AN ABSTRACT OF THE THESIS OF

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Title ABSORPTION AND METABOLISM OF IRON AS RELATED

TO THE COTTON FUR SYNDROME IN MINK

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Abstract approved

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"Cotton fur" (CF) in mink (Mustela vison) is characterized by lack of pigment in the underfur of dark mink and is part of a syndrome including hypochromic, microcytic anemia and substandard growth, resulting from an iron deficiency. Such symptoms are produced by feeding rations containing raw Pacific hake (Merluccius productus), even though the ration contains adequate iron.

The research reported herein concerns two phases of the CF abnormality; first a study using purifed diets to determine if iron deficiency per se results in failure of pigment formation, and second, studies of iron metabolism using ^{59}Fe as a tracer to determine why iron is not utilized by mink fed hake-containing rations.

Standard dark mink kits were fed either a basal iron-deficient diet or the basal diet with 15 ppm of added iron. Chemical analysis

of the diets showed that they contained 4.6 and 19.7 ppm of iron, respectively. Blood data illustrated that a hypochromic, microcytic anemia was present in those animals receiving the iron-deficient diet. After the winter furring cycle, all mink on the deficient diet exhibited unpigmented underfur and those receiving the adequate diet had normally pigmented underfur; establishing the necessity of iron as a chromotrichial nutrient.

In a subsequent trial four groups of mink received purified diets with 0, 5, 10, and 15 ppm of added iron. Results supported those of the previous one, with 75 and 33 percent CF incidence in the 0 and 5 ppm groups respectively, and no CF in either the 10 or 15 ppm groups. Limited data showed that 70 percent of the ^{59}Fe present in mink hair was located in the melanin granule, indicating that iron may be an integral part of the melanin molecule.

Eight trials involving 37 individual iron balance trials were conducted using ^{59}Fe to provide information on the second objective. ^{59}Fe was administered in feed or stomach tubed and iron absorption was determined from blood and excretory data.

Ferrous citrate-^{59}Fe was given in either raw Pacific hake or sole based rations. From blood data, iron absorption was 1.4 percent for the raw hake-fed mink versus 11.2 percent for mink receiving sole. Due to uncontrollable fecal ^{59}Fe contamination of urine, plasma radioactivity was used to determine if the iron was

being absorbed and excreted in the urine.

To ensure complete ingestion of the tracer dose, mink were stomach tubed with ^{59}Fe in a water filtrate of hake, and controls received the radioisotope in distilled water. Iron absorption was greater (16.3%) in presence of raw hake filtrate than with distilled water (11.6%). Mink fed raw hake containing rations absorbed 7.5 percent and those fed rations of cooked hake absorbed 6.7 percent of the ^{59}Fe given. ^{59}FeSO$_4$ was given in cooked and raw hake suspensions and iron absorption was 23.2 percent for the cooked hake and less (12.3%) for raw hake with iron added immediately, as compared to 15.9 percent for iron allowed to incubate with raw hake.

^{59}Fe-hemoglobin was adminstered in cooked and raw hake suspensions and absorption of iron was completely stopped in the mink given raw hake, but only lowered (5.2%) in those given cooked hake. ^{59}FeCl$_3$ was given with cooked and raw hake suspensions. Mink receiving cooked hake absorbed 16.6 percent and those receiving raw hake absorbed only 2.9 percent, suggesting that the factor present in raw hake specifically interferes with the utilization of ferric iron.

Form of iron, presence of other feedstuffs, and length of incubation of added iron were found to influence the utilization of iron in presence or absence of raw hake. ^{59}Fe activity of blood

components indicate that in every case where iron utilization is impaired by the presence of raw hake, interference is at the absorptive level. Time of feed passage through the gastrointestinal tract as determined by excretion of radioactive iron for the 37 observations made was 185 ± 22 minutes with a range of 59-360 minutes.

ABSORPTION AND METABOLISM OF IRON AS RELATED
TO THE COTTON FUR SYNDROME IN MINK

by

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GENERAL INTRODUCTION

The mink (Mustela vison) is aboriginal to the North American

Continent, and in the wild state inhabits most of the forested areas

where there is permanent fresh water. Its range extends from

Florida to Alaska and from California to Quebec.

As far back as February of 1871, the New York Post published

an account of a new animal industry--ranch raising of mink for mar-

ket (Ashbrook, 1948). In the early 1920's, a keen interest in mink

raising developed. However, the industry remained in the experi-

mental stage until the early 1930's when results demonstrated that

these fur animals could be produced profitably. Mink farmers have

improved the quality of the animals by controlled selective matings,

proper and regular feeding, and intelligent management. During the

past twenty years, the color of mink fur has unquestionably become

the most important single fur attribute (American Fur Breeder, 1965).

Ranch mink production in the United States has increased from

slightly less than three million in 1954 to seven and one-half million

in 1964. During 1964, Oregon ranked sixth among the top ten mink

producing states. Production was 371,400 ranch mink pelts, which

amounts to a $4,500,000 industry in the state of Oregon alone

(National Board of Fur Farm Organizations, 1965).

In the wild state, mink, being carnivores, consume a diet consisting mainly of birds, fish, mice, and other rodents. Mink ranchers, therefore, have a problem of feed supply uncommon to other livestock producers. Previously, horsemeat composed a large part of the mink ration. Now slaughterhouse by-products, chicken offal, turkey offal, and fish, especially in coastal areas, comprise the major portion of the ration.

The feeding of certain species of fish leads to a fur anomaly known as "cotton fur" (CF) which is characterized by a light colored underfur. The production of CF reduces the value of dark mink pelts, and since standard dark pelts are selling at an all-time high (1964), the occurrence of CF greatly detracts from the profitability of this important segment of mink ranching. CF is part of a syndrome, including anemia and substandard growth rate, which results from an iron deficiency in the body.

This thesis concerns two phases of the CF abnormality: first, a study to determine if iron deficiency per se results in the failure of pigment formation, and secondly, studies of iron metabolism using ^{59}Fe as a tracer in an attempt to determine why dietary iron is not utilized by mink fed the causative fish diets.

