AN ABSTRACT OF THE THESIS OF

GARY EDWARD COSTLEY (Name) for the DOCTOR OF PHILOSOPHY (Degree)

in ANIMAL NUTRITION (Major) presented on JANUARY 5, 1970 (Date)

Title: INVOLVEMENT OF FORMALDEHYDE IN DEPRESSED IRON ABSORPTION IN MINK AND RATS FED PACIFIC HAKE (MERLUCCIUS PRODUCTUS)

Abstract approved: Redacted for Privacy

Dr. Floyd M. Stout

Inclusion of substantial levels of raw-frozen Pacific hake (Merluccius productus) in diets of ranch-raised mink (Mustela vison) causes a large percentage of animals to develop an abnormal condition termed "cotton fur" (CF), characterized by depressed body weight, microcytic-hypochromic anemia and a failure of under-fur pigmentation. Observations by several groups of researchers over the past decade indicate that this syndrome is the manifestation of an iron deficiency in the mink and among several causes may be induced by a heat-labile factor present in certain species of marine fish. Further observations suggest that the CF-causative factor in hake and other fish species is related to an antibacterial property of these fish.

A series of feeding trials and tracer-iron absorption experiments with more than 650 experimental animals, including both rats

and mink, were conducted to determine possible causes of this induced iron deficiency. Feeding black, Long-Evans, laboratory rats diets based on raw-frozen Pacific hake resulted in decreased body weights, hematocrit levels and degree of hair pigmentation. Absorption of ^{59}ferric chloride in mink was significantly lowered when administered in the presence of raw-frozen as compared to either cooked-frozen or raw-unfrozen eggs of Pacific hake. These results parallel earlier observations that the factor in Pacific hake responsible for its antibacterial activity was present in raw-frozen but not raw-unfrozen Pacific hake and support observations that the CF-causative and antibacterial properties of Pacific hake are related.

Significant depressions in the absorption by rats of ^{59}ferric, ^{59}ferrous and hemoglobin iron-59 were demonstrated when it was administered in preparations of raw-frozen, vs. either cooked-frozen or raw-unfrozen, whole Pacific hake. Similarly, mink absorbed significantly less ^{59}ferric and ^{59}ferrous iron in raw-frozen than in cooked-frozen Pacific hake extracts. These data show that Pacific hake in the raw-frozen state depresses absorption of iron-59 in both rats and mink independently of iron form and provide substantial evidence that the anemia in Pacific hake-fed rats is a result of a failure of normal iron absorption.

Norwegian researchers reported trimethylamine oxide, a

normal, physiologically important constituent of a wide variety of marine fish, was anemiogenic when fed to mink. This observation, coupled with findings by Japanese workers that trimethylamine oxide is converted in the fish tissues to dimethylamine and formaldehyde by the actions of an enzyme present in the cod pyloric caeca, led to the hypothesis at this station that either trimethylamine oxide or one of its breakdown products was responsible for depressed iron absorption in animals fed Pacific hake.

Trials with rats showed that added trimethylamine oxide or formaldehyde significantly depressed absorption of ^{59}ferric, ^{59}ferrous and hemoglobin iron-59 in preparations of either cooked-frozen or raw-frozen Pacific hake while trimethylamine and dimethylamine had no effect. Similar results were obtained when trimethylamine oxide and formaldehyde were added to either raw or cooked, chicken eggs.

Additions of sodium bisulfite to either raw-frozen hake extracts, cooked-frozen hake extracts containing added formaldehyde or mink diets including raw-frozen Pacific hake significantly increased absorption of ^{59}ferric and ^{59}ferrous iron in rats. Sodium bisulfite is known to react with the free aldehyde group of formaldehyde; consequently, it is assumed that this group is involved in the observed depression of iron absorption. These findings together with the fact that trimethylamine oxide is widely distributed in

marine organisms suggests that formaldehyde and not trimethylamine oxide is responsible for the effects of Pacific hake on iron absorption. In vitro experiments indicate that trimethylamine oxide reacts directly with both ferric and ferrous solutions forming insoluble iron hydroxide precipitates; whereas, formaldehyde has no detectable, chemical effect. Experiments also indicate that formaldehyde administered either in water or in extracts of raw rockfish had no effect on iron absorption in rats; however, supplemental trimethylamine oxide lowered iron absorption independently of substrate used. Addition of acetaldehyde to cooked-frozen Pacific hake had no significant effect upon iron absorption in rats suggesting that not only the aldehyde group but also the specific organic compound is of importance.

It is hypothesized that formaldehyde depresses iron absorption by interfering with normal absorption mechanisms, possibly by reacting with protein components of the gastro-intestinal tract. It is further theorized that some other unidentified compound(s) is necessary for formaldehyde to affect iron absorption since formaldehyde was without effect when administered in water solutions. Additionally, it is conceivable that raw rockfish carcass contains factors, possibly bisulfites, which are able to overcome the effects of formaldehyde on lowering iron absorption. Collectively, these experiments suggest that formaldehyde naturally occurring in

raw-frozen but not cooked-frozen or raw-unfrozen Pacific hake significantly depresses absorption of ^{59}ferric, ^{59}ferrous and hemoglobin iron-59 in rats and mink and consequently is responsible for the CF-causative properties of this fish species.

Involvement of Formaldehyde in Depressed Iron Absorption in Mink and Rats Fed Pacific Hake (Merluccius productus)

by

Gary Edward Costley

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1970

APPROVED:

Redacted for Privacy

Associate Professor of Animal Science
in charge of major

Redacted for Privacy

Head of Department of Animal Science

Redacted for Privacy

Dean of Graduate School

Date thesis is presented January 5, 1970

Typed by Donna L. Olson for Gary Edward Costley

ACKNOWLEDGEMENTS

The author wishes to extend his most sincere appreciation to his major professor, Dr. F. M. Stout, for his constant encouragement, sound advice and never ending willingness to help throughout the tenure of my graduate program and more recently for the several hours spent aiding in the preparation of this manuscript. The contribution of Dr. J. E. Oldfield to the completion of this program is gratefully acknowledged.

Most sincere thanks are also offered Drs. F. L. Hisaw, P. H. Weswig and J. E. Parker, for the time and effort spent while serving on my graduate committee.

The assistance of Nancy Wehr both in terms of chemical analysis and the contribution of new and original ideas to the research is acknowledged. Appreciation is extended Bill Amberg of the Small Animal Laboratory and John Adair of the Experimental Fur Farm for their assistance in physical set up of experiments as well as the care of experimental animals.

Thanks are extended to the National Science Foundation for providing funds through a traineeship for both research and living expenses. Additionally, the research was supported by funds from the National Institute of Health, grant number AM 08210 "Factors Associated with Aberrant Iron Metabolism".

To my wife, Cheryl, I extend a most sincere thanks for her financial assistance, constant encouragement and willingness to give of herself without which this program could not have been accomplished.

TABLE OF CONTENTS

Page

LIST OF TABLES

INVOLVEMENT OF FORMALDEHYDE IN DEPRESSED IRON ABSORPTION IN MINK AND RATS FED PACIFIC HAKE (MERLUCCIUS PRODUCTUS)

INTRODUCTION

The research presented in this thesis deals with elucidation of an abnormality of iron metabolism which occurs when certain marine fish are included in diets of ranch-raised mink (Mustela vison). This aberrant iron metabolism precipitates a condition popularly known as "cotton fur" (CF) which is characterized by a microcytic-hypochromic anemia, depressed body weight and most strikingly by the failure of underfur pigmentation. The work dates from earlier studies by Stout, Oldfield and Adair (1960a, b) which demonstrated that inclusion of raw Pacific hake (Merluccius productus) or Atlantic whiting (M. bilinearis) in mink rations resulted in an iron-deficiency state leading to CF symptoms. It was subsequently reported by Bailey (1967) and also Havre, Helgebostad and Ender (1967) respectively, that inclusion of raw Pacific hake and coalfish (Gadus virens) resulted in significant depressions of iron-59 absorption.

The research of this thesis was performed with basic objectives of determining the nature and mechanism of action of the factor(s) responsible for iron deficiency as induced by feeding

CF-causative, marine fish. Results of this research have generally been meaningful and several key questions have been answered. The scope of the work includes two feeding experiments and 15 iron absorption trials employing various forms of iron-59. In all, more than 650 experimental animals have been used. Although important points have been established, the research has probably raised more questions than it has answered and hopefully will serve as a basis for further work in this area.

REVIEW OF LITERATURE

The CF abnormality in mink was described as early as 1929 by Seton (1929). In the past this abnormality resulted in large, annual, economic losses to the fur industry. In searching for a genetic basis to this abnormal condition of the fur, Hummon and Bushnell (1943) indicated that inbreeding of descendants of CF mink did not result in production of CF symptoms when the animal received "standard" mink rations. It was reported by Ender and Helgebostad (1947) that pigmentation failures occurred in weanling foxes fed diets high in marine fish. These researchers subsequently supplemented these fish diets with rice starch, glucose, Fe, Cu, Co, Zn and Mn; vitamins A, D, E, K, C, thiamine and pantothenic acid and were not able to overcome the CF condition. In later work Ender and Helgebostad (1955) reported that the CF symptoms in mink fed marine fish seemed to be intensified when the diet contained high levels of marine fats. From this they concluded that fat peroxides were acting to destroy certain B-vitamins which are necessary for normal pigment formation.

Stout, Oldfield and Adair (1960) identified two specific fish species, Pacific hake and Atlantic whiting, to be causative of CF if fed raw to mink. Their data further indicated that the CF incidence paralleled the level of these fish species present in the diet and

inferred the presence of a heat-labile, CF-causative factor in the fish, since cooking the fish prior to feeding completely removed its CF-causative action. Additionally, these workers reported that the causative factor seemed to be concentrated in the viscera of the causative fish since evisceration reduced the CF incidence in animals receiving hake and completely removed it in whiting-fed domestic mink. In a subsequent paper Stout, Oldfield and Adair (1960b) showed that hake and whiting-fed CF animals exhibited a microcytic, hypochromic anemia which could not be prevented by parenterally administered B vitamins, parenteral copper, oral lysine and tyrosine or oral iron but would respond to parenteral iron. These data suggested that the causative fish, hake and whiting, were rendered dietary iron unavailable. Similar results had been reported by Helgebostad and Martinsons (1958) who observed that 16 mg of organic iron, injected weekly, would prevent anemia in mink fed raw coalfish. Unlike the results of Stout et al. these workers presented data which suggest that oral iron supplementation was effective in reducing the CF incidence in raw coalfish-fed mink.

Stout, Oldfield and Adair (1960) and Helgebostad (1961) have made observations suggesting that fish-induced anemia is influenced, to a certain extent, genetically. It was noted that anemia produced in mink kits raised at the Oregon station on diets containing raw Pacific hake was particularly widespread among certain litters while

kits from other litters developed normally. Additionally, Stout (1966) suggested that certain families of mink become anemic and develop CF symptoms while being fed "standard" mink rations not containing known causative fish. Later it was reported by Gjonnes and Helgebostad (1965) that silver fox and rats fed the same fish diets appeared to be less susceptible to anemia than mink. Helgebostad (1966) found a higher incidence of anemia and CF symptoms in aleutian than in standard dark mink again suggesting the importance of genetics in this primarily nutritional disorder.

Working independently, Bailey at Oregon (1967) and Havre, Helgebostad and Ender in Norway (1967) provided evidence for the validity of the thesis of Stout that CF-causative fish species present in the ration of mink were in fact inducing an iron deficiency by rendering dietary iron unabsorbable. These workers demonstrated that both raw Pacific hake and raw coalfish significantly depress the absorption of ^{59}ferric chloride. Bailey's data, although not extensive, further suggest that ^{59}ferric and hemoglobin iron-59 are adversely affected by raw Pacific hake while ^{59}ferrous iron is not.

