THE UNIVERSITY OF MANITOBA

A STUDY OF

THE HYDROLYSIS *IN VITRO* AND CERTAIN BIOLOGICAL EFFECTS OF SOME ECGONINE ALKALOIDS FOUND IN *ERYTHROXYLON COCA*

by

RICHARD ROSCOE

A THESIS

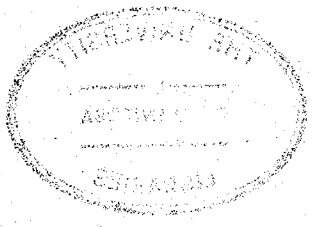
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

WINNIPEG, MANITOBA



October, 1979

A STUDY OF

THE HYDROLYSIS *IN VITRO* AND CERTAIN BIOLOGICAL EFFECTS OF SOME ECGONINE ALKALOIDS FOUND IN *ERYTHROXYLON COCA*

BY

RICHARD MAURICE HENRY ROSCOE

A thesis submitted to the Faculty of Graduate Studies of
the University of Manitoba in partial fulfillment of the requirements
of the degree of

DOCTOR OF PHILOSOPHY

ABSTRACT

Investigations were completed concerning the hydrolysis of cocaine and certain biological effects of selected ecgonine alkaloids derived from coca leaf. The alkaloids were cocaine, ecgonine methylester, benzoylecgonine, and ecgonine.

Analytical and hydrolysis procedures were developed to evaluate the hydrolysis of cocaine in a variety of alkaline media. In non-enzymatic systems of pH greater than 11 ecgonine methylester was found to be the major intermediate in the hydrolysis of cocaine to ecgonine. Cocaine was shown to rapidly hydrolyze in these systems, including those of the alkaline materials used in the chewing of coca leaf, i.e., ishku. The rapid hydrolysis of cocaine to ecgonine proceeds via two parallel pathways, one involving benzoylecgonine as an intermediate and the other, greatly favored at pH values greater than 11, ecgonine methylester.

Measurements of the energy metabolism of rats indicated that significant depression of respiratory quotients occurred in rats fed a low protein-high carbohydrate diet containing cocaine, ecgonine methylester, or benzoylecgonine. Ecgonine was without effect. Since available data showed that cocaine did not change the urinary nitrogen output of rats maintained on the low protein diet, the changes in respiratory quotient point to changes in the relative utilization of fat and carbohydrate for energy metabolism. The depressed respiratory quotients would therefore indicate an increased relative utilization of fat in the rats. The results of the metabolic studies involving ecgonine methylester in rats receiving low protein-high carbohydrate diets were confirmed by results of whole body analysis studies in mice similarly treated. The increased

relative utilization of fats found in rats was consistent with the reduced whole body triglyceride levels found in the mice. When administered concurrently with oral xylose test solutions, cocaine increased the absorption of xylose while ecgonine decreased xylose absorption.

A preliminary single-rat experiment showed that ecgonine methylester is excreted in the urine of the rat in larger amounts than benzoylecgonine following the oral administration of cocaine while benzoylecgonine is the major urinary metabolite following subcutaneous cocaine administration.

The results of the present research provide good evidence that cocaine alone is not responsible for the biological effects associated with coca chewing. The hydrolysis products of cocaine, in particular ecgonine methylester, must play a greater biological role than previously realized in coca chewing.

ACKNOWLEDGEMENTS

At this time I wish to thank Dr. Trieste G. Vitti for his thoughtful and expert direction, supervision and assistance during my period of studies at the Faculty of Pharmacy. The receipt of a Medical Research Council Studentship Award, a Teaching Assistantship from the Faculty of Pharmacy, and a Geigy Pharmacy Scholarship was greatly appreciated. I thank the members of the Faculty, Support Staff, and Student Body of the Faculty of Pharmacy, Department of Chemistry, and the Health Sciences Centre for the advice, counsel, and technical assistance they afforded me during my program.

The results presented in Table 19, page 101, are based on data graciously provided by R. Boni and F. Burczynski who performed the nitrogen balance study.

This acknowledgement is made complete when I express my deepest gratitude to my wife Barbara and my children, Robyn, Jodi, and Jamis for all of their laughter and all of their tears. Thank-you.

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I INTRODUCTION

I - A Erythroxylon coca

1. Pharmacognosy

Erythroxylon coca is a hardy shrub growing to a height of about 1 meter in the mountainous uplands of South America, primarily in the Andes of Peru and Bolivia, and to a lesser extent in the mountains of Mexico, the West Indes and Java (1, 2). The coca plant with its delicate tea-like leaves is cultivated in the valleys of these regions at altitudes of about 6000 meters above sea level. The crops are cared for in large plantations with leaf harvesting taking place several times annually.

The species of coca which is found in the South American regions contains the greatest amounts of cocaine, corresponding to about 0.6% of the dry weight of the leaf material (3). The leaves contain at least 14 alkaloids of the ecgonine, tropane, tropeine, and hygrine classes (4). The ecgonines, including cocaine, are the most abundant.

2. Coca Chewing

Approximately half the populations of Peru and Bolivia are coca chewers or coqueros (5). This represents about 6 million people in these two countries alone. Coca chewing is practiced in Ecuador, Chile, and Colombia as well, where coca is also extensively cultivated.

The origin of coca chewing is lost in history (6). Two thousand years ago coca chewing formed an integral part of many religious and medical practices. It was performed in these ancient times by tribal priests and nobility in various ceremonies, but today coca chewing is not restricted to the upper class. In fact, coca chewing is practiced primarily by the lower class (7), who chew between 30 and 200 grams of the dry leaf daily. In Peru alone this represents 8.5 million kilgrams a year (8).

The coca chewers place about 10 to 20 grams of the leaf into their mouths and masticate this material into a bolus, swallowing whatever components are extracted into the saliva. No expectoration takes place during the chewing process. The chewing takes place several times during the day but usually before, during, and after meals. In practice, the chewers add a second material to the bolus of coca leaf in the mouth. Depending upon the locale, either a burned limestone substance is added in powder form by means of a saliva-wetted stick or a bite is taken from a hard cake-like substance composed mostly of ashes from a burned plant material. The burned limestone substance is called ishku and the caked ash substance is called llipta. Both of these materials reportedly produce alkaline conditions in the mouth and are believed to aid in the extraction of cocaine from the leaf bolus. More coca leaf and alkaline material is added as the chewing process continues. The daily dose of cocaine if all the cocaine in the leaf material chewed were absorbed unchanged could amount to 500 to 1000 mg.

Early research on coca chewing has been biased from the point of view that the chewing was seen as an effort on the part of the native to obtain cocaine and thereby experience some desirable salutary effect. The coca chewer has been viewed as a poorly nourished, overworked, and inadequately clothed individual who is able to endure the hardships of his life through an addiction to cocaine (2). Efforts to prove that this is the case have not been successful. Early research reports that blood levels of cocaine in the chewers following coca chewing are very low or non-existent (9). More recent investigations, however, report significant blood levels of cocaine following coca chewing (10). Much controversy exists as to whether it is indeed the cocaine that is responsible for the effects produced by

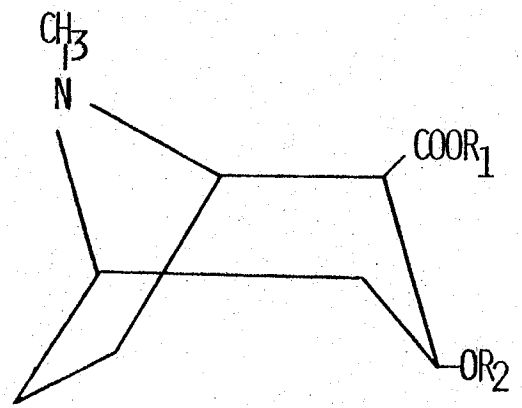
chewing coca leaf. In fact, Mortimer postulated in 1901 that certain metabolic breakdown products of cocaine may play an important role in the physiological and metabolic effects of coca chewing (11). Cocaine is unstable at the alkaline pH most likely existing in the coca leaf bolus, which should lead to rapid hydrolysis to benzoylecgonine and ecgonine (12, 13). Unfortunately, however, early work on the recovery of coca alkaloids from the urine of coca chewers does not differentiate among the alkaloids assayed (14). The alkaloids found may not have been cocaine alone.

Several facts are known concerning the practice of coca chewing. The coca chewers have a difficult life at high altitudes being exposed to low oxygen tensions and cold temperatures yet they appear to function and work adequately. They subsist on diets that are high in carbohydrates but adequate in vitamins and minerals. These diets are low in fats and protein as compared to diets in areas of high standards of living. The coca chewer consumes large amounts of coca leaf regularly. There is no evidence, however, that addiction occurs to or that highs result from this coca chewing. Nonetheless, the popular belief persists that the coca chewer is able to dull his senses against his hardships and even possibly lift his spirits above his meagre existence in a harsh and demanding environment.

I - B Cocaine and Related Ecgonine Alkaloids

1. Chemistry

Cocaine is an ecgonine alkaloid closely related to the three ecgonine alkaloids: benzoylecgonine, ecgonine methylester and ecgonine shown in Figure 1 (3). Cocaine is an organic base with a pKa of 8.39. It is very soluble in chloroform and almost insoluble in water. As the



ECGONINE ALKALOID	R_1	R_2
COCAINE	$-CH_3$	$-COC_6H_5$
BENZOYL-ECGONINE	$-H$	$-COC_6H_5$
ECGONINE METHYLESTER	$-CH_3$	$-H$
ECGONINE	$-H$	$-H$

H- HYDROGEN, CH_3- METHYL, COC_6H_5- BENZOYL.

Figure 1. The ecgonine alkaloids.

hydrochloride, cocaine is very water soluble. Hydrolysis of the methyl ester function in cocaine produces benzoylecgonine with a pKa of 11.80 which has an appreciable water solubility even as the free base. Hydrolysis of the benzoyl ester function in cocaine produces ecgonine methylester with a pKa of 9.16 and solubility characteristics similar to cocaine (3). Hydrolysis of both ester functions in cocaine produces ecgonine. Ecgonine is amphoteric with a pKa of 10.91 and is very water soluble (3). Cocaine and benzoylecgonine and usually isolated as the free base while ecgonine methylester and ecgonine usually as the hydrochloride salts.

Cocaine is prepared from extracts of the coca leaf in high yields (15). It is usually supplied commercially as the hydrochloride salt. Benzoylecgonine, ecgonine methylester and ecgonine are prepared from cocaine because their isolation from the natural source is very inefficient. These four alkaloids have been separated and quantitated by a number of different procedures including paper (16), thin layer (17), ion-exchange (18), gas (19), and high-pressure liquid (20) chromatography. Spectrophotometric (21, 22) and immunological methods (23, 24) have also been employed. The structure, stereochemistry and important physicochemistry properties of the ecgonines have been worked out for some time (25-27).

2. Pharmacology

Cocaine was introduced into Europe after the Spanish conquest of South America in the sixteenth century but was ignored until the early eighteenth century when cocaine was popularized by such figures as Sigmund Freud and Sir Arthur Conan Doyle's Sherlock Holmes (1). It seemed that up to the early 1900's every medicinal remedy sold contained

significant amounts of cocaine. The ever popular beverage Coca-Cola was no exception, originally marketed as a remedy for headaches and hang-overs. De-cocainized coca leaf material is still employed in the secret recipe of Coca-Cola today.

Cocaine possesses a variety of pharmacological actions (28, 29). The layman knows of cocaine's central nervous system stimulation which is the basis for its high abuse potential. The medical profession uses cocaine primarily for its local anaesthetic properties. Cocaine is an excellent local anaesthetic and is the forerunner of all local anaesthetics. It blocks nerve impulse transmission along nerve fibres and also blocks the neuronal re-uptake of endogenously released norepinephrine. The localized vasconstriction produced by cocaine's blockage of the re-uptake of norepinephrine prolongs the anaesthetic effect of cocaine and also keeps surgical fields free of blood. This accounts for cocaine's use today as a local anaesthetic in ophthalmic surgery. Other local anaesthetics lack this intrinsic action of cocaine and require specific vasoconstrictors in their formulation. The action of cocaine to block the neuronal re-uptake of neurotransmitters is employed as a tool in pharmacological research into the mechanism of action of new autonomic and psychotropic agents.

Cocaine administered to man and animals has been shown to produce elevated body temperatures, increased metabolic rates, increased heart rates, increased respiratory rates, elevated blood pressures, elevated blood sugars, anorexia, and generalized vasoconstriction and central nervous system stimulation. Most of these effects are brought about by adrenergic stimulation produced or enhanced by cocaine. Rather than a

tolerance, a sensitivity develops to repeated doses of cocaine with the initial response being produced by progressively smaller amounts of cocaine (30). Physiological dependence on cocaine does not occur but rather psychological dependence or habituation. With respect to the central nervous system stimulation, the drug-induced effects are subject to wide and varied interpretation on the part of the subject with the actual effect ranging from simple mood elevation to total euphoric excitement (31, 32). This range of effects is dependent not only upon dose and route of administration but also upon the individual and his/her environmental circumstance.

It has been thought that cocaine acts only through the adrenergic system but it has been shown that cocaine can affect the cholinergic system as well and that the resulting effects are very dose dependent (33).

The pharmacological effects produced by cocaine following oral administration have not been fully investigated. Some recent preliminary data suggest that cocaine produces the same and sometimes greater central effects when administered orally as compared to the intranasal route of administration (34). The validity of these results is in some doubt because of the lack of proper controls in the study. The reported effects are short lived and to be maintained cocaine must be administered repeatedly regardless of the route (1).

Cocaine has been shown to cause hepatotoxicity upon chronic administration to mice (35). When cocaine was administered to rats as part of their diet, changes were observed in the levels of certain liver enzymes and serum globulins regardless of whether the diet was low or high in

protein (36). The growth of these animals was not affected by the cocaine added to the diet. The rats fed the low protein diet alone developed histologically confirmed fatty livers while those fed the low protein diet supplemented with cocaine displayed no similar histology. Cocaine inhibits cellular respiration, an effect believed to be mediated through the blockade of entry of two-carbon fragments into the citric acid cycle (37, 38).

Cocaine has been shown to produce in animals elevations in serum lactate and triglycerides, which appear to be mediated by cocaine's enhancement of the action of endogenously released norepinephrine (39-41). In most instances cocaine has been shown to enhance the action of norepinephrine. Some research of muscle physiology, specifically involving creatine synthesizing and phosphorylase enzyme activity, showed that cocaine inhibited the stimulating action of norepinephrine in this system (42).

The pharmacology of benzoylecgonine is not completely known. Benzoylecgonine appears to be without local anaesthetic activity (29); it is capable of producing central nervous system stimulation only when administered intracisternally (43). Little is known about the pharmacology of ecgonine methylester. It has been shown to be without local anaesthetic and central nervous system activity in animals (29, 44).

Ecgonine, the ultimate product of cocaine hydrolysis, would be expected to be devoid of pharmacological activity, but apparently this is not the case (45). When ecgonine was investigated as a mixture with small amounts of cocaine in a ratio of 10 parts ecgonine to 1 part cocaine, a pronounced anticholinergic effect was observed in isolated

rat duodenum preparations (46). One report states that ecgonine, administered subcutaneously, was able to improve the physical performance of mice treated with depressant drugs or surgical brain lesions (47). Ecgonine, however, has been found to have no local anaesthetic action (29).

Available pharmacological and biochemical studies concerning the effects produced by coca chewing have been crude and poorly controlled (48, 49). The measured increases in oxygen consumption, heart and respiratory rates, and body temperatures were attributed to the adrenergic action of cocaine. One report indicates that the measured effects were enhanced when the coca leaf was chewed with alkaline material and reduced with dilute hydrochloric acid (50). Clinical differences found between coca chewers and non-coca chewers have included reductions in serum protein and cholesterol and reduced haemoglobin and haematocrit levels in the chewers (51, 52). These differences have been attributed to the coca worsening an already poor nutritional state in the native (53). One fact obvious throughout these studies is that no effort was made to determine if cocaine was directly responsible for any of the reported observations. Most importantly, much of this early work was biased in its conception trying to show coca chewing detrimental to the welfare of the chewer. Many such studies were sponsored by organizations whose main efforts to solve the coca problem were directed towards the eradication of the practice of coca chewing; that coca chewing could possibly produce beneficial effects was a foreign expectation.

3. Hydrolysis and Metabolism

Cocaine has been known to be unstable in alkaline media; it has been suggested that it hydrolyzes to benzoylecgonine and ecgonine (13, 21).

It is thought that ecgonine methylester appears as a cocaine hydrolysis product only in systems incorporating esterase enzymes. Ecgonine methylester has been found to be formed when cocaine is administered to animals or incubated with serum preparations (54-56). The accepted overall hydrolysis profile of cocaine is depicted in Figure 2. The hydrolysis pathway from cocaine to ecgonine in systems involving esterase enzymes results in both benzoylecgonine and ecgonine methylester being produced as intermediates, while the hydrolysis of cocaine in non-enzymatic systems is thought to produce only benzoylecgonine en route to ecgonine (12). It has been reported that not all mammalian esterases can hydrolyze cocaine. Hydrolysis of cocaine has been observed in plasma but this hydrolysis can be prevented by pre-treatment of the plasma with physostigmine which blocks cholinesterase activity but is without effect on other esterases (54, 57). Cocaine is most stable at a pH of 2.0 (58). Earlier studies of the hydrolysis of cocaine which reported the disappearance of cocaine to be slow in alkaline solutions were likely in error. The analytical techniques employed then were unable to differentiate other ecgonine alkaloids from cocaine (59). The ability of animal urine to hydrolyze cocaine to benzoylecgonine and ecgonine has not been fully investigated (60). Whether cocaine undergoes hydrolysis when crossing biological membranes is not known.

Benzoylecgonine and ecgonine methylester have not been studied to determine their hydrolysis characteristics. Following the administration of benzoylecgonine to animals, ecgonine has been found in the urine (43). Ecgonine cannot be further hydrolyzed (61).

Some work has been reported on the hydrolysis of coca leaf material

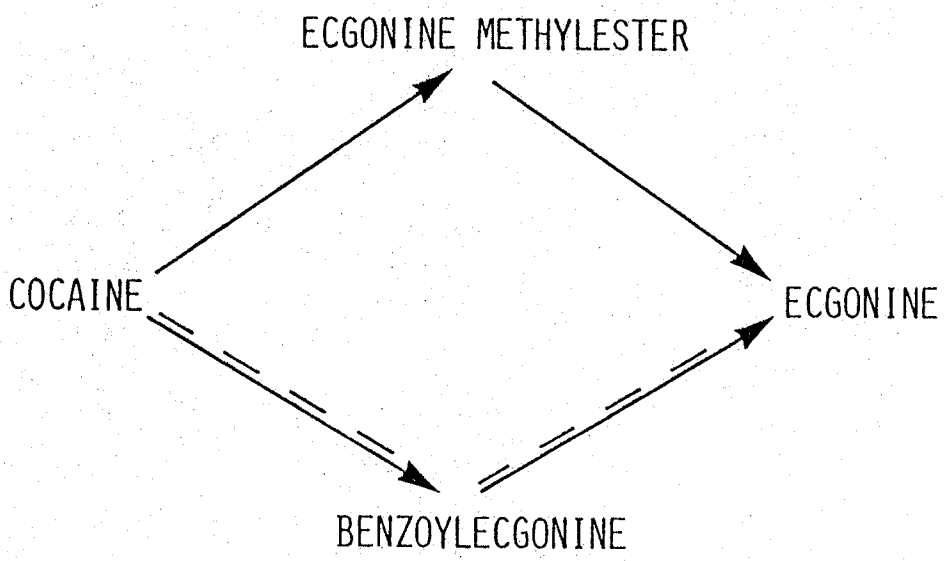


Figure 2. The accepted hydrolysis pathways of cocaine in enzyme —— and in non-enzyme --- systems.

in alkaline media (62). This work showed that the cocaine released from the leaf material is subject to hydrolysis. In spite of this, the age-old belief persists to this day that the alkaline materials added to the coca leaf during the chewing process serve only to aid in the extraction of cocaine (6).

The metabolism of cocaine is coming under intense investigation by many researchers. As more information is compiled it is obvious that the metabolism of cocaine is not simple (63). All known and proposed metabolites of cocaine are presented in the structural scheme of Figure 3 (63). Most data on cocaine metabolism has been obtained in animal studies. Similar research in man is confounded by strict governmental controls on cocaine and by the considerable risk of acute toxicity associated with its use. Because cocaine is a drug subject to abuse, most research in man is concerned with forensic matters, such as proof of cocaine use. Much scientific effort has been spent on providing incontrovertible evidence for the identification of cocaine metabolites in the urine of cocaine abusers. Such research has led to significant advances particularly in the area of analytical methodology (16-23).

Cocaine is metabolized primarily by ester hydrolysis and N-demethylation. There is some evidence for both ecgonine- and phenyl ring-hydroxylation(s) but the resulting molecular species have not been absolutely identified. The principal urinary metabolites of cocaine identified in animals are benzoylecgonine and ecgonine; very little unchanged cocaine is found in the urine. When tissues are examined, a variety of N-demethylated metabolites are found, particularly in the liver and brain (64). Although no hard evidence exists to suggest that the tropane ring

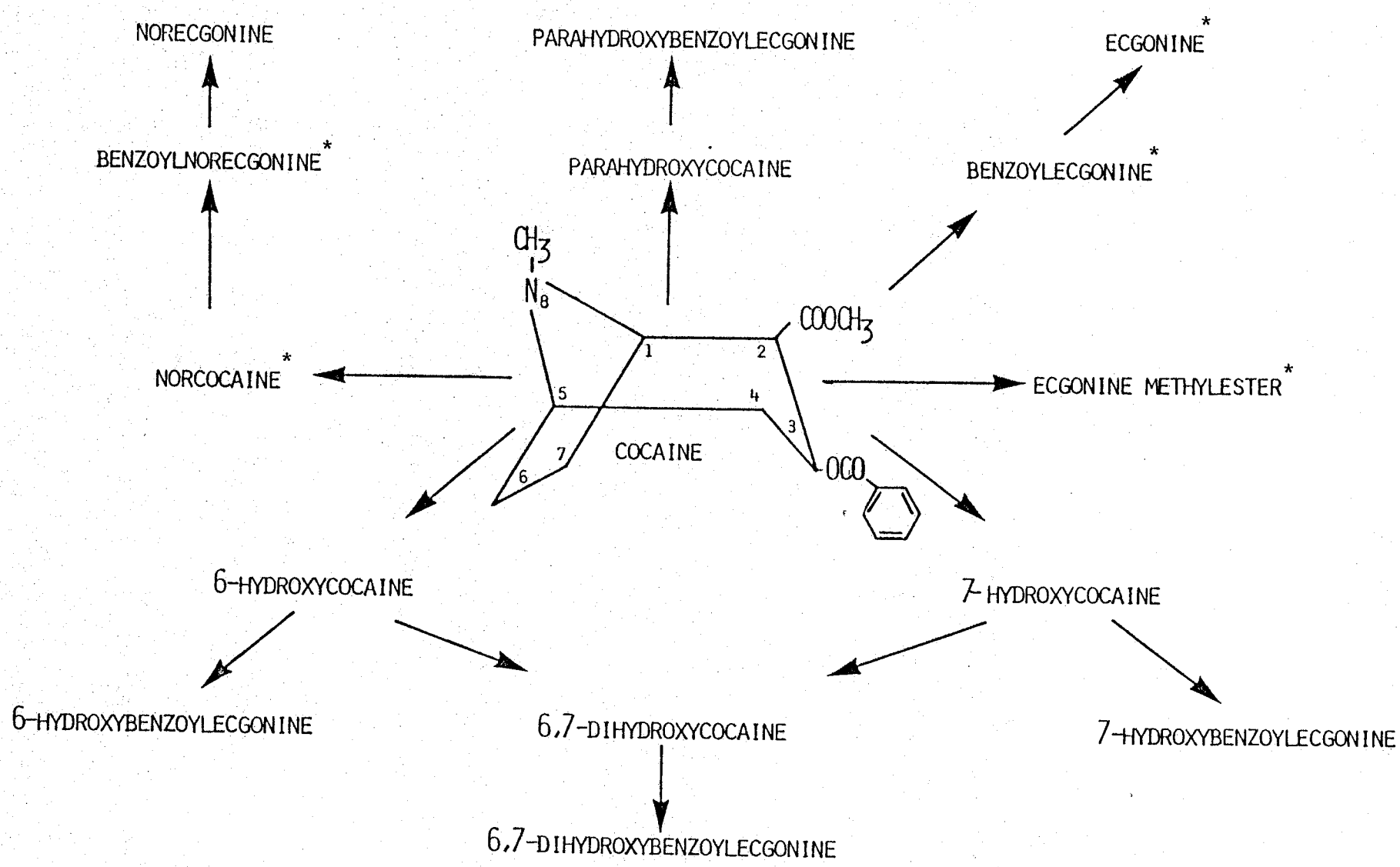


Figure 3. Scheme showing cocaine with known* and proposed metabolites.

structure of the ecgonine alkaloids undergoes radical transformation, this must be considered a possibility as studies involving well controlled procedures measuring total urinary and fecal metabolites could not account for all of the cocaine dose (65). The polar metabolites of cocaine, e.g., benzoylecgonine and ecgonine, do not undergo hepatic or other tissue N-demethylation prior to excretion. It has been found that the N-demethylating enzymes involved in cocaine metabolism are not inducible but they can be inhibited (66). In animal studies in which the N-demethylation was inhibited there was no evidence of increased central nervous system stimulation following cocaine administration indicating a possible role for cocaine metabolites. The ecgonine alkaloids that have been specifically investigated have plasma half-lives ranging from 0.5 to 1.5 hours (62, 63, 65). These alkaloids have different tissue distributions because of different polarities. Cocaine is rapidly absorbed following intranasal, oral, and topical administration (67-69).

It is clear that there is still much more to be studied. As is often the case with medicinal agents that have been in use for many years, the picture of cocaine is far from complete. It has been in use for thousands of years and yet new information continues to appear regularly regarding the unique chemical and biological properties of this alkaloid. As long as cocaine continues to be a drug of abuse and as long as the "coca problem" among Andean natives remains unsolved, cocaine will continue to demand attention. Both the medical community and governmental agencies are becoming increasingly aware of the desperate need for a full and valid appraisal of cocaine to help define its potential as a useful or detrimental chemical entity for society.

CHAPTER II RESEARCH OBJECTIVES

II - A General

Coca chewing, as practiced by the natives of the Andes, involves mastication of the coca leaves with added alkaline substances and swallowing the saliva-extract. Little is known of the chemical fate of the cocaine released from the bolus of chewed material. Cocaine is considered to be unstable in acid or alkaline solutions, and early work (12, 13, 45) suggests that the chemical hydrolysis of cocaine leads to the products benzoylecgonine and ecgonine. This work, however, was seriously hampered by the lack of specificity in the assay methodology used. Ecgonine methylester has been identified as a product of cocaine hydrolysis but only in enzyme systems involving cholinesterase (12). In the present research the hydrolysis of cocaine in various media *in vitro* will be investigated with particular attention to identifying the compounds produced. An attempt will also be made to characterize the kinetic behavior of the various products involved in the hydrolysis process. An in-depth study of the hydrolysis of cocaine *in vitro* should provide some insight into the fate of cocaine upon its being exposed to the various agents during the coca chewing process beginning with those in the bolus and continuing with those occurring throughout the gastrointestinal tract.

In considering the poor stability of cocaine in alkaline media, one could propose that the biological effector in coca chewing may not all be cocaine but possibly some hydrolysis product of cocaine, alone or together with minimal amounts of cocaine. The pharmacological effects of cocaine following parenteral administration are fairly well known

(28, 29, 44) but the effects following oral administration are only now coming under investigation (10, 34). One could certainly expect that the biological effects of orally administered cocaine or of cocaine exposed to alkaline media (as occurs in coca chewing) would be substantially different from effects arising from parenteral cocaine. The biological effects observed in coca chewing could well be mediated by one or more of the hydrolysis products of cocaine following absorption across the intestine.

In the past, various models have been proposed to explain the phenomenon of coca chewing. Traditional models depend on cocaine acting in a variety of ways. Some suggest that the Andeans chew coca to effect a drug induced escape from their menial existence, or that the coca enables them to cope with their harsh environment by dulling their sensibility to pain, hunger, and fatigue, or even somehow to improve their physical performance (2, 5, 70-72). In Bolton's model, it is proposed recognizing that the Andean native normally suffers from chronic hypoglycemia, that the cocaine obtained from coca chewing dulls his hunger pains and provides him with ready energy (73).

The most recent model, the ecgonine model proposed by Burchard (6), shifts the emphasis from cocaine to ecgonine as the possible mediator of the effects associated with coca chewing. According to Burchard, in spite of the native's dependence on a diet high in carbohydrate and low in fat and protein, ecgonine formed from cocaine through some process during coca chewing brings about an improved food energy utilization sufficient for the native to cope with his hostile environment. The underlying mechanism is not fully explained by Burchard except that ecgonine by virtue of certain structural similarities it shares with atropine, an

anticholinergic agent, stimulates carbohydrate metabolism in states of limited caloric intact (6).

It would seem that Burchard's ecgonine model has real merit. The concept that ecgonine may exert biological effects by virtue of its structural similarities to atropine has limited significance. Burchard's model, however, is successful in questioning the sole importance of cocaine as the mediator of the biological effects of coca chewing. As a background argument in support of this model, if all the cocaine contained in the coca leaf chewed in one day were absorbed unchanged, the coca chewer (because "reverse-tolerance" develops to certain non-central pharmacological effects of cocaine; elevation of blood pressure, increased heart rate, etc.) would eventually suffer acute toxicity. Also, there is no evidence for the development of addiction as a part of the coca chewing practice. Cocaine cannot be singly involved. All this suggests that cocaine has only a minor, if any at all, direct role in the biological effects of coca chewing.

Besides ecgonine, however, the other possible hydrolysis products of cocaine must also be taken into account in explaining the biological effects of coca chewing. It is difficult to conceive how ecgonine alone among the several hydrolytic products of cocaine, especially since its highly polar nature would argue against it, could elicit the effects originally attributed to cocaine. The present investigation has been undertaken primarily to evaluate in intact laboratory animals firstly the effects of cocaine and its hydrolytic products (administered individually as feed-adjuncts) on the relative utilization of dietary carbohydrates and fats, and secondly their effects on the intestinal absorption of carbohydrate substrate material.

II - B Ecgonine Alkaloids - Hydrolysis

As has already been explained, cocaine released during the coca chewing process runs a gauntlet of chemical agents, each of which is capable of causing hydrolytic cleavage of the cocaine molecule: the added alkaline substance (ishku or llipta), salivary enzymes, stomach acid and pepsin, and intestinal buffer-fluid enzymes. The hydrolysis profiles in vitro of cocaine, ecgonine methylester and benzoylecgonine will be evaluated in a variety of acid and alkaline media designed to mimic the in vivo condition. The hydrolysis will be carried out at 37°C.

Inasmuch as hydrolysis of the ester function is a highly time-dependent process, the kinetics of the hydrolysis of each of the several alkaloids will be elaborated. Kinetic models will be developed, and the applicability of a single general model will be tested. Of particular interest is whether or not the hydrolysis of cocaine can be represented by the sum of the two first-order ester-hydrolysis reactions. Wherever possible, theoretical hydrolysis profiles based on theoretical equations using the best estimates of the reaction rate constants will be fitted to the experimentally derived hydrolysis data.

Since it would be of tremendous interest to know whether any of the alkaloids chemically derived from cocaine through hydrolysis could be formed in the intact animal, a preliminary study will be conducted in the rat to determine which of the ecgonine alkaloids appears in the urine following the administration of cocaine. Both oral and subcutaneous administration will be tried. Particular interest will be not only in whether cocaine, ecgonine methylester, benzoylecgonine, or ecgonine will be found in the urine but also in what relative amounts.

Available extraction-assay methodology will not be directly applicable for the determination of the several alkaloids expected as products in the hydrolysis reaction and as metabolites in the urine of the cocaine-treated rat. The gas chromatographic procedure developed by Moore (74) with the capability of determining cocaine, benzoylecgonine, and ecgonine in single samples of cocaine bulk drug material will be refined for the simultaneous determination of these three alkaloids together with ecgonine methylester.