PART 1: IRON AS A CHROMOTRICHIAL FACTOR

The early Greeks and Romans believed that Mars, the god of war, had infused iron with force and strength and that these properties could be imparted therapeutically to persons who suffered from weakness. The progression of discoveries which led from this empiricism to the identification of iron in hemoglobin, and to the recognition that hypochromic, microcytic anemia is the principal manifestation of iron deficiency, is an interesting part of history.

Iron is present in living organisms in extremely small amounts and performs a number of biological functions essential to life (Bothwell and Finch, 1962). The functions of iron in oxygen transport and oxidative processes are so basic to life that investigators from many scientific disciplines have been attracted to a study of one or another phase of iron metabolism. A previously unknown function, the involvement of iron in pigment formation, is the area explored in the three experiments conducted. The objective of Experiment 1 was to determine if mink, fed purified, iron-deficient rations would exhibit lack of underfur pigment, characteristic of CF. Experiment 2 was designed to verify Experiment 1 and further determine iron requirements for maintaining normal fur color and blood values. The objective of Experiment 3 was to gain an insight on the function of iron in hair pigmentation by determining if iron

was an integral part of the pigment.

Review of Literature

Cotton Fur in Mink

In a letter written by Henry Bertman in 1924, the "cotton-fur" (CF) abnormality was considered an obscure disease and described thus: "These Mink, derive their name 'cotton' from their fur, especially the under fur, which according to the stage of their sickness, may be gray, light, and finally white; not unlike cotton in colour and texture. The outer fur never shows any perceptible evidence of the condition of the under fur, but on brushing aside the outer fur, one readily sees the white under-coloured fur which characterizes the Cotton Mink" (Seton, 1929, p. 520).

In giving instructions for the ranch raising of mink, Patton (1925, p. 198) had these words of advice: "Care should be taken never to allow what is known as the 'cotton mink' to enter the herd, because these animals produce furs of lesser value. It is not one of a species of mink, but merely a colortype variety produced by an unknown cause."

Seton (1929, p. 520) further discusses the cotton mink: "Among the curious and worthless freaks that have no reference to season, place, or age...distinguished from the normal Mink

by having the under fur of flimsy texture, and drab or white instead of dark brown or warm gray." CF is thus described as a failure of underfur of dark haired mink to become darkly pigmented.

Kennedy (1947) described CF as a result of a nutritional deficiency and noted that a hypochromic, microcytic anemia was present and hemoglobin values were 40 percent below normal in mink with CF. Adair (1955) reported that CF mink were experimentally produced when an attempt was made to utilize Pacific hake (Merluccius productus) and whiting (Merluccius bilinearis) for mink feed. Stout, Oldfied, and Adair (1960a) further described the nature and cause of the CF abnormality in mink. They showed that the incidence of CF was directly related to the proportion of hake and whiting in the diet and that thorough cooking of hake prior to mixing in the diet led to complete elimination of CF development. They also found that evisceration of the causative fish eliminated CF incidence in the case of whiting and lowered it in the case of hake.

Hummon and Bushnell (1943) reported studies of cotton mink in which they attempted to produce cotton mink kits by mating CF adults. No kits were produced that exhibited CF; however, they fed a non-causitive diet and the CF which they studied was not accompanied by anemia. Stout, Oldfield, and Adair (1960a) demonstrated that there was a genetic tendency toward higher or lower

incidence of CF and anemia when known causative diets were fed.

Helgebostad and Martinsons (1958) reported that 16 milligrams
of organic iron, injected weekly, would prevent the anemia which
accompanied CF. They also found that daily oral doses of 40 milli-
grams of iron gave some relief. The type of oral iron used was not
stated in the reference. Conversely; Stout, Oldfield, and Adair
(1960b) reported that there was no relief of anemia or CF when
animals were fed up to 88.1 milligrams of iron (as a ferroglycine
sulfate complex) per kilogram of ration. But they did find that
intramuscular injections of 25 milligrams of iron (as ferric hydrox-
ide) per week gave complete prevention of both anemia and CF.

Nutritional Basis of Achromotrichia

Frost (1948) reviewed the relation of nutritional deficiencies
to graying. Achromotrichia has been attributed to deficiencies of
pantothenic acid, p-aminobenzoic acid, folic acid, biotin, ribo-
flavin, choline, inositol, cystine, lysine, copper, and zinc; how-
ever, iron was not mentioned as a cause. A further review related
achromotrichia in the presence of anemia to nutritional deficiencies
of pantothenic acid, copper, lysine, and folic acid (Stout, Oldfield,
and Adair, 1960b).

Lerner and Fitzpatrick (1950) have presented evidence show-
ing that copper is essential for pigmentation in mammals.

Copper-deficient diets resulted in pigmentation failure in rats,

cats, rabbits, and cattle. Addition of trace amounts of copper salts

to the deficient diet restored pigmentation. However, an interesting

point is that copper plus iron were more effective in restoring pig-

mentation than was copper alone.

Rothman and Flesch (1943) isolated an iron-containing pigment

from human red hair. Their data indicate that it was a complex,

phenolic iron compound in which the phenolic hydroxyl group is

attached to a heterocyclic ring containing nitrogen and a single

complexed ferric ion. This iron-containing pigment was not found

in the red hair of animals or in human hair other than red. Its

complete extraction did not change the original color of the hair to

any marked degree; however, it corresponded with the disappearance

of light brown granules from the cortex of the hair.

Stout, Oldfield, and Adair (1960b) pointed out that a physio-

logical deficiency of iron could result in the failure of pigment

formation in mink fur. Normal pigmentation could be restored by

intramuscular injections of iron.

Lack of feather pigmentation has been attributed to iron de-

ficiency in Rhode Island Red chicks (Hill and Matrone, 1961) and

New Hampshire chicks (Davis, Norris, and Kratzer, 1962), which

was not related to the copper level of the diet.

Cusack and Brown (1964 and 1965) have reported achromo-trichia in black rats when they were fed purified diets deficient in iron. They found a variation among individuals in the time of onset and severity; but after eight weeks on the iron-deficient diet, graying was pronounced in all test animals.