Leekley and Stout in unpublished data (1968) observed that Alaskan pollock (Theragra chalcogrammus) were CF causative if frozen prior to being fed to mink but did not cause anemia if fed fresh (unfrozen). This observation is of interest when considered with the report of Stout, et al. (1969) which suggests that raw-frozen

but not raw-unfrozen Pacific hake has broad-spectrum antibacterial properties. Additionally, Stout has made numerous observations over the last decade which indicate that the antibacterial and CF-causative properties of Pacific hake are related or perhaps caused by the same, unidentified factor.

Reviews by Stout, Oldfield and Adair (1960a, b) and Stout (1966) suggest the existence of three separate causes of iron deficiency in mink all of which can lead to development of CF. These include the iron deficiency induced from the inclusion of various, raw, marine fish in mink diets, a genetically caused iron deficiency exhibited by certain families of mink and a secondary iron deficiency resulting from inclusion of rancid fats in the diets of mink. Stout and Bailey (1969) provide substantial evidence that the pigmentation failure of CF is a symptom of iron deficiency which develops in pigmented, iron-deficient animals regardless of the specific cause of the deficiency.

EXPERIMENTAL

Objectives

The primary objective of this research was to determine how Pacific hake interferes with normal iron metabolism by identifying compounds and mechanisms of action and to relate this back to the practical problem of CF observed in mink.

General Methods

In order to use rats for short duration, iron absorption experiments preliminary research was conducted to determine the effect of Pacific hake on laboratory rats. General techniques used in all iron absorption trials were similar, with major modifications occurring in sample preparation. Thus, these procedures will be presented here with modifications being specified where appropriate.

Iron Absorption

Whole Body Counting

A whole body counting technique for measuring absorption of radioactive iron in laboratory rats as reviewed by Riebes, Conrad and Crosby (1967) was employed in some of the earlier experiments. This involved administration of iron-59 to rats that had been fasted

for 12 hours. Radioactivity contained in the entire animal body was determined after both one hour and seven days following dose administration employing an Armac whole body liquid scintillation counter[1] (Appendix 1). Percent iron absorption was calculated using the following formula where N.C.P.M. is net counts per minute and standard represents an aliquot of the material administered to the animals:

$$\text{Percent Absorption} = \frac{\dfrac{\text{N.C.P.M.-Rat (7 days after dosing)}}{\text{N.C.P.M.-Standard (7 days after dosing)}}}{\dfrac{\text{N.C.P.M.-Rat (1 hour after dosing)}}{\text{N.C.P.M.-Standard (1 hour after dosing)}}}.$$

Measuring the activity of the standard both at one hour and at seven days obviates the necessity of calculation of decay rates.

Determination of Blood Specific Activity

A second, more rapid procedure for estimating iron absorption was employed because of availability and accuracy of counting equipment. It was recognized that this technique measures iron uptake by the blood only, but since this is highly related to total iron absorption it will be referred to as absorption throughout the remainder of this thesis. Accumulated data show the correlation coefficient between

---

[1] Packard Instrument Company, Des Plaines, Ill.

iron absorption as determined by whole body counting and by blood specific activity to be .95. Previously fasted rats or mink were dosed either by stomach tubing or feeding with a preparation of iron-59 and radioactivity was determined in a sample of blood 48 hours after dose administration. Generally one ml containing .25 microcuries of iron-59 was administered per 100 grams of body weight; this varied in some instances and will be specified in appropriate trials. Feed was offered one hour after dosing, and animals were allowed feed and water ad libitum for the remaining 48 hours of the trial. In most of these experiments the isotope was added to the material being tested 10-12 hours prior to dose administration. Blood samples were obtained by heart puncture, weighed to the nearest 10 mg in shell vials, placed in auto-gamma tubes and counted in a Packard Auto-Gamma Spectrometer[2] (Appendix 1) to an accuracy of one standard deviation (Wang and Willis, 1965). Iron absorption was estimated by the following calculations:

$$\text{Percent Absorption} = \frac{(\text{N. C. P. M./g blood})\ (\text{body weight})\ (0.08)}{\text{N. C. P. M. total dose}} .$$

Blood and dose samples were counted on the same day so correction for radioactive decay was unnecessary. The .08 figure in the formula

---

[2] Packard Instrument Company, Des Plaines, Ill.

represents the average blood composition of the rat body (Altman and Dittmer, 1961). Determination of blood volumes for individual animals would have been desirable but in view of time required was not possible; however, with the extremely high correlation coefficient (.95) between iron absorption determined by whole body and blood counting the figure does not create a large error.

During the experiments rats were housed singly in standard, battery-type rat cages. Pens were equipped with special aluminum trays permitting total collection of excreta. Rats used in iron absorption trials were offered feed for eight hours only each day for a minimum of two weeks prior to use with the intent of decreasing the stress caused by fasting prior to isotope administration. This feeding procedure was adopted from methods employed by Van Potter (1968) to decrease variation within treatment groups in enzyme assay experiments. Mink trials were conducted similarly to rat experiments with points of variation noted under specific trials.

Antibacterial Assays

It was reported by Stout, Oldfield and Adair (1968) that Pacific hake possessed a wide-spectrum antibacterial property, and observations by Stout (unpublished) over the past ten years suggest that this antibacterial action is identical or closely related to the depressing effects of hake on iron metabolism. In view of this, antibacterial

assays, as decresibed by Cooper (1963), using Serratia marcescens as a test organism were employed throughout the experimental work as a possible indicator of presence and strength of the CF-causative factor(s) present in various hake preparations.

Fish Preparation

Preparation of the fish varied from trial to trial, but specific treatments were generally employed after the fish had been stored. For example, the cooked, frozen material was prepared by cooking fish that had been stored in the frozen state. Additionally, levels of chemical additives employed in Part 2 were based on levels naturally occurring in various forms of Pacific hake as analyzed in this laboratory.

Statistical Evaluation

Iron absorption data collected were subjected to analysis of variance and least significant difference comparison of means (Snedecor, 1950).

Part 1: Effects of Dietary Pacific Hake on Iron Absorption in Rats and Mink

Specific Objectives

The experimental objective of this initial phase was to determine the effects of ingested, raw-frozen Pacific hake on the absorption of iron-59 in rats and mink. More specifically answers to the following questions were sought:

1) Do laboratory rats develop symptoms similar to cotton fur in mink when fed diets containing raw-frozen Pacific hake?
2) Does raw-frozen Pacific hake decrease absorption of various forms of labelled iron, including ^{59}ferric chloride, ^{59}ferrous citrate and hemoglobin iron-59 in rats and mink?
3) Are the effects on iron absorption of raw-frozen and raw-unfrozen Pacific hake similar in rats and mink?
4) What compound(s) in Pacific hake might be responsible for its CF-causative properties?

In order to provide experimental answers to these questions, six trials were conducted. These experiments are presented according to objectives and results and are not necessarily in the sequence conducted.

Methods, Results and Discussion

Trial 1 (Feeding Rats)

Gjonnes and Helgebostad (1965) failed to observe anemia in rats fed diets based on raw, gutted coalfish. This did not agree with their earlier observations with mink and foxes and suggested that laboratory rats might not be affected by CF-causative fish species. To test the validity of these findings an experiment here was designed to evaluate the effects of feeding raw and cooked Pacific hake to laboratory rats.

Thirty-six, black, Long-Evans rats (18 males and 18 females) weighing 35 $\pm$ 5 g were divided into equal groups by sex and randomly placed on one of the three diets presented in Table 1.

Table 1. Composition of experimental rations. (Trial 1)

Item	Percent as fed		
	Control	Cooked hake	Raw hake
Standard lab chow[1]	100	--	--
Raw Pacific hake	---	--	93
Cooked Pacific hake[2]	---	93	--
Corn starch	---	3	3
Cellulose[3]	---	2	2
Lard	---	1.75	1.75
Vitamin Premix[4]	---	0.25	0.25

[1]Standard Purina Lab Chow, Ralston Purina Company, St. Louis, Missouri.
[2]Heated by boiling in an open container to an internal temperature of 190°F.
[3]Alphacel, N.B.C.
[4]Vitamin premix for laboratory rat and mouse rations, Nutritional Biochemicals Corp., Cleveland, Ohio.

Animals were housed two per pen in standard, battery-type rat pens and fed ad libitum daily. Body weights were taken weekly, and hematocrit levels were measured on alternate weeks after the fifth week using blood collected by clipping a toenail. Samples of hair from the back were collected ten weeks after the start of the experiment so that the intensity of hair pigmentation could be determined by a method involving analysis of melanin concentration (Costley, 1968). Results of this trial are given in Table 2.

Table 2. Final weights, hematocrit and melanin values of rats fed experimental, hake diets. (Trial 1)

Experimental group	Final body weight (g)	Hematocrit (%)	Melanin value of hair*
Control	$321^{a} \pm 82$	$44.7^{a} \pm 1.4$	$60^{a} \pm 6$
Cooked hake	$300^{b} \pm 92$	$43.5^{a} \pm 1.8$	$60^{a} \pm 11$
Raw hake	$269^{c} \pm 62$	$38.7^{b} \pm 3.3$	$36^{b} \pm 10$

12 animals per treatment group.
Means in the same column bearing different superscripts are significantly ($P < .01$) different.
$\pm$ figure represents standard deviation.
*Optical density of aqueous suspensions of melanin granules per mg of hair.

These data indicate that feeding raw Pacific hake to black rats significantly depressed the melanin value of the hair. Furthermore, a significant decrease ($P < .01$) in final body weight of both groups receiving hake was noted when compared to the control group. Animals fed cooked hake were significantly heavier ($P < .01$) at the end

of the experiment than rats fed raw hake.

These results show that rats fed diets composed of high levels of raw Pacific hake develop anemia, fail to make optimal gains and exhibit decreased hair pigmentation which are symptoms similar to those seen in mink. The greying of fur first became evident after six weeks of feeding and was most pronounced after ten weeks. Decreased weight gains by hake-fed rats might be explained in that the diet was approximately 66 percent water, and consequently animals were not able to consume enough feed to meet their basic requirements for energy.

Although rats do appear to be adversely affected by feeding raw Pacific hake, severity of the resultant anemia is not as great as in mink fed hake. This could possibly reflect a species difference in iron requirements or in food passage rates. The rate of food passage in the mink is considerably more rapid than in rats (Bailey, 1967) and presumably could decrease the animal's ability to absorb iron.

These results do not agree with those of Gjonnes and Helgebostad (1965) who suggest that the rat is not affected by the feeding of coalfish (a CF-causative species). One important difference between this experiment and that of Gjonnes and Helgebostad was that they fed gutted fish whereas whole fish were used here. This could be a key reason for the observed difference in view of the work of Stout, Oldfield and Adair (1960) which showed that evisceration of Pacific

hake and Atlantic whiting greatly decreased their CF-causative properties. Additionally, it should be pointed out that Gjonnes and Helgebostad used white rats and thus could not evaluate the effect of the fish on hair pigmentation. In summary, these results suggest that the pigmented, laboratory rat could be an effective pilot animal for further research related to iron deficiency as induced by feeding Pacific hake.