II - C Ecgonine Alkaloids - Biological Effects

1. Energy Metabolism

In the harsh Andean environment, the coca chewer must sustain himself on a diet high in carbohydrate and low in both fat and protein (75, 76). It is not clear what the overall picture of energy metabolism is in such a state of food deprivation nor how metabolic homeostasis is preserved. In some as yet unexplained way coca seems to provide protection against the obviously inadequate diet. Once each of the intermediates and/or products of cocaine hydrolysis has been identified by the procedures outlined in the hydrolysis study, evaluation of the nutritional effects can be carried out. Accordingly, a comprehensive study will be carried out in the rat of the effects produced by cocaine and each of its possible hydrolysis products on energy metabolism, more specifically upon the relative utilization of carbohydrate and fat as metabolic substrates. Overall metabolic effects in the intact animal can be best and most easily assessed in terms of oxygen consumption and respiratory quotient. Respiratory quotient values become elevated with increased utilization of carbohydrate, and become depressed with increased

utilization of fat (77-79). For the purpose of this research, measurements of oxygen consumption and carbon dioxide production need not be basal but will be standardized and taken without bias in animals with respect to time and manner of treatment.

Growing rats (100-150 g) will be maintained on specially prepared semi-synthetic diets high in carbohydrates and low in both fat and protein, but adequate in all other nutrients. Since coca chewing is practiced just prior to, during, and just after meals, it will be appropriate to administer the test alkaloids to the rats on their daily diet (6). Paired-feeding of the control rats according to the food intake of the treated rats will be followed to make allowance for possible changes in the amount of food consumed, brought about by the added alkaloid. Measurements of weight changes, food intake, and respiratory gas exchange will be taken daily for each test animal.

2. Intestinal Carbohydrate Absorption

The intestinal absorptive mechanisms of the coca chewer are subjected to extremely high carbohydrate loads because of high carbohydrate diets. Little is known of whether coca chewing helps in the transport of carbohydrate substrate across the intestine. A study will be performed in rats to investigate the effects of selected ecgonine alkaloids upon the intestinal absorption of carbohydrates.

The xylose absorption test will be employed. It is doubly suited for this study in that it is a well established test-indicator of the intactness of intestinal absorption (80), and it uses xylose as the test substance which has been shown to share with glucose the same transport mechanisms in the intestine (81). The latter characteristic enables

xylose absorption test results to be interpreted specifically as a measure of carbohydrate absorption. This is particularly advantageous because of the need to evaluate how the intestine handles high loads of carbohydrate in the diet.

3. Mouse Whole Body Composition

Measurement of respiratory quotients is a reliable, direct method for making an overall assessment of the relative utilization of carbohydrates and fats as substrates for energy metabolism. Alone, however, respiratory quotient estimates can only be used to detect metabolic shifts from one substrate to another. In circumstances wherein nitrogen balance is maintained, changes in respiratory quotients indicate the direction in which metabolism is proceeding: that is, toward more or less carbohydrate metabolism than fat metabolism. But changes in respiratory quotients offer little information as to how the switch comes about, or what changes in the composition of body tissues might accompany such changes. Direct examination of the levels of carbohydrate, fat and protein in the body would help pinpoint the substrate as energy source.

In the present research, the energy metabolism studies will be supplemented by a separate study of the possible effects of selected ecgonine alkaloids on the whole body composition. The laboratory mouse will be chosen as the test animal. The small size of the mouse will permit circumventing some rather difficult technical problems associated with the required homogenization procedure. The basic methodology to be followed for determining whole body composition will consist of whole mouse homogenization in the ultrasonic/grinding apparatus, aliquot sampling of the homogenate, and assay of the aliquots for carbohydrate (as glucose

and glycogen), fat (as triglycerides), and nucleic acid-protein (as total nitrogen). The total body nitrogen value will be important to correct the values of the other components for possible differences in mouse body weights.

Significantly, the approach presently described for determining mouse whole body composition is a novel one. Little data are available on the principal individual body components of the mouse. Relevant literature as a whole originates in nutrition-oriented research in which gross estimates are made instead; fat is determined in terms of total ether-extractable fat and carbohydrate simply by difference, i.e., as what is not accountable as fat, protein, mineral, and water. Protein, in terms of total nitrogen, is usually the only accurately determined component.

CHAPTER III EXPERIMENTAL

III - A Ecgonine Alkaloid Assay

1. Materials and Equipment

Cocaine hydrochloride (Lot #81046/4801) was obtained from Allen Hanbury Chemicals, Toronto, Ontario, Canada and samples removed for use were stored under vacuum over phosphorus pentoxide. N, O-bistrimethylsilylacetamide (BSA) and Silyl-8 column conditioning agent were purchased from Pierce Chemical Company, Rockford, Illinois, USA and stored at 4°C. Gas chromatographic column packing materials were purchased from Chromatographic Specialties Ltd., Brockville, Ontario Canada. Chloroform was distilled fresh prior to use. All other chemicals and solvents were reagent grade and were used without further purification. All aqueous solutions were prepared in doubly glass-distilled water.

Gas chromatographic analyses were performed in a Hewlett-Packard Model HP 5711 gas chromatograph (GC) equipped with flame ionization detectors and a temperature programmable oven. Two-meter long by 2 mm internal diameter glass columns were used in the GC.

2. Methods

Ecgonine methylester hydrochloride, benzoylecgonine, and ecgonine hydrochloride were synthesized according to established procedures (90, 91) and stored until use under vacuum over phosphorus pentoxide. The melting point of each of the alkaloids was determined and compared to its literature value. The Rf values obtained for the alkaloids were verified in established thin layer chromatographic systems.

The GC method employed was a modification of Moore's GC method (74). The glass columns used in the GC were pre-rinsed with BSA and then packed

with the coated solid support materials; minimal amounts of silanized glass wool were used to hold the packings in place (82). For the hydrolysis studies Gas Chrome Q was used as the solid support while Chromosorb W-HP was employed for the urinary excretion study. Both solid supports were 80-100 mesh and were coated with 10% OV-101 by the solvent evaporation technique. The packed columns were conditioned and maintained with periodic 50 µl injections of Silyl-8. Although the Chromosorb W-HP solid support was also tried in the urinary excretion study in an effort to obtain cleaner chromatograms, the performance of the two columns in terms of resolution, response, and retention times was identical for the alkaloids and the internal standards.

The operating conditions of the GC included gas flow rates of nitrogen carrier gas at 40 ml/min with air and hydrogen optimized at 240 and 30 ml/min, respectively. The injector port and detector block temperatures were both maintained at 300°C and the oven temperature programmed from 170°C to 250°C at 16°/min. The attenuator settings were 1.6×10^{-9} and 4×10^{-10} for the hydrolysis and the urinary excretion study, respectively.

For the assay procedure in the hydrolysis study, a methanolic solution containing cocaine HCl, benzoylecgonine, ecgonine methylester HCl, and ecgonine HCl was prepared each at a concentration of 0.5 µg/µl calculated as the free base. A separate methanolic solution of theophylline at 0.75 µg/µl was prepared for use as the internal standard. When not in use these solutions were stored at 4°C. To elaborate the standard assay curve 100 µl of 0.6 N HCl in 2-propanol was added to each of six separate 15 ml assay tubes equipped with Teflon-lined screw caps. Ten, 25, 50,

100, and 200 µl of the stock alkaloid standard solution were added to five of the tubes. To the sixth tube 200 µl of methanol were added; this sample was used as the reagent blank. To each of the tubes 100 µl of the theophylline internal standard solution (0.75 µg/µl) were added. Each tube was taken to dryness at 50°C under a stream of nitrogen and the residues obtained taken up in 50 µl of BSA. The tubes were tightly capped, mixed and heated in a water bath at 70°C for 10 minutes. Following centrifugation and vortexing of the tube contents 1-2 µl of the BSA solution were chromatographed. Standard assay curves were drawn for cocaine, benzoylecgonine, ecgonine methylester, and ecgonine by plotting the peak height ratios of the individual cocaine[a], benzoylecgonine-trimethylsilyl (-TMS), ecgonine methylester-TMS, and ecgonine-TMS peaks to the theophylline-TMS peaks versus the original amount of the corresponding alkaloid in the sample tubes, namely: 5, 12.5, 25, 50, and 100 µg.

The procedure followed for the extraction of alkaloids in the hydrolysis studies was a modification of the method of Misra, Nayak, Bloch and Mule (44). To 250 µl of the hydrolysis sample in a 15 ml tube, 5 ml of chloroform/2-propanol 2:1 were added, followed by 250 mg of anhydrous potassium carbonate. The tube was vigorously hand shaken 15 times and then shaken for 3 minutes on the flat-bed shaker. The tube was centrifuged to separate the contents into two phases. Four milliliters of the upper organic phase were transferred to a fresh tube containing 100 µl of 0.6 N HCl in 2-propanol. Following the addition of 100 µl of the theophylline internal standard solution (0.75 µg/µl) the tube contents

---

[a]Cocaine is unreactive to BSA

were taken almost to dryness at 50°C under nitrogen. The sides of the tube were washed down with 1 ml of the extracting solvent and the tube was taken to dryness. Following dissolution of the residue obtained in 50 µl of BSA, the samples were processed in the GC in the same fashion as were the standards. Recoveries of each alkaloid in 250 µl aqueous samples containing alkaloids at 20, 50, 100, 200, and 400 µg/ml concentrations were determined. From these recoveries working standard extraction-assay curves were elaborated for use in the assay of samples obtained in the hydrolysis studies.

Separate standard assay curves for the alkaloids were employed for the urinary excretion study. A methanolic solution of the four alkaloids together was prepared at a concentration of 0.2 µg/µl of each alkaloid calculated as the free base. The internal standard was caffeine prepared in methanol at a concentration of 0.15 µg/µl. To establish standard assay curves for cocaine, benzoylecgonine, ecgonine methylester, and ecgonine 100 µl of 0.6 N HCl in 2-propanol were added to five separate sample tubes. Ten, 25, 50, 100, and 200 µl of the stock alkaloid standard solution (0.2 µg/µl) were added to the tubes. To each of the tubes were added 100 µl of the caffeine internal standard solution (0.15 µg/µl) and the tube contents were evaporated to dryness at 50°C under nitrogen. The residue obtained was taken up in 50 µl of chloroform/methanol 1:1. The tube contents were mixed and 1-2 µl of the solution was analyzed in the GC. Upon injection of these underivatized samples the cocaine, ecgonine methylester, and caffeine will separate on the column. Standard assay curves for cocaine and ecgonine methylester were drawn by plotting the peak height ratios of the cocaine and ecgonine methylester to the caffeine

peaks versus the original amount of each alkaloid in the sample tubes.

The final solutions remaining from the cocaine and ecgonine methylester GC analysis were evaporated to dryness as described above. The residues obtained were taken up in 50 µl of BSA. The tube was tightly capped, mixed, and heated at 70°C for 10 minutes. Following centrifugation and mixing of the tube contents, 1-2 µl of the BSA solution were chromatographed on the GC column which had been pre-flushed with 25 µl injection of BSA. Standard assay curves for benzoylecgonine and ecgonine were established by plotting the peak height ratios of the benzoylecgonine-TMS and ecgonine-TMS peaks to the caffeine peaks versus the original amount of the alkaloids in the sample tubes.

The extraction-assay procedure for the alkaloids in the urine was quite involved. For the extraction of cocaine, benzoylecgonine, and ecgonine methylester from rat urine, 5 ml of chloroform were added to 1 ml of rat urine in a 15 ml tube followed by the addition of 50 µl of a saturated solution of potassium carbonate. The tube was capped and the contents vigorously hand shaken 15 times and shaken for 3 minutes on the flat-bed shaker and centrifuged as described previously. The upper aqueous layer was aspirated off and discarded; 4 milliters of the lower organic layer were transferred to a fresh tube containing 100 µl of 0.6 N HCl in 2-propanol. To each tube 100 µl of the caffeine internal standard solution (0.15 µg/µl) were added and the tube contents were taken almost to dryness at 50°C under nitrogen. The sides of the tube were washed down with 1 ml of chloroform and the tube contents were evaporated to dryness. The residue obtained was taken up in 50 µl of chloroform/methanol 1:1; the resulting solution was mixed and 1-2 µl were

chromatographed for the determination of cocaine and ecgonine methylester. The final solution remaining from the cocaine and ecgonine methylester GC analysis was evaporated to dryness and the residue obtained taken up in 50 µl of BSA. The tube was capped, mixed, and heated at 70°C for 10 minutes. Following centrifugation and mixing of the tube contents, 1-2 µl of the BSA solution were chromatographed in the GC column which had been pre-flushed with a 25 µl injection of BSA for the determination of benzoylecgonine.

The extraction of ecgonine from rat urine was performed on a separate 1 ml rat urine sample. To the 1 ml rat urine sample in a 15 ml tube, 5 ml of chloroform/2-propanol 2:1 were added followed by the addition of 1.2 g of anhydrous potassium carbonate. The tubes were capped, shaken, and centrifuged as previously described. Four milliters of the upper organic layer were transferred to a fresh tube containing 100 µl of 0.6 N HCl in 2-propanol and the tube contents were evaporated almost to dryness. The sides of the tube were washed down with 1 ml of extracting solvent and the contents evaporated to dryness. The residue obtained was taken up in 50 µl of methanol/water 1:1 and the solution obtained was mixed and streaked in its entirety onto thin layer chromatography plates coated with silica gel G. The plates were developed in system 'N' of Noirfalise and Mees (83) and the silica gel with the band corresponding to ecgonine was scraped into a fresh tube and extracted with 5 ml of methanol by shaking for 5 minutes on the flat-bed shaker. Following centrifugation of the silica gel-extract suspension, four milliters of the methanol supernatant were transferred to a fresh tube containing 100 µl of 0.6 N HCl in 2-propanol. To the tube 100 µl of the caffeine internal standard

solution (0.15 µg/µl) were added and the contents of the tube taken almost to dryness, the sides of the tube washed down with 1 ml of methanol, and the contents evaporated to dryness. The residue obtained was taken up in 50 µl of BSA, the tube tightly capped, mixed, and heated at 70°C for 10 minutes. Following centrifugation and mixing of the tube contents, 1-2 µl of the BSA solution was chromatographed for the determination of ecgonine.

Recoveries of each alkaloid from blank rat urine spiked with each alkaloid at concentrations of 2, 5, 10, and 20 µg/ml were determined. From these recoveries working standard extraction-assay curves were elaborated for use in the assay of rat urine samples obtained in the urinary excretion study.

III - B Ecgonine Alkaloid Hydrolysis

1. Materials and Equipment

All chemicals and solvents used were as previously described in the assay section. The tubes used for the hydrolysis were 15 ml round bottom Pyrex^{R} tubes equipped with Teflon-lined screw caps. A Thermolyne temperature controlled water bath set at 37°C was used.

2. Methods

The concentration range of the ecgonine alkaloids in the hydrolysis study was chosen to duplicate as closely as possible the concentration that would occur in the gastric fluid if 10-30 g of coca leaves were chewed and all the extractable alkaloid content was swallowed. Gastric fluid volume usually ranges between 50 and 100 ml (84). If all of the alkaloids from the leaf material were released during the chewing process the resulting total alkaloid concentration would be 2 to 3 mg/ml, or in

terms of the individual ecgonine alkaloid possible present, roughly 500 µg/ml. Also, because coca chewing is practiced at or near mealtime when gastric fluid volume would be somewhat increased, the concentration of each alkaloid chosen for each hydrolysis test was somewhat reduced to 400 µg/ml.

Cocaine HCl, benzoylecgonine, and ecgonine methylester HCl solutions were prepared separately in methanol at a concentration of 10 µg/µl. To a separate tube, 400 µl of the methanolic solution of the alkaloid under study was added and the solution evaporated to dryness. To the alkaloid residue 10 ml of the hydrolysis medium under investigation, warmed to 37°C in the water bath were added. The tube was inverted several times and placed in the water bath at 37°C. At timed intervals, the tube was removed, inverted twice, and a 250 µl sample was taken and placed in a fresh 15 ml tube and immediately processed through the hydrolysis study extraction and assay procedure. The addition of the organic solvent and potassium carbonate terminated the hydrolysis. Each hydrolysis sample was run in duplicate simultaneously.

The hydrolysis media studied included distilled water, 0.1 N HCl, simulated gastric fluid USP, pH 7.3 phosphate buffer, simulated intestinal fluid USP, pH 10 phosphate buffer, pH 11 glycine buffer, 0.01 N and 0.04 N solutions of NaOH and $Ca(OH)_2$, the supernatants from 4 mg/ml aqueous dispersions of ishku and llipta substances both prepared at 37°C. Determinations were made of some of the constituents and pH values of these supernatant ishku and llipta solutions.

At each sampling time the amounts of each of the alkaloids found present in the hydrolysis medium were calculated as the percentage of

the total micromolar (μM) composition of ecgonine alkaloids in the hydrolysis solution.

A kinetic model was elaborated to describe the hydrolysis concentration-time profile for each alkaloid. Integrated equations were derived using the technique of Laplace Transforms from the differential equations describing the rate of hydrolysis of the several components in the kinetic model. To obtain estimates of the first-order formation rate constants for benzoylecgonine and ecgonine methylester a unique procedure was devised whereby the overall first-order disappearance rate constant of cocaine was partitioned between the two formation rate constants by an iteration process on the basis of the peak ratios obtained experimentally between benzoylecgonine and ecgonine methylester. The integrated equations were employed to elaborate theoretical concentration-time profiles for each of the components in the hydrolysis model which were then fitted to the experimental concentration-time data.

III - C Ecgonine Alkaloid Urinary Excretion

1. Materials and Equipment

All chemicals and solvents used were as previously described. One large male Sprague Dawley rat (ca. 600 g) maintained on regular Purina Rat Chow^{R} was used for this preliminary study. A stainless steel metabolic cage equipped for the separation of urine and feces was employed.

2. Methods

A stock solution of cocaine HCl in normal saline was prepared at a concentration of 10 mg/ml calculated as the free base and stored before use at 4°C. Following an overnight fast the rat was dosed intragastrically at 20 mg/kg with the cocaine solution. One week later, again following an

overnight fast, the rat was dosed subcutaneously at 10 mg/kg with the same cocaine stock solution. In both experiments the rat was placed in the metabolic cage, and urine was collected for the next 24 hours in a 25 ml graduated cylinder containing 1 ml of 0.1 N HCl. Each collection was split into 2.5 ml fractions and kept frozen at -40°C until assayed. The several urine samples were assayed for ecgonine alkaloids by the urine extraction and assay procedure.

III - D Rat Energy Metabolism

1. Materials and Equipment

The ecgonine alkaloids studied were those previously described. Semi-synthetic diet components including vitamin-free casein, D(+) crystalline sucrose, dextrin (white, technical), vitamin diet fortification mixture, Alphacel, and Jones-Foster biological salt mixture were purchased from ICN Pharmaceuticals Inc., Cleveland, Ohio, USA. Mazola Corn Oil was used as the dietary fat component. A Hobart mixer was employed for the mixing of the semi-synthetic diets. The regular rat diet was Purina Laboratory Chow^{R} with a labelled percent caloric composition of 24% protein and 9% fat. Male Sprague Dawley rats, 100-140 g, were used. Compressed air was hospital grade and was used as purchased without further treatment. Oxygen and carbon dioxide analyses were performed in a Micro-Scholander Gas Analyzer manuractured by Phipps and Bird Co., Richmond, Virginia, USA.

The metabolic chamber used for the energy metabolism measurements is presented in Figure 4. It was constructed from a glass desiccator of about 2500 ml capacity. The lid of the desiccator was modified to incorporate a 5 mm inlet hose nipple and also a combination 5 mm outlet hose

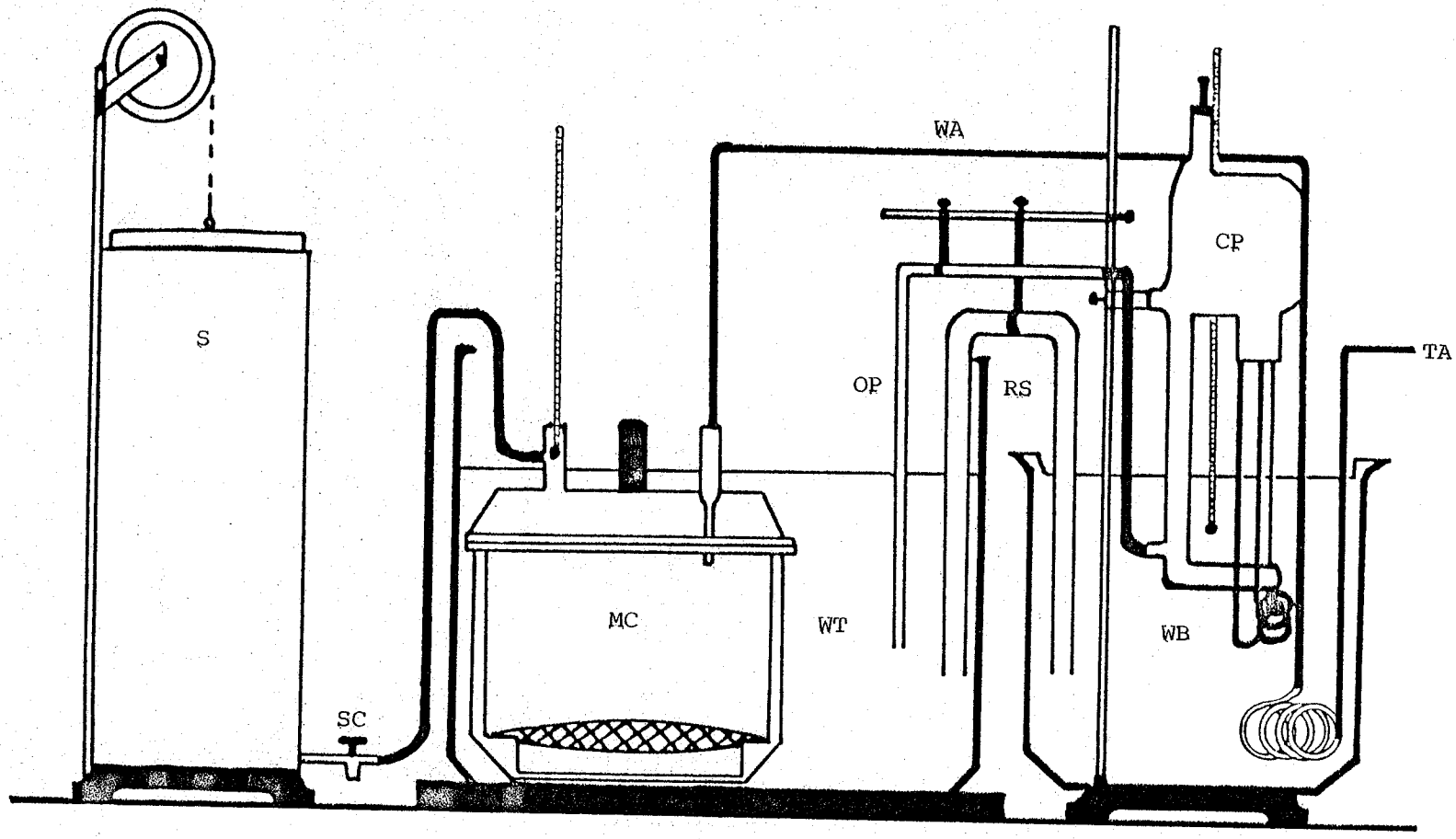


Figure 4. Diagramatic representation of the apparatus employed for the energy metabolism measurements in rats. Key: S = spirometer, SC = 3-way stopcock, MC = metabolic chamber, WT = large cylinder of water, OP = heated water outlet from water bath, WA = heated air from water bath, RS = return siphon to water bath, CP = heating/ circulating water pump, WB = water bath assembly, TA = from tank air supply.

nipple ground-glass thermometer fitting. The floor of the chamber was made from aluminum screening material and was positioned over a 3500 g lead weight at the bottom of the desiccator. Water proof grease was used as a seal between the lid and bottom of the desiccator. The lid was held in place with two spring clamps. Air was supplied to the chamber from a tank of compressed air at a rate of 250 ml/min set with a regulator-needle valve arrangement at the air tank. The rate was checked at both the inlet and outlet ports of the chamber with a bubble meter. The air was warmed prior to entering the chamber by passage through a glass coil immersed in a 30°C water bath. The outlet air from the metabolic chamber was collected in a 6 liter spirometer. The stainless-steel bell of the spirometer was weight suspended in the spirometer cylinder. The water jacket around the spirometer was covered with a 1 cm layer of heavy liquid petrolatum. To make measurements on a rat, the animal was sealed in the metabolic chamber, and the whole unit lowered into the large water bath. The water was circulated between the large cylinder and the 30°C water bath by means of a temperature controlled circulating heating pump and a large capacity return siphon. The large water bath was positioned on a 5 cm thickness of woven fiber pad. This arrangement minimized noise and vibration in the chamber.

2. Methods

Semi-synthetic diets were prepared according to the formulations presented in Table 1. The sucrose supplied was in granular form and was powdered with a hand grinder prior to formulation. The Purina Laboratory Chow Pellets were also powdered in this manner. The diet mix incorporating the test alkaloids was prepared with a mortar and pestle in 200 g

Table 1. Compositions of low and high protein semi-synthetic diets.

INGREDIENT	LOW PROTEIN			HIGH PROTEIN		
	GRAMS	KCAL	% TOTAL KCAL	GRAMS	KCAL	% TOTAL KCAL
CASEIN	86	390	10	212	950	25
DEXTRIN	624	2500	65	521	2080	53
SUCROSE	159	590	16	136	500	13
CORN OIL	31	270	7	31	270	7
VITAMINS	20	70	2	20	70	2
MINERALS	50	-	-	50	-	-
CELLULOSE	30	-	-	30	-	-
TOTAL	1000	3820	100	1000	3870	100

amounts at 1 mg/g of test alkaloid. The bulk semi-synthetic diets were prepared in 2500 g amounts with the Hobart mixer. Mixtures composed of both the semi-synthetic and powdered Purina diets were also prepared at percent compositions of 25%, 50%, and 75% of semi-synthetic diet. The semi-synthetic/Purina diet mixtures were employed to phase the rats from 100% Purina pellets to 100% semi-synthetic over a 5-day period. Prior to use all diet mixtures were stored at 4°C.

Separate trials of 12 rats each (6 control and 6 treated) were completed with the following:

1. Cocaine/Low protein semi-synthetic diet,
2. Ecgonine methylester/Low protein semi-synthetic diet,
3. Benzoylecgonine/Low protein semi-synthetic diet,
4. Ecgonine/Low protein semi-synthetid diet,
5. Cocaine/High protein semi-synthetic diet.

The rats for each trial in the metabolic study were brought into the laboratory in groups of 12 rats each at a weight of 120 ± 10 g. They were individually caged in a temperature-light controlled room. Fresh water was provided at all times throughout the study. Purina pellets were offered to the rats for the first 24-hour period. Subsequently, they were offered food in weighed amounts of 25 g, in powdered form, beginning with the 100% powdered Purina pellets on Day 1, then the 25%, 50%, and 75% semi-synthetic/powdered Purina mixtures on Day 2, Day 3, and Day 4, respectively. Finally, on Day 5 the rats were fed the 100% semi-synthetic diet for 3 days. During this period the rats were allowed to eat ad libitum. After the third day of 100% semi-synthetic diet, the rats were weight-paired into two groups of six rats each. The groups

were randomly assigned by a coin-toss to either the control or treated group of the trial. The treated group continued to receive the semi-synthetic diet ad libitum, now spiked with test alkaloid at 1 mg/g. The control group was pair-fed with 100% semi-synthetic diet to match the food consumption of their weight-mates in the treated group. All rats were weighed, fed, and freshly watered prior to 9:00 AM on each day of the trial.

On each day, beginning with the end of the first 24-hour period on the 100% semi-synthetic diet (Day 5), metabolic measurements were made on each rat in the trial always at the same clock-time between 9:00 AM and 4:00 PM. The rat was placed in the metabolic chamber and the chamber was sealed, clamped, and lowered into the large water-bath. A 10-minute period was permitted for the animal to acclimatize and the temperature to reach equilibrium at 30°C. A 3-way stopcock valve was opened to allow the collection of all outlet air from the metabolic chamber into the spirometer. The collection period was 15 minutes long as timed by a stopwatch, which resulted in collecting 3700 to 3800 ml of outlet air. At the end of the 15-minute collection, the 3-way stopcock valve was positioned to allow for containment of the collected outlet air in the spirometer while allowing the continued flow of outlet air from the metabolic chamber to exhaust into the room air. The volume of air collected in the spirometer was recorded and the rat was removed from the metabolic chamber. The chamber was cleaned and the next rat was placed in the chamber and readied for measurement. The order of the rat measurements was randomly assigned on the first day and this order was followed subsequently throughout the trial. While the next rat was

acclimatizing, the sampling valve of the spirometer was opened and the first liter of collected air from the previous rat was allowed to escape; by means of a Bailey bottle the next 30 to 40 ml of the collected air was sampled. The sampled air was analyzed for oxygen and carbon dioxide content by the micro Scholander method (85). At the beginning of each day of metabolic measurements, the entire procedure was run without a rat; this served as the baseline measurement of the oxygen and carbon dioxide content of the tank air. The oxygen consumption, carbon dioxide production, and respiratory quotient of each animal was calculated each day of the metabolic test period.

III - E Xylose Absorption in Rats

1. Materials and Equipment

The ecgonine alkaloids used were the same as those previously specified. D(+) xylose (Lot #722505) was obtained from Fisher Scientific Co. Ltd., Winnipeg, Manitoba, Canada. All other chemicals and solvents were reagent grade and were used as purchased without further purification. The rats used were male, Sprague Dawley rats weighing between 100 and 200 g. All absorbance measurements of solutions were made with a Bausch and Lomb Spectronic 20 Spectrophotometer. The solutions for absorbance measurements were prepared in specially selected Bausch and Lomb Spectronic 20 test-tubes.

2. Methods

All rats in the study received the xylose test solutions intragastrically. The xylose test solution was prepared by dissolving D(+) xylose in water to make a solution of 100 mg/ml xylose such that a dose of 5 ml/kg provided a xylose dose of 500 mg/kg. The xylose test solutions containing

additional materials, i.e., alkaloids, for simultaneous administration were prepared as follows:

1. Xylose test solutions with cocaine HCl, benzoylecgonine, ecgonine methylester HCl, or ecgonine HCl were prepared in water such that a dose of 5 ml/kg of the solution provided 500 mg/kg xylose and 10 mg/kg of the alkaloid calculated as the free base,
2. Xylose test solution with atropine sulfate was prepared in water such that a dose of 5 ml/kg provided 500 mg/kg xylose and 0.15 mg/kg atropine calculated as the free base,
3. Double-strength xylose test solution was prepared in water such that a dose of 5 ml/kg provided 1000 mg/kg xylose.

The three trials of the xylose study were:

1. Four pairs of rats were randomly selected from each of the trials of the metabolic study. After Day 17 of the metabolic trial the rats, following an 18-hour fast, received the xylose test solution without additional materials at a dose of 5 ml/kg intragastrically. Each rat was placed in a metabolic cage and urine was collected for the next five hours,
2. Fourteen rats within a narrow weight range were brought into the laboratory and individually caged in the temperature-light controlled room. The rats were provided with fresh water daily and phased

into the 100% semi-synthetic low protein diet as previously described. After the second day of receiving the 100% semi-synthetic diet, the rats were deprived of food for 18 hours and then grouped into seven pairs. The pairs were randomly assigned to the control or 1 of 6 treated groups. The control pair was processed as in xylose absorption trial 1. Each treated pair received 1 of the 6 xylose test solutions which contained additional materials for simultaneous administration (cocaine, benzoylecgonine, ecgonine methylester, ecgonine, atropine, or double-strength xylose). Following the intragastric administration of the respective solutions, the 7 pairs of rats were processed as in xylose absorption trial 1.

3. Twenty-four rats were brought into the laboratory and handled similarly to those in xylose absorption trial 2 up to the stage of pairing. In this trial the 24 rats were weight-grouped into 8 groups of 3 rats each. In each group the individual rats were randomly assigned either to be a control or to receive 1 of 2 different treatments. One treatment was the xylose test solution with cocaine and the other treatment was the xylose test solution with ecgonine. The control group received the xylose test solution without additional materials. Following the intragastric administration of the test solutions the rats were processed as in xylose absorption

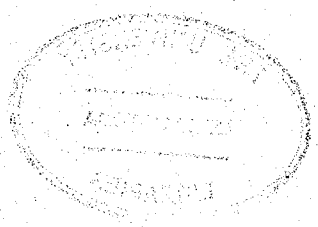
trial 1.