Experiment 1: Iron-deficient and Adequate Purified Diets in Relation to Cotton Fur

Methods and Materials

Two groups of four standard dark mink kits were used to determine the effect of feeding an iron-deficient diet on fur pigmentation. Each group consisted of two males and two females and each animal had a littermate of the same sex in the opposite group. One group received the basal purified diet listed in Table 1; whereas, a control group received the basal diet with 74.6 milligrams $FeSO_4 \cdot 7H_2O$ (15 ppm of iron) added per kilogram of ration.

This ration was developed at Cornell University (Warner, Travis, and Bassett, 1964) and modified as follows: (1) Two parts Alphacel and one part Solka-Floc were used as a source of purified cellulose. Alphacel, alone, was unsatisfactory in that the ration tended to become hard and lumpy after being stored in the refrigerator; whereas a homogeneous mixture was difficult to obtain when

Table 1. Basal purified diet.

Ingredient	Parts
Vitamin-free casein	30.0
Sucrose	39.0
Lard (unstabilized)	10.0
Cottonseed oil[1]	9.0
Alphacel[2]	3.33
Solka-Floc[3]	1.67
Choline dihydrogen citrate	0.66
L-arginine	0.05
DL-methionine	0.025
L-cystine	0.025
Vitamin mixture[4]	1.00
Mineral mixture[5]	3.19

[1]Wesson Oil, The Wesson Oil Company, New Orleans, Louisiana.

[2]Nutritional Biochemicals Corporation, Cleveland, Ohio.

[3]Brown Company, Boston, Massachusetts.

[4]Vitamin Mixture	/kg diet	[5]Mineral Mixture	/kg diet
Thiamine HCl	10 mg	KCl	107 mg
Pyridoxine HCl	10 mg	NaCl	6.62 g
Calcium Pantothenate	15 mg	ZnCl	62 mg
Riboflavin	20 mg	KI	5.25 mg
Niacin	40 mg	K_2HPO_4	12.81 g
i-Inositol	250 mg	$CaHPO_4 \cdot 2H_2O$	3.15 g
Para aminobenzoic acid	500 mg	$CaCO_3$	11.69 g
Menadione	25 mg	$CuSO_4 \cdot 5H_2O$	78.6 mg
Folic acid	2 mg	$MnSO_4 \cdot 1H_2O$	190 mg
Biotin	0.5 mg	$MgSO_4$	3.10 g
Vitamin E	40 IU	$Na_2MoO_4 \cdot 2H_2O$	3.78 mg
Vitamin D_3	1200 IU	Na_2SeO_3	0.56 mg
Vitamin A powder	12,000 IU		
L-ascorbic acid	99 mg		
Vitamin B_{12} (0.1% triturate)	0.04 mg		
Sucrose -- Added to make 10 g total			

Solka-Floc was used alone. The indicated combination gave a homogeneous mixture that solved the difficulties noted with the individual types. (2) Since Vitamin E was added to the diet, the synthetic antioxidant Santoquin (ethoxyquin) was omitted. (3) The quantity of added iron was inadvertently reduced from 150 ppm, as used at Cornell University, to 15 ppm.

Six kilograms of each diet were mixed at a time, which was sufficient to feed a group approximately two weeks. The mineral and vitamin mixtures were prepared separately by blending the ingredients of the respective mixtures and then passing them through a fine copper screen several times. After homogeneous mixtures were obtained, they were placed in brown glass jars for storage. The mixing procedure for the whole ration was as follows: All ingredients, except lard and cottonseed oil, were weighed and mixed in a 15 gallon plastic tub by passing through a fine copper wire screen until homogeneous. Melted lard and cottonseed oil were then blended into this dry mixture. Mixed diets were placed in plastic bags and stored at four degrees Centigrade until used.

Animals, previously fed conventional, high-fish diets, were fed once daily in glass jars from July 30, 1964 to February 19, 1965. Feed was offered ad libitum, but level of feeding was adjusted according to the amount of feed each animal would consume daily. Distilled water (<0.1 ppm Fe) was continuously supplied by

an automatic, iron-free watering system consisting of glass, plastic, and brass components. Animals were housed separately in cages 10 x 12 x 24 inches, which were constructed of zinc-plated, copper-coated, steel wire covered with 0.010 inch vinyl.

Initially and at the termination of the test period, blood samples were taken for evaluation of changes in the overall blood picture. Three milliliters of blood were withdrawn by cardiac puncture from unanesthetized animals and mixed with an anticoagulant of potassium and ammonium oxalate prepared as described by Davidsohn and Wells (1962, p. 67). However, the anticoagulant solution was lyophilized[1] prior to use. Midway through the experimental period, blood was also withdrawn by clipping a toenail for micro-hematocrit determination. Hemoglobin was determined by the cyanmethemoglobin procedure (Davidsohn and Wells, 1962 p. 73), hematocrit by the microhematocrit method (Davidsohn and Wells, 1962, p. 93), and red blood cell counts were made using an auto-cytometer (Fisher Model 85).

Animals were weighed (to the nearest 10 grams) in a box trap on a Model 4700 Chatillon Scale. The feeds were wet ashed for iron determination according to the method of Kennedy (1927). The iron content of the two diets and distilled water was quantitatively

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[1] Thermovac, Model FDC-10-JDV Freeze Drier, Thermovac Industries Corporation, Copiaque, New York.

determined by using a Techtron Model AA-3 Atomic Absorption

Spectrophotometer.[2]

Results

Growth curves of males and females from both groups are

shown in Figure 1. The initial weight loss, due to the failure of

the animals to eat, was probably a consequence of the major change

in physical nature of the diet.

An error made in calculating the copper requirement resulted

in a supply of ten times more copper than required for the first

month. Initially, stools were dark blue-green in color and after

lowering the copper to the appropriate level, they became light

yellow-orange in color. A blue-green stool is one of the character-

istic symptoms of copper toxicity (Kaye, 1961). The animals

apparently suffered no lasting adverse affects from the excess

copper, but it could be one reason for the initial anorexia.

As evident from the growth curves, animals receiving the

complete ration exhibited a more rapid growth pattern than those

receiving the iron-deficient ration. Chemical analysis of the two

diets showed that the iron-deficient diet contained 4.6 ppm of iron

and the basal diet contained 19.7 ppm of iron.