Trial 2 (Iron Absorption--Rats)

A second pilot experiment was conducted to determine if aqueous extracts of raw Pacific hake decrease the absorption of ^{59}ferric chloride or ^{59}ferrous sulfate in rats. Raw, whole Pacific hake were finely ground and the liquid expressed with a Carver laboratory press and divided into two equal volumes. One was left in the raw state while the other was autoclaved for 30 minutes at 15 psi. ^{59}Ferric chloride or ^{59}ferrous sulfate were added to the hake extracts 12 hours prior to administration to rats by stomach tubing. Iron absorption was determined using the whole body counting technique described previously.

Results of the experiment given in Table 3 show no significant differences between absorption of either form of iron from raw or cooked Pacific hake. As expected, significantly more ($P < .05$) ferrous iron was absorbed than ferric.

Table 3. Absorption by rats of ^{59}ferric chloride or ^{59}ferrous sulfate in presence of raw or cooked Pacific hake extracts. (Trial 2)

Type of iron	Mean absorption	
	Raw hake %	Cooked hake %
^{59}Ferric chloride	$12.0^{a} \pm 6.3$	$7.3^{a} \pm 4.7$
^{59}Ferrous sulfate	$20.9^{b} \pm 6.0$	$22.8^{b} \pm 4.8$

Two animals per treatment group.
Means in the same row or column bearing different superscripts are significantly ($P < .01$) different.
$\pm$ figure represents standard deviation.

These results were difficult to interpret when compared with those of trial 1. A possible explanation is the CF-causative factor of Pacific hake was inactivated during the sample preparation procedure. To confirm this, antibacterial assays were run on the materials used in the experiment. Results of these assays showed that the raw hake extract had lost its antibacterial activity previously present;although reasons for this loss were not apparent. Excessive variation and too few animals per treatment group were further detriments to the experiment; consequently it was repeated.

Trial 3 (Iron Absorption--Rats)

This trial was designed to test results obtained in trial 2 and to determine if differences existed between the effects of Pacific hake

on ferric and ferrous iron. Four groups of three each Long-Evans rats were stomach tubed with either ^{59}ferric chloride or ^{59}ferrous sulfate in the presence of either raw or cooked extracts of Pacific hake. These extracts were prepared as previously and assayed for antibacterial activity prior to administration. However, results of the assays, unlike those of trial 2, indicated that the raw fish contained substantial amounts of antibacterial activity. As a possible means of reducing within treatment variation, rats were placed on an eight-hour feed day. Results are presented in Table 4.

Table 4. Absorption of ^{59}ferric chloride and ^{59}ferrous sulfate by rats stomach tubed with raw or cooked Pacific hake extracts. (Trial 3)

Form of iron	Mean absorption	
	Raw hake %	Cooked hake %
^{59}Ferric chloride	$6.2^{a} \pm 2.4$	$15.2^{b} \pm 5.4$
^{59}Ferrous sulfate	$7.1^{a} \pm 3.6$	$8.4^{a} \pm 3.7$

Three animals per treatment group.
Means in the same row or column bearing different superscripts are significantly ($P < .05$) different.
$\pm$ figure represents standard deviation.

These data indicate that raw Pacific hake significantly depressed ($P < .05$) absorption of ^{59} ferric chloride but had no significant effect upon the absorption of ^{59}ferrous sulfate, although a slight decrease was noted. These results generally are in agreement with

the findings of Bailey (1967) which suggest that raw hake does not affect ferrous sulfate to the same extent that it affects ferric chloride.

Experimental animals were bled by heart puncture 48 hours after dosing and again immediately after the seven-day, whole-body activity was determined. Using these blood samples, iron absorption figures were calculated using three different methods: i.e., whole-body radioactivity, seven-day, blood activity and 48-hour, blood activity. They are presented in Table 5.

Table 5. Absorption of iron-59 as determined using three methods of measurement and the correlations between these methods. (Trial 3)

Animal number	Iron absorption		
	Whole body activity %	7 day blood activity %	48 hour blood activity %
1	14.6	12.9	10.2
2	13.9	12.0	10.3
3	16.9	16.0	13.5
4	6.2	5.9	5.5
5	6.9	6.3	4.9
6	5.4	5.1	7.3
7	8.0	7.7	7.3
8	8.8	8.0	7.6
9	8.4	7.4	7.0
10	6.5	6.0	5.8
11	7.7	7.5	7.2
12	7.1	6.4	6.0
Mean	9.2	8.4	7.7

	Correlation coefficient
Whole body vs. seven-day, blood activity	.995
Whole body vs. 48-hour, blood activity	.951
Seven-day vs. 48 hour blood activity	.990

These results show that an extremely high correlation exists between whole-body counting and blood-activity levels. Subsequent absorption trials using rats employed the 48-hour, blood activity determination as it permitted faster accumulation of data with no loss of accuracy.

Trial 4 (Iron Absorption--Mink)

Considerable data collected here over the past ten years have indicated that the CF-causative property of Pacific hake is related to or caused by the factor(s) responsible for the observed antibacterial activity of hake tissues (Stout, unpublished). This coupled with the knowledge that raw Pacific hake prior to freezing does not exhibit antibacterial properties (Stout, 1969), suggested that fresh-unfrozen hake may not be CF-causative either. Consequently, this experiment was conducted to determine if differences occurred in absorption of iron by mink receiving raw-unfrozen and raw-frozen Pacific hake. Fresh-unfrozen Pacific hake were delivered to the laboratory and assayed for antibacterial activity. Three days after being frozen, eggs and associated tissues present in female fish showed presence of antibacterial activity, and thus this tissue was used for the iron absorption experiment. On the fourth day the frozen eggs were divided and one half left raw and the other half cooked by autoclaving as before. The three forms of eggs (raw-unfrozen,

raw-frozen and cooked-frozen) were again assayed for antibacterial activity with significant amounts found only in the raw-frozen eggs. A slight zone of inhibition was also noted in the cooked material. Suspensions containing 75 percent egg tissues and 25 percent water were prepared, with ^{59}ferric chloride added 12 hours prior to stomach tubing. Nine, standard, dark, female mink which were anemic were selected from a group that had been fed a diet containing 50 percent raw-frozen Pacific hake for approximately six months. The animals were fasted for 12 hours prior to dosing by stomach tube with one ml per 100 grams of body weight with one of the above egg tissue preparations. After administration of the dose the mink were placed in pens which were equipped with plastic bags allowing total excreta collection. Food was offered to these animals approximately one hour after stomach tubing,and they were subsequently fed ad libitum. Seven days after dosing, the mink were bled by heart puncture; the blood was counted to an accuracy of one standard deviation in the whole body counter,and iron absorption was calculated as previously. These data show iron absorption to be 0.96% for the raw-frozen, 2.04% for the cooked-frozen, and 9.78% for the raw-unfrozen Pacific hake groups. Statistical evaluation of these data indicates that iron in the presence of raw-frozen suspensions is absorbed significantly poorer ($P < .10$) than in the cooked-frozen material and both of these are absorbed significantly poorer ($P < .01$) by animals

than the unfrozen material. These findings agree with the observation that fresh-unfrozen fish do not possess antibacterial activity and therefore provide further evidence for a definite relationship between the CF-causative and antibacterial properties of Pacific hake.

Differences in iron absorption between mink receiving cooked and raw hake were not as great as expected; however, it is possible that the amount of heat involved did not remove all of the CF-causative factor from the eggs, and as a result iron absorption was depressed but not to the same extent as in the raw-frozen fish. This is consistent with the observation that the cooked eggs caused a slight zone of inhibition in plates of Serratia marcescens.

Trial 5 (Iron Absorption--Rats)

Objectives of this experiment were to study the effects on absorption of ^{59}ferric, ^{59}ferrous and hemoglobin iron-59 of Pacific hake in three forms: raw-frozen, raw-unfrozen and cooked-frozen. Methods were similar to those used in previous experiments except that the method of dose administration was changed. Each of the three groups of experimental animals was fed one form of fish for three days prior to isotope administration. On the fourth day, after being fasted for 12 hours, the rats were fed a test dose ($1 \pm .05$ grams) of one of the fish preparations containing 0.5 microcuries of either ^{59}ferric chloride, ^{59}ferrous citrate or hemoglobin iron-59. In

all, this factorial experiment involved nine experimental groups of five rats each. Radioactivity which was not consumed was accounted for by weighing back uneaten feed. However, this correction was found unnecessary as the animals ate the hake well. A 0.1 to 0.5 gram sample of the labelled feed was accurately weighed and counted in the Packard Auto-Gamma Spectrometer. Radioactivity as net counts per minute in the total dose for each rat was calculated from these sample counts and weights of material consumed by the animals. Iron absorption was determined using the 48-hour, blood levels of iron-59.

Hemoglobin iron-59 was prepared as follows: from three, 150-gram, Long-Evans, male rats three ml of blood were taken by heart puncture on each of three days prior to an intraperitoneal injection of approximately 50 microcuries of ^{59}ferrous citrate. These rats were again bled by heart puncture 48 hours after the iron-59 injection and approximately nine ml of blood were removed from each. Collected blood was centrifuged at 3000 rpm for 30 minutes; the plasma was removed, and the red blood cells remaining were washed with distilled water to hemolize them, and the resultant hemoglobin solution was completely dried by lyophilization. The dry preparation was then reconstituted with ten ml of distilled water, and the specific activity as disintegrations per minute (dpm) per ml of solution was determined to be 4.6 microcuries of iron-59 per ml.

This was considered sufficient activity to allow iron absorption trials to be run with reasonable counting statistics.

In order to obtain the best possible comparison between the three forms of fish, absorption trials utilizing the raw-unfrozen hake were conducted one week earlier than those using the frozen fish. This allowed the same fish, which had been previously ground, to be used for experimental groups receiving either frozen or unfrozen tissues. The isotopes were added to fish preparations one hour prior to dosing. Results of the experiment are given in Table 6.

Table 6. Absorption of three forms of iron-59 when fed with raw-frozen, cooked-frozen or raw-unfrozen Pacific hake. (Trial 5)

Type of iron	Mean absorption		
	Raw-frozen %	Cooked-frozen %	Raw-unfrozen %
^{59}Ferric chloride	$7.4^{a} \pm 1.7$	$15.2^{c} \pm 6.3$	$11.6^{d} \pm 2.1$
^{59}Ferrous citrate	$4.8^{a} \pm 1.6$	$14.4^{e} \pm 2.2$	$11.0^{d} \pm 1.7$
^{59}Hemoglobin	$1.0^{b} \pm 0.6$	$3.9^{e} \pm 1.3$	$3.5^{e} \pm 1.3$

Five animals per treatment group.
Means in the same row or column bearing different superscripts are significantly different ($P < .05$).
$\pm$ figure represents standard deviation.

These results indicate that raw-frozen Pacific hake significantly decreased ($P < .05$) absorption of all three forms of labelled iron as compared to either the cooked-frozen or raw-unfrozen forms of

hake. Unlike in trial 4, the cooked-frozen hake-fed animals absorbed more iron than did the ones fed raw-unfrozen hake. Significantly less ($P < .05$) hemoglobin iron was absorbed than either ferric or ferrous iron; however, there was no significant difference in absorption of the two inorganic iron forms as the result of any of the three fish treatments. Repeatability of values within treatment groups in this experiment was good and,in contrast to the thesis of Bailey (1967),indicates that absorption of ferrous iron is reduced by presence of raw-frozen Pacific hake, at least in this trial where rats were used as the experimental animal.

Trial 6 (Iron Absorption--Mink)

This experiment was conducted to establish whether raw Pacific hake extracts depress absorption of both ^{59}ferric and ^{59}ferrous iron in domestic mink. The experiment thus served to test the thesis of Bailey (1967) that ferrous iron absorption in mink is not affected by raw Pacific hake while ferric iron is.

