Only 3% is excreted in the urine, the rest being transformed in the body. The rate of transformation varies from 10 - 30% an hour in different individuals.
A major step in the metabolism of Pyramidon in man involves demethylation to form 4-aminoantipyrine, an active analgesic and antipyretic. This compound is acetylated in large part to N-acetyl 4-aminoantipyrine, which seems to be pharmacologically inert. A minor fraction of Pyramidon is converted to 4-hydroxyantipyrine, which is excreted in a conjugated form. About 50% of the Pyramidon is unaccounted for.

The analgesic and antipyretic activity of Pyramidon is exerted only in part through 4-aminoantipyrine; it is probable that the parent compound is also an active analgesic.

Fig. 28—TRANSFORMATION OF AMINOPYRINE IN MAN

Boreus and Sandberg concluded, following their experiments with acetophenetidin and N-acetyl p-aminophenol, that the latter compound was found to exert about the same analgesic and antipyretic action as acetophenetidin but to have a lower toxicity and a smaller methemoglobin producing effect.
Barbour, one of the original researchers of antipyretics, attempted to evaluate the clinical signs of aspirin antipyresis in normal, and fevered individuals. He noted that acetylsalicylic acid did not seem to have any effect on persons with normal temperature values. However, there was a definite increase in the amount of heat loss when this antipyretic was administered to the individuals with elevated temperatures. This was accomplished by peripheral vasodilation, hydremia, polypnea and especially by an increase in the production of perspiration by the body.

Guerra and Barbour, working with monkeys, discovered essentially the same thing. Further research by Guerra and Brobeck showed a marked liability of temperature regulation in monkeys following the establishment of lesions in the anterior and anterolateral hypothalamus. This, however, did not alter the rate of decline of fever produced by yeast injection following aspirin administration.

As in the case of enzyme models, additional research has been carried out, but the above mentioned projects represent the highlights of the programs carried out to date and indicates the current thinking on the activity at the molecular and clinical levels.

There have been no projects to date attempting to determine if the liver has any effect on these antipyretic compounds with respect to the formation of enzyme models.
CHAPTER THREE

EXPERIMENTAL PROCEDURE

1. ANTIPYRETIC CONSTITUENTS OF THE TESTING PROGRAM*

A. 4-Aminoantipyrine

1. Molecular Structure

\[ \text{\begin{align*}
        \text{N} & \quad \text{N} \\
        \text{C} & \quad \text{C} \\
        \text{N} & \quad \text{C} \\
        \text{CH}_3 & \quad \text{CH}_3
    \end{align*}} \]

2. Molecular Weight - 203.2

B. Acetamidoantipyrine (4-acetyl aminoantipyrine)

1. Molecular Structure

\[ \text{\begin{align*}
        \text{N} & \quad \text{N} \\
        \text{C} & \quad \text{C} \\
        \text{N} & \quad \text{C} \\
        \text{CH}_3 & \quad \text{C} \quad \text{CH}_3
    \end{align*}} \]

2. Molecular Weight - 245.2

C. N-Acetyl p-aminophenol

1. Molecular Structure

\[ \text{\begin{align*}
        \text{N} & \quad \text{N} \\
        \text{C} & \quad \text{C} \\
        \text{O} & \quad \text{N} \\
        \text{C} & \quad \text{CH}_3
    \end{align*}} \]

2. Molecular Weight - 151.2
D. 3-Hydroxyacetanilide
1. Molecular Structure

2. Molecular Weight - 151.2

E. P-acetphenetidine
1. Molecular Structure

2. Molecular Weight - 171.2

F. Acetoacetanilide
1. Molecular Structure

2. Molecular Weight - 177.2

G. Acetylsalicylic Acid
1. Molecular Structure

2. Molecular Weight - 180.15
II. TESTING PROCEDURE

A. Introduction

These seven antipyretic compounds were tested for their ability to catalyze the decarboxylation of acetoacetic acid. The reaction products are acetone and carbon dioxide. The rate of the reaction is determined by measuring the pressure differential produced by the carbon dioxide gas emitted.