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[2] Techtron Pty. Limited, Melbourne, Australia.
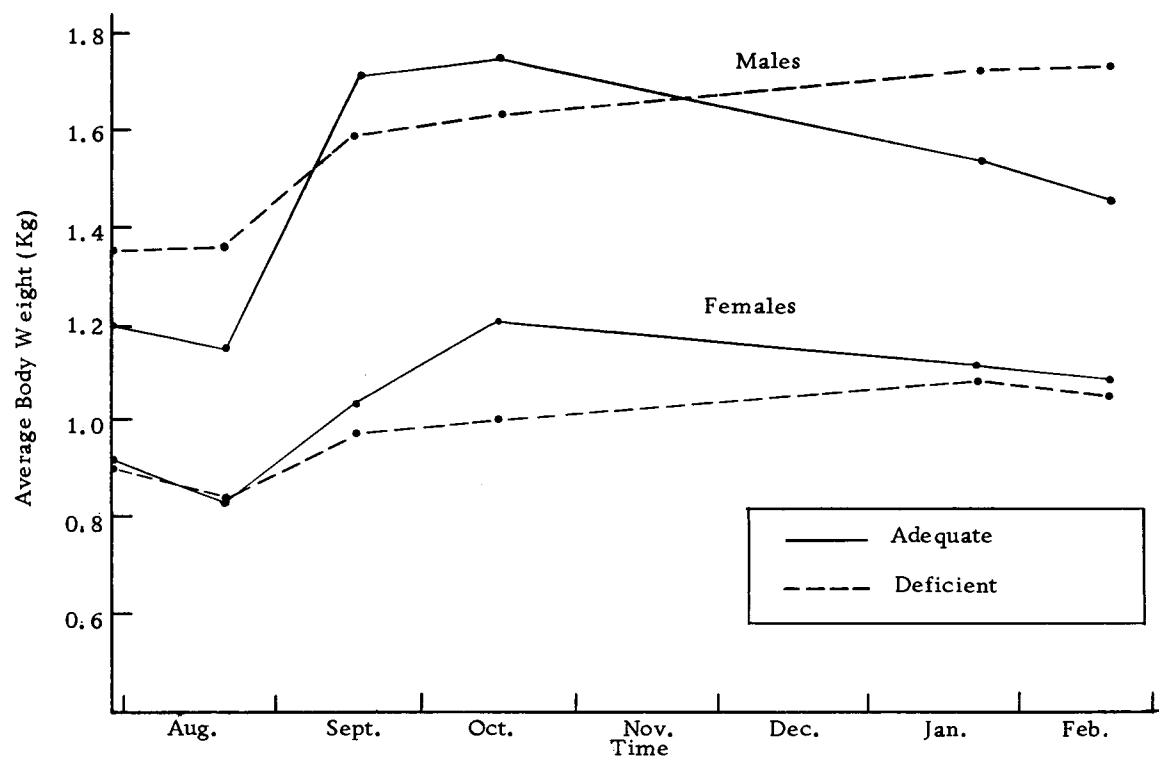
Figure 1. Growth curves of mink fed diets
adequate and deficient in iron.

The final weight loss of the control group is a consequence of an unexplained decrease in feed consumption of animals fed the control diet. During the period of December through February, average daily consumption per mink was as follows: Control males, 55 grams; control females, 59 grams; iron-deficient males, 80 grams; and iron-deficient females, 66 grams. The feed offered was adjusted daily to the level of the previous days consumption; therefore, it appears that the desire for feed was greater in the iron-deficient group. Since there were no body weights taken in November and December, it cannot be determined whether this weight loss occurred rapidly or gradually.

Fur growth in mink occurs in two cycles. In spring and fall, the old fur is shed and replaced by new growth. The summer coat is relatively thin as compared to the much heavier winter coat. At the start of the experimental period, all of the animals had darkly pigmented summer coats. The effect of iron deficiency on underfur pigmentation was later quite evident. Examination on November 16 and until termination of the experiment revealed that in every case, the mink on the iron-deficient ration exhibited the unpigmented underfur which is characteristic of CF (see Figure 2). However, all mink receiving the iron-adequate ration had normally pigmented underfur.

Blood data listed in Table 2 show the dramatic effect of the
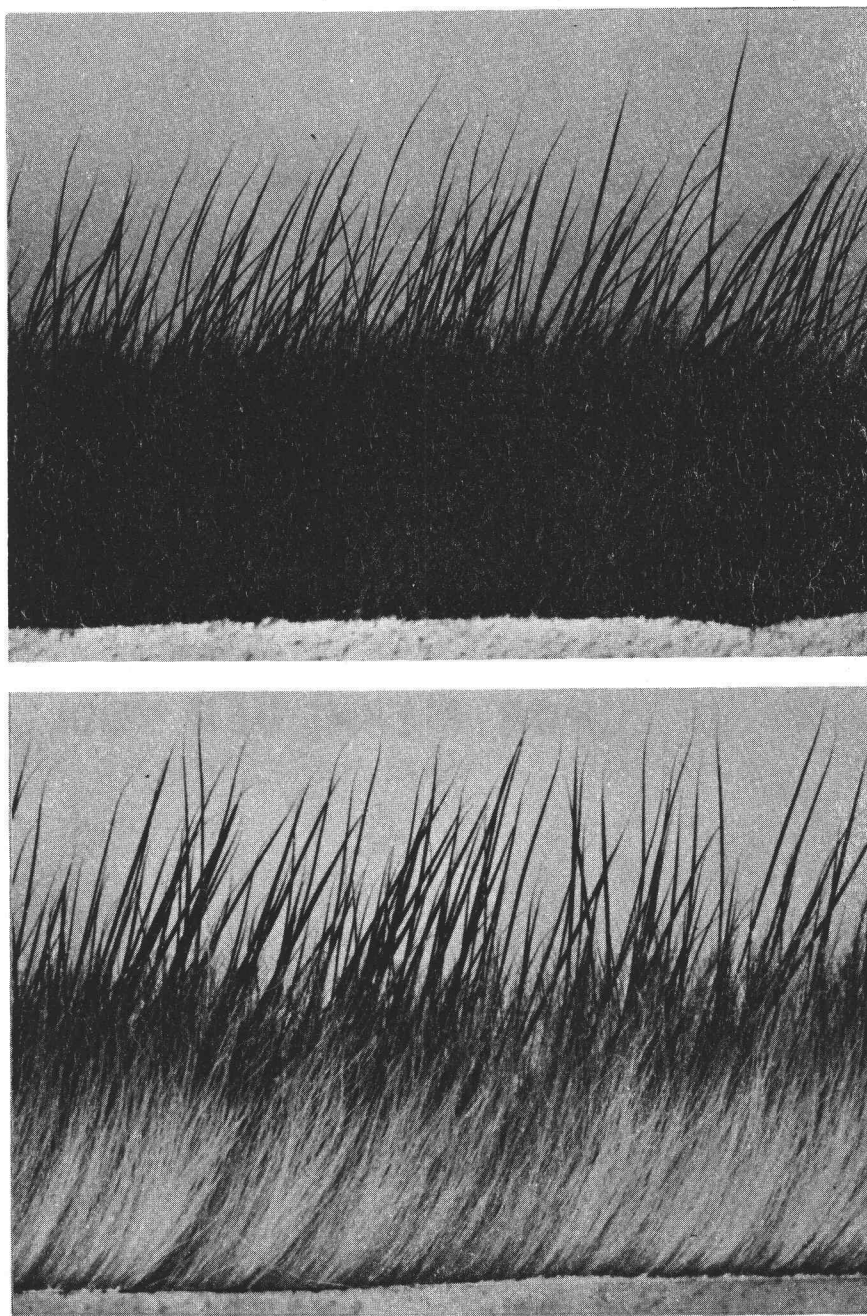
Figure 2. Cross-section of mink pelts showing (upper) darkly pigmented fur of mink fed purified diet adequate in iron and (lower) unpigmented underfur of mink fed iron-deficient purified diet.