Methods used in this experiment were similar to those of the previous absorption trial with mink (trial 4) except that blood was counted in the Packard Auto-Gamma Spectrometer. Pacific hake extracts administered in this trial were prepared by pressing raw Pacific hake in a food press as in trial 2. Half of this extract was cooked by autoclaving for 30 minutes at 15 psi. The cooked hake

extracts thus obtained did not exhibit antibacterial activity; whereas, significant amounts were found in the raw hake extracts. One-fourth microcurie of iron-59 per ml of solution was added to each of the hake extracts approximately eight hours prior to stomach tubing the mink with eight ml each. Results of the experiment are given in Table 7.

Table 7. Effects of raw and cooked Pacific hake extracts on the absorption of the ^{59}ferric and ^{59}ferrous iron in mink. (Trial 6)

Type of iron	Mean absorption	
	Raw hake %	Cooked hake %
^{59}Ferric chloride	$3.4^{a} \pm 1.7$	$8.9^{b} \pm 3.7$
^{59}Ferrous citrate	$1.3^{a} \pm 0.8$	$9.3^{b} \pm 4.6$

Three mink per treatment group.
Means in the same row or column bearing different superscripts are significantly different ($P < .05$).
$\pm$ figure represents standard deviation.

Results indicate that the absorption of both ^{59}ferric and ^{59}ferrous iron by mink is significantly lower ($P < .05$) in the presence of raw than in cooked hake extracts. These observations support the results of trial 5 using rats. They do not, however, agree with the observation of Bailey (1967) that raw Pacific hake did not affect absorption of ferrous iron in mink. Reasons for these differences are unknown; however, an important difference in

technique is that Bailey added the tracers immediately prior to dosing while, in this experiment, they were added approximately eight hours before dosing. This period of incubation may be of importance in allowing ferrous iron to be converted to the ferric state. Nevertheless, from a practical standpoint, these data indicate that ration supplementation with either ferric or ferrous iron would not overcome the CF-causative actions of Pacific hake.

Part 2: Effects of Formaldehyde and Trimethylamine Oxide on Iron Absorption in Rats and Mink

During the course of experimental work presented in Part 1, significant literature citations were discovered which altered the direction of subsequent research. Shewan (1951) while studying the biochemistry of fish found that various methylamines, including: trimethylamine oxide (TMAO), trimethylamine (TMA) and dimethylamine (DMA) were widely distributed in various fish of marine origin. Additionally, he observed that although concentrations of these compounds vary considerably, they are uniformly high in marine fish tissues and low in meat products. Apparently TMAO is a product of choline breakdown in vivo (Dechezlepretre, Portet and Cheymol, 1967) while DMA and TMA are produced both from the degradation of TMAO and the catabolism of proteins (Shewan, 1951).

Amano, Yamada and Bito (1963) discovered the biological formation of formaldehyde (FA) in the tissues of Japanese cod (Gadus macrocephalus), Alaska pollock (Theragra chalcogrammus) and Japanese hake (Heragrammos stellan), all members of the cod (Gadidae) family. These workers suggest that gadoid fish species have an enzyme, concentrated in the pyloric caeca, which is capable of converting TMAO to FA and DMA. Further, they found rich concentrations of TMAO but no FA in such marine fish as rockfish, salmon, herring, tuna, shark and squid indicating that the conversion

of TMAO to FA may be specific for gadoid fish. In subsequent papers Amano and Yamada (1964, 1964b) and Yamada and Amano (1965, 1965b, 1965c) accumulated convincing evidence for the existence of this enzyme system. Furthermore, they have tested tissues of other fish species as well as other biological materials, such as beef tissue, cheese, eggs, etc., and have not been able to duplicate the actions of the enzyme present in tissues of gadoid fishes. During the course of these experiments, they found levels of TMAO, TMA, DMA and FA to vary greatly with storage. Upon storage for up to two weeks at temperatures of 1 to 4° C, the TMAO level would decline with a corresponding increase in DMA and FA levels. In unpublished research, Stout (1969) employed the methods of Bricker and Johnson (1945) to analyze FA in chromotropic acid solutions by colorimetric procedures and has found FA levels as high as 500 ppm in frozen and as low as 20 ppm in fresh, Pacific hake. He has concluded that antibacterial activity of raw-frozen Pacific hake is the result of its FA concentration.

TMAO was reported by Ender and Helgebostad (1968) to be anemiogenic when fed at relatively high levels over a three month period to mink. They suggest that TMAO reacts with ferrous salts to form an insoluble, and presumably nonutilizable, ferric hydroxide oxide precipitate.

With the knowledge of the immediately preceeding information,

part 2 of this thesis was undertaken in an attempt to determine whether TMAO or one or more of its breakdown products affect the absorption of iron in its various forms and to establish whether they are responsible, individually or collectively, for the CF syndrome induced by feeding raw Pacific hake to mink.

Specific Objectives

The second part of the research of this thesis was undertaken to answer the following specific questions:

1) Does the separate addition of TMAO, TMA, DMA or FA to cooked and raw preparations of Pacific hake alter iron-59 absorption by the animal?
2) Are the concentrations of these compounds, as they occur naturally in fish tissues, important in affecting iron absorption?
3) If one or more of these compounds does affect iron absorption, what physical or chemical means might overcome these effects?

The general methods used for this research were similar to those employed in Part 1, with specific exceptions listed where appropriate.

Methods, Results and Discussion

Trial 7 (Iron Absorption--Rats)

Since Amano and Yamada (1965) reported that formaldehyde (FA) is formed from trimethylamine oxide (TMAO) in the digestive tracts of certain gadoid fish,and since the CF-causative and antibacterial actions of Pacific hake appear related, it was speculated that either TMAO or FA might be responsible for the depressing action of Pacific hake on iron absorption. This trial was conducted to evaluate effects on absorption of hemoglobin iron when TMAO and FA were added to various preparations of Pacific hake. Fresh-unfrozen Pacific hake were obtained from Oregon coastal fishermen and delivered to the laboratory packed in ice. Upon assaying the fish, FA was present at approximately 300 ppm, which was a ten-fold increase over concentrations measured in a previous batch. Reasons for the elevated FA level are not known, but it is possible that the fish had been frozen before delivery. The fish were ground, subdivided, frozen and cooked as in trial 5. Unlike in trial 5, the fresh portion was held at refrigerator temperatures for one week while the other portion was frozen. Furthermore, instead of feeding the ground, whole fish to the rats, tissue juices were extracted by pressing,and extracts were administered to the animals via stomach tube. Hemoglobin iron-59 was prepared as before. In this and all

subsequent experiments the chemical additives were added to the fish tissue preparation at the same time as the isotopes. Results of the trial are presented in Table 8.

Table 8. Absorption of hemoglobin iron-59 administered to rats in extracts of Pacific hake, with water, TMAO or FA added. (Trial 7)

Additive	Mean absorption		
	Raw-frozen hake %	Cooked-frozen hake %	Raw-unfrozen hake %
Water (control)	$1.81^{a} \pm 0.44$	$1.86^{a} \pm 0.65$	$0.89^{d} \pm 0.24$
" + TMAO (1500 ppm)	$1.74^{a} \pm 0.51$	$1.13^{b} \pm 0.69$	$0.35^{e} \pm 0.07$
" + FA (300 ppm)	$1.05^{c} \pm 0.45$	$0.87^{c} \pm 0.52$	$0.18^{f} \pm 0.07$

Four animals per treatment group.
Means in the same row or column bearing different superscripts are significantly different ($P < .05$).
$\pm$ figure represents standard deviation.

Iron absorption data as affected by form of Pacific hake are probably not valid as they represent different cell constituents since the fish tissues were pressed after the several storage conditions. Furthermore, the elevated FA level present in the fresh-unfrozen fish confounds comparisons between fresh and frozen fish tissues. However, these results are of interest from the standpoint of the effects of the three chemical treatments employed. The data indicate

that both TMAO and FA significantly depressed ($P < .05$) iron absorption when they were added to Pacific hake extracts. Absorption of hemoglobin iron in rats receiving FA was significantly lower ($P < .05$) than in those receiving TMAO in any of the three fish preparations. These results are most interesting and suggest that the FA and possibly TMAO naturally present could be responsible for the observed depressing effects on iron absorption associated with feeding Pacific hake.

One question which arises is: "Does TMAO affect iron absorption per se, or does it serve only as a precursor to FA?" The measured level of TMAO naturally occurring in these frozen fish declined by approximately one-fifth during a 30 minute autoclaving (15 psi). A reduction of this magnitude does not appear to be sufficient to account for differences in iron absorption of cooked and raw fish-fed animals as observed in trials 3, 4, 5 and 6. Formaldehyde concentrations, however, were reduced ten-fold during an equal cooking period. Furthermore, it has been found that the TMAO level of numerous marine fish species that are non-CF-causative is as high as those occurring in Pacific hake (Shewan, 1951). These facts prompt the thesis that FA is responsible for the iron absorption depressing effect of Pacific hake and that naturally occurring TMAO either serves as a precursor to FA or has no effect. The objective of several of the trials following is to test this hypothesis.

Trial 8 (Iron Absorption--Rats)

This experiment was essentially the same as trial 7 in which the objective was to determine effects of FA and TMAO on iron absorption when added to various forms of Pacific hake. Unlike trial 6, ^{59}ferric chloride was used rather than hemoglobin iron-59. Additionally, the fresh fish preparations were used one week earlier than the frozen fish as in trial 5 so that comparisons between fish tissues would be meaningful. Another important difference is that the fresh-unfrozen hake used in this experiment contained only 23 ppm FA compared to 300 ppm present in the unfrozen fish used in trial 7.

Results of this experiment (Table 9) are in general agreement with those of trial 7. These data indicate a significant depression ($P < .05$) in absorption of ferric chloride when either TMAO or FA was added to preparations of raw-frozen, cooked-frozen or raw-unfrozen Pacific hake. The effects of FA are significantly greater ($P < .05$) than those of TMAO with all three forms of Pacific hake tested. Such results would be unexpected if TMAO were acting only as an FA precursor dependent upon an enzyme for the conversion as proposed by Amano and Yamada (1965). The conversion should not occur when the enzyme is destroyed by heating. Also the results confirm the findings of trials 4 and 5 where significant decreases were noted in iron absorption as affected by feeding frozen as

compared with unfrozen Pacific hake. As previously, data show a significant decrease ($P < .05$) in iron absorption by animals receiving raw as compared to cooked hake. FA and TMAO levels of the three hake extracts employed in this trial as determined chemically are given in Table 10.

Table 9. Absorption of ^{59}ferric chloride administered to rats in extracts of Pacific hake, with water, TMAO or FA added. (Trial 8)

Additive	Mean absorption		
	Raw-frozen %	Cooked-frozen %	Raw-unfrozen %
Water (control)	$5.36^{b} \pm 1.81$	$7.39^{a} \pm 2.67$	$13.9^{c} \pm 1.51$
" + TMAO (1500 ppm)	$2.54^{e} \pm 1.54$	$4.64^{d} \pm 1.52$	$4.61^{f} \pm 0.61$
" + FA (300 ppm)	$0.46^{g} \pm 0.15$	$2.35^{e} \pm 2.05$	$3.21^{h} \pm 0.68$

Four animals per treatment group.
Means in the same row or column bearing different superscripts are significantly different ($P < .05$).
± figure represents standard deviation.