In each trial the 5-hour collections of rat urine were diluted to 250 ml before being assayed for xylose.

Xylose determinations were made using the method of Roe and Rice (86) as modified for urinary samples (87). Xylose standards were prepared in saturated solutions of benozic acid at D(+) xylose concentrations of 0.1, 0.2, and 0.3 mg/ml. These solutions before use were stored at 4°C. Para-bromoaniline reagent was prepared fresh daily by dissolving 2 g of para-bromoaniline in 100 ml of a saturated solution of thiourea in glacial acetic acid. Diluted urine samples and standards were run in duplicate together with a duplicate set of reagent blanks. Duplicate samples of 0.5 ml of the diluted rat-urines and 0.5 ml of the xylose standards were pipetted into separate tubes. To each tube 2.5 ml of the para-bromoaniline reagent was added. The tubes were mixed and one set of urine samples and standards along with a reagent control was placed in the dark and the other set was placed in a water bath at 70°C for 10 minutes. The heated samples were cooled to room temperature with the aid of an ice bath and placed in the dark with the blanks for an additional 60 minutes. All the tubes were then mixed and the absorbance of the solutions read at 520 nm in the Spectrophotometer. A standard assay curve was established each day for the xylose standards from which the xylose concentrations of the diluted rat urine samples was calculated. Finally, the percent of the total administered dose of xylose excreted in the urine in the 5-hour collection period was calculated for each rat.

III - F Mouse Whole Body Composition

1. Materials and Equipment



All solvents and chemicals were reagent grade and were used without further purification. The ecgonine alkaloids were as previously described. The mice were male, albino mice obtained at 12 to 20 g body weight. A Polytron homogenizer was employed for homogenizing whole body tissue components. The Spectronic 20 spectrophotometer was used for all absorbance measurements. The Micro-Kjeldahl digestion-distillation apparatus was used for nitrogen determinations.

2. Methods

Three trials were planned in this study, one a procedural control to test the assay methodology and the others a cocaine and an ecgonine methylester trial:

1. Twelve mice were brought into the laboratory and housed together in a common plastic cage in the temperature-light controlled room. These mice were provided free access to water and Purina Laboratory Chow pellets. After two days these mice were killed and processed as a procedural control for the study.
2. Twelve mice were brought into the laboratory and individually housed in separate plastic cages in the temperature-light controlled room. These mice while having free access to water were phased into the low protein semi-synthetic diet as in the rat metabolic studies. After the third day of receiving the 100% semi-synthetic diet the mice were weight-paired into two groups of six mice each: one group was chosen randomly as the control group and the

other as the treated group. The mice in the treated group continued receiving the 100% low protein semi-synthetic diet ad libitum now spiked at 1 mg/g with ecgonine methylester. The mice in the control group were pair-fed with 100% semi-synthetic diet to match the food consumption of their weight-mates in the treated group. All mice in the trial were weighed, fed, and provided with fresh water daily throughout the experiment. After 10 days of test period all mice remaining were killed and processed as the ecgonine methylester trial.

3. Twelve mice were brought into the laboratory and handled in an identical fashion to those in whole body composition trial 2 but with cocaine as the test alkaloid in this third trial.

At the end of each trial when the mice were killed, each mouse was placed in a separate aluminum foil envelope and kept frozen at -40°C. At the time of analysis the frozen mouse was broken into four or five pieces and placed in a 125 ml Erlenmeyer flask and cold water was added to about 75 ml. The contents of the flask while kept cold in an ice-water slurry were homogenized with the Polytron homogenizer 30 seconds beyond the time when mouse pieces were no longer visible. The homogenate was poured into a 250 ml graduated cylinder kept in an ice-water slurry. The homogenate was brought to a final volume of 250 ml with cold water washings of the Erlenmeyer flask and the generator of the homogenizer. The graduated

cylinder was inverted several times prior to removal of an aliquot of the homogenate for assay.

For the determination of nitrogen, duplicate 0.5 ml samples of the homogenate were pipetted into Kjeldahl flasks and stored until assayed at -40°C. For the determination of triglycerides, duplicate 0.5 ml samples of the homogenate were transferred to 15 ml tubes placed in an ice-water slurry. For the determination of glucose and glycogen, duplicate 0.1 ml samples of the homogenate were transferred to 15 ml tubes also placed in the ice-water slurry.

The nitrogen content of the homogenate samples determined by the Micro-Kjeldahl procedure (88) was used to calculate the total body nitrogen of each mouse. The latter was expressed as a percent of dead body-weight.

The triglyceride content of the homogenate samples was determined by the method of Levy (89) as modified by Teitz (87). Standards of triolein were used which were prepared in 2-propanol at concentrations of 1, 2, 3, 4, and 5 mg/ml. Periodate reagent was prepared by dissolving 1.3 g of sodium meta-periodate in 100 ml of 0.88 M acetic acid. Acetylacetone reagent was prepared by diluting 1.5 ml of acetylacetone to 200 ml with 2 M ammonium acetate. To separate 15 ml tubes 0.5 ml of the triolein standards were added. A blank of 0.5 ml water was also included. To each of the 0.5 ml homogenate samples and the blank, 0.5 ml of 2-propanol was added while to each standard 0.5 ml water was added. To all tubes 3 ml of 2-propanol were added followed by 1 ml of 0.1 N H_2SO_4 and 2 ml of heptane in that order. The tubes were mixed for 20 seconds and the contents allowed to separate by standing for 10 minutes. To specially

selected Spectronic 20 test tubes 200 µl of the upper heptane layer of each assay tube were transferred, and 3 ml of 0.01 N alcoholic KOH was added. The tube contents were mixed and heated at 60°C for 10 minutes. Next, in order, 100 µl of the periodate reagent and 1 ml of the acetylacetone reagent were added to each tube. The contents were mixed and heated once more at 60°C for 10 minutes. Following cooling to room temperature, the absorbance of each solution was read at 412 nm on the Spectronic 20 spectrophotometer. A new standard curve was established at the time of each assay with the triolein standards, from which the triglyceride content of the homogenate samples was determined. The total triglyceride of each mouse expressed in terms of triolein was calculated as a percent of total dead body weight.

The total glucose and total glycogen content of the homogenate samples was determined by the method of Van Handel (90, 91). Aqueous standards of mixtures of glucose and glycogen were prepared at concentrations such that 100 µl provided 400/100, 300/200, 200/300, and 100/400 µg of glucose/glycogen. Anthrone reagent was prepared by dissolving 300 mg of anthrone in 200 ml of 14 M H_2SO_4. To separate 15 ml tubes 100 µl of the glucose/glycogen standards were added 100 µl of water in a separate tube was employed as a blank. To these tubes and to each of those separately prepared with the homogenate (0.1 ml), 200 µl of methanol were added followed by 50 µl of a saturated solution of sodium sulfate. The tube contents were mixed and centrifuged. One milliliter of a saturated solution of sodium sulfate in 66% ethanol was added to each tube; the tube contents were mixed, centrifuged, and the resulting supernatant transferred by Pasteur pipet to a second 15 ml tube. The 1 ml sodium sulfate/66% ethanol

extraction was repeated once more. The original tube now contained the glycogen adsorbed on the precipitated sodium sulfate and the second tube contained the glucose fraction. To each of the glycogen tubes 500 µl of 30% KOH were added. The mixed contents were heated to 100°C for 10 minutes. One milliter of 95% ethanol was added to each of the heated glycogen tubes; the contents were mixed and centrifuged and the resulting supernatant was aspirated off and discarded. The residue was taken up in 2 ml of water. All the glucose and glycogen tubes were mixed and 200 µl aliquots were transferred to specially selected Spectronic 20 test tubes and 3 ml anthrone reagent was added to each tube. The tube contents were mixed and heated at 90°C for 20 minutes. The tube contents were mixed and cooled to room temperature with the aid of an ice bath and the absorbance of the final solutions was read at 620 nm in the Spectronic 20 spectrophotometer. Standard concentration curves were established each time at assay with the glucose/glycogen standards, from which the amount of glucose and glycogen in each of the homogenate samples was calculated. Total glucose and total glycogen content of each mouse was expressed as a percent of dead body weight.

III - G Statistics

A least squares linear regression procedure was followed for the elaboration of all assay standard curves. The same procedure was followed to determine the best fitting line to the concentration-time data used for calculating the first-order rate constants for the hydrolysis of cocaine, benzoylecgonine, and ecgonine methylester each followed in a separate experiment.

Statistical analysis of animal data for intra-trial comparisons was

based upon the paired t-test, for inter-trial comparisons on the t-test for non-paired observations. All significant differences are reported as corresponding to a level of at least $p < 0.05$, although in some instances a higher level of significance was found between differences.

CHAPTER IV RESULTS

IV - A Ecgonine Alkaloid Assay

Benzoylecgonine, ecgonine HCl, and ecgonine methylester HCl were each prepared from cocaine HCl (92, 93) according to the reactions summarized in Figure 5. The melting point of each alkaloid: cocaine HCl (195°C), benzoylecgonine (195°C with decomposition), ecgonine methylester HCl (214°C with decomposition), and ecgonine HCl (246°C with decomposition) compared favorably with its literature value (3, 92, 93). The thin layer chromatography Rf values for cocaine HCl (0.82) and ecgonine HCl (0.15) were verified in system 'N' of Noirfalise and Mees (83) while the Rf values for benzoylecgonine (0.20) and ecgonine methylester HCl (0.62) were checked in the thin layer chromatography system of Wallace et al (17) and Taylor et al (54), respectively. When all four alkaloids were run in system 'N' of Noirfalise and Mees (83) it was found that separation could be achieved as schematically represented in Figure 6. Visualization of the alkaloid bands was achieved by an initial light spraying with Dragendorff's reagent followed in order by a light over-spray with concentrated H_2SO_4 and a 90-second exposure to I_2 vapours (17). The cocaine and the benzoylecgonine bands appear red-pink following the H_2SO_4 over-spray, while the ecgonine and ecgonine methylester bands appear red-orange. All alkaloid bands appear dark-brown following exposure to I_2 vapours.

The separation of the four ecgonine alkaloids: cocaine, benzoylecgonine, ecgonine methylester, and ecgonine using the gas chromatographic (GC) procedure described for the hydrolysis study was excellent. As can be seen from the sample chromatogram in Figure 7 the resolution of the

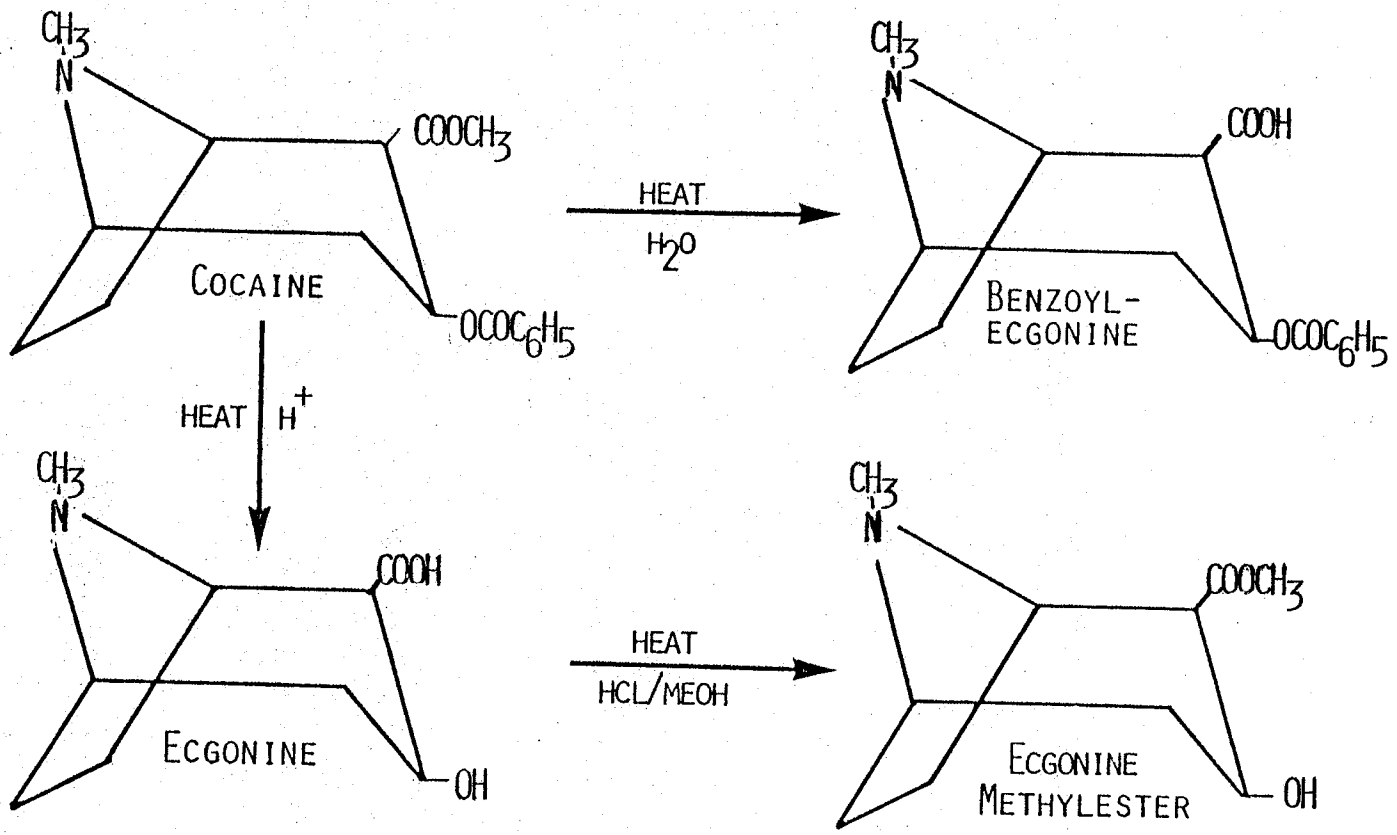


Figure 5. Synthesis of the ecgonine related hydrolysis products of cocaine.

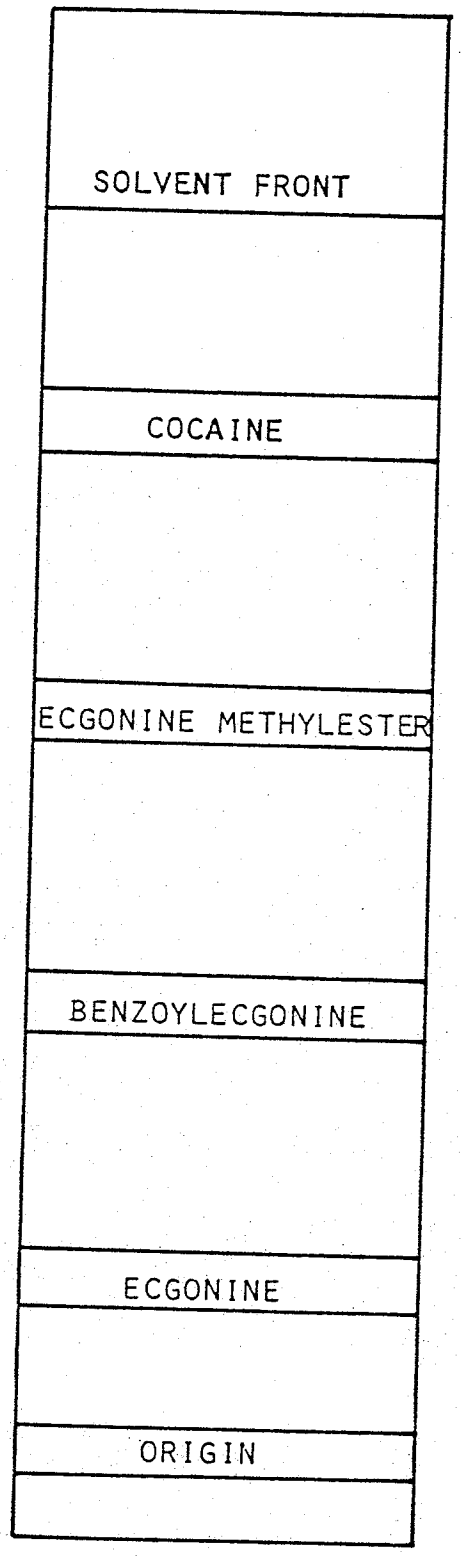


Figure 6. Schematic representation of the thinlayer chromatographic separation of the ecgonine alkaloids achieved in system N of Noirfalise and Mees (83).

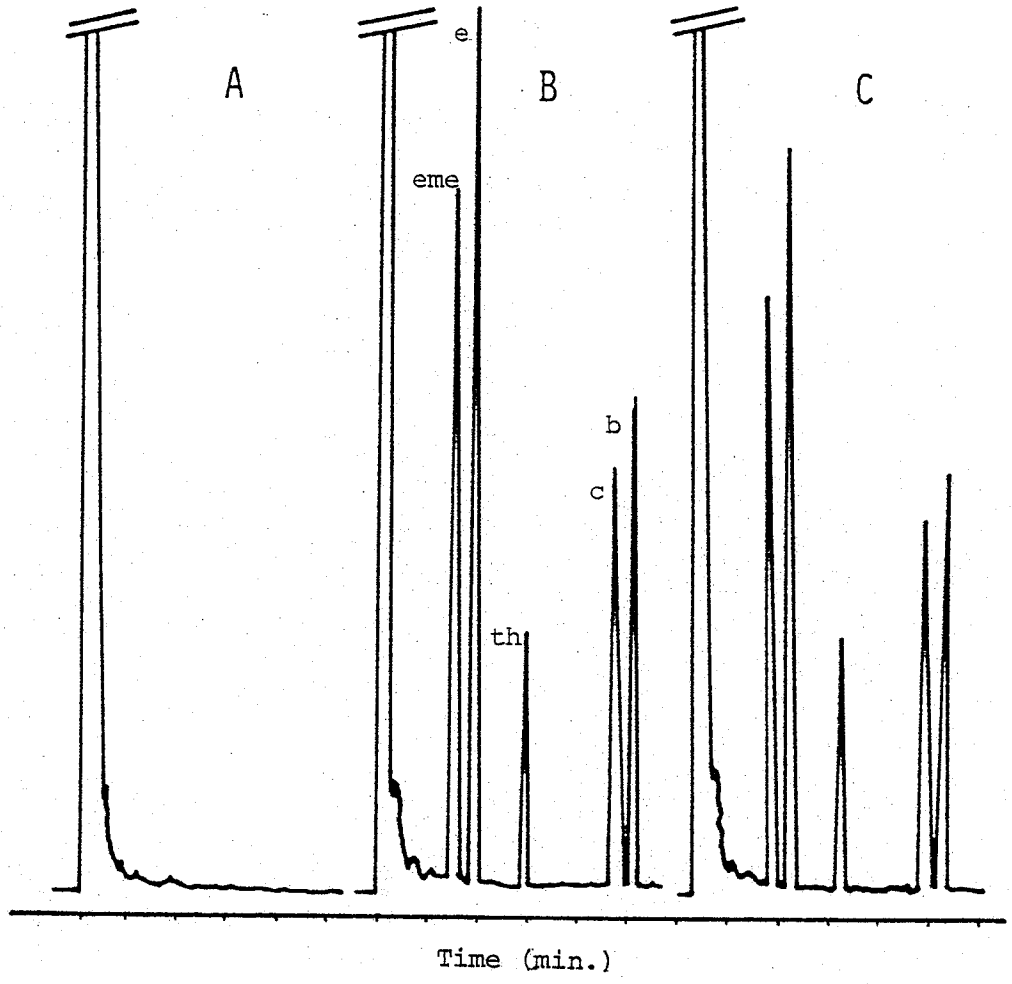


Figure 7. Sample gas chromatograms from the hydrolysis study assay procedure. Key: A- BSA blank: B- standards: (eme) ecgonine methylester-TMS, (e) ecgonine-TMS, (th) theophylline-TMS, (c) cocaine, (b) benzoyl-ecgonine-TMS. C- extract of hydrolysis sample.

several alkaloid peaks together with the theophylline peak representing the internal standard was very satisfactory. In these assays cocaine does not undergo trimethylsilyl-derivatization (-TMS) with N, O-bistrimethylsilylacetamide (BSA). The chromatographic data are presented in Table 2. Caffeine also does not derivatize but its behavior in this system, as expected, is very similar to the theophylline-TMS derivative.

The extraction recovery data for the alkaloids in the hydrolysis study are presented in Table 3. The working standard assay curves for the hydrolysis study were elaborated as shown in Figure 8. All curves were linear over the concentration range examined; each data point on the curves represents the average of five replicate determinations.

Samples of gas chromatograms of derivatized and non-derivatized urine extracts from the urinary excretion study assay procedure are presented in Figure 9. Once more the separation among the peaks was good and interference of extraneous peaks posed no problem in the procedures followed. The extraction recovery data for alkaloids in urine obtained by the procedure used in the urinary excretion study are presented in Table 4. The working standard assay curves used to determine the amount of alkaloid in the original urine samples are depicted in Figure 10. The curves were linear over the concentration range examined; each data point on the curve represents the average of five replicate determinations.

IV - B Ecgonine Alkaloid Hydrolysis

The hydrolysis profiles of cocaine in distilled water, 0.1 N HCl, and simulated gastric fluid USP are presented in Figures 11, 12, and 13, respectively. The profiles of cocaine hydrolysis in pH 7.3 phosphate

Table 2. Gas chromatographic data for the ecgonine alkaloids and the internal standards for the analytical procedures in the hydrolysis and the urinary excretion studies.

	ECGONINE METHYLESTER[a]	ECGONINE[a]	CAFFEINE	THEOPHYLLINE[a]	COCAINE	BENZOYL-ECGONINE[a]
RETENTION[b]	0.50	0.63	1.00	1.00	1.50	1.63
RESPONSE[c]	2.25	2.70	1.08	1.00	2.17	2.36

[a] As trimethylsilyl derivatives.
[b] Values relative to theophylline, actual times are X 3.2 minutes.
[c] Equimolar peak height responses.

Table 3. Recovery data for the extraction of ecgonine alkaloids in the hydrolysis study assay procedure.

	COCAINE	BENZOYL-ECGONINE	ECGONINE	ECGONINE METHYLESTER
AVERAGE PERCENT RECOVERY [a]	101.1	102.0	96.5	100.3
COEFFICIENT OF VARIATION [b]	3.1	2.3	3.8	2.0

[a] Five replicate determinations at each of the concentrations examined.

[b] Coefficient of variation = $\frac{\text{Standard Deviation}}{\text{Mean}}$ X 100.

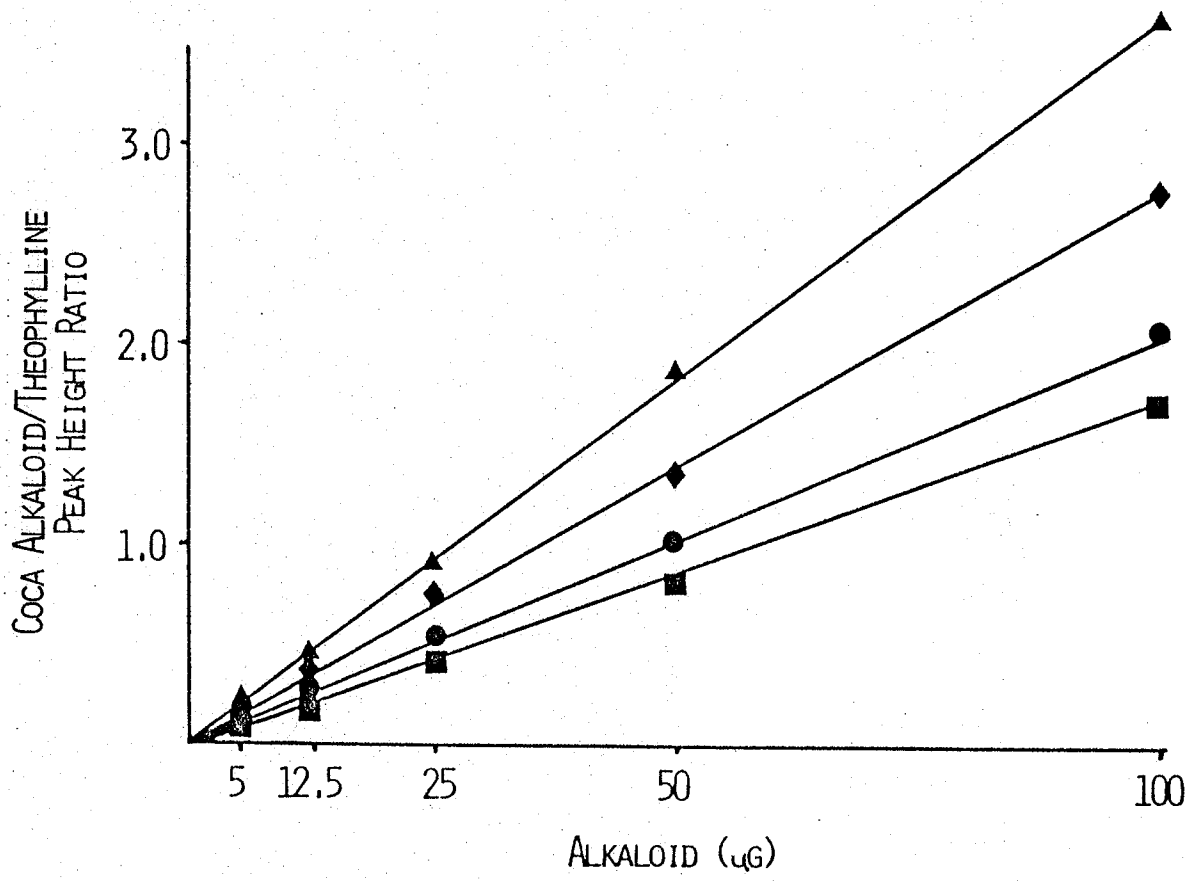


Figure 8. Working standard curves for the amount of ecgonine alkaloid in the original sample analyzed in the hydrolysis studies. Key: ■- cocaine, ●- benzoylecgonine, ◆- ecgonine methylester, ▲- ecgonine.

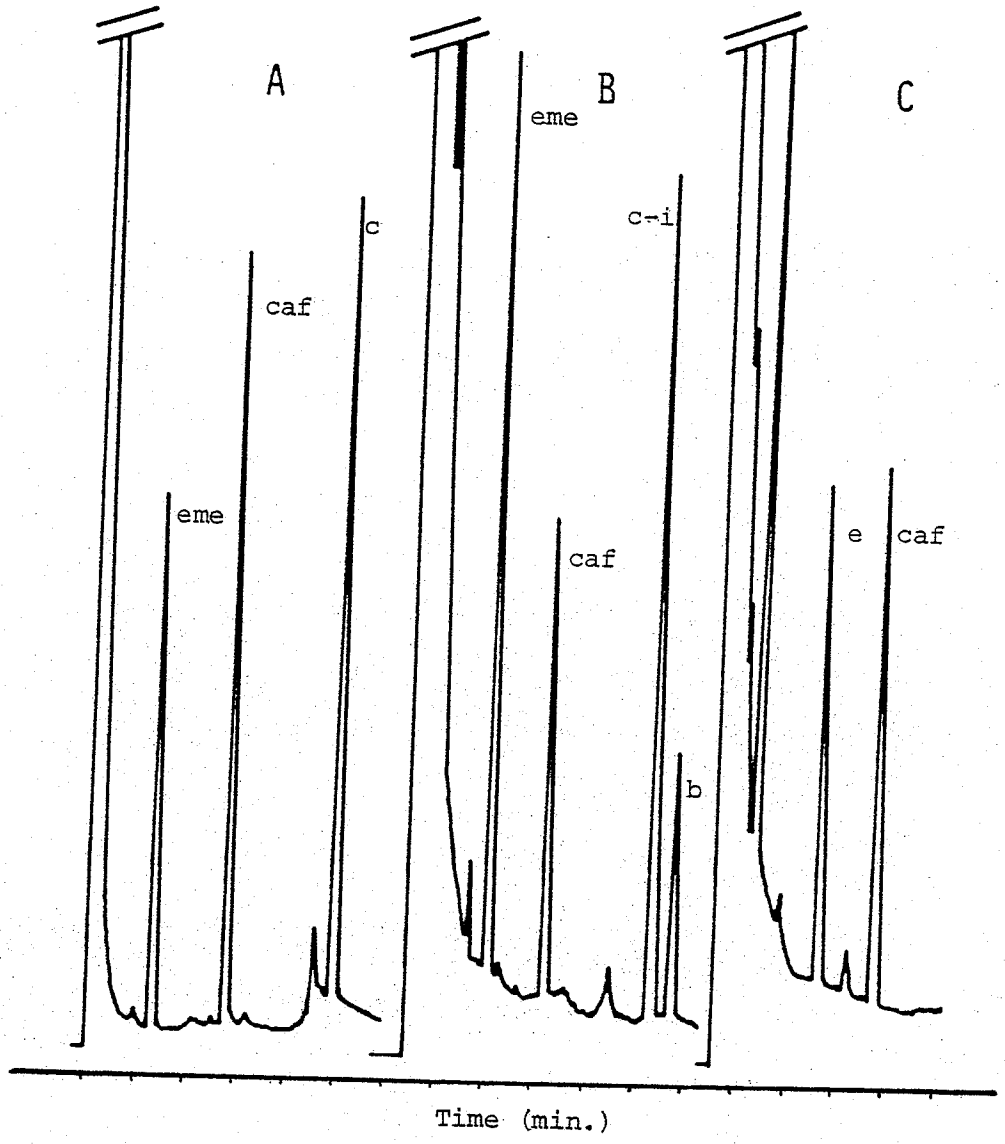


Figure 9. Sample gas chromatograms from the urine assay procedure. Key: A- urine extract: (eme) ecgonine methylester, (caf) caffeine, (c) cocaine; B- derivatized urine extract: (eme) ecgonine methylester-TMS, (caf) caffeine, (c-i) cocaine plus interfering substance, (b) benzoylecgonine-TMS; C- derivatized urine extract: (e) ecgonine-TMS, (caf) caffeine.

Table 4. Recovery data for the extraction of ecgonine alkaloids from urine in the urine excretion study assay procedure.

	COCAINE	BENZOYL-ECGONINE	ECGONINE	ECGONINE-METHYLESTER
AVERAGE PERCENT RECOVERY [a]	100.4	51.4	58.1	99.1
COEFFICIENT OF VARIATION [a]	3.1	5.5	5.9	3.2

[a] Five replicate determinations at each of the concentrations examined.