Table 2. Blood data of mink fed complete and iron-deficient purified rations (± values are standard deviations).

Period	Ration	Hemoglobin	Hematocrit	RBC million per mm^3	Mean corpuscular Hb. g x 10^{-12}	Mean corpuscular volume microns^3	Mean corpuscular Hb. Conc. %
Initial	Complete	18.2±0.7	46.3±2.2	7.10±1.4	25.9±1.8	66.0±5.2	39.3±3.3
(July 31, 1964)	Deficient	17.8±0.2	47.4±2.4	6.99±0.5	25.4±1.4	67.7±3.9	37.6±2.3
Intermediate	Complete		47.1±2.3				
(Sept. 26, 1964)	Deficient		37.5±2.9				
Final	Complete*	19.7±0.4	44.2±1.2	8.36±0.7	23.5±0.4	52.9±2.2	44.5±2.1
(Feb. 19, 1965)	Deficient	9.7±1.9	26.5±4.4	6.79±1.8	14.1±0.9	39.3±3.0	36.0±3.6

*One female was not included because of chronic blood loss as a result of chewing the end of her tail (first noted November 16). She showed a typical iron-deficiency anemia.

iron-deficient diet on the animals receiving it, and clearly illustrate that an iron deficiency was achieved. After 58 days on test, the average hematocrit value had been reduced by 20 percent in the iron-deficient group. Final values show that hemoglobin and hematocrit were reduced by an average of 51 and 40 percent respectively; whereas, erythrocyte numbers were reduced by only 19 percent. The observed anemia was hypochromic and microcytic as determined by calculating values for mean corpuscular volume and mean corpuscular hemoglobin concentration. This type of anemia is characteristic of an iron-deficient state.

Experiment 2: Further Studies with Purified Diets and Cotton Fur

Methods and Materials

Four groups of mink received graded levels of iron to obtain information relative to the dietary iron requirement for pigmentation and maintenance of normal blood values. Since the experiment of a year earlier indicated that 15 ppm of added iron was sufficient for both normal pigmentation and normal blood values, groups were set up to receive 0, 5, 10, and 15 ppm of added iron. Each group consisted of four animals (two males and two females) and were randomized as in Experiment 1. The animals received the experimental diet from August 6 until termination on November 3, at

which time fur color could be established.

The watering system was the same as in the earlier experiment except a plastic storage container was substituted for the glass one. Cages used in this experiment were chrome plated instead of vinyl clad as used previously. A substitution of cerelose (glucose) for sucrose and graded levels of iron were the only dietary changes. All animals were weighed monthly and blood samples were withdrawn and determinations conducted as described earlier. Feed was offered in plastic glasses instead of glass jars as used previously.

Results

The results of this experiment (Table 3) were rather inconclusive but did support results of the previous experiment. Two factors complicated this trial: first, the substitution of cerelose for sucrose and second, the fact that when the watering system was reassembled, a galvanized iron nut was unknowingly used, which subsequently rusted and supplied a small amount of iron to the mink. It was discovered and removed midway through the experimental period.

Figure 3. shows growth curves of all mink in the 15 ppm added iron group (25. 5 ppm Fe by chemical analysis). Only two survived the entire experimental period and it can easily be seen that growth

Table 3. Weights, blood values, and fur color (Experiment 2).

Added Iron (ppm)	Mink Serial No.	Initial Weight (g) (Aug. 6, 1965)	Final Weight (Nov. 3, 1965)	Blood Data (Nov. 3, 1965)		Fur Color (Nov. 3, 1965)
				Hemoglobin (g %)	Hematocrit (%)	
0	7678	1240	--**	--	--	CF (Band)
0	7679	1310	1690	14.1	37.5	CF (Band)
0	7680	820	770	17.7	48.0	Dark
0	7681	730	1090	14.6	40.5	CF (Base)
5	7682	1430	1640	18.5	46.5	Dark
5	7683	1180	1460	12.8	32.5	CF
5	7684	860	780	18.3	46.5	Dark
5	7685	720	--**	--	--	--*
10	7686	1210	--**	--	--	Dark
10	7687	1220	1720	18.4	48.5	Dark
10	7688	780	930	17.2	46.0	Dark
10	7689	720	980	18.3	45.0	Dark
15	7690	1370	--**	--	--	Dark
15	7691	1220	1330	17.0	45.0	Dark
15	7692	890	790	16.9	44.5	Dark
15	7693	680	--**	--	--	--*