Table 10. Concentration of naturally occurring FA and TMAO in Pacific hake extracts. (Trial 8)

Pacific hake extracts	TMAO ppm	FA ppm
Cooked-frozen	1075	57.2
Raw-frozen	1520	102.8
Raw-unfrozen	1610	23.6

These results, together with those of trial 7, provide further evidence that TMAO and FA are operating independently. There is a significant negative correlation ($r = -.51$); ($P < .01$) between the naturally occurring FA level and the percent iron absorption which suggests that the greater the FA concentration the lower the percent iron absorption. However, no significant correlation ($r = -.10$) existed between the naturally occurring TMAO concentration of the fish and percent iron absorption. This lack of a significant correlation, in view of the significant decrease in iron absorption when TMAO was added (trials 7 and 8), suggests that TMAO naturally present in tissues of Pacific hake may not be important in interfering with iron absorption, a point requiring further investigation.

Trial 9 (Iron Absorption--Mink)

This trial was conducted to confirm results of trial 8 using mink instead of rats as an experimental animal. Mink used in this experiment had been used in an earlier absorption experiment employing tracer iron, and it was thus necessary to subtract the original radioactivity of the blood in order to calculate iron absorption. Two animals were not administered tracers in this experiment. These animals were bled in conjunction with those receiving additional iron-59 so that control levels of blood during the experiment were available. During the seven days this experiment was in progress,

increases of 13 and 20 percent, respectively,in blood iron-59 of the two control animals were noted. This increase is indicative of an error in iron absorption as calculated for experimental animals since all of the increase in blood activity was incorrectly assumed to be the result of iron-59 absorbed from the gastro-intestinal tract. Nonetheless, the data indicate statistically significant differences between treatment groups which provides information relative to the objectives of the experiment. The radio tracers and chemical additives were added to the various hake preparations eight hours prior to dosing the mink by stomach tube.

The basic experimental design and results are presented in Table 11. These results indicate that TMAO and FA significantly depress ($P < .05$) the absorption of ^{59}ferric chloride in mink and agree with results of trial 8 in which rats were used as experimental animals. The results further indicate that 300 ppm FA had a greater depressing effect on iron absorption than 1500 ppm TMAO and substantiate earlier results with rats.

Table 11. Effects of TMAO and FA on the absorption of ^{59}ferric chloride in mink. (Trial 9)

	Mean absorption		
	Cooked-frozen hake	Cooked-frozen hake + TMAO (1500 ppm)	Cooked-frozen hake + FA (300 ppm)
Percent iron absorption	$12.8^{a} \pm 3.6$	$6.15^{b} \pm 3.2$	$2.63^{c} \pm 1.9$

Three mink per treatment group.
Means with different superscripts are significantly different ($P < .05$).
± figure represents standard deviation.

Trial 10 (Iron Absorption--Rats)

This experiment was conducted to determine the effects of derivatives of TMAO including FA, TMA and DMA upon iron absorption. The experiment was conducted using procedures as in trials 7 and 8. Levels of compounds added were based on levels naturally occurring in raw-frozen Pacific hake. Radio tracers and chemical additives were added to the fish extracts six hours prior to administration.

Results (Table 12) confirm earlier findings that FA significantly depresses ($P < .05$) absorption of ^{59}ferric chloride and ^{59}ferrous citrate and indicate that TMA and DMA have no depressing action upon iron absorption. These results together with those of earlier trials provide substantial evidence that under the experimental conditions of

this research the hypothesis of Bailey (1967) that ferrous iron is not affected by raw Pacific hake does not appear correct.

Table 12. Effects of FA, DMA, TMA added to cooked-frozen Pacific hake on the absorption of ^{59}ferric chloride and ^{59}ferrous citrate by rats. (Trial 10)

	Mean iron absorption			
	Chemical additives			
Form of iron	None	FA-300 ppm	TMA-300 ppm	DMA-300 ppm
		%	%	%
^{59}Ferric chloride	$8.31^{a} \pm 2.64$	$2.85^{b} \pm 1.90$	$7.97^{a} \pm 3.21$	$8.46^{a} \pm 1.93$
^{59}Ferrous citrate	$7.99^{a} \pm 1.84$	$3.21^{b} \pm 3.11$	$8.05^{a} \pm 3.11$	$8.13^{a} \pm 1.46$

Five animals per treatment group.
Means in the same row or column bearing different superscripts are significantly different ($P < .05$).
± figure represents standard deviation.

Trial 11 (Iron Absorption--Rats)

The objective of this trial was to determine the effects of various concentrations of FA and TMAO on depressing iron absorption. Techniques were similar to those of trial 8 where the test dose of cooked hake including tracers was fed to the animals after they had been fed the fish with additives included for three days.

The data in Table 13 indicate that additions of either TMAO or FA at concentrations as low as 100 ppm depress iron absorption equal to higher concentrations of these compounds. Such results are

not in strict agreement with the findings of trial 8 which indicated that a significant negative correlation exists between iron absorption and FA level of the fish. However, although results here are not statistically significant, a trend was observed which demonstrated a consistent decline in iron absorption with higher concentrations of FA and TMAO.

Table 13. Absorption of ^{59}ferric chloride fed together with cooked Pacific hake and various levels of TMAO or FA. (Trial 11)

Treatment	Mean iron absorption (%)
Cooked hake (control)	$4.01^{a} \pm 1.21$
" " + 100 ppm TMAO	$1.69^{b} \pm 0.31$
" " + 1000 ppm TMAO	$1.51^{b} \pm 0.51$
" " + 5000 ppm TMAO	$1.31^{b} \pm 0.43$
" " + 100 ppm FA	$1.90^{b} \pm 0.42$
" " + 500 ppm FA	$1.87^{b} \pm 0.48$
" " + 1000 ppm FA	$1.49^{b} \pm 0.71$

Five animals per treatment group.
Means bearing different superscripts are significantly different ($P < .05$).
± figure represents standard deviation.

The data from groups receiving TMAO suggests that TMAO present naturally in the fish does not alter absorption of iron by the animal. Additions of 100 ppm TMAO to cooked-frozen hake, analyzed to already contain 1070 ppm, resulted in a marked decrease in ^{59}ferric chloride absorption. However, additions of TMAO of 1000

and 5000 ppm failed to significantly reduce iron absorption in rats below the level of animals receiving 100 ppm. Such results would be unexpected if naturally occurring and supplemented TMAO produced a similar effect upon iron absorption. The same argument can be used for FA. The FA present in the cooked Pacific hake used in this experiment was only 20 ppm,and it is suggested that this is not in a free form. This is supported by the observation that FA measured in cooked Pacific hake was consistently higher when stronger digestion procedures were employed. When cooked fish, containing 43 ppm FA, was dialyzed and freeze dried, the measured FA was 612 ppm; yet this material did not exhibit antibacterial activity; whereas,FA levels as low as 100 ppm in raw fish result in detectable zones of inhibition on plates seeded with Serratia marcescens. These findings indicate that FA present in cooked Pacific hake is in a bound form and is not antibacterially active and presumably would not be active in depressing iron absorption.

Trial 12 (In Vitro Experiments)

These in vitro investigations were undertaken to answer some of the questions posed by the previous iron absorption experiments. Objectives here were to establish possible mechanisms of action of both FA and TMAO in decreasing iron absorption by animals. This research involved experiments to determine qualitatively if either

TMAO or FA have any chemical effects upon various types and strengths of iron solutions. Preparations of TMAO, FA and ferric and ferrous iron were used over a ten-fold range of concentrations. Additions of two ml of FA solution at each of ten concentrations to two ml of iron solution at each of ten concentrations failed to form precipitates, change the valence state or alter the amount of iron in solution indicating that FA does not have any detectable effect upon solutions of either ferric chloride or ferrous citrate. Mixture of solutions of TMAO and iron, either ferric or ferrous, caused the formation of yellow precipitates, the chemical nature of which are not known although large amounts of iron were indicated by atomic absorption analysis. Furthermore, it was found that ferric iron in the presence of TMAO no longer gave positive tests for either ferric or ferrous iron. However, upon adding ascorbic acid reactions for ferrous were again positive. These chemical changes are not understood; however, it is speculated that TMAO is forming some type of complex with iron which can be broken by additions of ascorbic acid. As mentioned earlier, Ender and Helgebostad (1968) suggest that TMAO reacts with ferrous sulfate forming an insoluble ferric hydroxide oxide. Results here, however, suggest that TMAO reacts with both ferric and ferrous iron.

These in vitro experiments indicate that TMAO has a chemical effect upon iron;whereas,FA apparently does not. Based on these

findings the following trial was conducted to establish if TMAO and FA have depressing effects on iron absorption when they are administered independently of Pacific hake in water solutions.

Trial 13 (Iron Absorption--Rats)

This experiment was designed to determine if the depressing effects of TMAO and FA on iron absorption, when administered with Pacific hake, occur if administered to rats in water solution. The experiment was conducted three times as results of the first trial were extremely variable because of a failure to include carrier (non-labelled) iron in the test dose. The absolute iron concentration in the radioisotope is very small,and thus slight differences in total iron absorption resulted in large percentage differences. As a result, the within treatment variation was extremely large and obscured possible differences occurring between experimental groups. The trial was conducted twice more using five animals per treatment group per experiment. In the second and third of these experiments unlabeled ferric chloride or ferrous citrate was added so that the final iron concentration of the solution was 200 ppm. Carrier and isotopic iron were added to the solutions four hours prior to administration by stomach tube.

Results (Table 14) indicate that TMAO in water solution significantly depressed ($P < .05$) the absorption of ^{59}ferric chloride and

^{59}ferrous citrate while FA in water solution had no significant effect.

Table 14. Absorption of ^{59}ferric chloride and ^{59}ferrous citrate in water alone or in water plus TMAO or FA. (Trial 13)

Form of iron	Mean iron absorption		
	(Control) %	Water + TMAO (1500 ppm) %	Water + FA (300 ppm) %
^{59}ferric chloride	$6.84^{a} \pm 3.02$	$4.04^{b} \pm 1.76$	$5.08^{ab} \pm 2.05$
^{59}ferrous citrate	$3.11^{c} \pm 1.30$	$2.32^{d} \pm 0.81$	$3.03^{c} \pm 1.34$

Ten animals per treatment group.
Means in the same row or column bearing different superscripts are significantly different ($P < .05$).
$\pm$ figure represents standard deviation.

These results concur and may be explainable by results of trial 12 where TMAO was shown to form insoluble precipitates with iron solutions;whereas,FA apparently does not. These data substantiate the idea that TMAO as naturally occurring in Pacific hake and as added chemically are not equivalent in their effects upon depressing iron absorption. In this experiment significantly more ($P > .05$) ferric iron was absorbed than ferrous iron by animals in all treatment groups. Reasons for this are not known,but this result does not agree with the commonly held belief that ferrous iron is better utilized than ferric.

Trial 14 (Iron Absorption--Rats)

Since FA did not lower iron absorption by animals when administered in water (trial 13) this experiment was conducted to determine if TMAO or FA affect iron absorption when fed with biological materials other than Pacific hake. Experimental procedures were basically the same as those employed in trials 7 through 10 in which isotopes included in various test materials were fed to rats. In this experiment chemical additives and isotopes were added to test preparations eight hours prior to feeding the test dose.

Results of the experiment (Table 15) present several interesting points. Additions of either TMAO or FA significantly depressed ($P < .05$) absorption of ^{59}ferric chloride in rats fed either raw-frozen or cooked-frozen Pacific hake and raw or cooked chicken eggs but had no significant effect when administered in raw rockfish carcass. Reasons for this latter observation were not understood and consequently were investigated further. Absorption of iron by rats receiving raw eggs was very low even when TMAO and FA were not included. This low level absorption is possibly due to presence of conalbumin in raw eggs which is known to reduce iron absorption (Tengerdy, Azari and Tengerdy, 1966). Rats fed raw-frozen Pacific hake absorbed consistently less iron than those receiving cooked-frozen Pacific hake which confirms results of earlier trials.