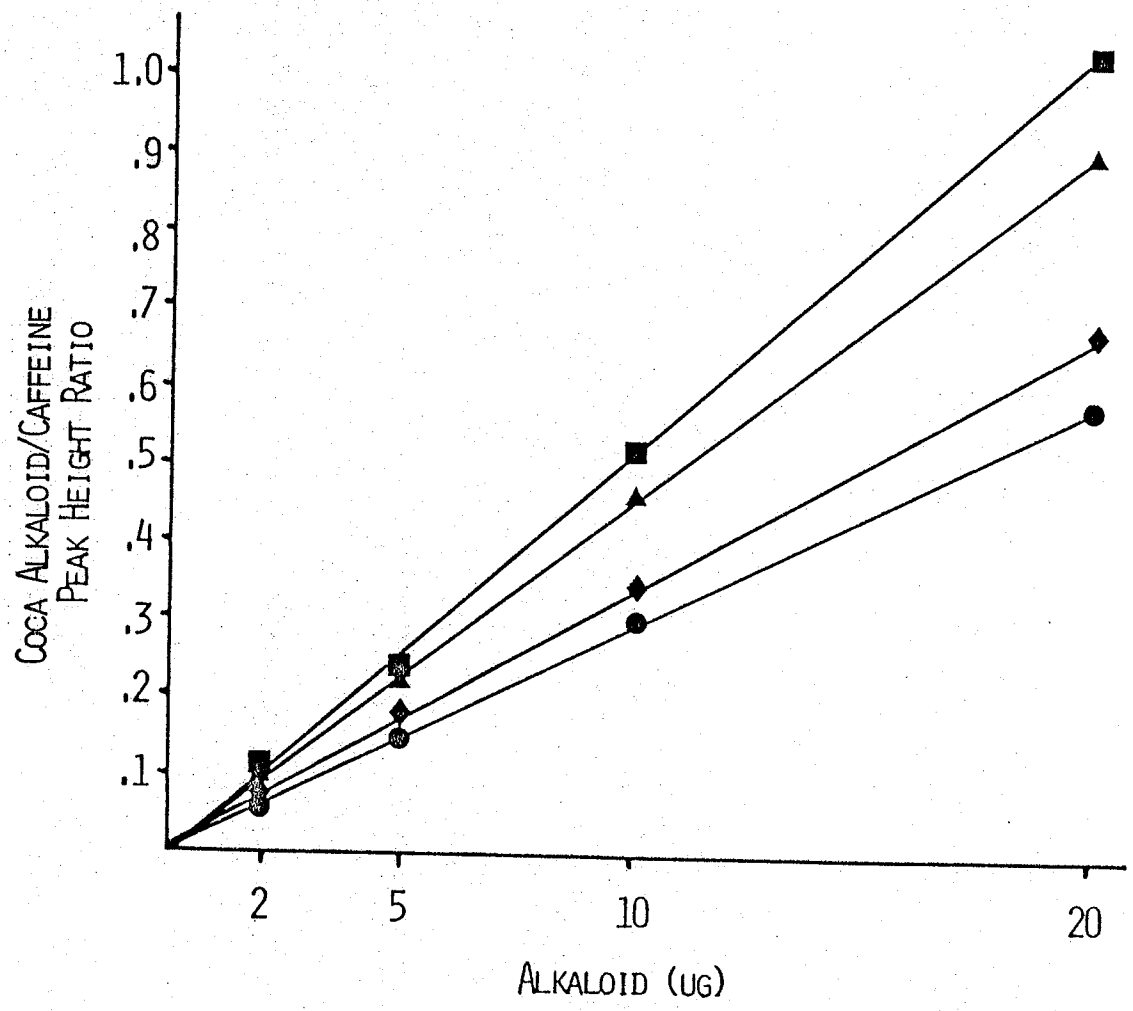


Figure 10. Working standard curves for the amount of ecgonine alkaloid in the original sample analyzed in the urinary excretion study. Key: ■- cocaine, ●- benzoylecgonine, ◆- ecgonine methylester, ▲- ecgonine.

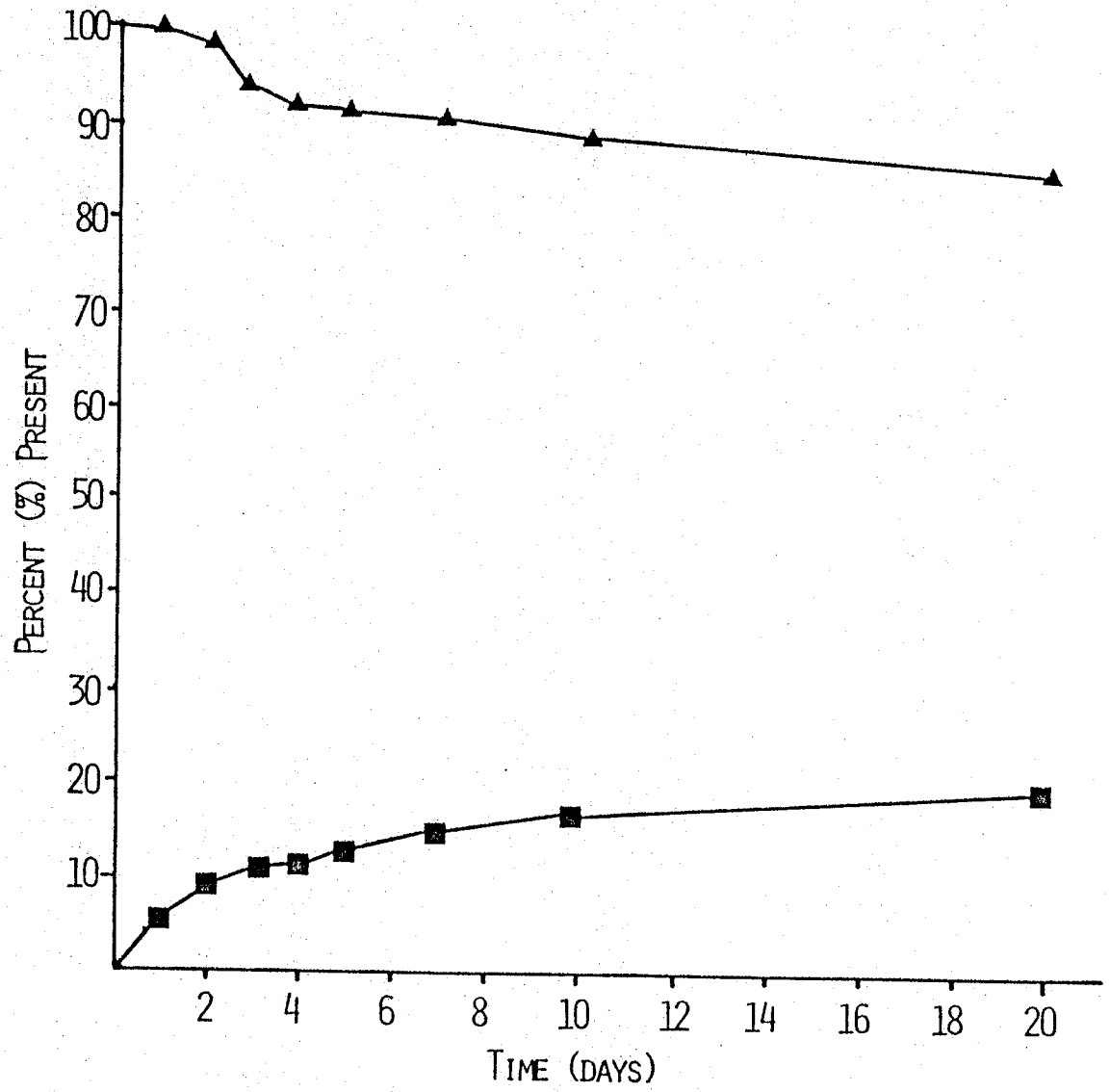


Figure 11. Cocaine hydrolysis in distilled water, pH 6.5, at 37° C.
Key: ▲- cocaine, ■- benzoylecgonine.

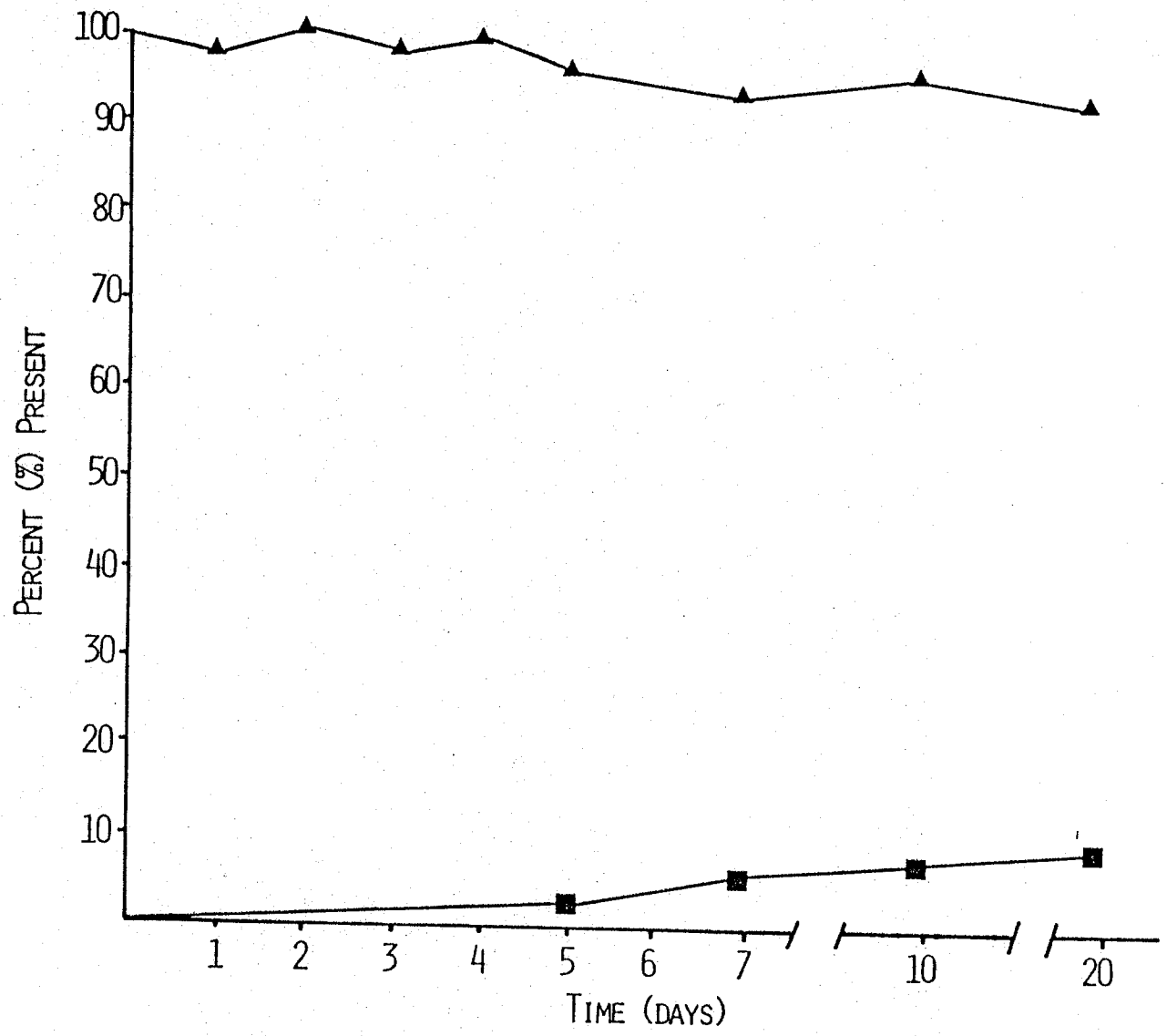


Figure 12. Cocaine hydrolysis in 0.1 N HCl, pH 1.2, at 37° C. Key: ▲- cocaine, ■- benzoylecgonine.

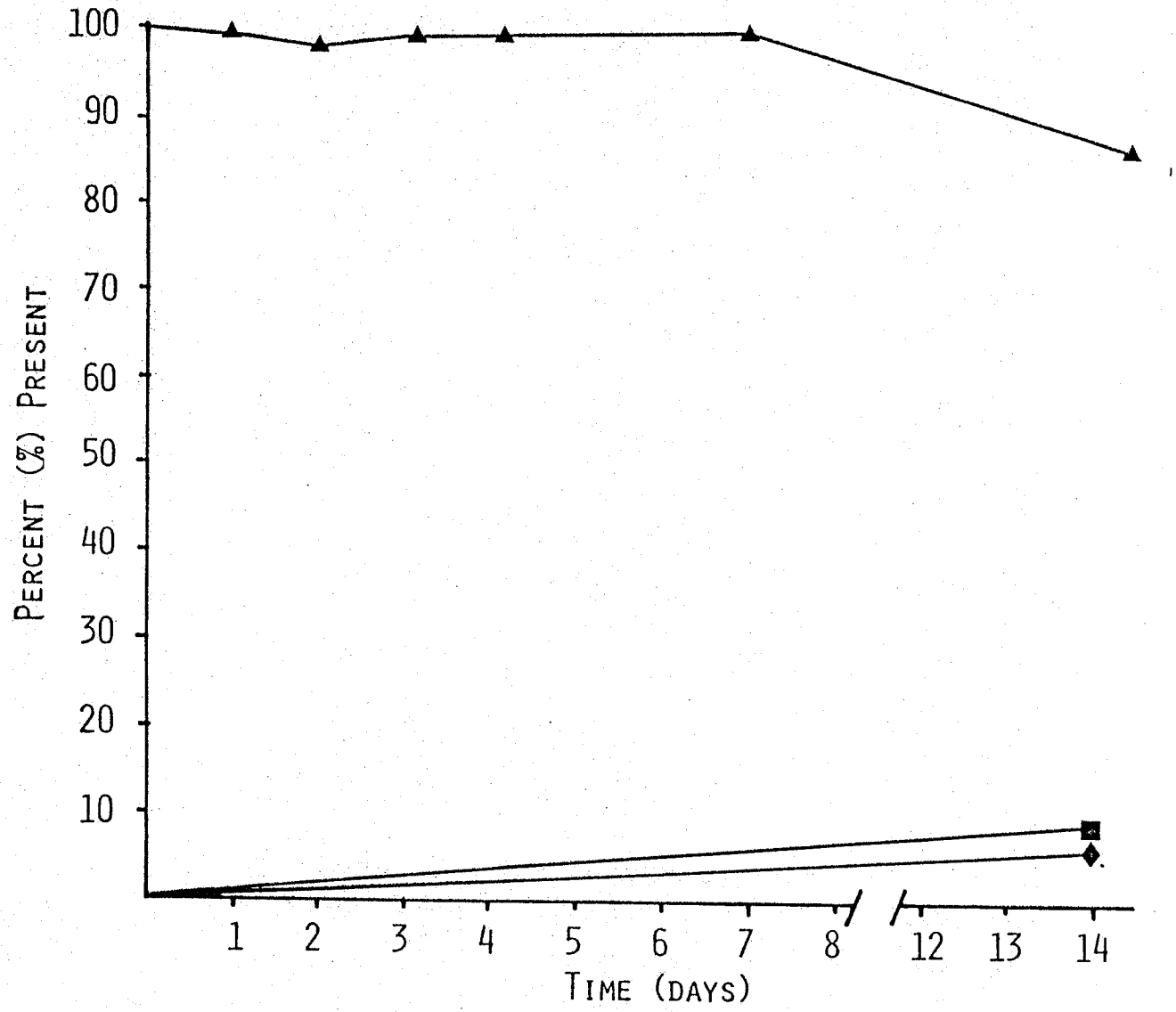


Figure 13. Cocaine hydrolysis in simulated gastric fluid USP, pH 1.2, at 37° C. Key: ▲- cocaine, ■- benzoylecgonine, ◆- ecgonine.

buffer and simulated intestinal fluid USP are depicted in Figures 14 and 15, respectively. The stability of cocaine is evident from its negligible disappearance in media of pH less than 7. On the other hand at pH 7.3 and 7.5 cocaine undergoes substantial hydrolysis with benzoylecgonine appearing as the major intermediate. The profiles did not change dramatically when the cocaine hydrolysis was carried out in pH 10 and pH 11 media as seen in Figures 16 and 17, respectively, with benzoylecgonine still being the predominant intermediate during hydrolysis. In the hydrolysis media consisting of NaOH 0.04 N or $Ca(OH)_2$ 0.04 N, both beyond pH 11, the cocaine hydrolysis was very rapid, as can be seen in the respective Figures 18 and 19. The major intermediate produced now as the hydrolysis of cocaine proceeds to ecgonine is ecgonine methylester.

To describe the hydrolysis of cocaine in strong alkaline media (pH >11) a kinetic model was elaborated showing the hydrolysis of cocaine proceeding along two parallel pathways both leading to ecgonine as the final product as depicted in Figure 20. Each pathway consists of two consecutive ester-hydrolysis reactions. By one pathway cocaine by hydrolytic splitting of its methylester function first produces benzoylecgonine which in turn by hydrolysis of its benzoylester function produces ecgonine. By the other pathway, the benzoylester function of cocaine is first hydrolyzed producing ecgonine methylester which in turn hydrolyzes to ecgonine. In this scheme k_1^1 is the first-order formation rate constant for ecgonine methylester from cocaine, k_1^2 for benzoylecgonine from cocaine, and k_2^1 and k_2^2 for ecgonine from ecgonine methylester and benzoylecgonine respectively. The sum of k_1^1 and k_1^2 is equal to the overall first-order rate constant (K) for the disappearance of cocaine.

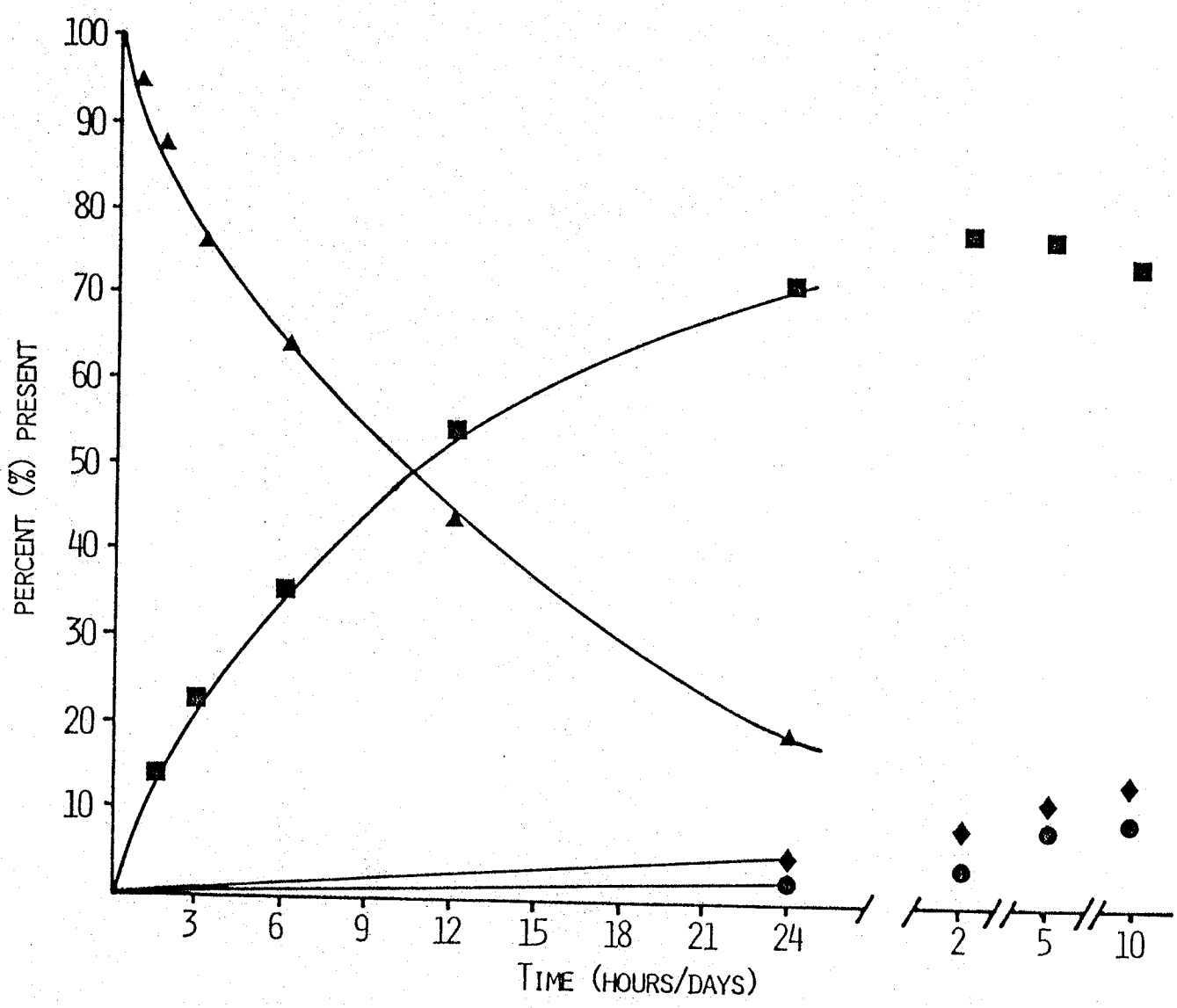


Figure 14. Cocaine hydrolysis in pH 7.3 phosphate buffer at 37° C. Key: ▲- cocaine, ■- benzoylecgonine, ●- ecgonine methylester, ◆- ecgonine.

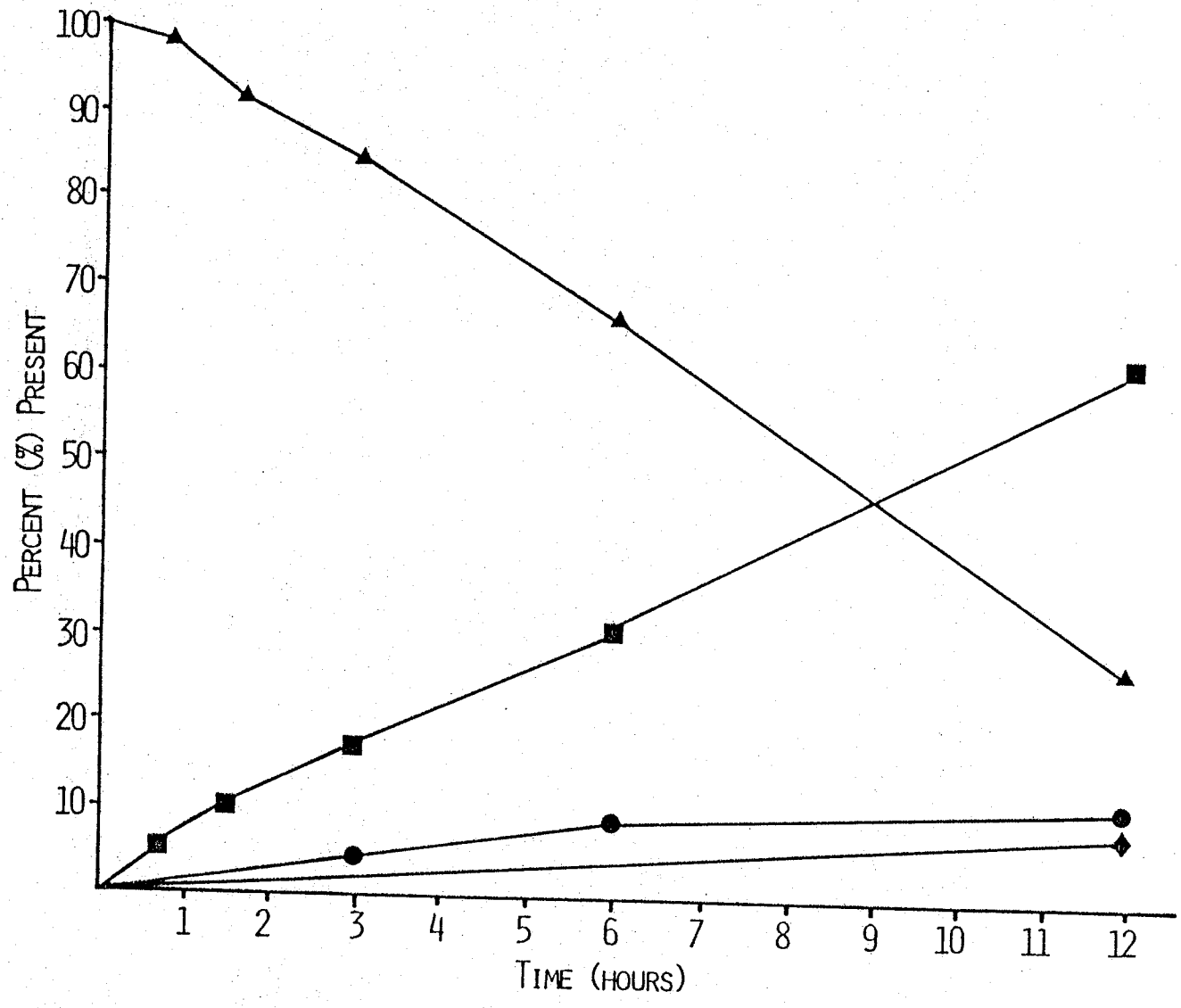


Figure 15. Cocaine hydrolysis in simulated intestinal fluid USP, pH 7.5 at 37° C.
Key: ▲- cocaine, ■- benzoylecgonine, ●- ecgonine methylester, ◆- ecgonine.

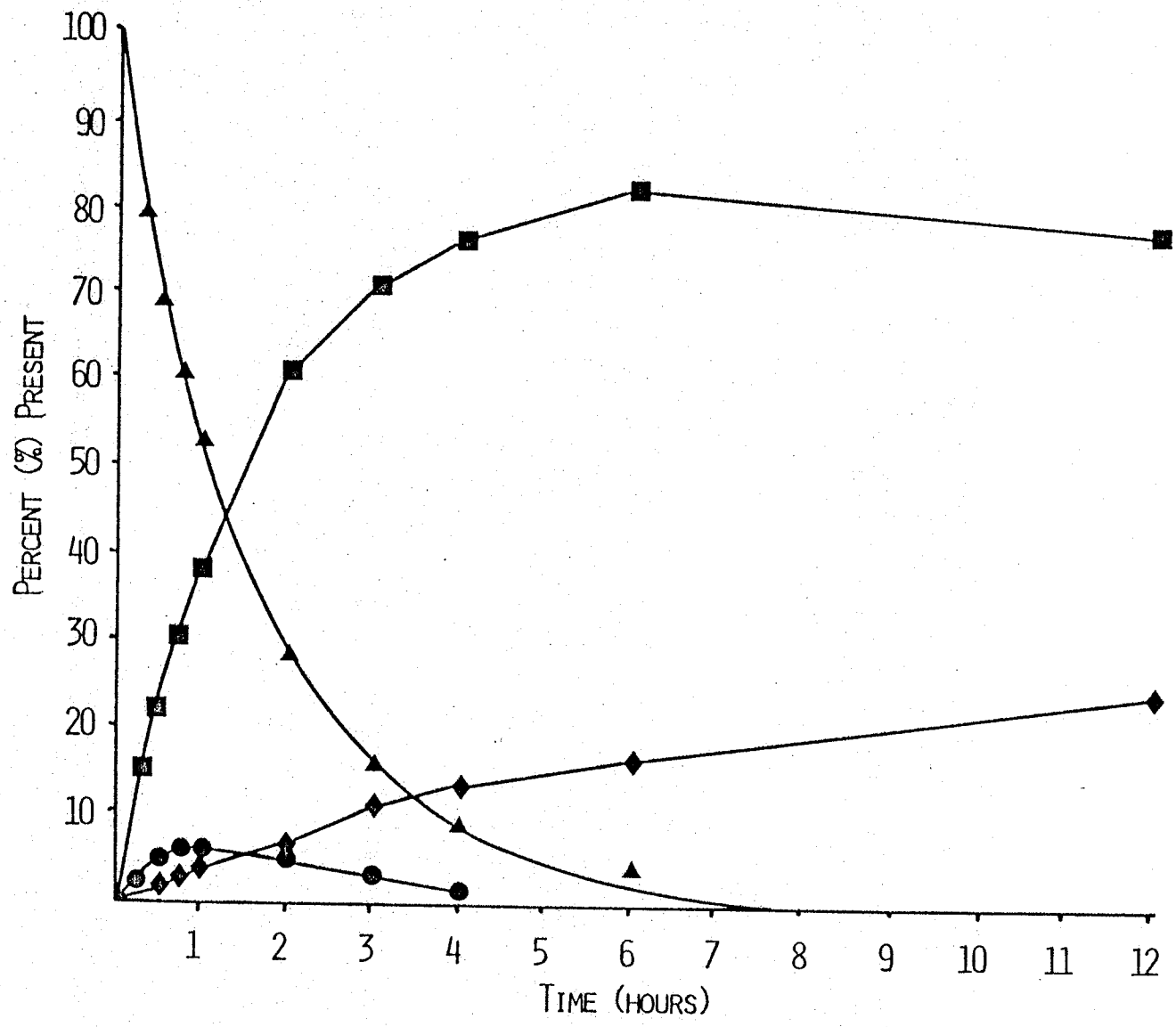


Figure 16. Cocaine hydrolysis in pH 10 phosphate buffer at 37° C. Key: ▲- cocaine, ■- benzoylecgonine, ●- ecgonine methylester, ◆- ecgonine.

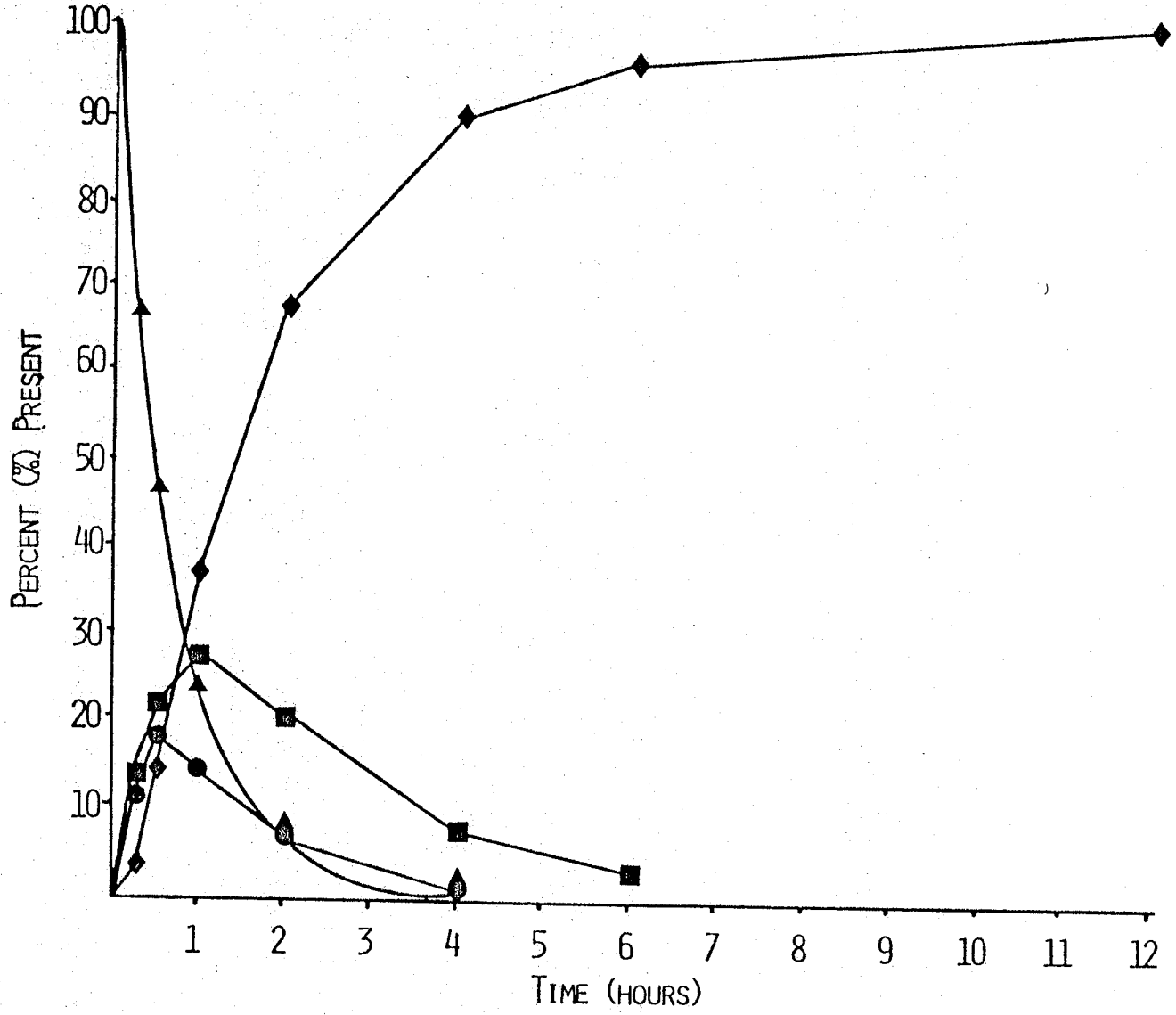


Figure 17. Cocaine hydrolysis in pH 11 glycine buffer at 37° C. Key: ▲- cocaine, ■- benzoylecgonine, ●- ecgonine methylester, ◆- ecgonine.