This is accomplished through the use of the Warburg Respirometer, an instrument which facilitates a high degree of control over the temperature of the solutions during the testing periods and ensures a uniform agitation of the flasks.

B. Stage One

1. Preparation of the Solutions

In this first stage, one half of the molecular weight, in milligrams, of each of the antipyretic compounds is dissolved in fifty milliliters of distilled water, making a 10mM solution. This is designated as solution A for purposes of explanation.

The acetoacetic acid is prepared by adding 1.0 milliliters of acetoacetate methyl ester and five milliliters of 2N NaOH to a fifty milliliter volumetric flask, diluted to volume and allowed to stand at room
temperature for two hours.

\[
\text{HC-C-C-C-O-C-CH \text{ di} \text{ alkali} \rightarrow HC-C-C-C-Na \text{ di} \text{ acid}}
\]

Fig. 29--REACTION FOR ACETOACETIC ACID PREPARATION

It was found that after a twenty-four hour period had elapsed, a considerable amount of spontaneous decarboxylation had taken place. If used, the solution would mimic the results present when catalysts is actually occurring. For this reason, a fresh solution of acetoacetic acid was prepared on testing days.

\[
\text{HC-C-C-C-OH} \rightarrow \text{HC-C-C-CH} + \text{CO}_2
\]

Fig. 30--SPONTANEOUS DECARBOXYLATION OF ACETOACETIC ACID

The only other constituent of the initial phase is sodium acetate buffer, which has been adjusted to a pH of 4.2 and is designated as solution B.

2. Preparation of the Warburg Flasks

The first two Warburg flasks, designated 1 and 1a, were prepared in the following manner: five milliliters of a 1:4 ratio of solution A to solution B were added to the main flasks, while one milliliter of acetoacetic acid
was placed in each side arm. The second pair of flasks, designated 2 and 2a, also contained one milliliter of acetoacetate in each of the side arms. However, to each main flask was added five milliliters of a 1:9 ratio of solution A to solution B. The third pair of flasks, designated 3 and 3a, acted as controls and contained five milliliters of distilled water in each main flask and one milliliter of acetoacetate in the side arms.

3. Procedure of the Initial Phase

The side arms of the flasks were stopped and attached to the six manometers, which were then secured to the side of the water bath drum, making certain that the water level was sufficiently high to cover the flasks. The solutions were equilibrated for five minutes in the water bath at 37 degrees. Following this, the contents of the side arms were added to the main flasks and the systems closed. The pressure differential produced by the carbon dioxide was recorded every five minutes for thirty minutes. The control figures were subtracted from the figures of the main test in order to accurately determine any catalytic activity present.

This procedure was carried out twice for six of the seven compounds. The only deviation from this program came in the case of P-acetophenetidine, where it was found that one half of molecular weight in milligrams would not dissolve in fifty milliliters of distilled water. In this case, ethyl alcohol was used as the solvent in the same quantity as the water had
been used for other preparations. It was necessary, therefore, to run an additional experiment using ethyl alcohol without the acetoacetic acid to determine the pressure differential produced by this compound alone. This figure was subtracted from the main test.

C. Stage Two

1. Preparation of the Solutions

As in the first step, one half of the molecular weight of each compound was used. This was added to twenty-five milliliters of distilled water along with one gram of glucose, one gram of yeast and five hundred milligrams of sodium phosphate. The contents were then placed in the fifty milliliter volumetric flask and diluted to volume. An aliquot (10 milliliters) was centrifuged immediately. The remaining, larger part of the solution was incubated at 37 degrees for twenty-four hours. Following incubation, this too was centrifuged. The supernatants of the centrifuged aliquot and the incubated sample were designated as solutions A and Al, respectively.

The acetoacetic acid and sodium acetate buffer preparations were identical to stage one.