Table 15. Absorption of ^{59}ferric chloride fed to rats in various biological materials with water, TMAO or FA added. (Trial 14)

Additives	Mean iron absorption				
	Raw-frozen hake %	Cooked-frozen hake %	Raw chicken eggs %	Cooked chicken eggs %	Raw rockfish carcass %
Water (control)	$1.37^{a} \pm 0.91$	$2.96^{a} \pm 0.91$	$0.76^{a} \pm 0.01$	$4.30^{a} \pm 0.66$	$2.49^{a} \pm 0.55$
" + TMAO (1500 ppm)	$0.49^{b} \pm 0.42$	$1.47^{b} \pm 1.21$	$0.16^{b} \pm 0.09$	$1.95^{b} \pm 0.61$	$2.42^{a} \pm 0.62$
" + FA (300 ppm)	$0.31^{b} \pm 0.07$	$0.96^{c} \pm 0.61$	$0.20^{b} \pm 0.09$	$0.91^{c} \pm 0.23$	$2.38^{a} \pm 0.47$

Five animals per treatment group.
Means in the same column bearing different superscripts are significantly different ($P < .05$).
$\pm$ figure represents standard deviation.

These results are potentially important in elucidating the mechanism of action of FA in depressing iron absorption. The effect of FA in reducing the absorption of iron when fed together with raw or cooked chicken eggs indicates that Pacific hake is not the only material in which FA is active. This considered together with the failure of FA in water solution to depress iron absorption by rats (trial 13) could mean that either raw, rockfish carcass or water lack some material(s) essential for FA to act or that they contain substance(s) which "inactivate" FA. The reason for failure of TMAO to depress iron absorption when fed in raw, rockfish carcass in this experiment is not known and does not agree with results of subsequent experiments.

Trial 15 (Iron Absorption--Rats)

Objectives of this experiment were to confirm results of trial 14 that iron absorption was unaffected by either TMAO or FA when fed in preparations of rockfish carcass and to establish effects of feeding TMAO or FA to animals for three days prior to isotope administration. Experimental methods employed were basically the same as those used in trials 11 and 13. Treatments included feeding raw, rockfish carcass alone or in combination with either TMAO or FA. Each treatment group consisted of 15 rats which had been fed one of the fish preparations for three days. On the fourth day each

group of 15 was subdivided into groups of five and received orally preparations of one of the preceeding three, fish treatments containing ^{59}ferric chloride. This experimental design made it possible to determine the effects of "prefeeding" TMAO and FA on iron absorption.

Results (Table 16) indicate that absorption of ^{59}ferric chloride was significantly depressed ($P < .10$) when TMAO was added to raw, rockfish carcass; whereas,addition of FA had no significant effect. The data further indicate that the feeding of either TMAO or FA in raw, rockfish carcass prior to isotope administration had no effect upon iron absorption. These results suggest that TMAO must be in direct association with the iron-59 in order to alter its absorption. Findings here do not agree with results of trial 14 with regard to the effect of TMAO fed in raw, rockfish carcass but do agree with previous observations (trial 12) that TMAO acts directly with iron.

Collectively, results here suggest that TMAO acts to depress iron absorption by reacting directly with iron and that this action is universal and independent of substrate. Reasons for failure of TMAO to depress iron absorption of animals in trial 14 are not known.

Results of the current trial and those of trial 14 indicate that FA had no significant effect upon iron absorption when fed in preparations of raw,rockfish carcass. Reasons why this should occur are not known since FA depresses the absorption of iron in other biological

materials including Pacific hake and chicken eggs. The possibility exists that a compound(s) present in rockfish carcass reacts with FA rendering it inactive.

Table 16. Effects of TMAO and FA on absorption of ^{59}ferric chloride fed to rats in preparations of raw, rockfish carcass (RRFC). (Trial 15)

	Mean iron absorption		
	Materials fed prior to isotope administration		
Materials fed with tracer dose	RRFC (control) %	RRFC + TMAO (1500 ppm) %	RRFC + FA (300 ppm) %
RRFC	5.39[a] ± 1.63	4.62[a] ± 1.42	4.96[a] ± 1.97
RRFC + TMAO (1500 ppm)	2.58[b] ± 1.11	2.63[b] ± 0.98	2.48[b] ± 0.99
RRFC + FA (300 ppm)	4.96[a] ± 1.56	5.08[a] ± 2.10	4.97[a] ± 1.46

Five animals per treatment group.
Means in the same row or column bearing different superscripts are significantly different ($P < .10$).
± figure represents standard deviation.

Trial 16 (Iron Absorption--Rats)

Sodium bisulfite is known to react with free aldehyde groups rendering them unavailable for other reactions (Morrison and Boyd, 1959). This experiment therefore was conducted to determine if

addition of this compound would overcome the depressing effect on iron absorption of raw-frozen Pacific hake or cooked-frozen hake plus FA.

The experiment involved administering ^{59}ferric chloride to rats by stomach tube in presence of one of the following extracts: (1) raw-frozen Pacific hake, (2) raw-frozen hake + sodium bisulfite, (3) cooked-frozen hake, (4) cooked-frozen hake + FA and (5) cooked-frozen hake + FA + sodium bisulfite. FA assays indicated that 216 and 52 ppm, respectively, were found in the raw-frozen and cooked-frozen extracts.

The basic experimental design and results are given in Table 17. Data here indicate that addition of sodium bisulfite to extracts of raw-frozen Pacific hake overcame the depressing action of the fish upon iron absorption. These data further show that sodium bisulfite does not increase iron absorption in animals receiving cooked fish extracts which supports previous findings that FA present in cooked fish is in an inactive form. The results add further credence to the theory advanced that naturally occurring TMAO is unimportant in the iron-absorption-depressing effect of hake; whereas, reactions between sodium bisulfite and TMAO are unexpected yet additions of bisulfite to raw frozen hake increased iron absorption to a level not statistically significantly different from the cooked-frozen + FA + $NaHSO_3$ group. However, addition of sodium bisulfite to raw-frozen

Pacific hake did not result in iron absorption equal to the cooked-frozen group. This suggests that either the sodium bisulfite was not present in high enough concentrations or not sufficiently in direct contact with FA to overcome all of its effects or that other compounds, possibly TMAO, in the raw-frozen fish acted to lower iron absorption. These results provide further evidence for the hypothesis that FA naturally occurring in raw-frozen hake is important in the CF-causative effect of the fish and point to the involvement of the aldehyde group.

Table 17. Effects of sodium bisulfite on absorption of ^{59}ferric chloride in extracts of raw-frozen or cooked-frozen Pacific hake. (Trial 16)

Treatment	Mean iron absorption %
Raw-frozen hake	$1.48^{a} \pm 0.98$
" " " + $NaHSO_3$	$3.15^{b} \pm 1.21$
Cooked-frozen hake	$4.80^{c} \pm 1.36$
" " " + FA	$0.97^{a} \pm 0.13$
" " " " + $NaHSO_3$	$3.96^{bc} \pm 1.84$

Five animals per treatment group.
Means bearing different superscripts are significantly different ($P < .05$).
$\pm$ figure represents standard deviation.

Trial 17 (Iron Absorption and Feeding Experiment--Rats, Mink)

To confirm the observed effect of sodium bisulfite in overcoming the depressing action of Pacific hake upon iron absorption the

following experiment was conducted. A group of anemic, adult, standard, dark mink, which had been fed a diet containing 50 percent of raw-frozen Pacific hake for over 18 months, were divided into two equal groups based on degree of anemia. One group continued to receive the Pacific hake diet while the other received a similar diet except that the cereal was supplemented with sodium bisulfite in equal molar concentrations with the 348 ppm FA assayed to be present in the diet. All animals were fed once daily, ad libitum, for two months; after 28 and 56 days of feeding, the mink were bled by clipping a toe nail and the hematocrit level determined. During this 56-day feeding period hematocrit levels of the mink remained essentially unchanged. Both control and sodium bisulfite-supplemented groups started with a mean hematocrit of approximately 39 percent and ended at values of 36.4 and 35.9, respectively. These results indicate that sodium bisulfite did not overcome the induced anemia. It should be pointed out, however, that these animals were in extremely poor condition as the result of being anemic for long periods of time. Additionally, it is possible that the sodium bisulfite in the cereal portion of the diet was not mixed well enough with the fish to allow direct contact between the bisulfite and the FA. A better experimental approach might have been to add the bisulfite directly to the ground fish prior to mixing the remainder of the diet. Also it is possible that young, growing animals would have made better

experimental animals since it would have been possible to determine the preventive rather than curative effects of bisulfite.

The second phase of the experiment involved an iron absorption trial in which rats were fed the two mink diets described above. This trial was conducted similarly to previous experiments when on the fourth day, after three days of receiving the ration, either ^{59}ferric chloride or ^{59}ferrous citrate was added to a small amount of the feed immediately before feeding. Iron-59 absorption was determined as before.

These results presented in Table 18 indicate that sodium bisulfite supplementation of mink diets containing 50 percent of raw-frozen Pacific hake significantly increased ($P < .05$) the absorption of both ^{59}ferric chloride and ^{59}ferrous citrate when fed to rats. Results here support earlier findings that sodium bisulfite is effective in overcoming the depressing effects of raw Pacific hake on iron absorption (trial 16). The data provide further evidence that absorption of both ^{59}ferrous and ^{59}ferric forms of iron is lowered in presence of raw Pacific hake. Results of this and several other trials taken together indicate that absorption of both ^{59}ferric and ^{59}ferrous iron is depressed in the presence of raw-frozen or cooked hake plus FA regardless of method of administration or incubation period. In this experiment both forms of radioactive iron were added just prior to administration to the animal.

Table 18. Effects of $NaHSO_3$ supplementation of Pacific hake-containing mink diets on absorption of ^{59}ferric chloride and ^{59}ferrous citrate in rats. (Trial 17)

Form of iron	Mean iron absorption	
	Raw Pacific hake diet %	Raw Pacific hake diet + $NaHSO_3$ %
^{59}ferric chloride	$1.34^{a} \pm 0.96$	$4.97^{b} \pm 1.35$
^{59}ferrous citrate	$1.64^{a} \pm 1.01$	$4.82^{b} \pm 1.73$

Five animals per treatment group.
Means in the same row or column bearing different superscripts are significantly different ($P < .05$).
$\pm$ figure represents standard deviation.

Trial 18 (Iron Absorption--Rats)

The experimental objective of this trial was to determine if acetaldehyde had effects on iron absorption similar to those of FA. The trial involved the stomach tubing of rats with ^{59}ferric chloride in preparations of cooked-frozen Pacific hake, cooked-frozen Pacific hake plus FA or cooked-frozen Pacific hake plus acetaldehyde.

It was proposed that since the aldehyde group of FA appeared to be involved in the depressing effect on iron absorption (trials 16 and 17) the aldehyde group of acetaldehyde might possibly have similar effects. Both isotopes and aldehydes were added to the fish preparations eight hours prior to dose administration.

Results (Table 19) indicate that acetaldehyde had no effect on

absorption of ^{59}ferric chloride, although they do demonstrate a significant depression of absorption in the presence of FA. These data therefore indicate that the action of FA on iron absorption is not a property common to all compounds containing a free aldehyde group.

Table 19. Effects of FA and acetaldehyde on absorption of ^{59}ferric chloride by rats. (Trial 18)

Mean Iron Absorption		
Cooked-frozen hake %	Cooked-frozen hake + FA (300 ppm) %	Cooked-hake + acetaldehyde (410 ppm) %
$9.24^{a} \pm 3.19$	$2.80^{b} \pm 1.83$	$9.91^{a} \pm 3.40$

Four animals per treatment group.
Means bearing different superscripts are significantly different ($P < .01$).
$\pm$ figures represents standard deviation.