*Died before fur color could be determined.
**Died before termination of experiment.
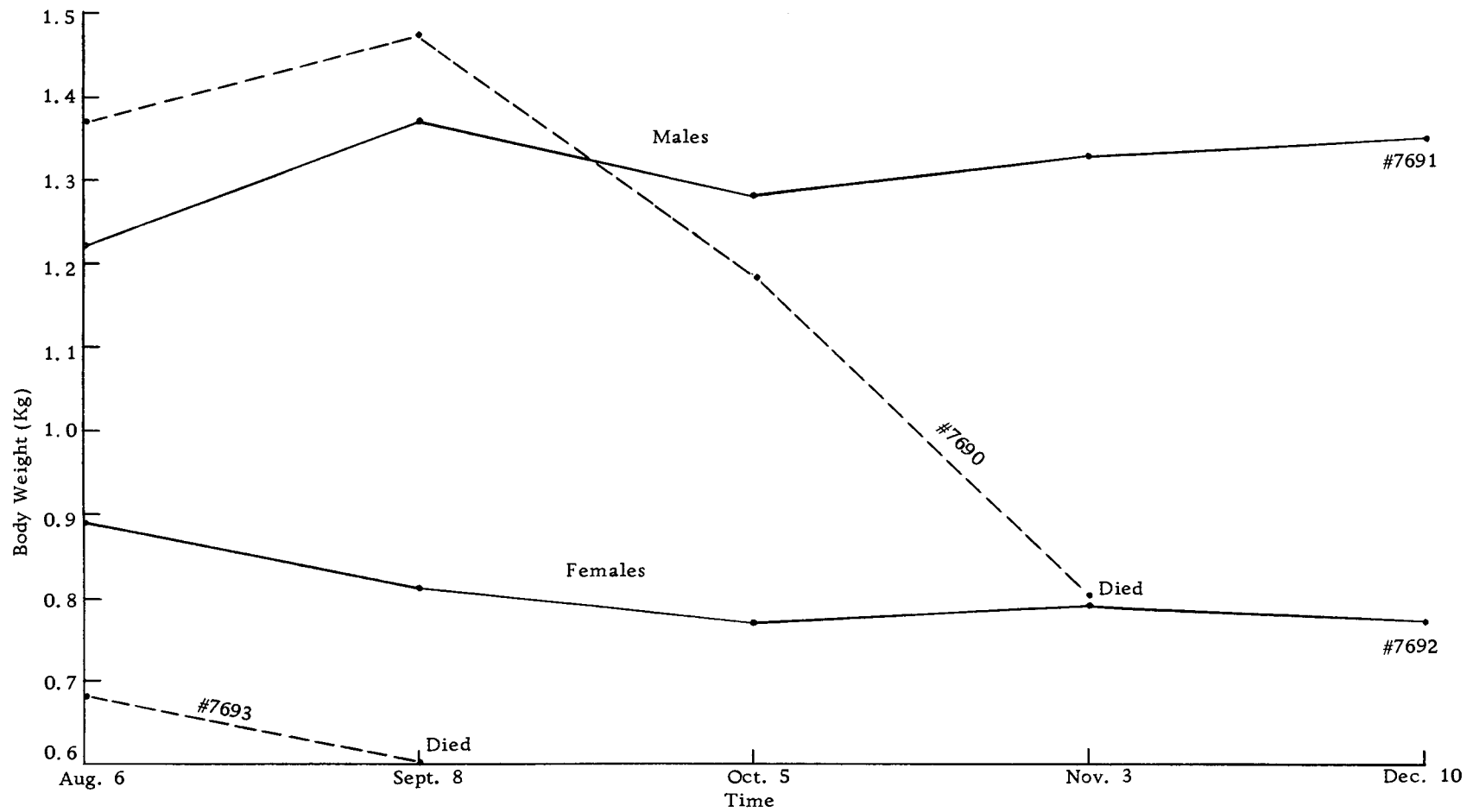
Figure 3. Growth curves of mink fed the iron-adequate purified diet (Experiment 2).

was poor, with gain and loss of weight erratic in all cases.

Final hemoglobin and hematocrit values and the incidence of CF are shown in Table 3. Notice that only anemic mink exhibited CF. Three out of four mink in the iron-deficient group exhibited CF and one out of three in the five ppm group; whereas, none of the mink in either the 10 ppm or 15 ppm groups exhibited CF.

At the termination of the experimental period, the survivors were killed, pelted, and autopsied. It was noted that many of the animals in this experiment showed ulceration of the stomach.

Discussion (Experiments 1 and 2)

Body Growth. Comparing the control group of Experiment 1 to the standard, conventional type mink ration (Group 64-1A) fed the same year (Adair, Stout, and Oldfield, 1965), shows that the females grew equally well on the purified diet, with a weight of approximately 1200 grams per animal. The males, however, did not grow as well, with a weight of 1765 grams as compared to 2139 grams per male, in the 64-1A group. But all four groups of animals in Experiment 2 showed lack of thriftiness and poor growth, regardless of the level of iron in the ration. Between groups in Experiment 2, initial body weights of males and females were comparable; however, the final weights were quite variable (Table 3). In Experiment 1, there were no deaths among eight experimental

animals even after feeding the purified rations for seven months, as compared to a death loss of five out of sixteen mink within a three month period in Experiment 2; indicating that something was basically wrong with the ration used in the latter experiment.

Many of the animals autopsied in Experiment 2 showed gastric ulcers with stages of ulceration ranging from a slightly reddened mucosa to raw and bleeding sores. Since none of the mink on the sucrose diet (Experiment 1) exhibited stomach ulcers, it is conceivable that those present in the mink of Experiment 2 were a consequence of the diet. Adair, Stout, and Oldfield (1966) fed a ration composed of approximately 30 percent cerelose to a group of 26 mink (Group 65-5A) and found that they were unthrifty and grew poorly. Eleven mink from that group died and some showed internal bleeding from stomach ulcers. These results support those of Experiment 2, and it may be justifiable to suspect that cerelose caused the observed ulcers and poor growth.

Cerelose has a molecular weight of 180, or approximately half the molecular weight of sucrose (342). Therefore, in a given weight, there would be nearly twice as many molecules of cerelose as of sucrose. A possible explanation for the observed stomach ulceration might be the increased osmotic pressure relationship in the stomach.