The only exception to the above preparations was, as in the first stage, P-acetphenetidine. The ethyl alcohol, however, would be impractical for the second stage, for it would interfere with the normal biochemical processes during incubation. Thus, one half of the molecular weight in milligrams of this compound was dissolved in one hundred milliliters of
water following the addition of yeast, glucose and sodium phosphate as with the other compounds.

In order to have the same number of milliequivalents reacting with the acetoacetic acid as the other compounds, it was necessary to double the amount of solution in the Warburg flasks. Thus, ten milliliters of 1:4 and 1:9 ratios of solution A Cr Al, to solution B were used.

2. Preparation of the Warburg Flasks

The flasks were prepared in the same manner as in stage one with respect to solution ratios and amounts. The only exception to this is the above mentioned P-acetphenetidine.

3. Procedure of the Second Stage

The deviation in the program from the first stage is a result of the incubation of the solutions. Each compound was first tested for catalytic activity following initial contact with the yeast. Here, an aliquot was centrifuged immediately and tested in the same manner as the compounds of stage one. The remaining part was first incubated for twenty-four hours and then centrifuged and tested. Thus, the catalytic activity of each compound was determined before and after the incubation process.

D. Stage Three

1. Preparation of the Solutions

In the final stage, liver was used in place of yeast. Thus, a liver weighing nine gram was removed from a freshly sacrificed rat and placed
in a Waring Blender together with ninety milliliters of water. The ingredients were then blended until a homogeneous mixture was obtained. This was divided into nine equal parts of ten milliliters each. Since the concentration of the mixture is one gram per ten milliliters, each part represents one gram of liver. They were stored in the freezer until such time as needed. The remaining preparations were identical to the ones of stage two.

2. Preparation of the Warburg Flasks

This was done in the same manner as stage two.

3. Procedure of the Third Stage

This was also the duplicate of the second stage.
A. SINGLE COMPOUND GRAPHS

- - - - - - - - - - - - -
Initial

- - - - - - - - - - - - -
Before Incubation

- - - - - - - - - - - - -
After Incubation

B. COMPARATIVE GRAPHS FOR AN ENTIRE STAGE

- - - - - 4-Aminoantipyrine

- - - - - Acetylsalicylic Acid

- - - - - Acetamidoantipyrine

- - - - - 3-Hydroxyacetanilide

- - - - - Acetoacetanilide

- - - - - N-Acetyl p-Aminophenol

- - - - - P-Acetphenetidine
CHAPTER FOUR

EXPERIMENTAL FINDINGS

1. Stage One

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### F. Acetyl - p - Aminophenol

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\[
\begin{align*}
5.1 & \quad 5.2 & \quad 4.7 & \quad 4.9 & \quad 0.8 & \quad 1.0
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\]
G. **P-Acetphenetidine** (using ethyl alcohol as the solvent rather than water)

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**P-Acetphenetidine**

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H. The following test is used to determine the amount of pressure caused by the ethyl alcohol during the experiment with P-Acetphenetidine. The contents of the flasks are as follows:

A. The Central Unit of Main Flask

a) Flasks numbered 1 and 1a contain 5ml. of the following solution:

(1) sodium acetate buffer adjusted to ph 4.2

(2) 1:5 dilution of ethyl alcohol.

b) Flasks numbered 2 and 2a contain 5ml. of the following solution:

(1) sodium acetate buffer adjusted to ph 4.2

(2) 1:10 dilution of ethyl alcohol.

B. The Side Arms - All of the flasks contain 1ml. of distilled water in the side arms.

<table>
<thead>
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<tbody>
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II. Stage Two

Note - The following differentiation is used in this stage:

Part A - Before incubation

Part B - After incubation

A. Aminoantipyrine. Part A

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4 - Aminoantipyrine. Part A

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## 4 - Aminoantipyrine, Part B

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STAGE II - 4-AMINOANTIPYRINE

DILUTION 1:5

TIME (minutes)

mm of PRESSURE
## B. Acetylsalicylic Acid. Part A

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### Acetylsalicylic Acid. Part A