Results here support the theory that FA is acting to depress iron absorption by interfering with the normal absorption mechanism rather than by acting directly upon the iron molecule. Further, the data suggest that the specific compound is of importance since formaldehyde and acetaldehyde differ only in the length of their carbon chains.

DISCUSSION

The area of iron metabolism is complex. During the last decade numerous review articles, original research publications and text books have been devoted to the subject, but only superficial gains have been made on some of the more basic problems. In spite of a substantial research effort in this area, the mechanism and control of iron absorption continue to elude our understanding. However, several factors are known to influence the absorption of iron, including: chemical nature of iron in the food; amount of iron present; acidity of the gastric juices; levels of other compounds such as phosphates, calcium, phytic acid, chelating agents (i.e. ethylenediaminetetra-acetate (EDTA) and others). The subject of iron absorption has been extensively reviewed in the following publications: Bothwell and Finch (1962), Crosby (1963) and Callender (1964). Research results presented in this thesis point to the fact that the abnormality in iron metabolism associated with the feeding of Pacific hake to mink is likewise not a simple matter although several key questions have been answered.

Although the original problem involved mink, much of the research reported herein was done with laboratory rats. Feeding rats a diet based on raw-frozen Pacific hake shows that symptoms similar to cotton fur (CF) in mink are produced in this species.

Anemia and greying of fur exhibited by these animals were similar to symptoms observed by Cusack and Brown (1964) who fed iron-deficient, purified diets to black, laboratory rats. These observations agree with the thesis of Stout (1960) who maintained that the pigmentation failure associated with feeding CF-causative fish to mink is actually a symptom of iron-deficiency. He suggested that iron is required in the biosynthetic process leading to normal pigment formation in hair. During periods of reduced iron availability animals preferentially use these iron supplies for higher priority systems including enzyme and respiratory pigment synthesis rather than for melanin formation.

Data in Part 1 collectively indicate that absorption of iron-59 is more than three times higher in presence of cooked-frozen than in raw-frozen Pacific hake (8.4 vs. 2.6 percent). This substantiates Bailey's research that the CF syndrome in mink as caused by feeding Pacific hake is the result of a failure in normal iron absorption from the digestive tract.

Iron absorption trials in Part 1 (Trials 1-6) provide substantial evidence that absorption of the three forms of iron tested, i.e. ^{59}ferric, ^{59}ferrous and hemoglobin iron-59, is lowered by inclusion of raw-frozen Pacific hake in diets of both rats and mink. Results here are not in general agreement with those of Bailey (1967) that suggest that ^{59}ferrous iron absorption in mink is not altered by

raw Pacific hake. However, specific methods employed in this research and in that of Bailey differed in several respects, including such things as incubation period and trial duration, which might account for observed differences. Nonetheless, under the experimental conditions composed here, numerous trials using substantial numbers of experimental animals show that all three forms of iron-59 are more poorly absorbed in the presence of raw-frozen Pacific hake. Results of Part 1 further show that raw-unfrozen Pacific hake does not significantly decrease absorption of iron-59, whereas raw-frozen hake does. Reasons for this were not known when the trials were conducted but became evident during the research of Part 2.

Absorption of hemoglobin iron-59 by experimental animals was consistently lower than either of the other iron forms. The relationship between the absorption of ferric and ferrous iron, however, was not always consistent which is not in agreement with the commonly held belief that ferrous iron is better absorbed than ferric iron (Bothwell and Finch, 1962). A possible reason for the variable results noted is that chemical treatments imposed varied from trial to trial, and it is possible that oxidizing or reducing agents present may have altered the valence form of the iron. In vitro experiments conducted, but not previously reported, suggest that gastric juices of the stomach and upper small intestine of fasted rats converted ferric to ferrous iron; whereas, the reverse was not true. This could help to

explain the lack of a consistent relationship between ferric and ferrous iron absorption since the feeding methods prior to tracer administration differed and depended upon specific objectives of the experiment. When wet feed ingredients, like hake or rockfish carcass, were fed, the absorption of ferric and ferrous iron tended to be similar; however, if the animals were fed standard laboratory chow the absorption of ferric iron seemed to be enhanced which suggests that the valence state of iron was affected by the specific contents of the gastro-intestinal tract.

The discovery that gadoid fish species contain an unusual enzyme system capable of converting trimethylamine oxide (TMAO) into formaldehyde (FA) and dimethylamine (DMA) originated with two Japanese scientists (Amano and Yamada 1963, 1964, 1964b and 1965; Yamada and Amano, 1965, 1965b) who were investigating the possibility that FA was being added to Japanese cod (Gadus macrocephalus) as a preservative. Their work eventually led to characterization of the enzyme system located in the pyloric caeca of these fish which converts TMAO to DMA and FA. Apparently, this conversion results from frozen storage as fresh (unfrozen) Pacific hake (a related species) contained little if any FA (Stout et al., 1969). Upon storage at freezing temperatures, the FA level increased to as high as 300 ppm with a corresponding decrease in the TMAO level.

The Japanese work considered together with a report by Ender

and Helgebostad (1968) that TMAO, widely present in marine fish (Shewan, 1951), was anemiogenic when fed to domestic mink led to the hypothesis here that TMAO and/or FA was (were) possibly the factor(s) in Pacific hake responsible for both its CF-causative and antibacterial actions. Upon laboratory investigation, however, Stout et al. (1969) found no antibacterial activity associated with TMAO. Additionally, only gadoid fish are CF-causative;whereas,TMAO is present in numerous other species of marine fish commonly fed to mink (Shewan, 1951). The other possibility, that FA as a breakdown product of TMAO was responsible for the CF-causative activity of Pacific hake,was considered likely since its antibacterial properties were well known (Wilson, 1958). Furthermore, FA had been found naturally occurring in the same fish species which were known to be CF-causative.

From this hypothesis, the research objectives presented in Part 2 were proposed to determine the relative effects of TMAO, TMA, DMA and FA on the absorption of various forms of iron-59. The initial iron absorption trials conducted here suggested that although FA had a greater effect on a molar concentration basis, both FA and TMAO significantly depressed the absorption of ^{59}ferric chloride, ^{59}ferrous citrate and hemoglobin iron-59 in rats, while TMA and DMA were without effect. Additionally, it was found that TMAO and FA significantly depressed iron absorption in mink. In

later trials evidence began to accumulate that although additions of TMAO to iron-59 solutions did decrease iron absorption by the animal, the TMAO naturally occurring in Pacific hake apparently had little if any effect. This point was substantiated by trial 9 in which the cooked hake used as a base material contained 1070 ppm TMAO. However, an additional 100 ppm reduced the iron absorption by half, although further additions (to 5000 ppm) had little more effect. These results, taken with in vitro experiments which indicate that TMAO added to iron solutions causes the formation of an insoluble and presumably nonutilizable iron precipitate, suggest that the TMAO present in fish preparations may be bound to other compounds or out of direct contact with the iron and thus does not reduce iron absorption.

Addition of FA to preparations of Pacific hake, either raw-frozen, raw-unfrozen or cooked-frozen, had consistent and significant depressing effects on the absorption of ^{59}ferric, ^{59}ferrous and hemoglobin iron-59. The mechanism of action of FA in causing this effect is not known, but the data do suggest that the aldehyde group of FA is involved. Sodium bisulfite, which specifically reacts with free aldehyde groups rendering them unavailable for further reaction (Morrison and Boyd, 1959), added to cooked hake with added FA overcame the depressing action of FA upon iron absorption. Addition of acetaldehyde to cooked hake extracts, however, had no

significant effect upon the absorption of iron-59, suggesting that not only the aldehyde group but other characteristics, possibly size, of the FA molecule are important in its effect on lowering iron absorption.

Results of the research presented in this thesis provide substantial evidence for the theory that the CF-causative property of Pacific hake and presumably other gadoid fish is caused by the presence of FA in the fish tissues. Additionally, the data indicate that although supplemented TMAO significantly depresses iron absorption, the TMAO naturally occurring in the fish is of little importance except as an FA precursor. Reasons for these conclusions are enumerated as follows:

(1) Significant negative correlations ($r = -.50$) exist between naturally occurring FA levels of fish tissues and iron-59 absorption; whereas, no significant correlation ($r = -.10$) exists between naturally occurring TMAO levels and iron absorption.

(2) TMAO is present at levels equivalent to those contained by CF-causative fish species in numerous marine fish commonly fed to mink (approximately 1000 ppm).

(3) CF-causative species are the only fish that have been found to contain the enzyme which converts TMAO to FA.

(4) Cooking of raw-frozen Pacific hake resulted in an 18 percent

average decrease in TMAO which does not appear sufficient to account for the three-fold increase in iron absorption by animals receiving cooked-frozen vs. raw-frozen Pacific hake.

(5) TMAO levels of raw-unfrozen Pacific hake were assayed to be in excess of 900 ppm and are comparable to levels found in raw-frozen hake, yet the raw-unfrozen hake has no effect upon iron-59 absorption. However, raw-unfrozen hake contains approximately 30 ppm FA compared to 300 ppm in the raw-frozen fish.

(6) Sodium bisulfite's addition to a raw-frozen Pacific hake diet or cooked-frozen hake plus FA resulted in significant increases in iron absorption and since this compound specifically reacts with aldehydes it is active here against FA present in the raw-frozen hake.

In vitro trials with FA and iron solutions failed to demonstrate physical effects or chemical changes in the valence state of the iron. Similarly, prefeeding of FA prior to administration of the test dose did not appear to alter the effects of the FA present in the dose. This indicates that for FA to lower iron absorption it must be administered in conjunction with the iron. Collectively, these observations suggest that FA exerts its effects through the animal and not on iron itself. This conclusion is supported by the experimental observation

that FA administered in water solution independent of hake suspensions does not affect iron-59 absorption.

Smith (1968) reported a significant decrease in intestinal motility when FA concentrations similar to those employed in this research were fed to laboratory rats. This explanation, for the present case, is not likely in view of the finding of Schode, Felster and Conrad (1969) who suggest that chemicals which decrease intestinal motility significantly increase the absorption of iron by prolonging the intestinal transit time. A more logical explanation of possible effects of FA in reducing iron absorption is that of Fozzard and Dominguez (1969) who present data which suggest that FA at levels as low as 200 ppm resulted in a decrease in the permeability of mammalian membranes to di- and/or tri-valent ions. This explanation would agree with the observation by Stout (1960) that feeding of Pacific hake to laboratory mice resulted in symptoms similar to manganese deficiency since if FA alters intestinal permeability, ions other than iron should be affected. However, data collected by Stout et al. suggest that iron injections are effective in completely preventing anemia and other CF symptoms in mink fed either Pacific hake, Atlantic whiting or Alaskan pollock. Such results would be unexpected if FA was acting to interfere with the absorption of di- and tri-valent ions generally.

The problem of a possible mechanism of action of FA is

further complicated by results of trials 13 and 14. In these experiments it was shown that although FA significantly depressed iron absorption when added to raw or cooked Pacific hake and raw or cooked chicken eggs it has no effect in water alone or when added to raw, rockfish carcass. This suggests that a factor(s) present in Pacific hake and chicken eggs is necessary for the effect of FA to be manifest. This satisfactorily explains the ineffectiveness of FA in water but does not explain the lack of effect in the presence of raw rockfish. It is possible that raw, rockfish carcass contains compounds, possibly bisulfites, which react with and render unavailable the aldehyde group of FA.