Fur Growth and Pigmentation. Stout, Oldfield, and Adair
(1960b) showed that the CF syndrome resulting from feeding Pacific
hake could be prevented by injections of iron, but the research
reported herein demonstrates unequivocally that the achromotrichia
of this syndrome results from an uncomplicated iron deficiency.
Since this work has been underway, Cusack and Brown (1964) have
shown that a symptom of iron deficiency in black rats is graying
or lack of pigmentation in the fur. As cited earlier, Lerner and
Fitzpatrick (1950) unknowingly may have been the first to produce
achromotrichia due to an iron deficiency, although they were spec-
ifically studying copper deficiency. Their diets were thought to
contain adequate iron, but since copper plus iron were more effec-
tive in restoring pigmentation than was copper alone, one might
conclude that the diets they used were also deficient in iron.

Results of Experiment 1 indicate that 15 ppm added iron
(19.7 ppm Fe by chemical analysis) is sufficient for normal
growth and maintenance of blood values. Results of Experiment 2
more closely identify the actual requirement and indicate that it
lies between five and ten ppm added iron in the ration. However,
the true picture is undoubtedly influenced as a result of the inad-
vertant iron contamination of the water. Therefore, the actual
requirement could be higher than indicated. Since 19.7 ppm of
iron is adequate for normal fur growth and hemoglobin levels,

150 ppm of iron in the diet as used by Warner, Travis, and Bassett (1964), is probably greatly in excess of the actual requirement. Nevertheless; in a conventional type ration, the iron requirement would be increased since most of the iron would be in forms other than ferrous, resulting in reduced utilization by the animal.

It is not known why iron deficiency causes achromotrichia, but several possible mechanisms of action are suggested. Iron could be an integral part of the pigment itself, or it could be necessary for activation of an enzyme system necessary for the formation of melanin, or both.

Several iron-containing pigments have been characterized. Rothman and Flesch (1943) isolated an iron-containing pigment from human red hair. Later, they (Flesch and Rothman, 1945) reported that this iron-containing pigment was actually a brown pigment that could be extracted from light brown granules in the hair cortex. Nickerson (1946) also indicated that iron was a part of the pigment when he isolated a component of melanin from red and buff feathers. Thus far, iron-containing pigments have been isolated only from human red hair and red and buff feathers, but have not been found in hair or feathers of other colors. Evidence indicates that these pigments are specific for the color of hair and feathers mentioned. Work has not been reported in which an attempt was made to determine if the iron-containing pigments

from hair and feathers are actually the same pigment.

Melanin is believed to be formed, via intermediates, by the action of tyrosinase on tyrosine (Lerner and Fitzpatrick, 1950). Copper is specifically required for the optimum activity of tyrosinase; however, Kaufman (1957) has shown that ferrous ions are required for the enzymatic conversion of phenylalanine to tyrosine.

The results of a study by Chanarin, Rothman, and Berry (1965) strongly suggest that iron deficiency in pregnancy may produce a secondary deficit of folic acid. Further, the studies of Velez et al. (1965) showed that patients with iron-deficiency anemia brought about by hookworm infestation had increased urinary formimoglutamic acid (FIGLU) excretion and megaloblastic anemia. All these metabolic defects were alleviated by iron therapy. These symptoms had been shown to be characteristic of folic acid deficiency (Luhby, Cooperman, and Teller, 1959). Chanarin, Bennett, and Berry (1962) also reported increased urinary FIGLU excretion in patients with iron deficiency.

With the finding recently (Vitale et al., 1966) that iron deficiency induces a secondary folic acid deficiency, notwithstanding that the diet provided abundant folate, the relationship between iron and fur pigmentation may possibly be explained. They found that iron was essential for optimum activity of the enzyme,

glutamate formimino-transferase, which is necessary for proper folic acid metabolism. Without the function of this enzyme, a folic acid deficiency results.

Frost (1948) and Rothman (1954) reviewed the relationship of achromotrichia to dietary deficiencies of pantothenic acid, folic acid and p-aminobenzoic acid (PABA) and suggested that pantothenic acid can be considered the chief anti-achromotrichial vitamin. In the absence of folic acid, the tissues cannot properly utilize pantothenic acid, and PABA acts only as a precursor for folic acid synthesis.

Tentatively, the achromotrichia that has been reported, due to deficiencies of folic acid, PABA, and iron, may ultimately be due to the inhibition of pantothenic acid utilization. Since iron-containing pigments have not been isolated from dark hair, it is possible that iron functions as an essential part of an enzyme system necessary for proper utilization of folic acid and subsequently pantothenic acid. However, iron could conceivably serve both enzymatic and structural functions. There is a need for further research to substantiate these suggested modes of action.

Experiment 3: Iron-59 Incorporation
into Melanin Granules

Methods and Materials

Animals used in Experiment 1 were transferred to a small animal facility at the University Radiation Center for isotope administration. Cages, watering system, and rations fed were the same as described in Experiment 1. The animals were injected intraperitoneally with ferrous citrate - ^{59}Fe and intramuscularly with Armidexan.[3] The amounts were calculated to be sufficient to bring body iron levels up to normal.

As a control, two animals fed the iron-deficient diet received no additional iron to determine if they would exhibit cotton fur when the spring furring cycle was completed. Animal numbers and respective doses are shown in Table 4.

The two females that died were the only females that received 50 microcuries of ^{59}Fe. This amounted to approximately five micrograms of iron, and since others have injected mink with 50 milligrams of iron, it appears that something besides iron overload was the cause of death. They died within a week after being injected with the second dose of 25 microcuries of ^{59}Fe; however, it is doubtful if death was due to exposure to radiation.

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[3] Armour Veterinary Laboratories, Chicago, Illinois. Ferric hydroxide dextran complex with 75 mg. of iron per milliliter.

Table 4. Results of Experiment 3. Iron injected, fur color, and radioactivity in hair and melanin.