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\[
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4.6 & \quad 4.2 \\
4.5 & \quad 0.3 \\
0.5 & \quad 0.0
\end{align*}
\]
### Acetylsalicylic Acid. Part B

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\end{align*}
\]
STAGE II - ACETYLSALICYLIC ACID

DILUTION - 1:5

TIME (minutes)

mm of PRESSURE

0  5  10  15  20  25  30
STAGE II - ACETYLSALICYLIC ACID

DILUTION - 1:10
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STAGE II - ACETAMIDOANTIPYRINE

DILUTION = 1:10

TIME (minutes)

mm. of
PRESSURE
### D. 3-Hydroxyacetanilide. Part A

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STAGE II - 3-HYDROXYACETANILIDE

DILUTION - 1:10

mm. of
PRESSURE

TIME (minutes)
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STAGE II - ACETOACETANILIDE

DILUTION 1:5

mm. of PRESSURE

TIME (minutes)
### F. N-Acetyl-\(p\)-Aminophenol. Part A

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STAGE II - N-ACETYL p-AMINOPHENOL

DILUTION 1:5

mm of PRESSURE

TIME (minutes)
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STAGE II - P-ACETPHENETIDINE

DILUTION 1:5

mm. of
PRESSURE

TIME (minutes)
### III. Stage Three

#### A. 4-Aminoantipyrine. Part A

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STAGE III - 4-AMINOANTIPYRINE

DILUTION - 1:5

mm. of PRESSURE

TIME (minutes)
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STAGE III - ACETYLSALICYLIC ACID

DILUTION - 1:5
STAGE III—ACETYLSALICYLIC ACID

DILUTION: 1:10

TIME (minutes)

mm of PRESSURE
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STAGE III - ACETAMIDOANTIPYRINE

DILUTION 1:10

TIME (minutes)

mm. of
PRESSURE

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### 3-Hydroxyacetanilide. Part B

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### Notes

- The table above lists the concentrations in parts per million (ppm) for various time points.
- The data is organized by flask numbers and time intervals in minutes.
- The concentrations for each time point are presented for different flasks (labeled 1, la, 2, 2a, 3, 3a).
- The values appear to decrease over time, indicating a possible reaction or degradation process.
- The data might be used for chemical analysis or reaction kinetics studies.
STAGE III - 3-HYDROXYACETANILIDE

DILUTION 1:5

mm. of PRESSURE

TIME (minutes)
STAGE III - 3-HYDROXYACETANILIDE

DILUTION - 1:10

mm of
PRESSURE

TIME (minutes)
### Acetoacetanilide, Part A

#### Flask Numbers

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#### Acetoacetanilide, Part A

#### Flask Numbers

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STAGE III-ACETOACETANILIDE

DILUTION 1:5

TIME (minutes)

mm. of PRESSURE
STAGE III - ACETOACETANILIDE

DILUTION 1:10

mm of PRESSURE

TIME (minutes)
### F. N-Acetyl -p- Aminophenol. Part A

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### P-Acetphenetidine. Part A

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\[
\begin{align*}
\text{9.0} & \quad \frac{9.9}{8.5} & \quad \frac{7.4}{4.5} & \quad \frac{4.4}{3.6} \\
\end{align*}
\]
### P-Acetphenetidine, Part B

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### P-Acetphenetidine, Part B

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| 10.6 | 11.3 | 9.0 | 10.3 | 4.4 | 5.1 |
STAGE III - P-ACETPHENETIDINE

DILUTION: 1:10
CHAPTER FIVE

DISCUSSION

The primary purpose of this body of research is to gain insight into the possible mode of action of antipyretic compounds. This was accomplished through a series of experiments designed to determine if certain of these compounds act as beta carboxylase models in the body, and further to see if incubation with yeast and/or liver has any effect on their activity in this respect.

The findings of stage one substantiate previous study and seem to support the concept of Akamatsu and Fukuda. These men were the first to observe that a prerequisite for a compound to act as a beta carboxylase model appears to be the presence of an acetoacetic residue within the structure of the compound. The free amino group in the alpha carbon position seems to be the key to the mechanism, as was stated earlier in the literature review, which may be to increase the affinity between the catalyst and substrate.