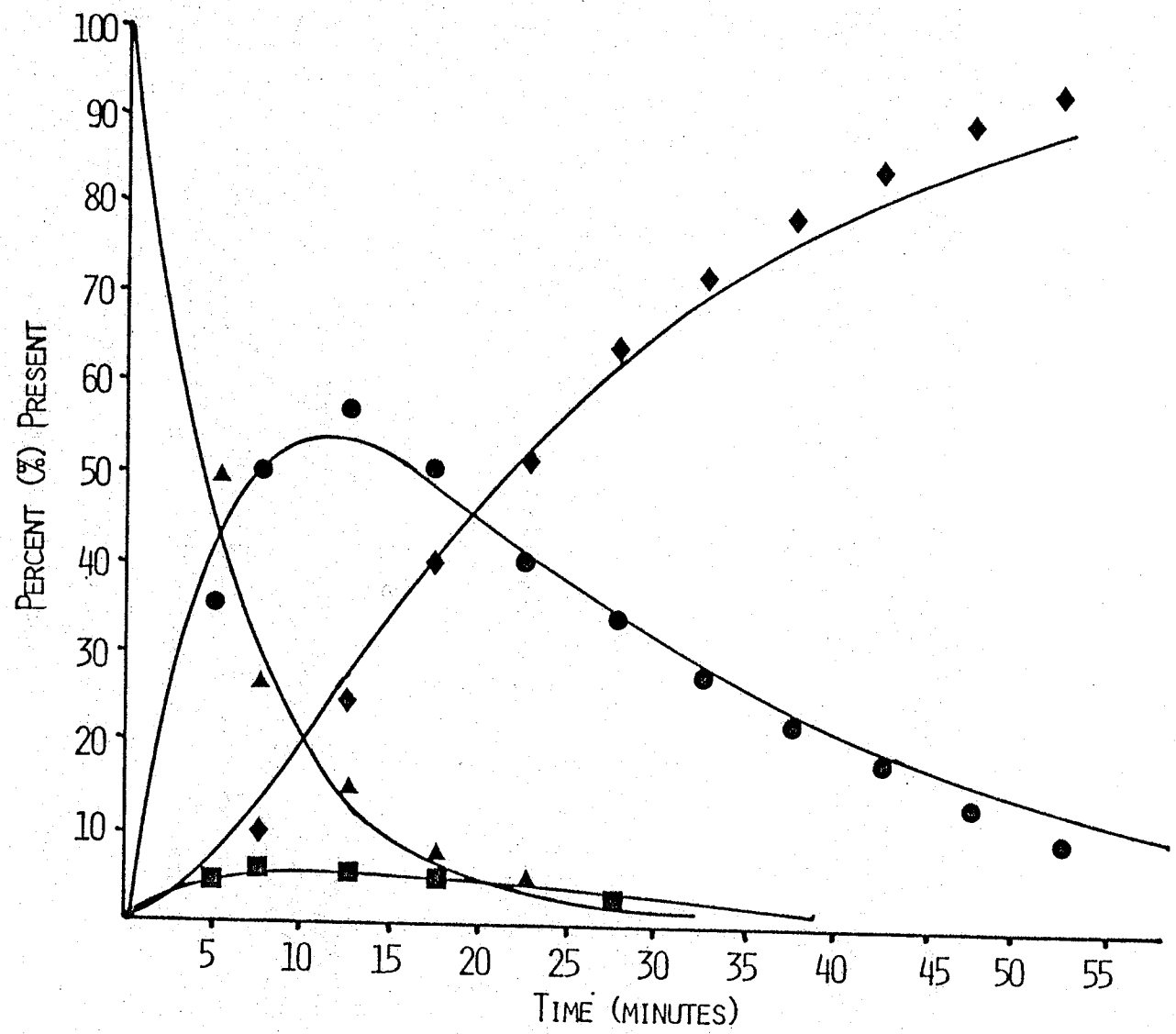


Figure 18. Cocaine hydrolysis in 0.04 N NaOH, pH 11.9, at 37° C. Key: ▲- cocaine, ■- benzoylecgonine, ●- ecgonine methylester, ◆- ecgonine representing experimental results and continuous lines representing theoretical profiles.

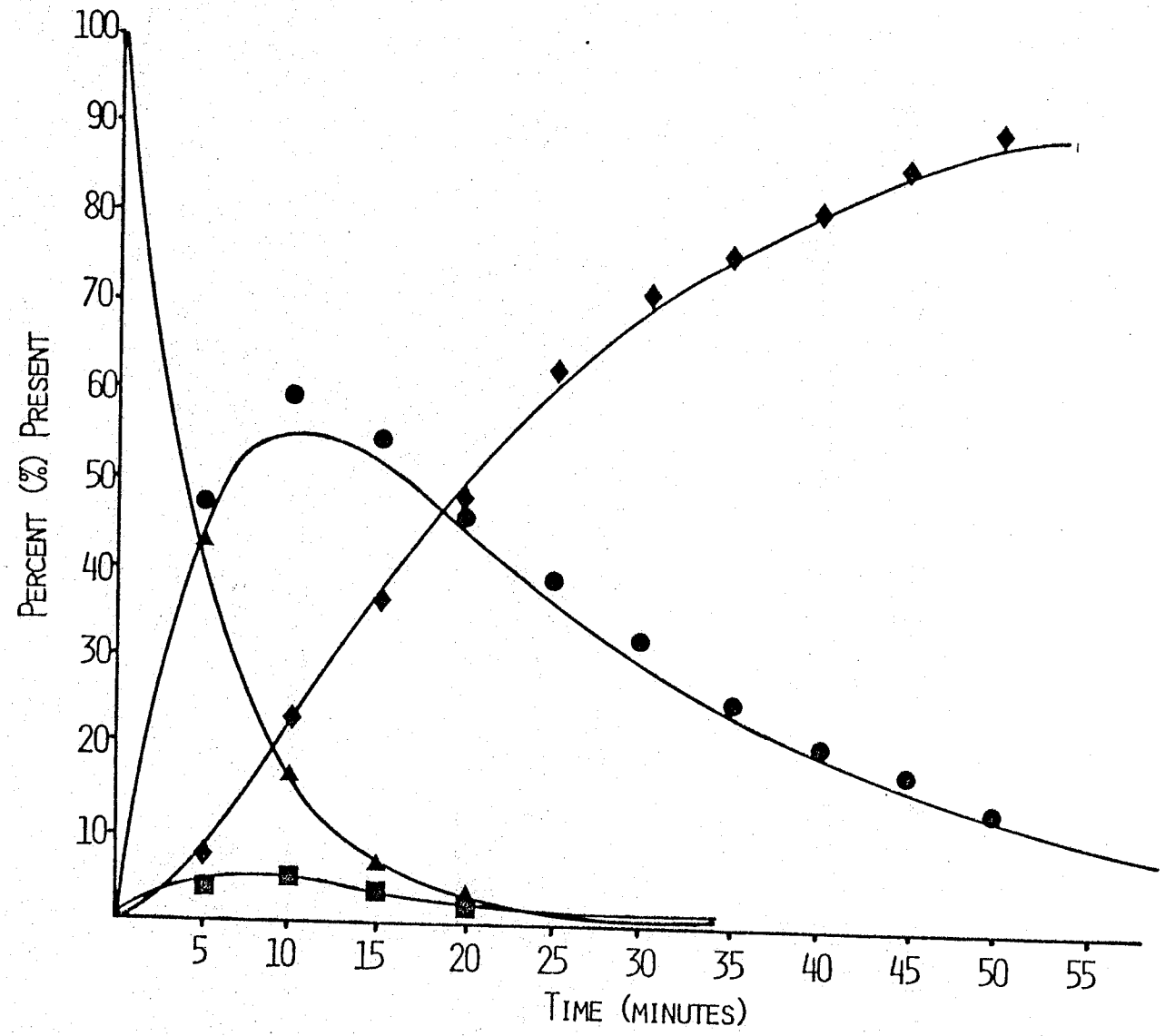


Figure 19. Cocaine hydrolysis in 0.04 N $Ca(OH)_2$, pH 11.8, at 37° C. Key: ▲- cocaine, ■ - benzoylecgonine, ●- ecgonine methylester, ◆ - ecgonine representing experimental results and continuous lines representing theoretical profiles.

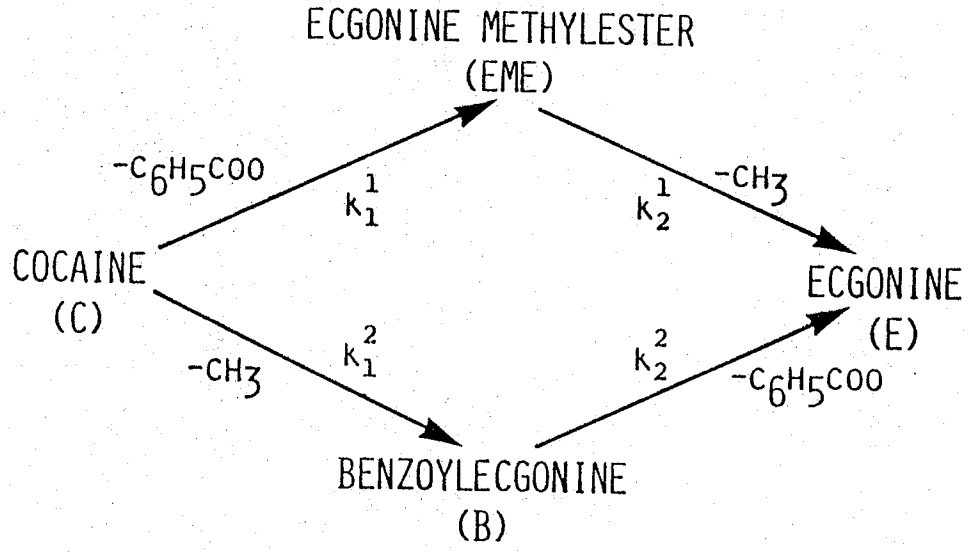


Figure 20. The kinetic model proposed for the hydrolysis of cocaine. All k's are first order rate constants.

Differential rate equations were developed applicable to the proposed model. These equations together with their corresponding integrated forms are shown in Table 5. K was directly calculated from the disappearance of cocaine in each of the hydrolysis media. The rate constants k_2^1 and k_2^2, were also obtained directly by following in separate experiments the hydrolysis of ecgonine methylester and benzoylecgonine each alone in each of the hydrolysis media under study. Obviously the hydrolysis data could not directly provide values for k_1^1 and k_1^2; estimates of these rate constants were obtained indirectly. After first fixing the value determined for K and the independently determined values of k_2^1 and k_2^2, the value of K was partitioned between k_1^1 and k_1^2 by an iterative process whereby their respective values became fixed upon obtaining two constants which when used to calculate theoretical peak heights of ecgonine methylester and benzoylecgonine yielded peaks both equal in proportion to their respective experimentally observed peak heights. By means of this approach, reasonable estimates were obtained for all four of the rate constants needed in the integrated equations used to fit theoretical profiles to the experimentally determined hydrolysis data.

Theoretical curve fitting to data was completed for the hydrolysis of cocaine in 0.04 N NaOH (Figure 18), 0.04 N $Ca(OH)_2$ (Figure 19), 0.01 N NaOH (Figure 21), 0.01 N $Ca(OH)_2$ (Figure 22), and ishku supernatant solution (Figure 23). In the figures showing the hydrolysis of cocaine in the strong alkaline media (pH>11), the continuous lines depict the profiles elaborated by the theoretical equations while the individual points represent the average of two separate experimentally determined alkaloid levels at the sampling time in that particular media. The

Table 5. Equations for the kinetic model describing the hydrolysis of cocaine as depicted in Figure 20.[a]

CHEMICAL SPECIES	DIFFERENTIAL FORM	INTEGRATED FORM
COCAINE (C)	$\frac{dC}{dt} = -K \cdot C$	$C = C^{o} \cdot e^{-Kt}$
ECGONINE METHYLESTER (EME)	$\frac{dEME}{dt} = k_1^1 \cdot C - k_2^1 \cdot EME$	$EME = \frac{k_1^1 \cdot C^{o}}{k_2^1 - K} \cdot (e^{-Kt} - e^{-k_2^1 t})$
BENZOYL-ECGONINE (B)	$\frac{dB}{dt} = k_1^2 \cdot C - k_2^2 \cdot B$	$B = \frac{k_1^2 \cdot C^{o}}{k_2^2 - K} \cdot (e^{-Kt} - e^{-k_2^2 t})$
ECGONINE (E)	$\frac{dE}{dt} = k_2^1 \cdot EME + k_2^2 \cdot B$	$E = \frac{k_1^1 \cdot C^{o}}{K \cdot (k_2^1 - K)} \left[k_2^1 \cdot (1 - e^{-Kt}) - K \cdot (1 - e^{-k_2^1 t}) \right] + \frac{k_1^2 \cdot C^{o}}{K \cdot (k_2^2 - K)} \left[k_2^2 \cdot (1 - e^{-Kt}) - K \cdot (1 - e^{-k_2^2 t}) \right]$

[a] All k's are first order rate constants and $K = k_1^1 + k_1^2$.

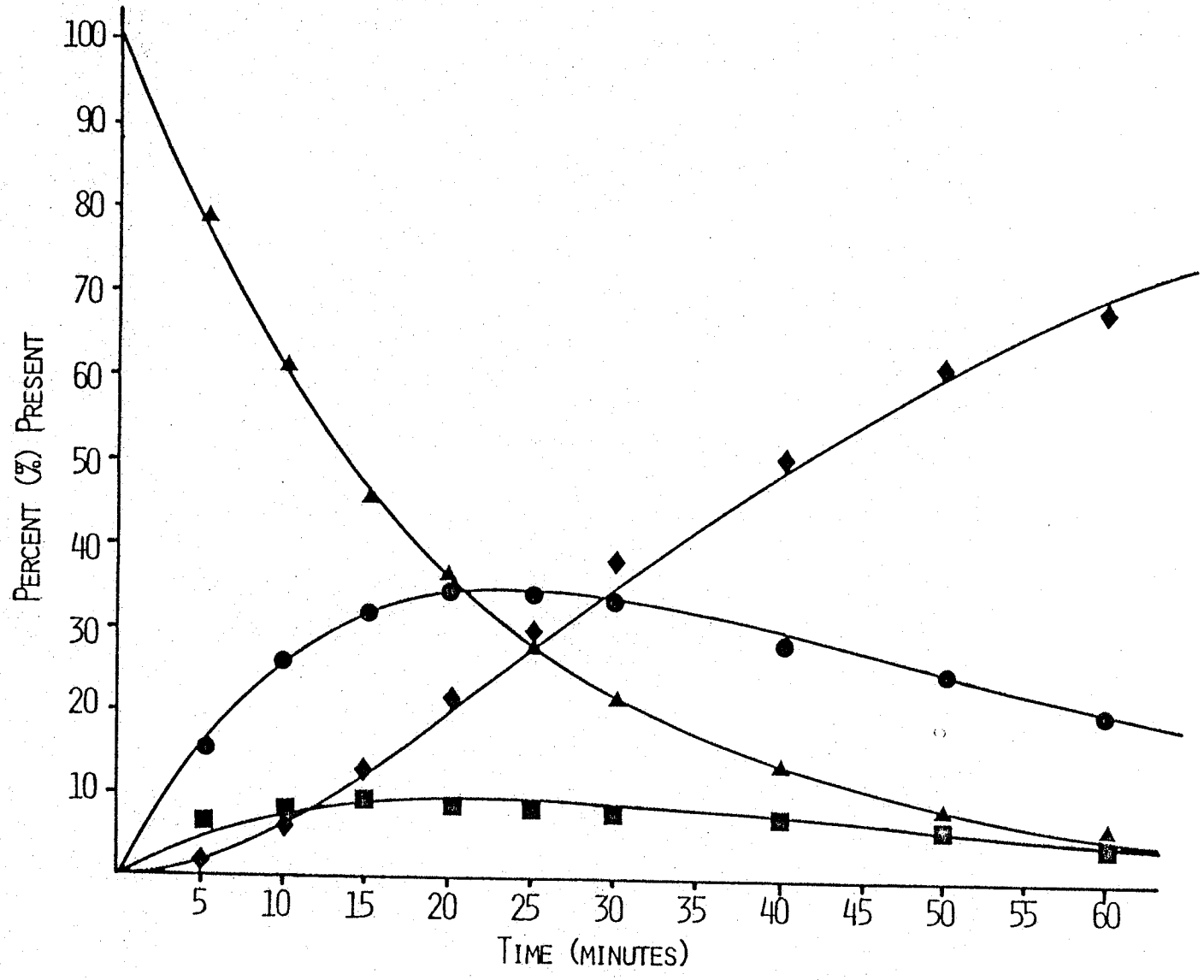


Figure 21. Cocaine hydrolysis in 0.01 N NaOH, pH 11.3, at 37° C. Key: ▲- cocaine, ■- benzoylecgonine, ●- ecgonine methylester, ◆- ecgonine representing experimental results and continuous lines representing theoretical profiles.

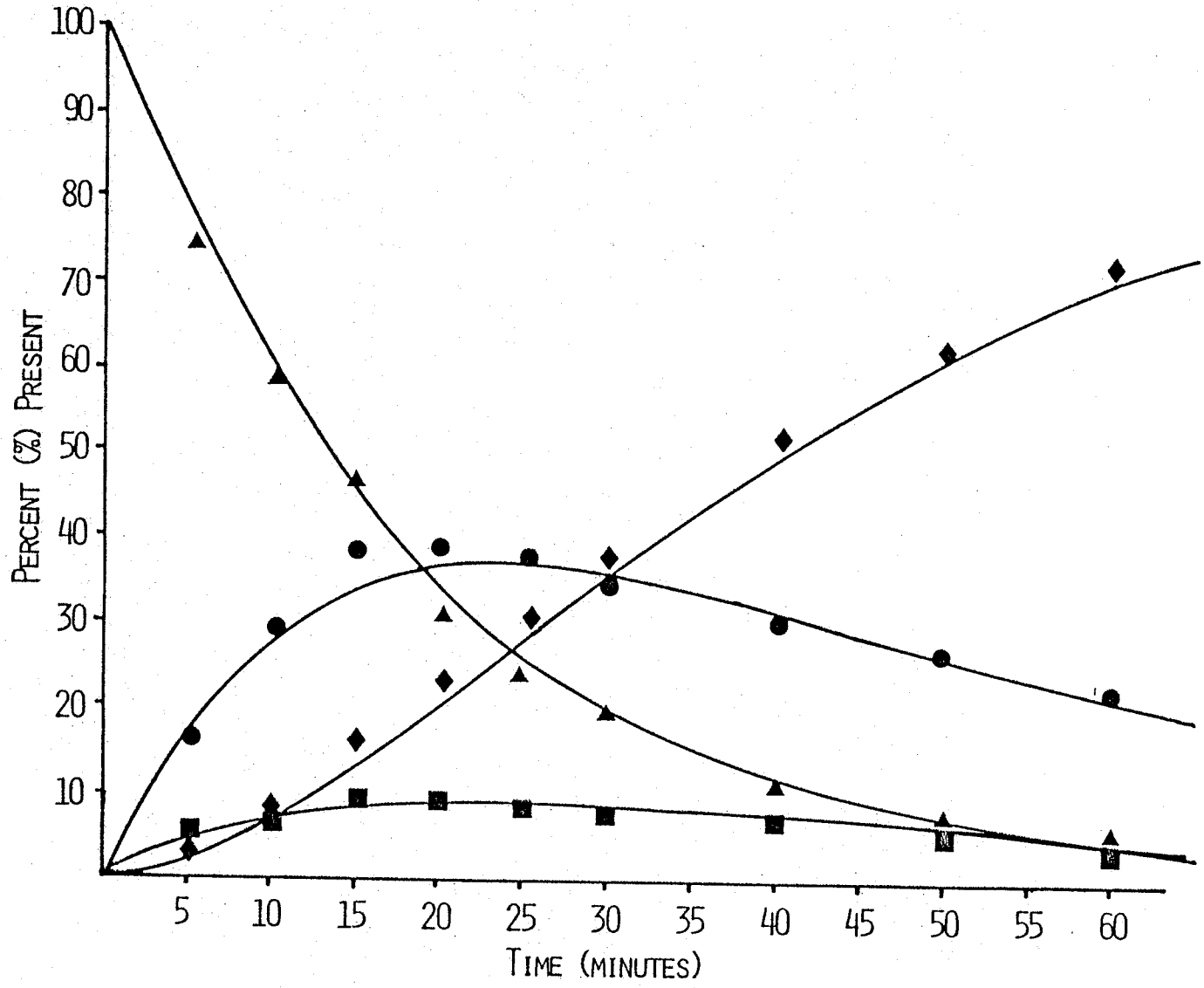


Figure 22. Cocaine hydrolysis in 0.01 N $Ca(OH)_2$, pH 11.1, at 37° C. Key: ▲- cocaine, ■ - benzoylecgonine, ● - ecgonine methylester, ◆ - ecgonine representing experimental results and continuous lines representing theoretical profiles.

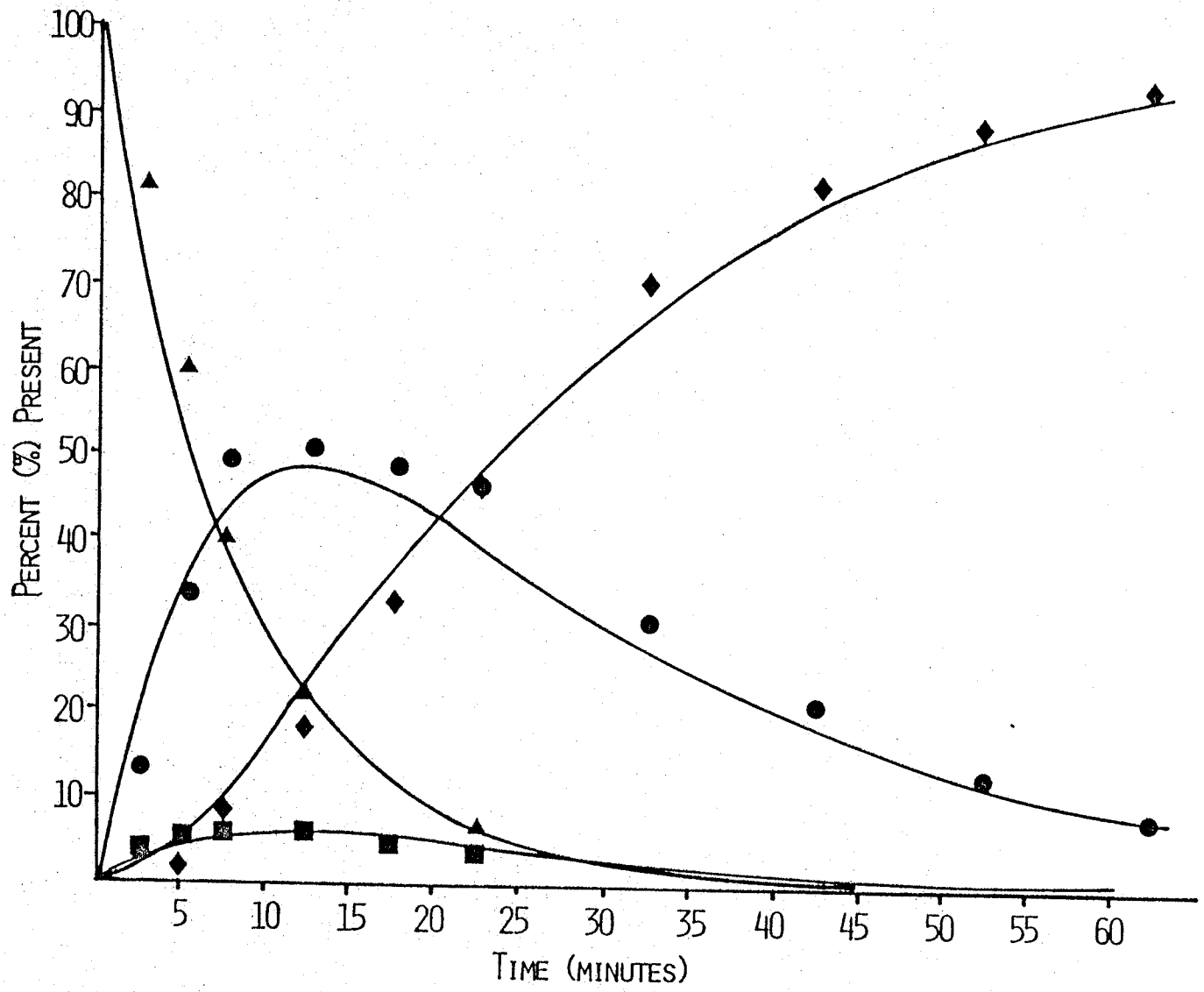


Figure 23. Cocaine hydrolysis in ishku supernatant solution, pH 11.7, at 37° C. Key: ▲- cocaine, ■- benzoylecgonine, ●- ecgonine methylester, ◆- ecgonine representing experimental results and continuous lines representing theoretical profiles.

depicted in Figures 18 and 19, respectively, is rapid, with ecgonine methylester appearing as the major intermediate. When the hydrolysis of cocaine was followed in these two media both at 0.01 N concentration as shown in Figures 21 and 22, respectively, the pattern of hydrolysis still favors the ecgonine methylester pathway, but the profiles look somewhat depressed and extended suggesting a slower hydrolysis of cocaine in these alkaline media of lower concentration.

Composition data characterizing the two alkaline substances, ishku and llipta, employed by coca chewers are included in Tables 6 and 7, respectively. The ishku substance, made from burned limestone ($CaCO_3$), is probably CaO with some unchanged $CaCO_3$. The llipta substance is more complex, having a high proportion of magnesium and potassium attesting to its plant origin. The llipta produced supernatant solutions that were not as alkaline as those resulting from the ishku substance.

The hydrolysis profiles of cocaine in the supernatant solutions of these two substances are presented in Figures 23 and 24. The profile of the ishku supernatant solution (Figure 23) is characteristic of the 0.04 N NaOH (Figure 18) and 0.04 N $Ca(OH)_2$ (Figure 19) hydrolyses, with rapid hydrolysis of the cocaine and with ecgonine methylester appearing as the major intermediate. On the other hand, the profile of the cocaine hydrolysis in the llipta supernatant solution (Figure 24) is characteristic of the hydrolyses of cocaine in the pH 10 (Figure 16) and pH 11(Figure 17) buffers in which cocaine is relatively slowly hydrolyzed and benzoylecgonine appears as the major intermediate.

Comparative values of the overall disappearance rate constant of cocaine (K) in the several media studied are presented in Table 8. It

Table 6. Some comparative characteristics of the supernatant solution obtained from a 4 mg/ml dispersion of ishku substance in water and a saturated (0.04 N) calcium hydroxide solution.[a]

	CALCIUM HYDROXIDE	ISHKU #1	ISHKU #2
CALCIUM (MEQ/L)	38.9	38.6	38.8
RESIDUE (MG)[b]	7.2	7.0	7.1
PH	11.8	11.6	11.7

[a] Calcium assays by the Clinical Chemistry Department, Health Sciences Centre, Winnipeg, Manitoba, Canada.

[b] Resulting from the evaporation of 5 ml of the supernatant solutions.

Table 7. Some chemical characteristics of the supernatant solutions obtained from 4 mg/ml dispersions of llipta substance in water.[a]

	LLIPTA #1	LLIPTA #2
CALCIUM MEQ/L	2.5	4.0
MAGNESIUM MEQ/L	24.0	20.0
SODIUM MEQ/L	2.0	2.0
POTASSIUM MEQ/L	74.0	50.0
pH	10.8	10.3

[a] Assays by the Clinical Chemistry Department, Health Sciences Centre, Winnipeg, Manitoba, Canada.

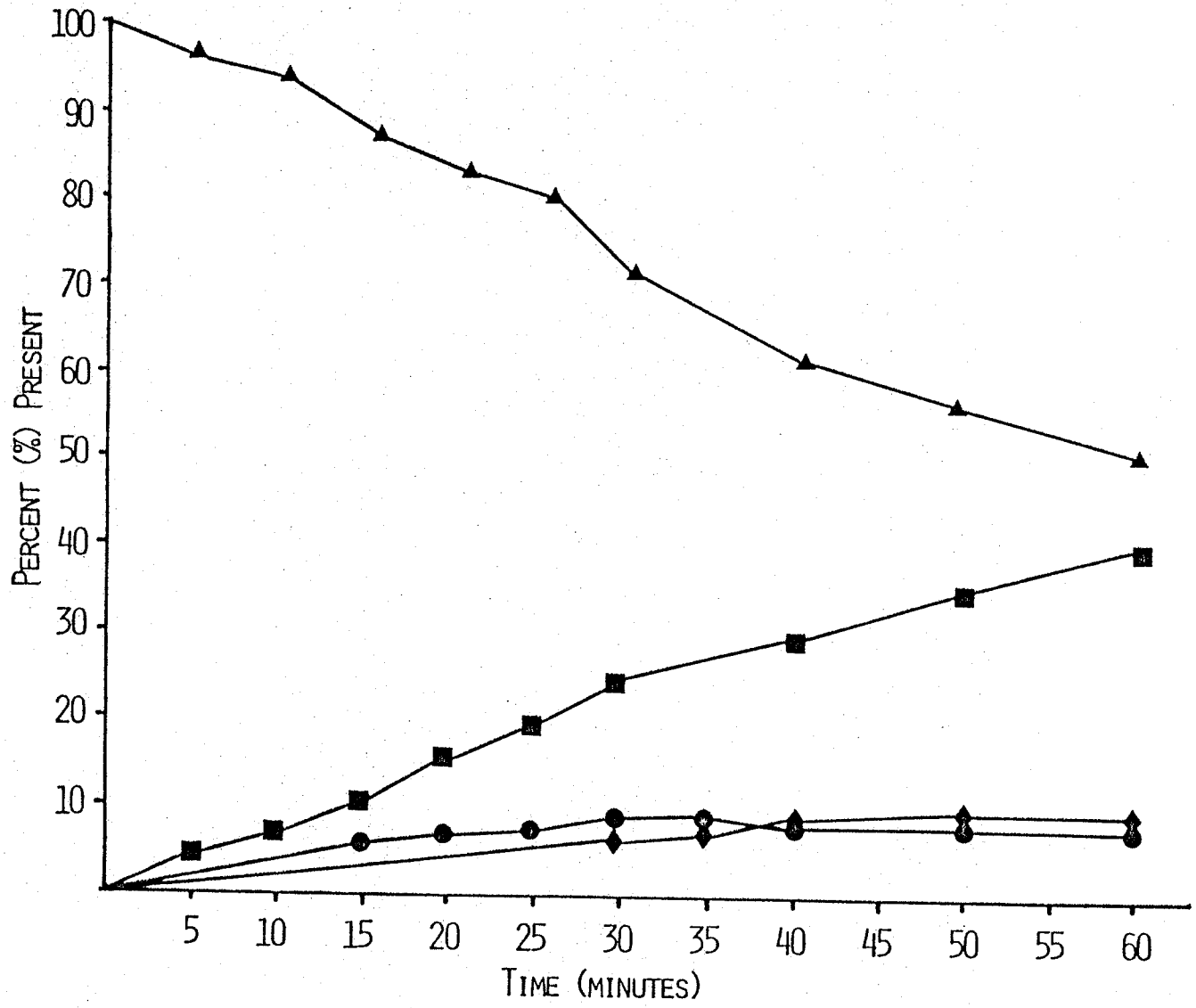


Figure 24. Cocaine hydrolysis in <u>llipta</u> supernatant solution, pH 10.7, at 37° C.
Key: ▲- cocaine, ■- benzoylecgonine, ●- ecgonine methylester, ◆- ecgonine.

Table 8. Comparison of cocaine first-order disappearance rate constants in different hydrolysis media studied.

SOLUTION	pH	K (HR^{-1})
HYDROCHLORIC ACID 0.1 M	1.2	.0001
SIMULATED GASTRIC USP	1.2	.0003
DISTILLED WATER	6.5	.0003
PHOSPHATE BUFFER 0.04 M	7.3	.0540
SIMULATED INTESTINAL USP	7.5	.1118
PHOSPHATE BUFFER 0.04 M	10.0	.6153
GLYCINE BUFFER 0.04 M	11.0	1.4534
SODIUM HYDROXIDE 0.04 M	11.9	9.5520

is obvious from the higher values of K that the hydrolysis of cocaine is more rapid in the more alkaline media. The data indicate that cocaine is stable at a pH less than 7. Above pH 7 its stability steadily decreases as the pH increases until at a pH greater than 11 the hydrolysis of cocaine is nearly complete in 25-30 minutes. When the concentrations of NaOH and $Ca(OH)_2$ media were each reduced to 0.01 N, the K values were reduced dramatically. The higher K values for the solutions of $Ca(OH)_2$ as compared to the values for the NaOH solutions at the same normality indicate a facilitative or possibly a catalytic role for Ca^{++} in the hydrolysis of cocaine. Some other interesting facts become apparent when one considers the rate constants of the different reaction steps involved in the hydrolysis of cocaine in various media (Table 9). Of the four possible hydrolysis steps the hydrolysis of the benzoylester function in the intact cocaine molecule (k_1^1) is the fastest reaction, while the hydrolysis of the methylester function in the intact cocaine molecule (k_1^2) is the slowest reaction. The hydrolysis of the benzoylester function in benzoylecgonine (k_2^2) is faster than either the hydrolysis of the methylester function in the intact cocaine molecule (k_1^2) or in ecgonine methylester (k_2^1).