Although the mechanism of action of FA in depressing iron absorption remains speculative its effects can be prevented, at least in iron absorption experiments with rats, by addition of sodium bisulfite as previously discussed. It should be pointed out, however, that addition of sodium bisulfite to rations could possibly result in other nutritional problems, e.g. palatability of rations containing sodium bisulfite at levels high enough to prevent FA-induced anemia is questionable.

Research methods employed in this thesis were in general successful. During the course of the investigations, several factors became evident which caused alteration of techniques, hopefully making results more repeatable. In initial iron absorption

experiments, it became evident that a minimum of four and preferably five animals per treatment group were necessary to compensate for the variation within treatment groups resulting from both actual differences in the animals themselves and differences due to experimental error. This within treatment variation was considerably reduced (mean standard deviations of 5.2 vs. 2.8) when the experimental animals used were preconditioned to fasting periods which was done by feeding on an eight hour day for a minimum of two weeks prior to use. Van Potter (1968) reported this technique reduced variability in enzyme assays with rats, and it appears to be equally effective in absorption studies. Additionally, it is realized that although absorption experiments employing iron-59 reflect the absolute absorption of non-labeled iron, they are not an actual measure of it.

Results of the research have been gratifying,and answers have been obtained to many of the basic questions. However, long-term feeding trials to confirm observations made in short-duration, iron absorption experiments would have been desirable. Some questions remain unanswered or require confirmation and many new questions have been raised pointing to the necessity of additional research in this area if a complete understanding of the problem is to be obtained.

SUMMARY

1. Feeding raw-Pacific hake diets to laboratory rats for 12 weeks decreased body weights, blood hematocrit levels and melanin granule concentrations in the hair.
2. Raw-frozen as compared with cooked-frozen Pacific hake significantly depressed the absorption by rats of ^{59}ferric chloride, ^{59}ferrous citrate or hemoglobin iron-59 when fed or administered by stomach tube under a variety of conditions as used in this research.
3. Raw-frozen as compared with cooked-frozen Pacific hake administered by stomach tube to mink depressed the absorption of either ^{59}ferric chloride or ^{59}ferrous citrate iron.
4. Fresh-unfrozen Pacific hake had no effect upon iron absorption in either rats or mink.
5. Trimethylamine oxide and formaldehyde when administered with Pacific hake significantly depressed the absorption of ^{59}ferric chloride, ^{59}ferrous citrate and hemoglobin iron-59.
6. Trimethylamine oxide naturally occurring in Pacific hake does not appear to lower iron absorption whereas naturally occurring FA does.
7. Trimethylamine and dimethylamine did not affect absorption of iron-59 by rats.

8. Addition of sodium bisulfite, which specifically reacts with free aldehydes, to hake preparations containing formaldehyde decreased the adverse effect on iron absorption.

9. Formaldehyde did not depress absorption of iron by rats when administered in the presence of water or raw, rockfish carcass.

10. Addition of acetaldehyde to cooked-frozen Pacific hake did not significantly affect absorption of ^{59}ferric chloride iron by laboratory rats.

BIBLIOGRAPHY

Amano, Keishi and Kinjiro Yamada. 1964. A biological formation of dormaldehyde in the muscle tissue of gadoid fish. Bulletin of the Japanese Society of Scientific Fisheries 30:430-435.

_____ 1964b. Formaldehyde formation from trimethylamine oxide by the action of pyloric caeca of cod. Bulletin of the Japanese Society of Scientific Fisheries 30:639-645.

Amano, Keishu, Kinjiro Yamada and Masamichi Bito. 1963. Detection and identification of formaldehyde in gadoid fish. Bulletin of the Japanese Society of Scientific Fisheries 29:695-696.

Altman, Philip L. and Dorothy S. Dittmer. 1961. Blood and other body fluids. Biological Handbook. Federation of American Societies for Experimental Biology. Washington, D. C. p. 114.

Bailey, David Eugene. 1967. Absorption and metabolism of iron as related to the cotton fur syndrome in mink. Master's thesis. Corvallis, Oregon State University. 79 numb. leaves.

Bricker, Clark E. and Helding R. Johnson. 1945. Spectrophotometric method for determining formaldehyde. Industrial and Engineering Chemistry 17:400-402.

Bothwell, Thomas H. and Clement A. Finch. 1962. Iron metabolism. 1st ed. Great Britain, J. and A. Churchill Ltd., London. 435 p.

Callender, Sheila T. 1966. Iron absorption. Proceedings of the Nutrition Society 26:59-62.

Callender, Sheila T. 1964. Digestive absorption of iron. In: Iron metabolism, ed. by F. Gross. Springer-Verlag, Berlin, 1964. p. 89-97.

Cooper, K. E. 1963. The theory of antibiotic inhibition zones. In: Analytical Microbiology, ed. by Frederick Kavanogh. New York and London, Academic Press. p. 1-183.

Costley, Gary Edward. 1968. An improved chemical method for measuring intensity of hair pigmentation. Master's thesis. Corvallis, Oregon State University. 58 numb. leaves.

Crosby, William H. 1963. Control of iron absorption by intestinal luminal factors. The American Journal of Clinical Nutrition 21:1189-1193.

Cusack, Ruth P. and W. Duane Brown. 1964. Achromotrichia in iron deficient rats. Nature 204:582-583.

Delchezlepretre, Par S., R. Portet and J. Cheymol. 1967. Toxicities comparees de al trimethylamine, de son oxyde el trimethylamine-oxide et de leur association. Medical Pharmacoloty 16:529-535.

Ender, Fredrick and Arne Helgebostad. 1947. Fôringens innflytelse på pelskvaliteten hos sølvrev. Norsk Veterinoer--Tidsskrift 12: 381-413.

_____ 1968. Studies on the anemiogenic properties of trimethylamine oxide, an etiological factor in fish-induced anemia in mink. Acta Veterinaria Scandinavica 9:174-176.

Fozzard, H. A. and G. Dominguez. 1969. Effects of formaldehyde and glutaraldehyde on electrical properties of cardiac purkinje fibers. The Journal of General Physiology 53:530-540.

Gjonnes, B. and Arne Helgebostad. 1965. Fish induced anemia in rats. Acta veterinaria Scandinavica 6:239-248.

Havre, G. N., A. Helgebostad and F. Ender. 1967. Iron resorption in fish-induced anemia in mink. Nature 215:187-188.

Helgebostad, A. 1961. Fôrtsatte studier over fôringsbetinget anemia hos mink. Norsk Pelsdyrblad 35:420-426.

_____ 1966. Anemia in standard mink and mutation mink. Norwegian Journal of Veterinary Medicine 18:347-350.

Helgebostad, A. and Fredrick Ender. 1955. Fôringens innflytelse på pelsutviklingen hos rev og mink. V. Marint felt årsak til misfarge i pelsen og røyting. Norsk pelsdyrblad 9:1-12.

Helgebostad, A. and E. Martinsons. 1958. Nutritional anemia in mink. Nature 191:1660-1661.

Helgebostad, A., B. Gjonnes and R. R. Svenkerud. 1961. Fôringsanemi hos mink. Symptomes, patologi og terapi. Norwegian Journal of Veterinary Medicine 13:593-603.

Hummon, O. J. and Frances R. Bushnell. 1943. Studies on cotton minks with reference to heredity and blood elements. American Fur Breeder 16:30-34.

Leekley, J. R. and Floyd Madison Stout. 1968. Unpublished.

Morrison, Robert T. and Robert N. Boyd. 1959. Organic chemistry. 1st ed. Allyn and Bacon, Inc., Boston. p. 632.

Riebes, Edmond E., Marcel E. Conrad and William H. Crosby. 1967. Gastrectomy and iron absorption: Effects of bleeding, iron loading and ascorbic acid in rats. Proceedings of the Society for Experimental Biology and Medicine 124:577-580.

Schode, Stanley G., Bertram F. Felsher and Marcel E. Conrad. 1969. An effect of intestinal motility on iron absorption. Proceedings of the Society for Experimental Biology and Medicine 130:757-761.

Seton, Ernest Thompson. 1929. Lives of game animals. Vol. II, Part 2. Garden City, Doubleday, Doran. 746 p.

Shewan, J. M. 1951. The biochemistry of fish. Biochemical Society Symposium. University Press, Cambridge 6:28-48.

Smith, G. C. 1968. Effects of formaldehyde on intestinal motility in the rat. Journal of Physiological Chemistry 34:156-158.

Snedecor, George W. 1950. Statistical methods. 4th ed. The Iowa State College Press, Ames, Iowa. 485 p.

Stout, Floyd Madison. 1960. Aberrant iron metabolism and the cotton fur abnormality syndrome in mink. Ph. D. thesis. Corvallis, Oregon State University. 94 numb. leaves.

_____ 1966. Factors associated with aberrant iron metabolism. Progress report to the National Institute of Health. Grant AM-08210. 15 p.

Stout et al. 1969. Personal communication.

Stout, Floyd Madison and David Eugene Bailey. 1969. Manuscript in preparation.

Stout, Floyd Madison, James E. Oldfield and John Adair. 1960. Nature and cause of the cotton fur abnormality in mink. The Journal of Nutrition 70:421-426.

_____ 1960b. Aberrant iron metabolism and the cotton fur abnormality in mink. The Journal of Nutrition 72:46-52.

_____ 1968. A new weapon in the struggle against bacteria. Oregon's Agricultural Progress, Agricultural Experiment Station. Oregon State University 15:2.

Tengerdy, Catherine, Parviz Azari and Robert P. Tengerdy. 1966. Immunochemical reactions of conalbumin and its metal complexes. Nature 211:203-204.

Wang, C. H. and David L. Willis. 1965. Radiotracer methodology in biological science. 1st ed. Prentice-Hall Inc. Englewood Cliffs, New Jersey. 381 p.

Wilson, G. S. 1958. Disinfection of fabrics with gaseous formaldehyde. Journal of Hygiene 56:488.

Yamada, Kinjiro and Keishi Amano. 1965. Studies on the biological formation of formaldehyde and dimethylamine in fish and shellfish. V. On the enzymatic formation in the pyloric caeca of Alaska pollock. Bulletin of the Japanese Society of Scientific Fisheries 31:60-64.

_____ 1965b. The biological formation of formaldehyde in cod flesh. In: The technology of fish utilization, ed. by Rudolf Kreuzer. Ludgate House, London, England 73-78.

_____ 1965c. Studies on the biological formation of formaldehyde and dimethylamine in fish and shellfish. VII. Effects of methylene and dimethylamine from trimethylamine oxide. Bulletin of the Japanese Society of Scientific Fisheries 31:1030-1037.

Yamada, Kinjiro, Katsuhiko Harada and Keishi Amano. 1968. Biological formation of formaldehyde and dimethylamine in fish and shell-fish. VIII. Requirement of cofactor in the enzyme system. Bulletin of the Japanese Society of Scientific Fisheries 35:227-231.

APPENDIX

APPENDIX I

Counter settings for measuring iron-59 in an Armac Whole Body Liquid Scintillation counter and a Packard Model 410A Auto-Gamma Spectrometer.

Item	Settings	
	Armac whole-body counter	Auto-Gamma spectrometer
(1) Counter efficiency	30 percent	10 percent
(2) Approx. background	1100 cpm	70 cpm
(3) High voltage switch	On	On
(4) Operating mode switch	Green mode	Automatic
(5) Analysis mode switch	Wide	Wide
(6) Lower baseline control	300	450
(7) Upper width control	900	800
(8) Attenuator switch	1	1
(9) High voltage tap switch	6	4
(10) High voltage fine control	175	100
(11) High voltage indicating meter	900	925
(12) Gain control	5 percent	---