4-aminoantipyrine is the only compound tested which has this structural component, and it was found to be the only one to exhibit substantial catalytic activity upon testing. This compound is ten to fifteen times more potent than any of the others tested.
Incubation with yeast, performed in the second stage, appeared to have a negligible effect on the catalytic activity of these compounds. Liver, in addition, produced results indicating a lack of influence of this tissue on these compounds with respect to affecting a transformation to a carboxylase model. In both cases yeast and liver, there was no significant alteration in the progress of the reaction which would indicate that either substance had changed these compounds in any way. It must be remembered, however, that the purpose of this program is very specific in nature, and this does not mean to imply that no change did occur. As a matter of fact, these compounds might still be found to act as enzyme models in the body with further investigation. It is very important to note at this time, however, that liver tissue does not cause a transformation of these antipyretic compounds to beta carboxylase models following twenty-four hours of incubation.

The specificity of enzyme models is exemplified by comparing the catalytic activity of 4-aminoantipyrine and acetamidoantipyrine with their structural arrangement. These compounds are very similar in structure, differing only in the nature of the group in the alpha carbon position. This difference is sufficient, however, to permit one, 4-aminoantipyrine, to act as a carboxylase model and not the other.

An attempt to correlate structural architecture with catalytic ac-
tivity as Kaneko did might be somewhat misleading for all of the compounds were found to be very similar in their ability to catalyze the decarboxyla-
tion reaction. The compounds listed according to their ability to catalyze the reaction are: 4-aminoantipyrine, P-acetphenetidine, acetoacetanilide, 3-hydroxyacetanilide, acetylsalicylic acid, N-acetyl p-aminophenol and acetamidoantipyrine.

4-aminoantipyrine is known to have harmful side effects which have caused its use to be reduced to a considerable degree. Knowing that this compound acts as a beta carboxylase model and there is definite evi-
dence that the others may not, a further research program might attempt to correlate this activity with the side effect produced. If successful, this might open the doors to finding the method of eliminating the cause of the side effects without damaging the antipyretic property. Another worthy area of investigation would be to determine if other biologic media can affect this transformation in these compounds to particular enzyme models.

Thinking along these lines of further research activity in the area of enzyme models, it should be noted that this concept of identifying the ac-
tion of therapeutie agents with that of an enzyme model is quite unique. Thus, the field is wide open in this regard. This concept is so general that it can and should be applied to many different pharmacologic agents as a possible mode of action.
CHAPTER SIX

SUMMARY

Experiments were performed on seven representative antipyretic compounds in an attempt to determine if these compounds act as beta carboxylase models, as would be evidenced by their ability to increase the rate of decarboxylation of acetoacetic acid. The rate of the reaction was determined by measuring the pressure differential produced by the CO₂ given off at 5 minute intervals for 30 minutes. This was accomplished through the use of the Warburg respirometer. The water bath of the apparatus was adjusted to 37°. Previous study has revealed that one of these compounds, 4-aminoantipyrine, does exhibit this property. Each of these compounds was also submitted first to yeast and then to liver incubation at 37° for 24 hours to determine if either substance affected a change in their chemistry to beta carboxylase enzyme models.

This experimental program revealed the following:

1. Six of the seven antipyretic compounds do not show substantial catalytic activity in the decarboxylation to a marked degree.

2. 4-Aminoantipyrine catalyzed the decarboxylation to a marked degree.

3. Incubation with yeast and liver appeared to have little effect on their ability to catalyze the reaction.
4. Little difference was noted in the rate of the decarboxylation with any of the compounds after either incubation process as compared with before incubation.
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APPROVAL SHEET

The thesis submitted by Dr. Jay Alan Mc Mahon has been read and approved by three members of the Department of Oral Biology.

The final copies have been examined by the Director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

[Signature of Advisor]

[Date]