Additional information can be obtained when ratios of some rate constants for the several reactions involved in the several media are examined. These ratios are presented in Table 10. The k_1^1/k_1^2 ratio relates to which pathway is preferred during the hydrolysis of cocaine to ecgonine, i.e., the ecgonine methylester pathway (k_1^1) or the benzoylecgonine pathway (k_1^2). Accordingly, since in each instance in Table 10 this ratio (k_1^1/k_1^2) is greater than one, the ecgonine methylester pathway

Table 9. Comparison of first-order rate constants (min^{-1}) corresponding to the different reaction steps involved in the hydrolysis of cocaine according to the model depicted in Figure 20 as obtained in various alkaline (pH>10) media.

SOLUTION	CONCENTRATION	pH	K	k_1^1	k_1^2	k_2^1	k_2^2
CALCIUM HYDROXIDE	0.04 N	11.8	.184	.162	.0227	.0477	.111
SODIUM HYDROXIDE	0.04 N	11.9	.159	.141	.0185	.0458	.0799
ISHKU	b	11.7	.138	.116	.0220	.0440	.0777
CALCIUM HYDROXIDE	0.01 N	11.1	.0549	.0433	.0116	.0315	.0395
SODIUM HYDROXIDE	0.01 N	11.3	.0490	.0381	.0109	.0325	.0368
LLIPTA		10.8	.0121	-	-	-	-

[a] $K = k_1^1 + k_1^2$.

[b] Supernatant solution resulting from 4 mg/ml dispersion in water.

Table 10. Comparison of ratios of some first-order rate constants for the reactions involved in hydrolysis of cocaine according to the model depicted in Figure 20 as obtained in various alkaline (pH>10) media.

SOLUTION	CONCENTRATION	$_{p}H$	k_1^1 / k_1^2	k_2^2 / k_2^1	k_1^1 / k_2^2	k_1^2 / k_2^1
CALCIUM HYDROXIDE	0.04 N	11.8	7.12	2.33	1.45	0.48
SODIUM HYDROXIDE	0.04 N	11.9	7.61	1.74	1.76	0.40
ISHKU	[a]	11.7	5.27	1.77	1.49	0.50
CALCIUM HYDROXIDE	0.01 N	11.1	3.74	1.25	1.10	0.37
SODIUM HYDROXIDE	0.01 N	11.3	3.49	1.13	1.03	0.34

[a] Supernatant solution resulting from a 4 mg/ml dispersion in water.

is favored over the benzoylecgonine pathway. The k_1^1/k_2^2 ratio shows rather unexpectedly that the hydrolysis of the benzoylester function in the intact cocaine proceeds at a faster rate than the hydrolysis of the same ester function in the less hindered benzoylecgonine. In a more expected fashion, the k_1^2/k_2^1 ratio indicates that the hydrolysis of the methylester function in the intact cocaine proceeds at a slower rate than the hydrolysis of the same ester function in ecgonine methylester. The values obtained for the ishku supernatant solution show that cocaine hydrolysis in this media proceeds in a manner very characteristic of the hydrolysis found in the more concentrated NaOH and $Ca(OH)_2$ solutions.

IV - C Ecgonine Alkaloid Urinary Excretion

The results of the preliminary rat experiment involving the oral and the subcutaneous administration of cocaine are presented in Table 11. The amounts of each alkaloid excreted in the urine of the rat for the first 24-hour period following dosing is expressed as a percent of the total cocaine dose administered. The total alkaloid excretion is greater following the subcutaneous route of administration than after oral administration. In the case of the subcutaneous route, the bulk of the alkaloid excreted was made up of benzoylecgonine, but in the case of the oral route ecgonine methylester. The amount of unchanged cocaine excreted in the case of this one rat studied was greater following the parenteral than after enteral administration. Nonetheless, following either route of cocaine administration the urinary excretion of ecgonine was minimal.

In the present research only the simple ester hydrolysis products of cocaine were of particular interest. For this reason no N-demethylated metabolite of cocaine was examined with respect to the analytical procedure employed. The possibility exists, therefore, that the BSA derivatives

Table 11. Twenty-four hour rat[a] urinary excretion of ecgonine alkaloids following cocaine administration.

ALKALOID	% TOTAL DOSE RECOVERED	
	INTRAGASTRIC[b]	SUBCUTANEOUS[c]
COCAINE	0.1	5.1
BENZOYL-ECGONINE	8.3	45.4
ECGONINE METHYLESTER	11.1	6.7
ECGONINE	1.4	2.1
TOTAL	20.9	59.3

[a] One rat, 600g
[b] 20 mg/kg cocaine given as the hydrochloride.
[c] 10 mg/kg cocaine given as the hydrochloride.

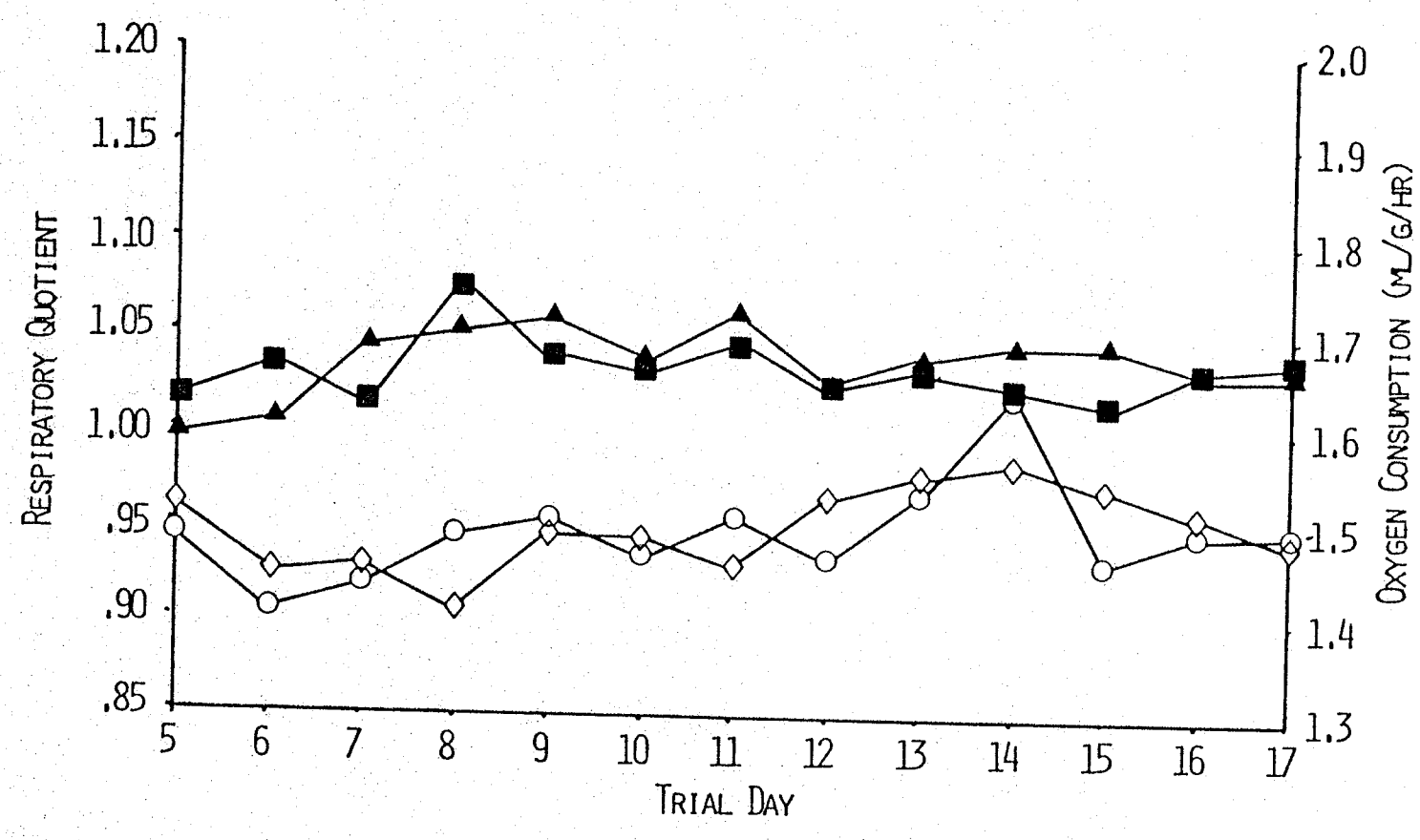


Figure 25. Profiles of daily respiratory quotients (Key: ■- control group, ▲- treated group) and oxygen consumptions (Key: ○- control group, ◇- treated group) for rats in the ecgonine/low protein trial.

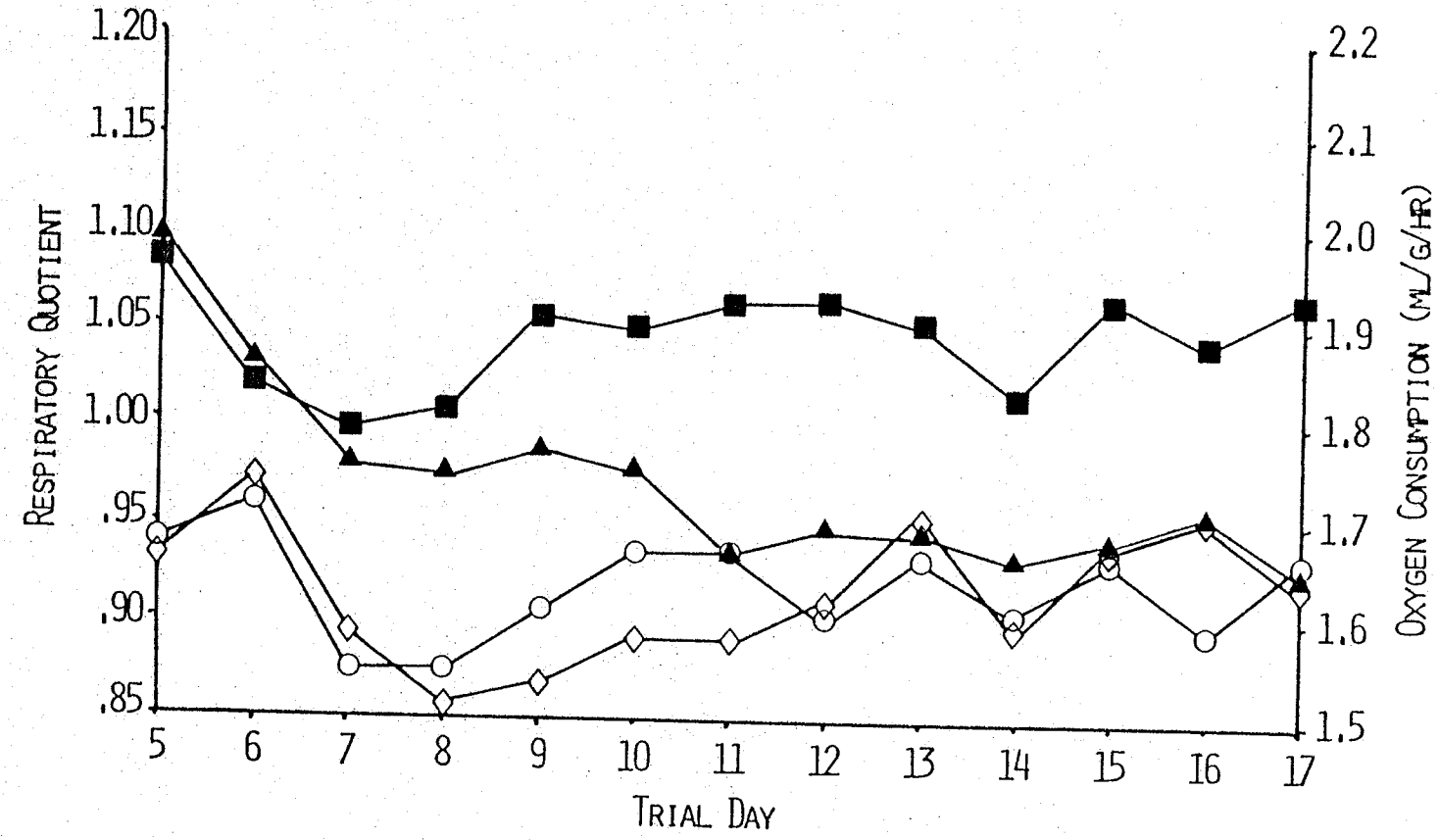


Figure 26. Profiles of daily respiratory quotients (Key: ■- control group, ▲- treated group) and oxygen consumptions (Key: ○- control group, ◇- treated group) for rats in the benzoylecgonine/low protein trial.

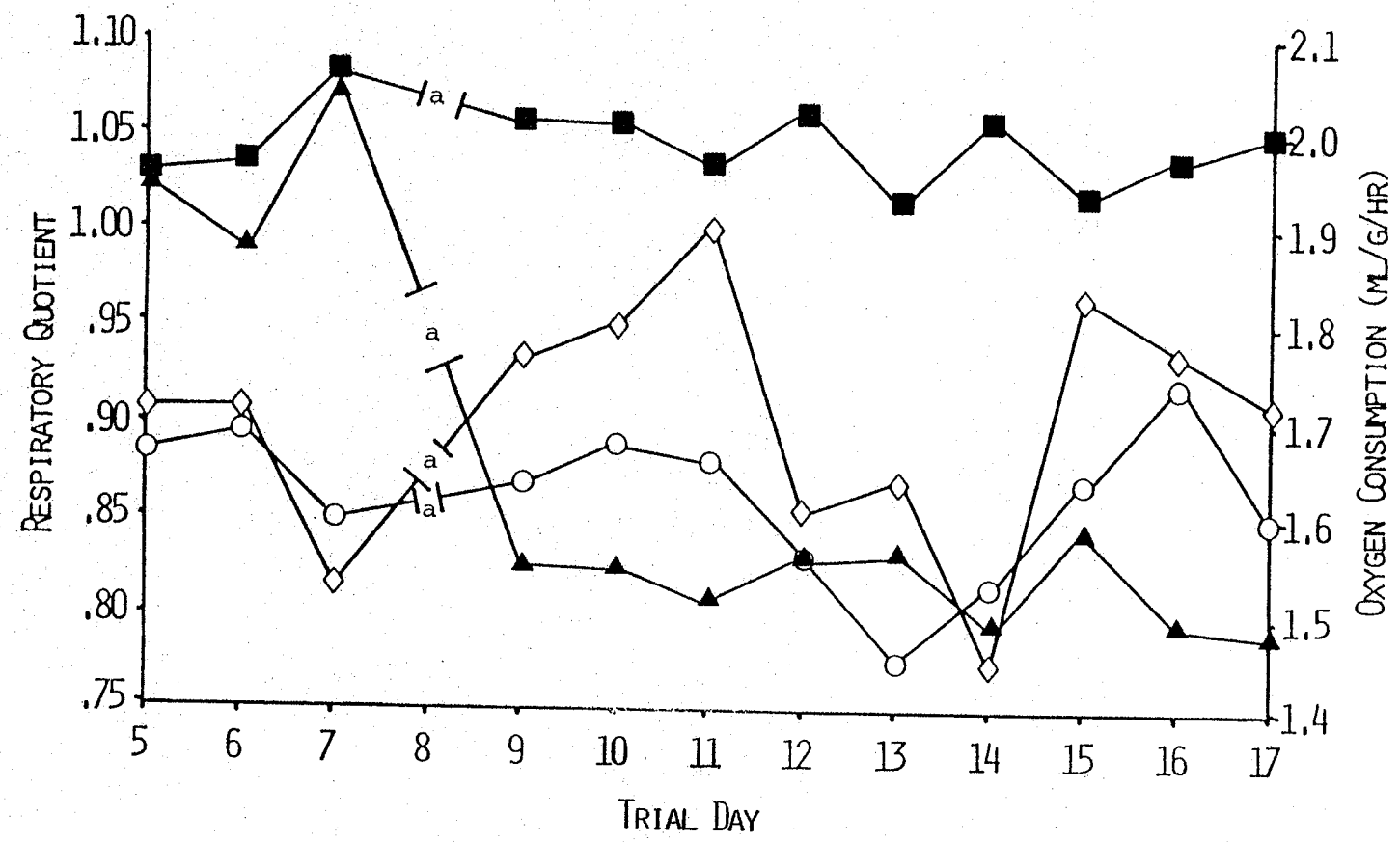


Figure 27. Profiles of daily respiratory quotients (Key: ■- control group, ▲- treated group) and oxygen consumptions (Key: ○- control group, ◇- treated group) for rats in the ecgonine methylester/low protein trial.

[a] Power failure in the laboratory.

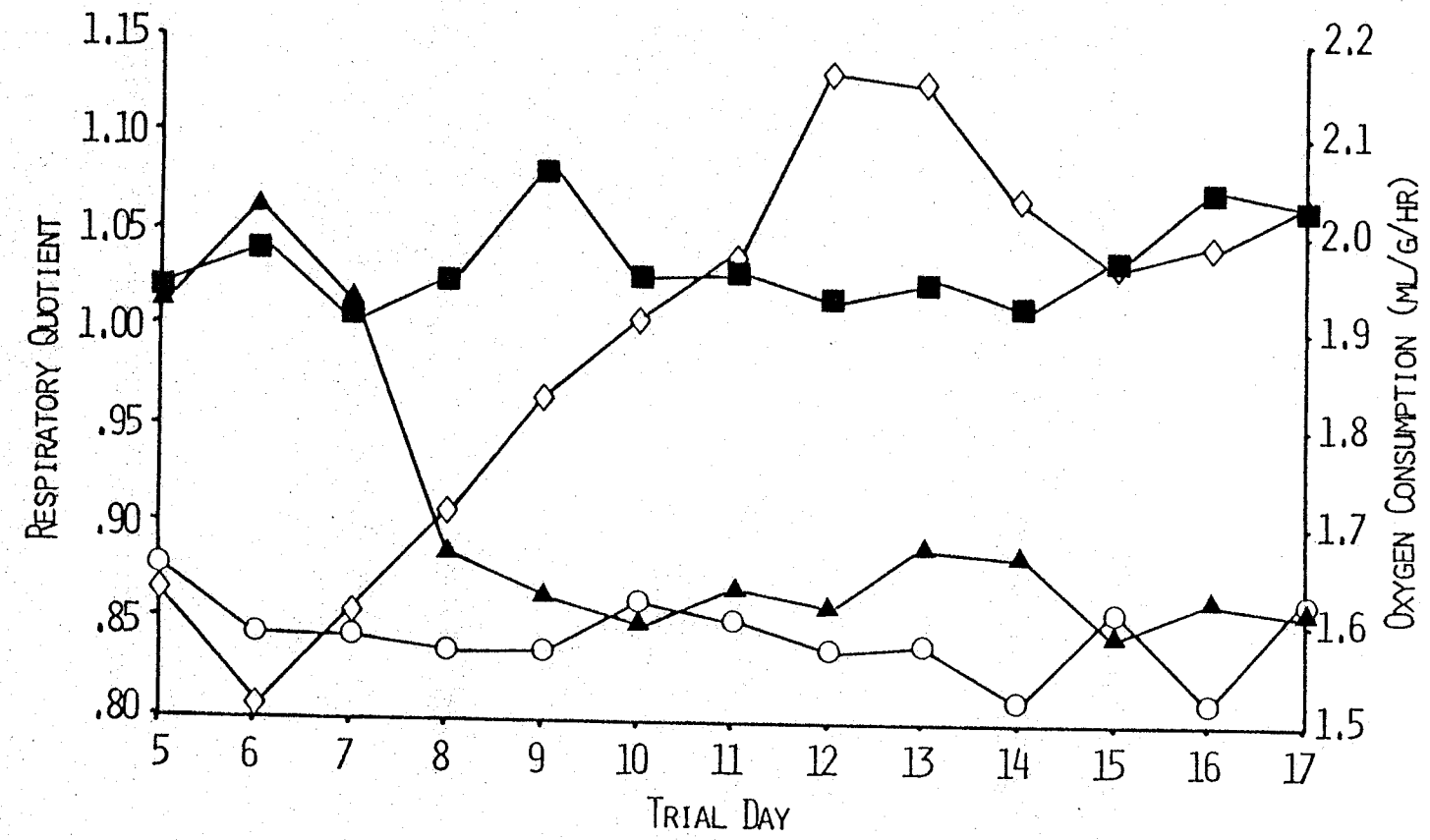


Figure 28. Profiles of daily respiratory quotients (Key: ■- control group, ▲- treated group) and oxygen consumptions (Key: ○- control group, ◇- treated group) for rats in the cocaine/low protein trial.

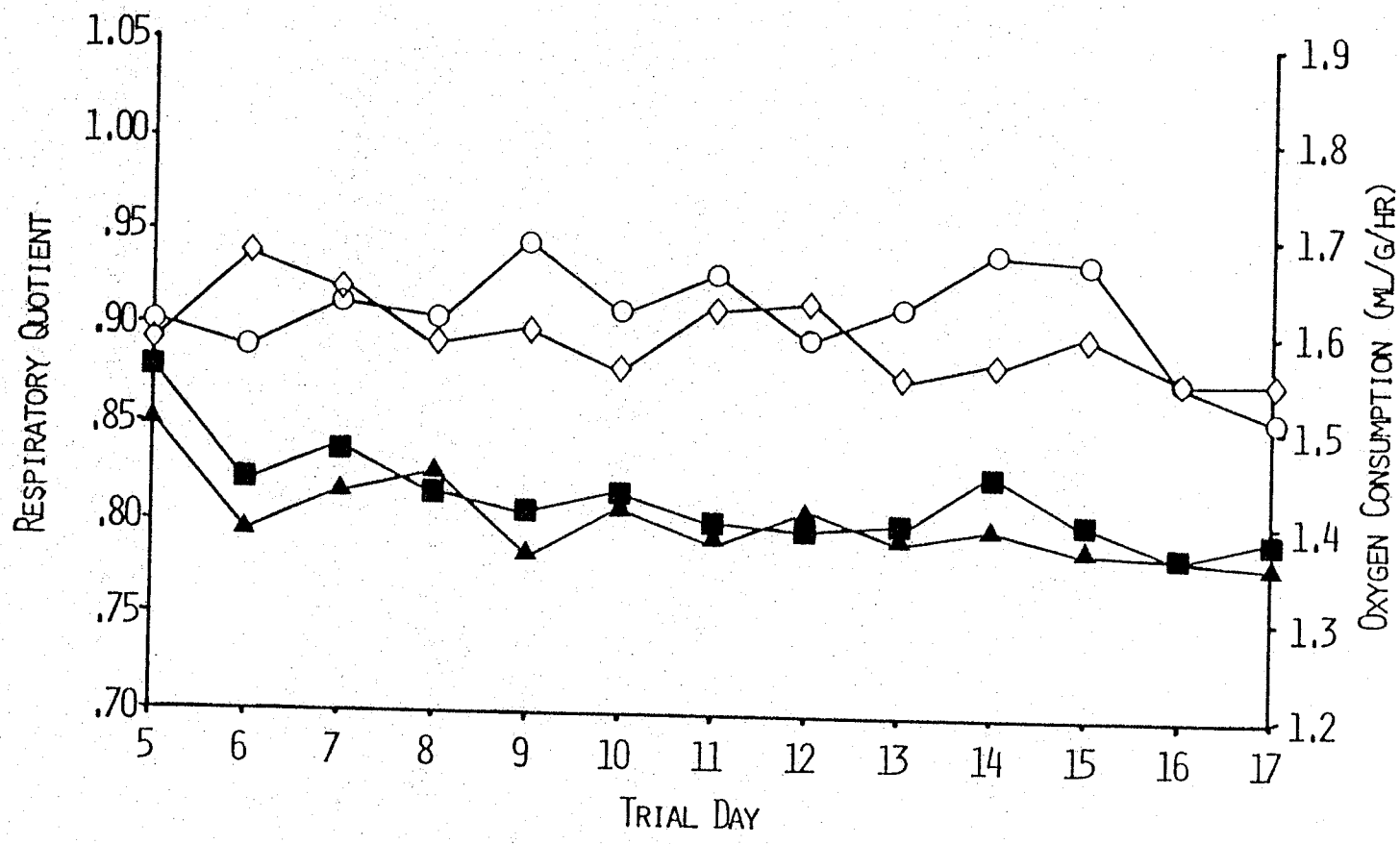


Figure 29. Profiles of daily respiratory quotients (Key: ■- control group, ▲- treated group) and oxygen consumptions (Key: ○- control group, ◇- treated group) for rats in the cocaine/high protein trial.

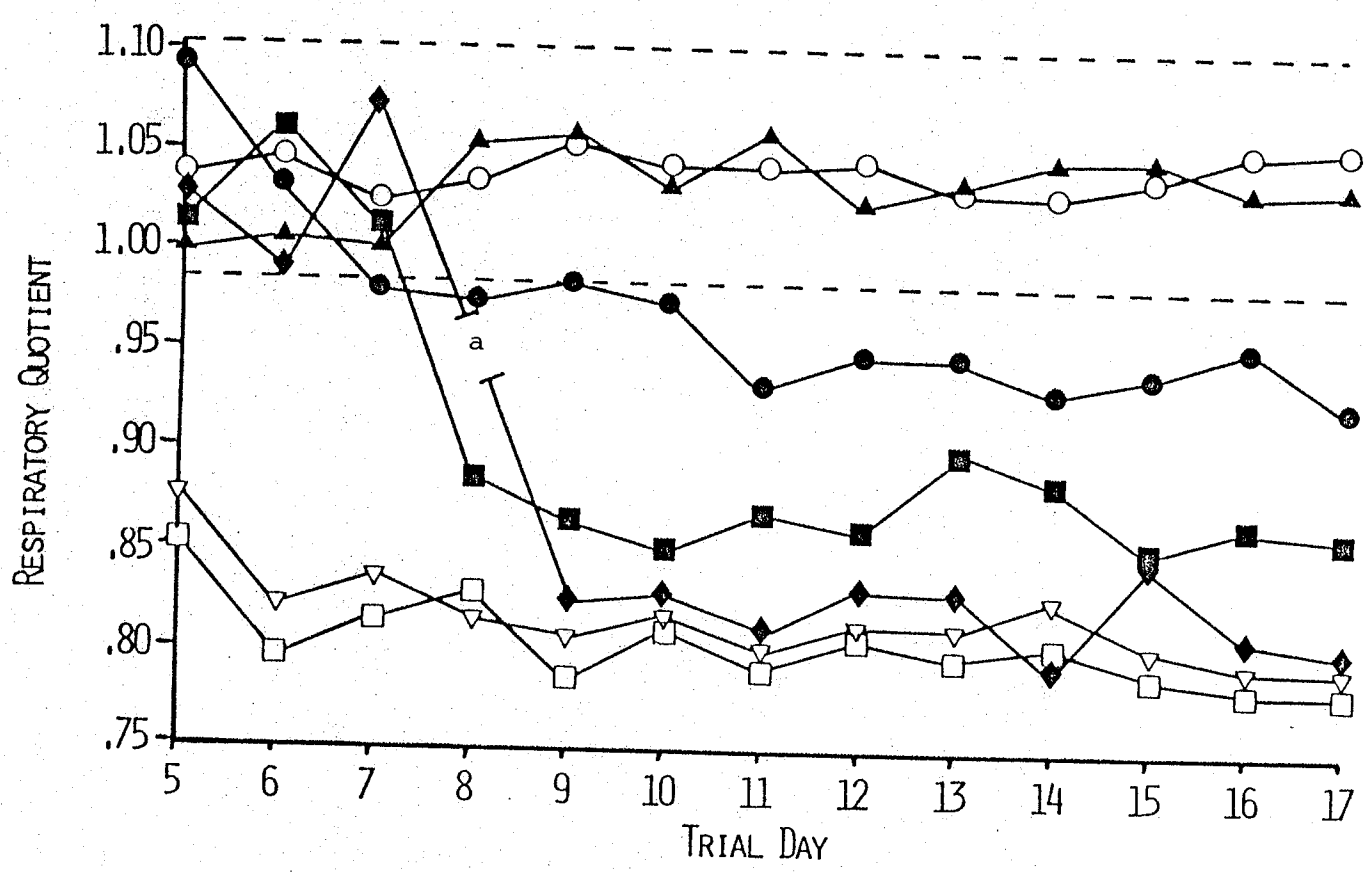


Figure 30. Profiles of daily respiratory quotients for rats in the different metabolic trials. Key: ○- control/low protein (=== - ± one standard deviation), ▲- ecgonine/low protein, ●- benzoylecgonine/low protein, ◆- ecgonine methylester/low protein, ▽- control/high protein, ■- cocaine/low protein, □- cocaine/high protein.

[a] Power failure in the laboratory.

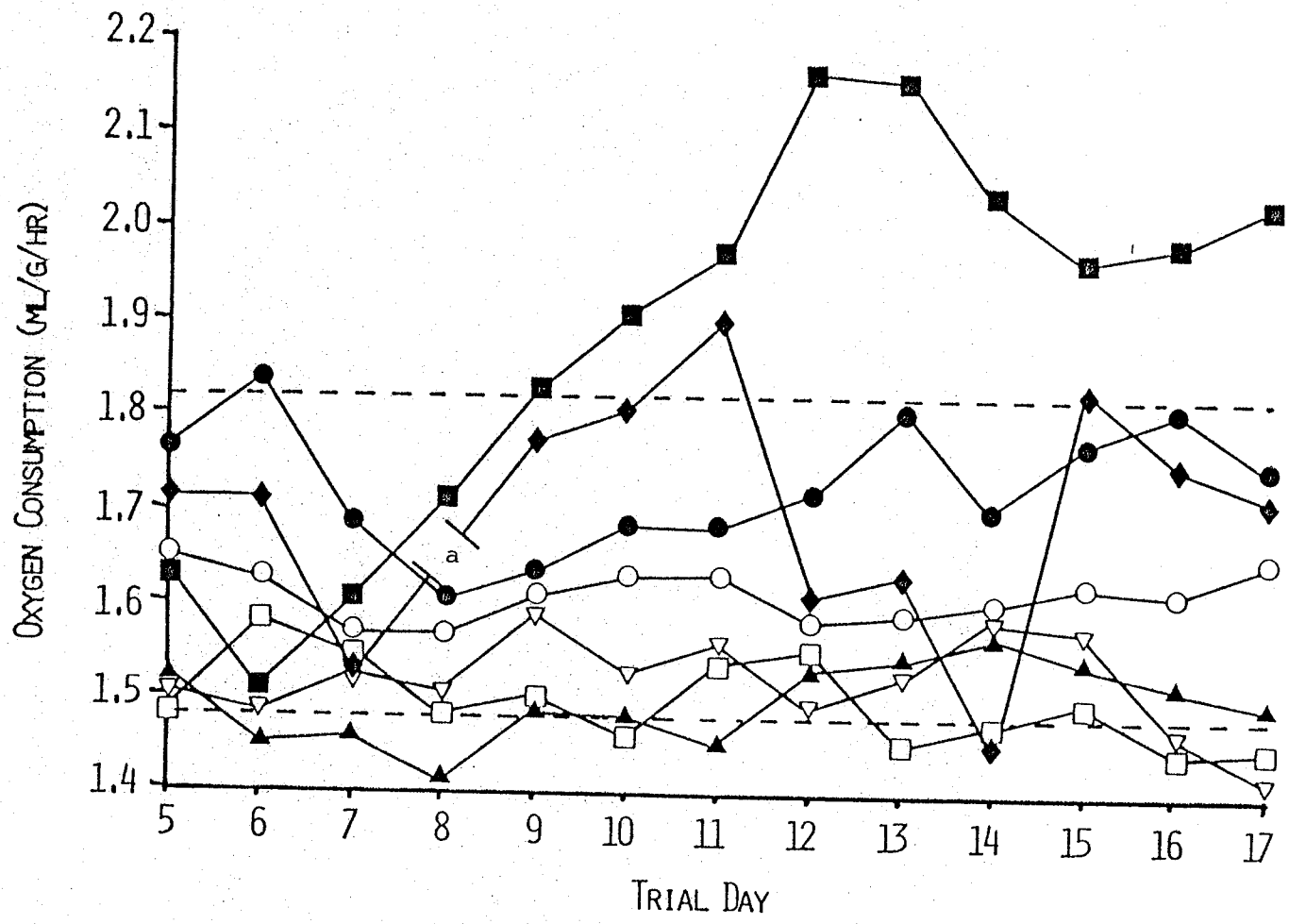


Figure 31. Profiles of daily oxygen consumptions for rats in the different metabolic trials. Key: ○- control/low protein (=== - ± one standard deviation), ▲- ecgonine/low protein, ●- benzoylecgonine/low protein, ◆- ecgonine methyl-ester/low protein, ▽- control/high protein, ■- cocaine/low protein, □- cocaine/high protein.

[a] Power failure in the laboratory.

or norcocaine, norbenzoylecgonine, norecgonine methylester, and norecgonine can be co-chromatographed with their non-demethylated counterparts.

IV - D Rat Energy Metabolism

The results of the metabolic trials are profiled in Figures 25 through 31. Summaries of the energy metabolism data together with some information concerning food intake and weight gain are presented in Tables 12 through 16. Other than hyperactivity observed in the rats of the treated group during the cocaine/low protein trial, no adverse effect was observed in any of the rats of the study. No animal died during any of the trials of the energy metabolism study. All rats grew regardless of which diet they received. The only trial in which there was a significant difference between the weight gain of the treated as compared to its pair-fed control was the ecgonine methylester/low protein trial in which the treated group gained significantly more weight over the experimental period (Table 14). This occurred in spite of the fact that food consumption, because of paired-feeding, was the same for both the control and treated groups. The treated group of the benzoylecgonine/low protein trial gained substantially more weight than its control but this gain was not significant. The treated groups of these two trials, the ecgonine methylester/ and the benzoylecgonine/low protein trials, were the only two groups of rats to show a decrease in carbon dioxide production as compared to their respective controls.

The oxygen consumptions of all alkaloid treated groups in each trial except that in the cocaine/low protein trial were not significantly different from that of their respective control groups. In the case of the cocaine/low protein trial the treated group had oxygen consumptions that were substantially greater than the control group (Table 15). This

Table 12. Summary of energy metabolism data for rats[a] over the experimental period (Day 8-17 incl.) in the ecgonine/low protein trial.

	CONTROL	TREATED
RESPIRATORY QUOTIENT	1.039	1.043
OXYGEN CONSUMPTION ML/G/HR	1.507	1.505
PERCENT WEIGHT GAIN	16.3	14.5
DAILY FOOD CONSUMPTION GRAMS	17.4	

[a] Six controls and six treated, pair-fed.

Table 13. Summary of energy metabolism data for rats[a] over the experimental period (Day8-17 incl.) in the benzoylecgonine/low protein trial.

	CONTROL	TREATED
RESPIRATORY QUOTIENT	1.048[b]	.952[b]
OXYGEN CONSUMPTION ML/G/HOUR	1.732	1.719
PERCENT WEIGHT GAIN	18.3	23.6
DAILY FOOD CONSUMPTION GRAMS	16.6	

[a] Six controls and six treated, pair-fed.
[b] Control and treated significantly different at $p < .05$.

Table 14. Summary of energy metabolism data for rats[a] over the experimental period (Day8-17 incl.) in the ecgonine methylester/low protein trial.

	CONTROL	TREATED
RESPIRATORY QUOTIENT	1.044[b]	.816[b]
OXYGEN CONSUMPTION ML/G/HR	1.611	1.721
PERCENT WEIGHT GAIN	5.8[b]	9.6[b]
DAILY FOOD CONSUMPTION GRAMS	11.0	

[a] Six controls and six treated, pair-fed.
[b] Control and treated significantly different at $p < .05$.

Table 15. Summary of energy metabolism data for rats[a] over the experimental period (Day8-17 incl.) in the cocaine/low protein trial.

	CONTROL	TREATED
RESPIRATORY QUOTIENT	1.038[b]	.867[b]
OXYGEN CONSUMPTION ML/G/HR	1.580[b]	1.978[b]
PERCENT WEIGHT GAIN	7.1	8.3
DAILY FOOD CONSUMPTION GRAMS	12.2	

[a] Six controls and six treated, pair-fed.
[b] Control and treated significantly different at $p < .05$.

Table 16. Summary of energy metabolism data for rats[a] over the experimental period (Day8-17 incl.) in the cocaine/high protein trial.

	CONTROL	TREATED
RESPIRATORY QUOTIENT	.804	.797
OXYGEN CONSUMPTION ML/G/HR	1.520	1.480
PERCENT WEIGHT GAIN	12.5	11.5
DAILY FOOD CONSUMPTION GRAMS	14.4	

[a] Six controls and six treated, pair-fed.

increase occurred in the only group which displayed hyperactivity.

The respiratory quotients of the alkaloid treated groups of the several trials: benzoylecgonine/low protein semi-synthetic diet (Table 13), ecgonine methylester/low protein semi-synthetic diet (Table 14), and cocaine/low protein semi-synthetic diet (Table 15), were significantly lower than the respiratory quotients of their respective control groups. The respiratory quotients between the treated and control groups of the ecgonine/low protein (Table 12) and of the cocaine/high protein (Table 16) trials were not significantly different.

When the ecgonine alkaloids are ranked with respect to their ability to lower the respiratory quotient of rats maintained on low protein semi-synthetic diets, ecgonine methylester produced the greatest significant depression followed in order by cocaine and benzoylecgonine; ecgonine was without measurable effect. What may be of significant physiological sig-ficance is the finding that neither the cocaine-elicited elevation of oxygen consumption or depression of the respiratory quotient found in the cocaine/low protein trial occurred in the cocaine/high protein trial.

The results of the daily determinations of the oxygen and carbon dioxide concentrations in the tank air used for the present metabolic studies are presented in Table 17. Duplicate determinations of the samples were performed in a matter of 5 to 10 minutes.

A sample of the calculation of the metabolic parameters is presented in Table 18.

Average daily urinary total nitrogen excretion data available from a separate trial involving rats on a cocaine/low protein semi-synthetic

Table 17. Summary of analyses by the Micro-Scholander method of tank-air used in the metabolic studies.

	TANK #1		TANK #2	
	CO_2	O_2	CO_2	O_2
PERCENT COMPOSITION	0.095	19.481	0.043	21.145
COEFFICIENT OF VARIATION	4.2	0.7	4.7	0.2
SAMPLES ANALYZED	70		39	

Table 18. Sample energy metabolism calculation.

TANK AIR	SAMPLE	COLLECTION
% CO_2 - 0.040	% CO_2 - 1.950	TIME - 15 MIN
% O_2 - 21.120	% O_2 - 19.100	VOLUME - 3780 ML
		RAT - 160 G

$$CO_2 \text{ PRODUCED} = 3780 \times \frac{(1.950 - 0.040) \text{ ML}}{100}$$

$$= 72.198 \text{ ML}$$

$$O_2 \text{ CONSUMED} = 3780 \times \frac{(21.120 - 19.100) \text{ ML}}{100}$$

$$= 76.356 \text{ ML}$$

$$\text{R.Q.} = CO_2 \text{ PRODUCED}/O_2 \text{ CONSUMED}$$

$$= 72.198 \text{ ML}/76.356 \text{ ML}$$

$$= 0.946$$

$$\text{STANDARD OXYGEN CONSUMPTION} = \frac{76.356 \text{ ML}}{.25 \text{ HR} \times 160 \text{ G}}$$

$$= 1.909 \text{ ML/G/HR}$$

Table 19. Average twenty-four hour urinary total nitrogen excretion by rats[a] over the experimental period (Day8-19 incl.) in separate cocaine/low protein trial.[b]

	CONTROL	TREATED
TOTAL URINARY NITROGEN (MG/24HR)	36.1	38.5
COEFFICIENT OF VARIATION	28.6	30.1

[a] Five controls and five treated, pair-fed.

[b] Results based on data provided through the courtesy of R. Boni and F. Burczynski who performed the nitrogen balance study.

diet (without energy metabolism measurements) are summarized in Table 19[a]. There was no significant difference between the 24-hour amounts of total nitrogen excreted in the urine of the cocaine treated group and that of its control group.

IV - E Xylose Absorption in Rats

A sample of the standard assay curve for xylose in urine samples is presented in Figure 32. The results were reproducible from assay to assay provided strict attention was given to experimental detail especially with regard to the heating period. The linearity was not affected but the slope of the standard assay curve would vary depending on differences in heating times.

Urine volume was quite uniform among the rats tested; all rats studied except two excreted between 1.5 and 3 ml of urine during the 5-hour collection period. No rat showed any visible signs of toxicity during any of the xylose absorption trials.

The results of the three xylose absorption trials are summarized in Tables 20 through 22. There was no significant difference between the treated and control groups of the trial in which rats were tested for xylose absorption at the end of the metabolic study (Table 20). In the second trial (Table 21), when the alkaloids were administered simultaneously with the xylose test solutions some differences became apparent between the treated and control groups with respect to the amount of xylose excreted in the urine in the first five hours following dosing. Atropine, cocaine, and ecgonine methylester each appear to increase the amount of xylose excreted, while ecgonine seems to decrease slightly

[a]Results presented in Table 19 are based on data provided through the courtesy of R. Boni and F. Burczynski who performed the nitrogen balance study.

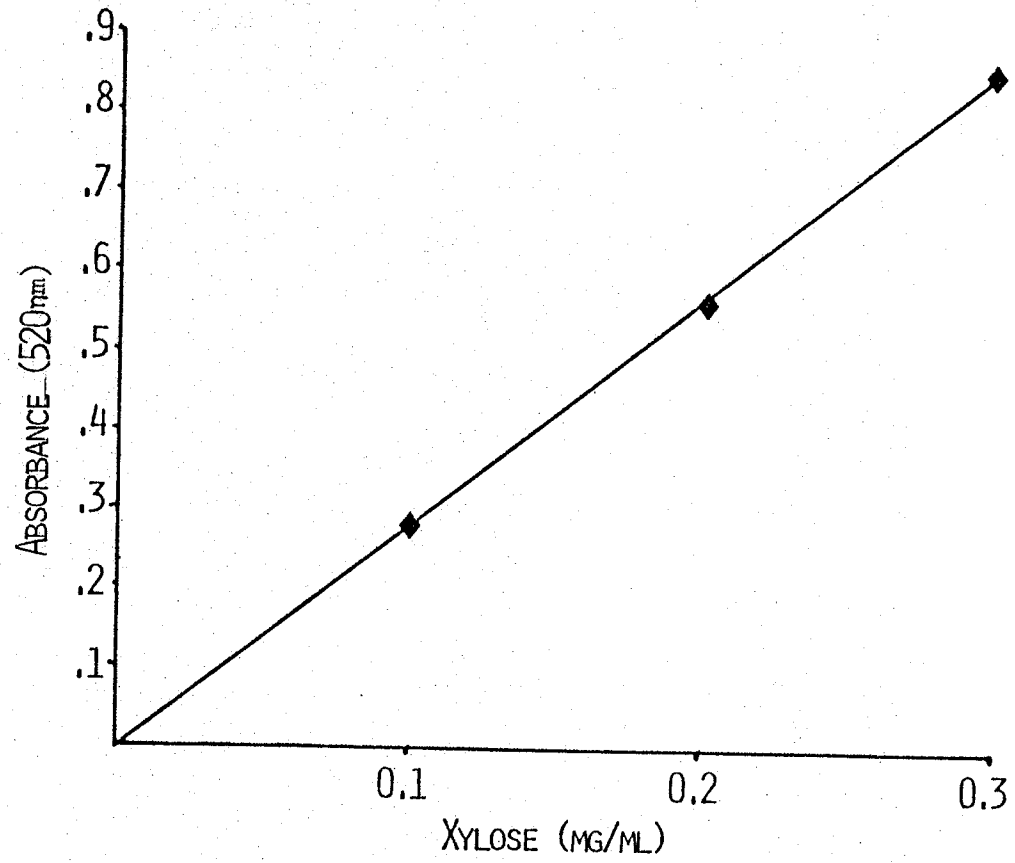


Figure 32. Sample standard curve for assay of xylose in urine samples of rats in xylose absorption studies.

Table 20. Summary of xylose urinary excretion data from xylose absorption trials in which the ecgonine alkaloids were administered in the diets prior to the xylose test.

TRIAL[a]	% XYLOSE DOSE EXCRETED[b]	
	CONTROL	TREATED
COCAINE-LOW PROTEIN	38.5	37.8
ECGONINE METHYLESTER-LOW PROTEIN	37.6	37.8
BENZOYL-ECGONINE-LOW PROTEIN	38.6	39.8
ECGONINE-LOW PROTEIN	41.5	39.4
COCAINE-HIGH PROTEIN	37.9	38.1

[a] Consist of 4 control and 4 treated pair-fed rats tested at the completion of each metabolic trial.

[b] In the first 5 hours following the xylose test dose.

Table 21. Summary of xylose urinary excretion data from xylose absorption trial[a] in which different individual alkaloids were incorporated into the xylose test dose for simultaneous administration.

	CONTROL	ATROPINE .15MG/KG	COCAINE 10MG/KG	ECGONINE METHYLESTER 10MG/KG	BENZOYL-ECGONINE 10MG/KG	ECGONINE 10MG/KG	XYLOSE 1.0G/KG
PERCENT XYLOSE EXCRETED[b]	37.4	47.2	44.6	43.2	36.5	35.6	21.9
COEFFICIENT OF VARIATION	4.8	4.4	4.9	5.8	6.0	5.9	5.0

[a] Two rats in control and each test group; all rats ate low protein semi-synthetic diet ad libitum for two days prior to xylose test.

[b] Calculated as a percent of the total xylose dose excreted in the first five hours following the xylose test dose.

Table 22. Summary of the xylose urinary excretion data from the xylose absorption trial[a] in which cocaine and ecgonine were incorporated into the xylose test dose for simultaneous administration.

	CONTROL	COCAINE 10MG/KG	ECGONINE 10MG/KG
PERCENT XYLOSE EXCRETED[b]	34.0	43.5[c]	30.2[c]
COEFFICIENT OF VARIATION	2.9	10.3	8.9

[a] Eight rats in control and each test group; all rats ate low protein semi-synthetic diet ad libitum for two days prior to xylose test.

[b] Calculated as a percent of the total xylose dose excreted in the first five hours following the xylose test dose.

[c] Significantly different from control at $p < .05$.

the amount of xylose excreted. Benzoylecgonine seems to produce little if any change. When the dose of xylose was doubled to 1000 mg/kg the absolute amount of xylose excreted was essentially the same as that excreted following the smaller, regular xylose dose. The differences observed between some of the individual groups in the second xylose absorption trial did not allow statistical evaluation because of the small number (only two) of rats making up each group. Consequently, the observed differences have been regarded as apparent rather than real.

In the third xylose absorption trial (Table 22) convincing evidence was obtained that cocaine significantly increased the percent total xylose dose excreted while ecgonine significantly reduced the percent total xylose dose excreted as compared to the control group. In each case the alkaloid was simultaneously administered with the xylose test solution.

IV - F Mouse Whole Body Composition

Sample standard assay curves for the determination of triglyceride, glucose, and glycogen in aliquots of original mouse whole body homogenate are shown in Figures 33, 34, and 35, respectively. These curves were obtained each day of analysis. They were reproducible provided, as was the case with the xylose assay, that strict attention was paid to the length of the heating periods. Once again the linearity was not destroyed but the slopes of the standard assay curves would change with variations in the duration of the heating periods. The size of the aliquots sampled from the homogenates were arrived at from estimates of fat, glucose, and glycogen in body tissues reported in the literature (94-96).

The results of the whole body mouse analyses for the first trial which was performed as a procedural control to check the methodology are

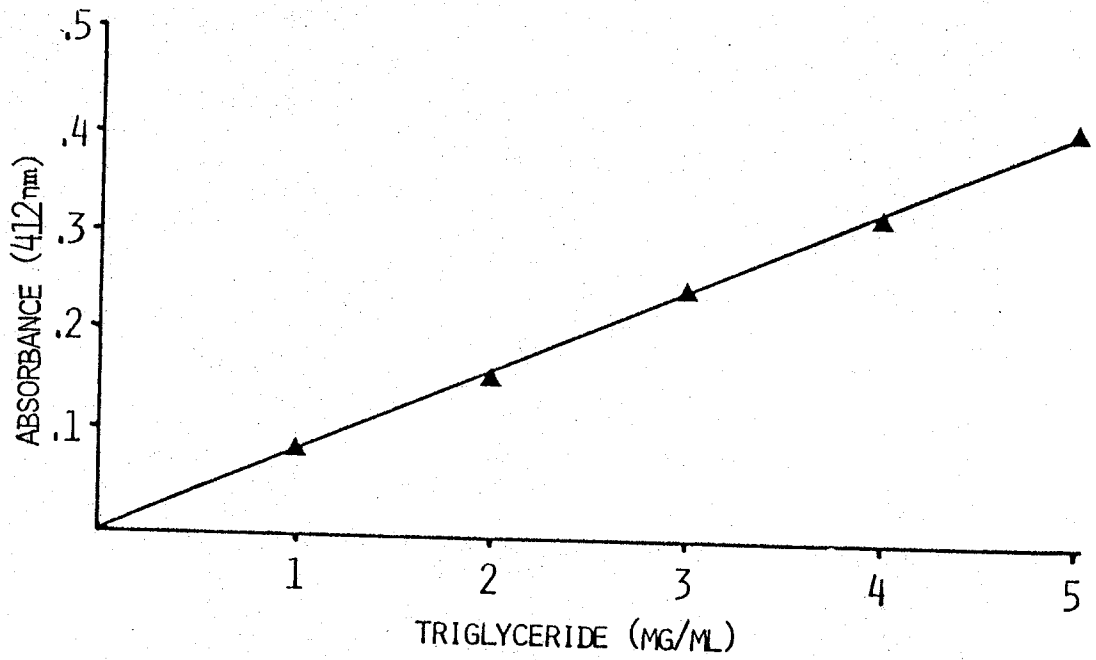


Figure 33. Sample standard curve for the assay of triglyceride in aliquots of homogenate in the mouse whole body analysis study.

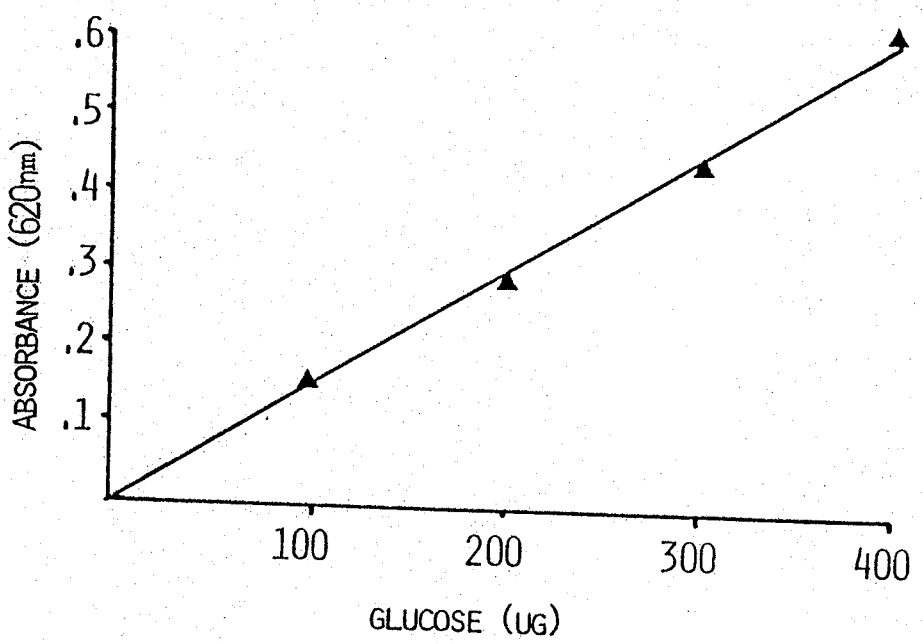


Figure 34. Sample standard curve for the assay of glucose in aliquots of homogenate in the mouse whole body analysis study.

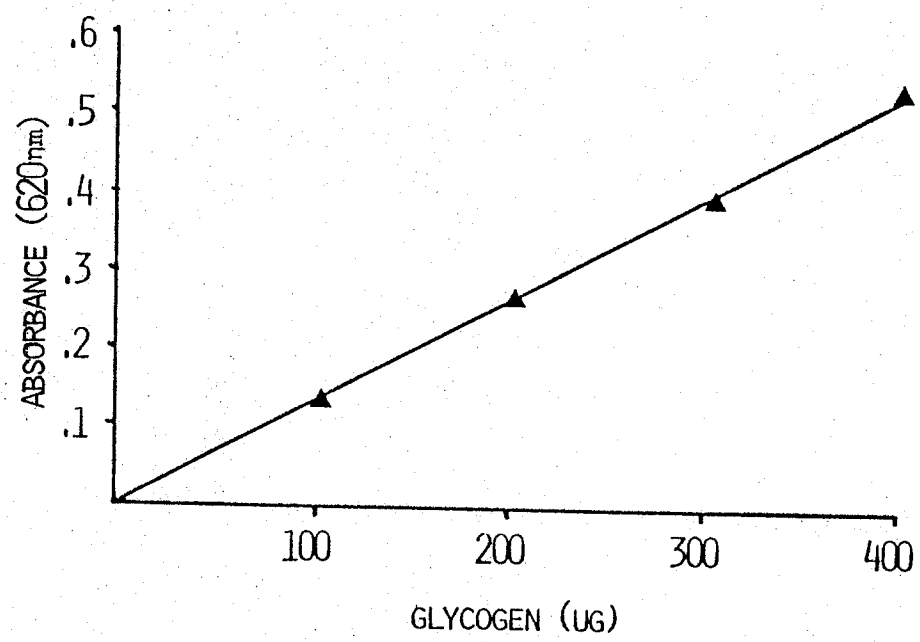


Figure 35. Sample standard curve for the assay of glycogen in aliquots of homogenate in the mouse whole body analysis study.

Table 23. Average whole body composition of twelve mice fed (ad libitum) a commercial high protein diet.

	BW	%TG	%GLU	%GLY	%N	TG/N	GLU/N	GLY/N	TG/GLU	TG/GLY
AVERAGE	23.6	4.53	.667	.345	3.00	1.51	.223	.115	6.80	13.2
COEFFICIENT OF VARIATION	12.5	4.8	4.4	8.8	4.1	6.2	5.6	8.4	8.3	11.5

Key: BW = dead body weight in grams, % = percent dead body weight, TG = triglyceride, GLU = glucose, GLY = glycogen, N = nitrogen.

presented in Table 23. The 12 mice in the trial were paired on a weight basis. One mouse in each pair was then assigned in a random manner to a hypothetical-control or hypothetical-treated group. Both groups were subjected to the whole body analyses for triglycerides, glucose, and glycogen content. When the resulting composition data was analyzed, there was no significant difference between the two hypothetical groups with respect to any of the three body components.

The treated mice of the cocaine/low protein trial all died before the end of the experimental period and were not subjected to the whole body analyses for any of the three body components.

The results of the whole body analyses of the mice in the ecgonine methylester/low protein trial are presented in Table 24. There were significant differences between the control and the ecgonine methylester-treated group in the calculated amounts of triglyceride and glycogen. The treated group had significantly lower triglyceride and significantly greater glycogen levels as compared to the control group. There was no difference between the control and treated groups with respect to any of the other assayed components. From Table 25 it can be seen that the differences between the control and treated groups with respect to triglyceride and glycogen remains significant when corrected for total body nitrogen. The calculated glucose/nitrogen ratio between the two groups was not significantly different. The calculated triglyceride/glucose and triglyceride/glycogen ratios between the control and treated groups were significantly different; this would be expected because of the difference between the two groups for the whole body content of triglyceride and glycogen.

Table 24. Average whole body compositions of mice[a] in the ecgonine methylester/low protein trial.

	BW		TG		GLU		GLY		N	
	CONTROL	TREATED	CONTROL	TREATED	CONTROL	TREATED	CONTROL	TREATED	CONTROL	TREATED
AVERAGE	19.1	18.7	6.45	4.65[b]	.531	.552	.332	.371[b]	2.91	2.85
COEFFICIENT OF VARIATION	12.0	12.8	7.9	13.1	10.9	13.2	10.7	10.7	4.9	4.6

[a] Six controls and six treated, pair-fed.
[b] Significantly different from control at $p < .05$.
Key: BW = dead body weight in grams, TG = triglyceride, GLU = glucose, GLY = glycogen and N = nitrogen each given in percent of dead body weight.

Table 25. Ratios calculated from average whole body composition data (Table 24.) obtained for mice[a] in the ecgonine methylester/low protein trial.

	TG/N		GLU/N		GLY/N		TG/GLU		TG/GLY	
	CONTROL	TREATED	CONTROL	TREATED	CONTROL	TREATED	CONTROL	TREATED	CONTROL	TREATED
AVERAGE	2.22	1.64[b]	.183	.194	.114	.131[b]	12.25	8.57[b]	19.7	12.7[b]
COEFFICIENT OF VARIATION	7.0	13.1	9.3	13.1	11.2	7.8	10.8	11.7	16.8	21.8

[a] Six controls and six treated, pair-fed.
[b] Significantly different from control at $p < .05$.
Key: TG = triglyceride, N = nitrogen, GLU = glucose, GLY = glycogen; each originally calculated as a percent of dead body weight.

CHAPTER V DISCUSSION

V - A Ecgonine Alkaloid Assay

Separation and quantitation of individual ecgonine alkaloids have been achieved in a number of ways for the study of these compounds in pharmaceutical samples (74), plant materials (97), and biological fluids (98). The analytical techniques used include spectrophotometry (99), thin layer- (100), gas- (101), and high pressure liquid chromatography (102), and immunological techniques (103). Until now, however, assays available for cocaine together with any of its several hydrolytic or metabolic products depended on techniques using radioisotope-labelled compounds (42, 54). In the present research, extraction and gas chromatographic procedures were successfully developed for the simultaneous assay of cocaine, benzoylecgonine, ecgonine methylester, and ecgonine.

The extraction procedures adopted in the present research to recover cocaine and the other ecgonine alkaloids in the hydrolysis and urinary excretion studies were modifications of reported methods (44, 100, 101). Efficient extraction of cocaine into organic solvents was achieved simply by adjusting the pH of aqueous solutions of biological fluids to approximately 7. Benzoylecgonine is best extracted at pH 9 to 10 with a mixed solvent system. To extract the amphoteric ecgonine, pH adjustment to 11-12 and use of a mixed-solvent system must be combined with salting out techniques. Little information is available regarding the extraction behavior of ecgonine methylester, but in the present research it was found to behave very much like cocaine.

In the hydrolysis studies, alkaloid concentrations were reasonably high and clean extracts were easily obtained. Simple pH adjustment and

salting-out (both achieved simultaneously by adding anhydrous potassium carbonate) resulted in high recoveries (~100%) of each of the alkaloids. Addition of the extracting solvent mixture prior to pH adjustment/salting-out with potassium carbonate was critical for maximum recoveries.

In the urinary excretion study in which the alkaloid concentrations were low and possible extraction of extraneous materials was of concern, the procedure was modified further. To extract cocaine, benzoylecgonine, and ecgonine methylester from urine samples, chloroform rather than the chloroform/2-propanol mixture was employed. The solvent was also added prior to adjusting the pH of the sample, but in this case a saturated solution of potassium carbonate was used to obtain pH 10. The recovery of both cocaine and ecgonine methylester was 100% but that of benzoylecgonine was only about 50%. This low recovery of benzoylecgonine was due to the use of a single rather than a mixed organic extracting solvent. Nonetheless, the recovery of benzoylecgonine was reproducible over the concentration range studied, with a coefficient of variation of less than 10%.

The extraction of ecgonine from urine samples was carried out by the combined procedure of pH adjustment to 11-12 and salting-out. Because the extracts were unsuitable for direct gas chromatographic (GC) analysis, a preliminary thin-layer chromatographic (TCL) clean-up procedure was instituted, reducing the overall recovery of ecgonine to 58%. This low recovery could be due to several factors related to the TLC procedure, including the incomplete scraping of the ecgonine bands, incomplete movement of the ecgonine from the origin, and/or incomplete extraction of the ecgonine from the silica gel. The recoveries of this

alkaloid, however, from spiked urine samples at the concentrations examined were reproducible with a coefficient of variation of less than 10%.

The GC procedure employed in this research was a modification of Moore's method (74) for benzoylecgonine and ecgonine in bulk drug samples of cocaine. It employed N, O-bistrimethylsilylacetamide (BSA) as the derivatizing agent and hexadecane and tetracosane as internal standards. In the present research BSA was also used for silylation but the two xanthines, theophylline and caffeine, were employed as internal standards because of their better chromatographic characteristics (Table 2). Because the extraction characteristics of the xanthines were different from those of the ecgonine alkaloids, the xanthine standards were incorporated just prior to derivatization rather than earlier in the assay procedure. In the course of developing the present GC procedure, an interaction was found to occur between theophylline and the ecgonine alkaloids (prior to BSA-derivatization) which resulted in reduced and variable theophylline peak heights. The interaction, possibly involving complex formation between the basic ecgonines and the acidic theophylline, was prevented by first adding the samples containing the ecgonine alkaloids to tubes containing 0.6 N HCl in 2-propanol, followed by the addition of the internal standard xanthine.

The lower limit of detection for each of the alkaloids in the GC procedure used was 20 ηg injected into the gas chromatograph. The working standard assay curves for each of the alkaloids for both the hydrolysis and the urinary excretion studies were reproducible from assay to assay (Figures 8 and 10). The coefficient of variation for the replicates at each of the data points on the curves was less than 10% over the

concentration range of 20 to 400 µg/ml of each alkaloid in the hydrolysis studies, and of 2 to 20 µg/ml of each alkaloid in the urinary excretion study.

V - B Ecgonine Alkaloid Hydrolysis

The chemical hydrolysis of cocaine has been reported to be slow and incomplete, finally leading to ecgonine with benzoylecgonine appearing as the only intermediate (45, 59). During the hydrolysis of cocaine in enzyme systems involving esterases, ecgonine methylester and benzoylecgonine have been reported as intermediates (43, 54-56). Ecgonine was still identified as the final product.

The present research included an in-depth study of the hydrolysis of cocaine, benzoylecgonine, and ecgonine methylester in strongly alkaline solutions of sodium hydroxide (0.04 N) and calcium hydroxide (0.04 N). The analytical procedure was able to account for all of the original cocaine on a molar basis in terms of the ecgonine alkaloids determined. For the first time it has been shown that ecgonine methylester appears as a major intermediate in the chemical or non-enzymatic hydrolysis of cocaine to ecgonine when the pH is greater than 11 (Figures 18 and 19). The hydrolysis of cocaine was complete within 30 minutes. The same rapid disappearance of cocaine was found in aqueous extracts of ishku (Figure 23) and ecgonine methylester is again the major intermediate. Accordingly, the exposure of cocaine to the alkaline ishku for even a short time during the coca chewing process would result in substantial hydrolysis of cocaine. This observation certainly would not support the earlier concept that the alkaline materials added to the coca leaf simply enhanced the extractability of cocaine from the leaf tissue.

Theoretical concentration-time profiles for the hydrolysis of cocaine at pH 11-12 (Figure 20) fitted well to the experimental data obtained. Cocaine hydrolyzes to ecgonine *via* two separate pathways, each consisting of two ester hydrolysis reaction steps. One pathway involves benzoylecgonine as the intermediate, while the other involves ecgonine methylester. The ecgonine methylester pathway is favored over the benzoylecgonine pathway by a factor of 7 to 8. An alternative hydrolysis model is not readily apparent to explain the alkaline hydrolysis phenomenon of cocaine.

According to the kinetic model developed for strongly alkaline systems, the hydrolysis of the benzoylester function in intact cocaine proceeds at a faster rate (Tables 9 and 10) than the hydrolysis of the same ester function in the apparently less hindered benzoylecgonine. A detailed study of the underlying mechanisms would be necessary to help explain the complex hydrolysis picture of cocaine.

When the concentration of the sodium hydroxide or the calcium hydroxide was changed from 0.04 N to 0.01 N, corresponding to a drop of only 0.4-0.6 pH units, the hydrolysis rate of cocaine was reduced approximately three-fold. The concentration-time profiles of the reaction components changed as well. There was a substantial reduction in the relative proportion of ecgonine methylester to benzoylecgonine. The new ratio was 3.5, which is approximately 50% as large as the corresponding ratio obtained at the higher concentration of either base.

The hydrolysis of cocaine was found to be faster in calcium hydroxide systems as compared to sodium hydroxide systems of the same normality. This finding suggests that calcium ions (Ca^{++}) could have some facilitative

or catalytic action in the hydrolysis of cocaine. It has been reported that Ca^{++} binds or complexes with certain of the ecgonine alkaloids (104). Perhaps Ca^{++} through such action favors the energetics of the formation of certain intermediates involved in the hydrolysis of cocaine resulting in more rapid cocaine hydrolysis.

In all hydrolysis media investigated having a pH less than 11, the hydrolysis of cocaine was slower and had a different profile than that in systems of pH greater than 11. Benzoylecgonine appeared as the major (Figures 14, 15, and 16), or the sole intermediate (Figures 11, 12, and 13) during the hydrolysis process to ecgonine. In pH 7.3 buffer cocaine hydrolysis proceeded at a moderate rate with only small amounts of ecgonine methylester being found in the system. In simulated intestinal fluid (pH 7.5) cocaine hydrolyzed faster than in the simple buffer system indicating some facilitative action on the part of the intestinal enzymes. Cocaine was found to be extremely stable in acid systems of pH 1 to 2, an observation previously reported by other workers (58). This stability of cocaine at low pH values is lost at elevated temperatures as is evident in some synthesis reactions involving cocaine. For instance, continued heating of an aqueous solution of cocaine at reflux yields benzoylecgonine almost quantitatively (92), while similar treatment of a highly acid solution of cocaine produces ecgonine (93).

In summary, in all systems studied of pH 11 or less, benzoylecgonine appears as the major intermediate in the hydrolysis of cocaine en route to ecgonine. Beyond pH 11 there is a dramatic change in the hydrolysis profile. Ecgonine methylester (not benzoylecgonine) now appears as the major intermediate. The latter occurs not only in hydrolysis systems

containing sodium hydroxide or calcium hydroxide, but also in supernatants of ishku.

V - C Ecgonine Alkaloid Urinary Excretion

In the preliminary study with one rat in the present research, the urinary excretion pattern of ecgonine alkaloids was different for the oral route as compared to the subcutaneous route of cocaine administration. When cocaine was administered intragastrically to the rat, the major urinary metabolite was ecgonine methylester, while following subcutaneous cocaine administration the major urinary metabolite was benzoylecgonine (Table 11). These results are in keeping with what has been reported by other researchers. The administration of cocaine to humans and animals by parenteral routes has been shown to result in substantial amounts of benzoylecgonine being excreted in the urine (44, 60). On the other hand, when cocaine was administered orally to humans the major urinary metabolite was ecgonine methylester (67). By either route of administration the amounts of ecgonine and unchanged cocaine excreted in the urine have been reported to be very small (44, 67).

It would appear that the oral ingestion of cocaine, especially if it occurs under the highly alkaline conditions of coca chewing, would result in substantial conversion of cocaine to ecgonine methylester. This ecgonine methylester, together with any produced during the intestinal absorption of cocaine, could result in substantial systemic absorption of ecgonine methylester. Thus, there is a definite possibility that a hydrolytic product could be responsible for some of the effects produced by oral cocaine administration. It will be necessary to obtain blood level data to confirm the presence of substantial amounts of cocaine

hydrolysis products following oral cocaine administration to support this possibility further. Results of the present research do demonstrate the susceptibility of cocaine to extensive chemical and metabolic conversion, supporting the concept that cocaine alone may not account for the biological effects seen in the South American natives who chew coca leaves.

V - D Rat Energy Metabolism

Closely standardized, reproducible procedures were developed to differentiate changes in energy metabolism between control and treated rats in each trial. The respiratory quotients (R.Q.) calculated from oxygen consumption and carbon dioxide production in the rat were taken as reliable indicators of the relative utilization of fat and carbohydrate as metabolic energy sources in the rat. The alkaloid-induced changes found in energy metabolism did not involve any intrinsic changes in the metabolism of protein since available data on daily urinary nitrogen excretion revealed that cocaine added to the low protein semi-synthetic diet of rats did not alter nitrogen balance (Table 19). R.Q. measurements indicate convincingly that cocaine, benzoylecgonine, and ecgonine methylester each produce changes in energy metabolism in the rat.

The average R.Q. value of 1.04 for four out of four control groups of rats in the low protein trials (Tables 12 through 16) indicate that carbohydrate utilization is taking place primarily for energy metabolism, while the R.Q. of 0.804 for the control group in the high protein trial indicates a mixed utilization of fat and carbohydrate. These values are comparable to those reported by others (105-108). Values for the oxygen consumption of all control rats in each trial ranged from 1.40 to 1.80 ml/g/hr and compared favorably to similar values reported in the

literature (109, 110).

In the trials with rats on the low protein semi-synthetic diet, the animals receiving ecgonine methylester (Figures 29 and Table 14), cocaine (Figure 28 and Table 15), or benzoylecgonine (Figure 26 and Table 13) 1 mg/g diet had significantly depressed R.Q. values when compared to their respective controls. The average R.Q. values obtained for the treated groups were 0.816, 0.867, and 0.952, respectively. The relative changes in values suggest an enhanced fat utilization in the treated animals as compared to their controls. One would not need to postulate any direct effect of the ecgonine alkaloids to explain the observed changes in energy metabolism. These could be explained in terms of the effects of endogenously released catecholamines on metabolism. The catecholamines per se exert a calorigenic effect in man and animals marked by an increase in oxygen consumption and an increase in oxidizable substrate; there is also an increase in the breakdown of triglycerides in adipose tissue (28, 111-113). The catecholamines can elevate blood glucose and lactate, but inhibit insulin secretion and depress the peripheral uptake of glucose (28). There is considerable evidence that cocaine enhances many of these catecholamine effects, e.g., elevation of free fatty acids, etc. (28). Consequently, cocaine and possibly any one of the ecgonine alkaloids, by enhancing the effects of the endogenous catecholamines, could be expected to produce substantial changes in the energy metabolism of animals or man, possibly through an improved utilization of fat.

The oxygen consumption of the rats receiving the low protein semi-synthetic diet spiked with cocaine or ecgonine methylester was increased compared to that of their controls. Benzoylecgonine seemed to produce no

change in oxygen consumption. The carbon dioxide production of the rats receiving the low protein semi-synthetic diet spiked with ecgonine methylester or benzoylecgonine was decreased compared to that of their controls. These results are consistent with the fact that the treated groups in these two trials were the only groups of rats which showed a substantial increase in body weight during the experimental period, compared with their controls. Ecgonine added to the low protein semi-synthetic diet produced no measurable change in the energy metabolism of the rat.

The degree to which each of the ecgonine alkaloids, ecgonine methylester, cocaine, and benzoylecgonine as diet adjuncts depressed the R.Q. values of the rats could be a function of several interesting factors. Their individual absorption, distribution, metabolism, and excretion characteristics following oral administration would influence the final biological effects exerted by each. Differences in the pharmacokinetic characteristics among ecgonine methylester, cocaine, and benzoylecgonine could certainly modify their effects on energy metabolism. For example, it is not unexpected that ecgonine, the final product of cocaine hydrolysis, is without measurable effect on energy metabolism (Figure 25 and Table 12). Its highly polar nature which would lead to rapid urinary excretion and limited tissue distribution, precludes any significant pharmacological effect.

Cocaine added to the high protein semi-synthetic diet, produced in the rats neither the depression of the R.Q. nor the elevation of the oxygen consumption (Figure 29 and Table 16) that was found in the cocaine treated rats in the low protein trial. One could speculate that cocaine was without any apparent effect on the enhanced energy metabolism of the

rats on the high protein semi-synthetic diet because the animals were not under any nutritional stress due to the adequacy of the diet. Therefore, the endogenous levels of catecholamines (which presumably would be elevated under the stress of a low protein diet) were reduced to a point where any enhancement by cocaine was without measurable effect. It could also be argued that the higher dietary level of protein enabled the rat to metabolize and eliminate the cocaine more rapidly than when the rat received the low protein diet. As a result, the cocaine and/or its hydrolytic/metabolic products would remain in the system for too short a time to produce any measurable biological effect (114, 115).

The Andean coca chewers live under conditions in which they are stressed both nutritionally by their low protein diets, and environmentally by the cold temperatures and reduced oxygen tensions in their mountain habitat. These factors combined could result in increased levels of endogenous catecholamines, the metabolic effects of which, particularly with respect to increased catabolism of fat, could be enhanced by the action of the cocaine, and/or ecgonine methylester, and/or benzoylecgonine produced by the chewing of the coca leaf.

V - E Xylose Absorption in Rats

In the xylose absorption trial involving rats selected for testing at the termination of the metabolic trials (Table 20), no difference was found between the treated and control groups in the percent xylose dose excreted. The treated rats had received the individual alkaloids in their daily diets. This could indicate that there was no lasting alkaloid-induced change in the absorptive capacity of the intestine. It could also indicate that in the animals (fasted for 18 hours prior to being subjected to

the xylose absorption test), no alkaloid was left to exert any measurable effect on xylose absorption. The latter explanation is supported by the fact that the ecgonine alkaloids have short biological half-lives (<1.5 hr) (62, 63, 65).

In the separate preliminary trial in which a limited number of rats received the xylose test solution simultaneously with the alkaloids, some evidence was obtained that some of the alkaloids can produce direct effects on xylose absorption (Table 21). Cocaine and ecgonine methylester seemed to increase, while ecgonine seemed to decrease xylose absorption. Benzoylecgonine had little effect upon the percent xylose dose excreted. Atropine was included as an experimental control because of its known positive effect on the absorption of xylose (80), an effect so demonstrated in this trial.

These preliminary results were sufficiently encouraging to warrant a more definitive trial involving only cocaine and ecgonine. The data obtained (Table 22) confirmed that in the dose administered cocaine significantly increased, while ecgonine significantly decreased, the percent xylose dose excreted in the urine, provided the alkaloids were administered simultaneously with the xylose test solution. The mechanisms by which cocaine increases xylose absorption in the rat as measured by xylose urinary excretion may have their basis in the adrenergic action exerted by cocaine on intestinal motility (28). Cocaine decreases intestinal motility, which by permitting longer contact time between the xylose and the intestinal mucosa would result in greater xylose absorption. In a similar fashion, atropine through its anticholinergic action on the intestine is believed to slow intestinal transit and thereby enhances

xylose absorption (80). The mechanism behind the decreased absorption of xylose produced by ecgonine is not readily apparent.

As already indicated, convincing evidence has been obtained in the xylose absorption trial that cocaine increases whereas ecgonine decreases the intestinal absorption of xylose (Table 22). The increased xylose absorption is specifically interpreted as corresponding to an increase in carbohydrate absorption (81). Thus, the increase in the relative utilization of fat over carbohydrate produced by cocaine and ecgonine methylester, as found in the energy metabolism trials, probably does not result from a reduced absorption of carbohydrate. On the other hand, the lack of any effect of ecgonine on fat utilization could be a consequence of its ability to reduce carbohydrate absorption. The possibility that the absorption of dietary fat may be affected by one or more of the several alkaloids remains unexplored.

V - F Mouse Whole Body Composition

In the procedural control of the mouse whole body composition trial, there was little mouse-to-mouse variation in the body components examined. The value of the whole body nitrogen agreed with the 3% value reported in other studies (91, 95). Direct comparison of the levels found for whole body constituents with corresponding literature values is difficult because most available composition data relate to levels of triglyceride, glucose, and glycogen (94) in isolated tissues of organs such as liver, muscle, or kidney.

All of the mice receiving the low protein diet containing cocaine at a level of 1 mg/g died before completion of the experiment; all were dead within seven days. It has been reported that cocaine produces severe

hepatotoxicity in mice (35, 116). It has also been reported that cocaine produces severe fatal hepatic necrosis in mice pre-treated with 3-methylcholanthrene or phenobarbital (117), although no mention was made of the dose or route of administration of the cocaine. It was suggested that the causative agent was a metabolite of cocaine which was specifically toxic to mice but not to rats, rabbits, or guinea pigs. The mice used in this present research were not pre-treated with 3-methylcholanthrene or phenobarbital, but they were maintained on a low protein diet. This diet may have enhanced their susceptibility to hepatotoxic agents such as cocaine (118).

In the mouse whole body composition trial involving the low protein diet with ecgonine methylester (Table 24), there was no difference between the control and ecgonine methylester group in the body levels of either glucose or nitrogen. Whole body levels of glycogen and triglycerides, however, were both significantly different between the control group and the ecgonine methylester-treated group (Table 24). The whole body glycogen level for the ecgonine methylester-treated group was 0.371% and for the control group was 0.332%. The triglyceride level of the treated group was 4.65% while that for the control group was 6.45%. The lower value for triglyceride in the treated group approached the 4.53% value observed in mice which received the high protein Purina diet in the procedural control trial. In considering these effects of ecgonine methylester upon stored body fat (triglyceride) and carbohydrate (glycogen) in the treated mice, it seems that the ecgonine methylester increases the utilization of fat and conserves carbohydrates. Ecgonine methylester may bring about this metabolic shift in a manner similar to cocaine by

enhancing the metabolic effects of endogenous catecholamines, which are known to spare glucose by increasing fat utilization (28, 111-113).

CHAPTER VI SUMMARY AND CONCLUSIONS

The combined analytical and hydrolysis techniques developed in this research permitted an in-depth evaluation of the hydrolysis of cocaine in a variety of *in vitro* systems. The results showed that contrary to accepted beliefs, ecgonine methylester is produced as an intermediate during the non-enzymatic alkaline hydrolysis of cocaine to ecgonine. The results also showed that cocaine is relatively resistant to hydrolysis at a pH of less than 7, but that it undergoes rapid hydrolysis in systems of pH greater than 11, including those systems employing the alkaline materials, e.g., *ishku*, with which coca leaves are chewed in certain mountainous regions of South America. This rapid hydrolysis proceeds from cocaine to ecgonine *via* two parallel hydrolysis pathways, one involving benzoylecgonine as the intermediate and the other ecgonine methylester. The ecgonine methylester pathway is greatly favored over the benzoylecgonine pathway at pH values greater than 11.

Cocaine as well as each of its hydrolysis products was evaluated with respect to its possible effects on the overall metabolic economy of laboratory animals. Rats and mice were maintained on diets comparable in composition to diets sustaining the South American coca chewers. Significant depression of respiratory quotients was found in rats fed the low protein-high carbohydrate diets containing cocaine, ecgonine methylester, or benzoylecgonine. Ecgonine methylester produced the greatest depression of the respiratory quotient, followed in turn by cocaine and benzoylecgonine. Ecgonine was without effect. Since, as shown by available data, cocaine added to the low protein diet of the rat does not change the excretion of nitrogen, the respiratory quotients measured in

similarly treated rats were reliable indicators of the relative utilization of fat and carbohydrate by the rats. The depressed respiratory quotients were indicative of increased relative utilization of fat.

The results of the metabolic studies involving ecgonine methylester in rats on low protein-high carbohydrate diets were confirmed by results of whole body analysis studies in mice similarly treated. The increased relative utilization of fat as indicated by depressed respiratory quotients in rats was reflected in the reduced whole body triglyceride levels in the mice. Ecgonine methylester added to a semi-synthetic diet that is low in protein and high in carbohydrate appeared to restore whole body constituents to levels comparable to those found in mice receiving a balanced diet of protein, carbohydrate, and fat.

In a preliminary single-rat experiment the oral administration of cocaine showed that the urinary metabolite profile following this route of administration was substantially different from that found following the subcutaneous administration of cocaine. The difference was in the relative amounts of benzoylecgonine and ecgonine methylester excreted. Ecgonine methylester is excreted in larger amounts than is benzoylecgonine following the oral administration of cocaine while benzoylecgonine is the major metabolite following the subcutaneous administration of cocaine. Following either route of administration, very little cocaine was excreted unchanged in the urine.

The xylose absorption test did not reveal any ecgonine alkaloid effect upon the absorption of xylose in rats tested following an 18-hour fast after the completion of the metabolic studies. When administered concurrently with the xylose test solution, cocaine increased the amount

of xylose absorbed while ecgonine decreased the amount absorbed. Because cocaine increased the absorption of xylose, the depression of the respiratory quotient found in the cocaine treated rats could not be due to a reduced availability of dietary carbohydrate as might result from a reduced intestinal absorption. This could possibly be the case with ecgonine which had no effect on respiratory quotient but did have a negative effect on the absorption of xylose. The possiblity that both cocaine and ecgonine could affect fat absorption was not ruled out.

In considering the practice of coca chewing by Andean natives in the light of the results obtained from the present investigation, one may be permitted certain speculations about the fate of the cocaine in the coca leaf material and its possible role in explaining the biological effects associated with its use. The cocaine released from the leaf is most likely subject to extensive hydrolysis in the gastrointestinal tract prior to its absorption. The hydrolysis products will include, depending on a variety of factors, varying proportions of cocaine, benzoylecgonine, ecgonine methylester, and ecgonine. Their effects upon the intestinal absorption of dietary carbohydrate will depend on the net proportion present of the stimulating components such as cocaine, and the inhibiting components such as ecgonine. In the coca chewer subsisting mainly on a low protein-high carbohydrate diet, it can be expected that the several ecgonine alkaloids released and/or formed through coca chewing will produce a net increase in the relative utilization of fat over carbohydrate as the primary source of metabolic energy. These metabolic effects may well be brought about through an enhancing effect of the ecgonine alkaloids upon the metabolic effects of endogenous

catecholamines. One should see in the Andean native a decreased respiratory quotient and a reduction in deposition of body fat.

It would appear that any model proposed to explain the phenomenon of coca chewing must take into account cocaine together with its hydrolysis products. The model does not need to include addiction to any of the coca leaf constituents. These constituents must be regarded as serving some essential function possibly in some role as dietary supplements. The chewing of coca leaf affords the Andean native a source of necessary vitamins present in the leaf material, and the latter, together with the added alkaline substance, provide much needed mineral supplements. Finally, as suggested from the results of the present investigation, the facilitative action of cocaine together with its hydrolytic products on carbohydrate absorption and fat utilization (in preference to carbohydrate utilization) suggests a central, vital role of ecgonine alkaloids in the normalization of the overall body metabolism of the Andean native. This speculation provides the most plausible explanation of the coca chewing phenomenon to date. It can be concluded that cocaine alone is not responsible for the biological effects associated with coca chewing. The hydrolysis products of cocaine, in particular ecgonine methylester, most certainly play a greater biological role than heretofore realized in coca chewing.

REFERENCES

1. Gay, G.R., Inaba, D.S., Sheppard, C.W., and Newmeyer, J.A. Clin. Toxicol. 8, 149, (1975).

2. Gutierrez-Noriega, C., and Von Hagen, V.W. Scientific Monthly Feb. 81, (1950).

3. The Merck Index, 8th Edition, Merck and Co. Inc., Rathway, N.J., U.S.A., (1968).

4. Martin, R.T., Econ. Botany 24, 422, (1970).

5. Zapata-Ortiz, V., Int. J. Addict. 5. 287, (1970).

6. Burchard, R.E., in Cannabis and culture, (Ed. Vera Rubin), Mouton Press, The Hague, (1975).

7. Verzar, F., Am. J. Clin. Nut. 3, 363, (1955).

8. Gutierrez-Noriega, C., Am. Indig. 9, 143, (1949).

9. Monge, C.M., Bull. Narc. Oct., 13, (1952).

10. Holmstedt, B., Lingren, J., Rivier, L., and Plowman, T., J. Ethnopharmacology, 1, 69, (1979).

11. History of Coca. (W. Golden Mortimer), and/or Press, San Francisco, (1974).

12. Werner, von G., Planta Med. 9, 293, (1961).

13. Werner, von G., Hoppe-Zeyler's Z. Physiol. Chem. 348, 1151, (1967).

14. Ciuffardi, C.T., Rev. Sandidad. Pol. 34, 943, (1950).

15. Toffoli, F., and Avico, U., Bull. Narc. Oct., 27, (1965).

16. Majlat, P., and Bayer, I., J. Chromatog. 20, 187, (1965).

17. Wallace, J.E., Hamilton, H.E., Schwertner, H., and King, D.E., J. Chromatog. 114, 433, (1975).

18. Mule, S.J., Bastos, M.L., Jukofsky, D., and Saffer, E., J. Chromatog. 63, 289, (1971).

19. Koontz, S., Besemer, D., Mackey, N., and Phillips, R., J. Chromatog. 85, 75, (1973).

20. Jatlow, P.I., Van Dyke, C., Barash, P., and Byck, P., J. Chromatog. 152, 115, (1978).

21. Strait, L.A., Aird, R.B., and Weiss, S., J. Pharmacol. Exp. Ther. 73, 363, (1941).

22. O'Brien, K.P., and Sullivan, R.C., Bull. Narc. Apr., 35, (1970).

23. Van Dyke, C., Byck, R., Barash, P., and Jatlow, P., Clin. Chem. 23, 241, (1977).

24. Mule, S.J., Bastos, M.L., and Jukofsky, D., Clin. Chem. 20, 243, (1974).

25. The Alkaloids, vol. 1, 6, 12, 13 (Ed. R.H.F. Manske and H.L. Holmes), Acadian Press, N.Y., (1950, 1960, 1970, 1971).

26. The Alkaloids, vol. 1, (Ed. K.W. Bentley), Interscience Press, N.Y., (1957).

27. Chemistry of the Alkaloids, (Ed. S.W. Pelletier), Van Nostrand Rhinehold, N.Y., (1970).

28. The Pharmacological Basis of Therapeutics, (Ed. Goodman and Gilman), MacMillan, N.Y., (1975).

29. The Alkaloids, vol. 5, (Ed. R.H.F. Manske and H.L. Holmes), Acadian Press, N.Y., (1958).

30. Collier, H.O.J., Nature 220, 1327, (1968).

31. Kosman, M.E., and Unna, K.R., Clin. Pharmacol. Ther. 9, 240, (1967).

32. Peterson, D.I., and Hardinge, M.G., J. Pharm. Pharmacol. 19, 810, (1967).

33. Kopanyi, T., and Feeney, G.C., Science 129, 151, (1959).

34. Van Dyke, C., Jatlow, P., Ungerer, J., Barash, P., and Byck, R., Science 200, 211, (1978).

35. Shuster, L., Quimby, F., Bates, A., and Thompson, M.L., Life Sci. 20, 1035, (1977).

36. Ramos-Aliaga, R., and Arroyave, G., Arch. Latinamer. Nut., 69, (1969).

37. Ryman, B.E., and Walsh, E.O.F., Biochem. J. 58, 111, (1959).

38. Ryman, B.E., and Walsh, E.O.F., Nature 172, 679, (1953).

39. Hardman, J.G., and Mayer, S.E., J. Pharmacol. Exp. Ther. 148, 29, (1965).

40. Lundholm, L., and Mohme-Lundholm, E., Acta. Pjarmacol. Toxicol, 15, 257, (1959).

41. Graham, M.H., Abboud, F.M., and Eckstein, J.W., J. Pharmacol. Exp. Ther. 143, 340, (1964).

42. Matsui, T., and Kuriaki, K., Am. J. Physiol. 196, 461, (1959).

43. Misra, A.L., Giri, V.V., Patel, M.N., Alluri, V.R., Pontani, R.B., and Mule, S.J., Res. Com. Chem. Path. Pharmacol. 13, 579, (1976).

44. Misra, A.L., Nayak, P.K., Bloch, R., and Mule, S.J., J. Pharm. Pharmacol. 27, 784, (1975).

45. Nieschulz, von O., and Schmersahl, P., Planta Med. 17, 178, (1969).

46. Levy, J., and Zissman, E., J. Physiologie 42, 499, (1950).

47. Nieshulz, von O., Arzneim. Forsch. 21, 275, (1971).

48. Zapata-Ortiz, V., Rev. Med. Exp. 3, 132, (1944).

49. Gutierrez-Noriega, C., and Zapata-Ortiz, V., Rev. Farmacol. Med. Exp. 1, 2, (1948).

50. Chamochumbi, N., Rev. Farmacol. Med. Exp. 2, 94, (1949).

51. Buck, A.A., Sasaki, T.T., Hewitt, J.J., and Macrae, A.A., Am. J. Epidemiol. 88, 159, (1968).

52. Buck, A.A., Sasaki, T.T., Hewitt, J.J., and Macrae, A.A., Bull. Narc. Oct. 23, (1970).

53. Goddard, D., de Doddard, N., and Whitehead, P.C., Int. J. Addict. 5, 165, (1970).

54. Taylor, D., Estevez, V.S., Englert, L.F., and Ho, B.T., Res. Com. Chem. Path. Pharmacol. 14, 249, (1976).

55. Blashako, H., Himms, J.M., and Strimblad, B.C.R., Brit. J. Pharmacol. 10, 442, (1955).

56. Stewart, D.J., Inaba, T., Tang, B.K., and Kalow, W. Life Sci. 20, 1557, (1977).

57. Jatlow, P., Barach, P.L., Van Dyke, C., and Byck, R. Clin. Res. XXIV (3): 255A, (1976).

58. Murray, J.B., and Al-Shoura, H., J. Pharm. Pharmacol. 28, 24P, (1976).

59. Sanchez, G.C., and Guillen, A., Rev. Farmacol. Med. Exp. 2, 209, (1948).

60. Valanju, N.N., Baden, M.M., Valanju, S.N., Mulligan, D., and Verma, S.K., J. Chromatog. 81, 170, (1973).

61. Misra, A.L., Vadlamani, N.L., Bloch, R., Nayak, P.K., and Mule, S.J., Res. Com. Chem. Path. Pharmacol. 8, 55, (1974).

62. Sanchez, G.C., and Guillen, A., Rev. Farmacol. Med. Exp. 2, 8, (1949).

63. Nayak, P.K., Misra, A.L., and Mule, S.J., J. Pharmacol. Exp. Ther. 196, 556, (1976).

64. Mule, S.J., Casella, G., and Misra, A.L., Life Sci. 19, 1585, (1976).

65. Misra, A.L., Patel, M.L., Alluri, V.R., Mule, S.J., and Nayak, P.K., Xenobiotica 6, 537, (1976).

66. Estevez, V.S., Ho., B.T., and Englert, L.F., Res. Com. Chem. Path. Pharmacol. 17, 179, (1977).

67. Inaba, T., Stewart, D.J., and Kalow, W., Clin. Pharmacol. Ther. 23, 547, (1978).

68. Van Dyke, C., Barash, P.G., Jatlow, P., and Byck, R., Science, 191, 859, (1976).

69. Dvorchik, B., Miller, S.H., and Graham, W.P., J. Chromatog. 135, 141, (1977).

70. Gutierrez-Noriega, C., Am. Ind. 12, 111, (1952).

71. Hanna, J.M., Human Biol. 42, 1, (1970).

72. Hanna, J.M., Human Biol. 43, 200, (1971).

73. Bolton, R., Ethnology 12, 227, (1973).

74. Moore, J.M., J. Chromatog. 101, 215, (1974).

75. Collazos, C., White, H.S., Huhnemann, R.L., Reh, E., White, P.L., Castellanos, A., Benites, R., Bravo, Y., Loo, A., Moscoso, I., Caceres, C., and Dieseldorf, A., J. Am. Dietetic Assn. 30, 1222, (1954).

76. Mazess, R.B., and Baker, P.T., Am. J. Clin. Nut., 15, 341, (1964).

77. The Textbook of Medical Physiology, (Ed. Guyton), Saunders Co., Philadelphia, (1971).

78. Comparative Animal Physiology, 2nd Edition, (C.L. Prosser, and F.A. Brown), Saunders, N.Y., (1961).

79. General Endocrinology, 4th Edition, (C.D. Turner), Saunders Co., Philadelphia, (1964).

80. Gray, G.M., Am. J. Clin. Nut. 26, 121, (1973).

81. Saloman, L.L., Allims, J.A., and Smith, D.E., Biochem. Biophys. Res. Comm. 4, 123, (1961).

82. Brochmann-Hansen, E., and Svendsen, A.B., J. Pharm. Sci. 51, 1095, (1962).

83. Noirfalise, A., and Mees, G., J. Chromatog. 31, 594, (1967).

84. Documenta Geigy, 7th Edition, (Ed. K. Deim, and C. Lentner), J.R. Geigy, S.A., Basle, Switzerland, (1970).

85. Scholander, P.F., J. Biol. Chem. 167, 235, (1947).

86. Roe, J.H., and Rice, E.W., J. Biol. Chem. 173, 507, (1948).

87. Fundamentals of Clinical Chemistry, 2nd Edition, (Ed. N. Teitz), Saunders Co., Philadelphia, (1976).

88. Association of Official Agricultural Chemists, Methods of Analysis, 8th Edition, Washington, D.C., 144, (1955).

89. Levy, A.L., Ann. Clin. Lab. Sci. 2, 474, (1972).

90. Van Handel, E., Anal. Biochem. 11, 256, (1965).

91. Van Handel, E., Anal. Biochem, 11, 266, (1965).

92. Findlay, S.P., J. Am. Chem. Soc. 76, 2855, (1954).

93. Bell, M.R., and Archer, S., J. Am. Chem. Soc. 82, 4642, (1960).

94. Fanelli, F.T., and Kaplan, M.L., J. Nut. 108, 1491, (1978).

95. Leveille, G.A., and Chakrabarty, K., J. Nut. 93, 546, (1967).

96. Parker, R.J., Personal communication, Faculty of Agriculture, Department of Animal Science, University of Manitoba, Winnipeg, Manitoba, Canada, (1979).

97. Aynilian, G.H., Duke, J.A., Gentner, W.A., and Farnsworth, N.R., J. Pharm. Sci. 63, 1938, (1974).

98. Bastos, M.L., Jukofsky, D., and Mule, S.J., J. Chromatog. 89, 335, (1974).

99. Woods, L.A., Cochin, J., Fornefeld, E.J., McMahon, F.G., and Seevers, M.H., J. Pharmacol. Exp. Ther. 101, 188, (1951).

100. Bastos, M.L., and Hoffman, D.F., J. Chromatog. Sci. 12, 269, (1974).

101. Fish, F., and Wilson, W.D.C., J. Chromatog. 40, 164, (1969).

102. Graffeo, A.P., Lin, D.C.K., and Foltz, R.L., J. Chromatog. 126, 717, (1976).

103. Kaul, B., Millia-, S.J., and Davidow, B., J. Pharmacol. Exp. Ther. 199, 171, (1976).

104. Misra, A.L., and Mule, S.J., Res. Comm. Chem. Path. Pharmacol. 11, 663 (1975).

105. Bramante, P.O., J. Appl. Physiol. 16, 982, (1961).

106. Grad, B., Am. J. Physiol. 174, 481, (1953).

107. Conrad, M.C., and Miller, A.T., Am. J., Physiol. 186, 207, (1956).

108. Krog, H., Monson, M., and Irving, L., J. Appl. Physiol. 7, 349, (1955).

109. Cuthbertson, W.F.J., Proc. Nut. Soc. 16, 70, (1957).

110. Clarke, H.E., Coates, M.E., Eva, J.K., Ford, D.J., Milner, C.K., O'Donoghue, P.N., Scott, P.P., and Ward, R.J., Lab. Animals, 11, 1, (1977).

111. Himms-Hagen, J., Pharmacol. Rev. 19, 367, (1967).

112. Catecholamines, (Ed. H. Blaschko, and E. Muscholl), Handb. Exp. Pharmak., Vol. 33. Springer-Verlag, Berlin, 97, (1972).

113. Porte, D., and Robertson, R.P., Am. Soc. Exp. Biol. 32, 1792, (1973).

114. Conney, A.H., Pantuck, E.J., Kuntzman, R., Kappas, A., Anderson, K.E., and Alvares, A.P., Clin. Pharmacol. Ther. 22, 707, (1977).

115. Kato, R., and Tanaka, A., J. Biochem. 63, 406, (1968).

116. Evans, M.A., and Harbison, R.D., Pharmacologist 18, 142, (1976).

117. Evans, M.A., Pharmacologist 20, 182, (1978).

118. Campbell, T.C., and Hayes, J.R., Fed. Proc. 35, 2470, (1976).