Iron Content of Ration	Mink Serial No.	Sex	Iron Injected		Fur Color	Radioactivity		Percent of ^{59}Fe in Pigment
			April 20, 1965	May 6, 1965		cpm/g hair	cpm in melanin/g hair	
15 ppm	7127	M	25 μc	25 μc	Dark	4880	3410	69.9
15 ppm	7128	M	25 μc	--	Dark	--	--	--
15 ppm	7129	F	25 μc	25 μc	--*			
0 ppm	7131	M	25 μc + 37.5 mg	25 μc	Dark	4210	2990	70.9
0 ppm	7132	M	--	--	CF	--	--	--
0 ppm	7133	F	25 μc + 37.5 mg	25 μc	--*			
0 ppm	7134	F	--	--	CF	--	--	--

*Died before fur color could be determined.

On June 18, 1965, the three surviving animals that had received ^{59}Fe were sheared on the shoulders and hips. Individual hair samples were defatted by refluxing in ether for one and one-half hours, allowed to dry, and then ground with a micro Wylie Mill[4] using a #60 mesh screen.

Radioactivity of one gram of the resultant ground sample was counted to determine the specific activity of ^{59}Fe in the hair. To determine if the ^{59}Fe present in the hair was actually a part of the pigment, the melanin granules were isolated by digesting the hair with liquid urea according to the methods of Lea (1954), with the following modifications: A 30 milligram sample of ground hair was digested with four grams of urea at 190 degrees Centigrade for 15 minutes. This digestion mixture was diluted with distilled water and the melanin granules filtered off on a sterilizing Seitz Filter. The filter paper containing the melanin granules was then carefully folded and placed in a plastic counting tube for counting in the auto-gamma spectrometer. The counting procedure was the same as described in Part 2.

Results and Discussion

Results are shown in Table 4. After counting the hair samples

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[4] Model 4276M, Arthur H. Thomas Co., Philadelphia, Pennsylvania.

from animals 7127, 7128, and 7131 it was determined that the ^{59}Fe

activity was too low to accurately determine the activity of isolated

melanin granules in the samples from 7128; therefore, only hair

from the other two mink was used.

It is interesting to note that the two mink kept on the iron-

deficient diet did exhibit CF even after they had gone through the

spring furring cycle. It has been shown (Stout, Oldfield, and Adair,

1960b) that CF usually occurs when there is a growth stress on the

animals, as well as the stress of fur growth; but, these data indicate

that CF can result even at maturity if iron deficiency is severe.

Assuming that there was equal incorporation of the labeled

and non-labeled iron, calculations give a value of 43 ppm of iron

in mink fur as compared with 40 to 60 ppm of iron in red hair as

found by Rothman and Flesch (1943). This value is also comparable

to the value of 27.1 ppm of iron given for the iron content of black

human hair (Rothman, 1954), and 22 to 64 ppm of iron from samples

of black rat hair (Cusack and Brown, 1964).

These data (Table 4), though extremely limited, suggest that

a substantial part of the iron in hair (70%) is actually a part of the

pigment. Here too, there is a need for further research since

data are so few.

PART 2: IRON METABOLISM IN MINK AS AFFECTED
BY RATIONS CONTAINING PACIFIC HAKE
USING IRON-59 AS A TRACER

Feeding raw Pacific hake to mink produces iron-deficiency

symptoms, including: CF; hypochromic, microcytic anemia; and

retarded growth; notwithstanding that the ration contains adequate

iron. Iron-59, a radioactive isotope of iron, was used as a tracer

to determine why mink fed such rations are unable to utilize dietary

iron for body functions. This objective was pursued by feeding

^{59}Fe labeled rations containing either cooked or raw hake, and

determining the radioactivity of collected excreta. From this, it

was intended to explain whether dietary iron was being excreted in

the feces or if it was absorbed and later excreted via the kidneys.

Due to unresolvable contamination problems, this approach was

modified after Trials 1 and 2 and blood ^{59}Fe levels were specifi-

cally studied.

With the production of radioactive isotopes, researchers

have been presented with an extremely useful tool for the study of

body metabolism. The radioactive isotopes are "marked" elements

which behave precisely like their nonradioactive replicas in the

physiology of the body, but can be readily recognized as distinct

entities wherever found. Fortunately, ^{59}Fe has a relatively long

half-life (45 days) which allows ample time for prolonged study of

iron metabolism. This isotope also gives assurance that when found in tissues or fluids, it is the iron introduced and not previously absorbed body iron. When radioactive iron is fed, one can readily distinguish between the newly acquired iron and that present in the body, even though the inert iron exceeds the radioactive iron by many hundred times.

Iron-59 is formed by the bombardment of iron-58 with neutrons. It decays with the emission of beta rays and relatively strong gamma rays. Iron-59 was chosen instead of one of the other radioisotopes of iron because it is a gamma emitter which greatly simplifies sample preparation.

Review of Literature

Result of Feeding Pacific Hake to Mink

The effect of feeding Pacific hake to mink has been studied and reported by Stout, Oldfield, and Adair (1960a), who found that incidence of CF in mink was directly related to the proportions of hake in the diet. Evisceration of hake reportedly lowered the CF incidence and by thorough cooking of hake prior to mixing in the diet, CF was completely eliminated. From this, they concluded that hake contained a heat-labile CF-causing factor. Later, the same authors (1960b) showed that iron deficiency was responsible

for the observed CF and anemia. They also indicated that since

the hake ration contained sufficient iron (114 ppm), there must be

a factor present in hake that inhibits the utilization of the dietary

iron. Earlier work in Norway, which they cited, indicated that CF

was produced when diets composed primarily of "coal fish" (Gadus

virens) were fed.

Helgebostad and Ender (1961) fed coal fish either raw or

boiled, with or without ferrous sulfate, to female mink and kits,

and studied blood and fur characteristics. Their results show that

CF and anemia were severe in those animals that received the raw

coal fish diet and that females produced fewer kits per litter.

Hemoglobin and hematocrit levels were reduced by approximately

50 percent and kits per litter were reduced to 80 percent of normal.

The animals receiving the raw coal fish plus iron, had improved

blood values (75% of normal), decreased incidence of CF, and

increased number of kits per litter (90% of normal). There was

no anemia or CF among the animals fed the boiled coal fish diets,

and females produced larger kits with lower mortality rates. The

authors concluded that coal fish contain an iron-binding, anemia-

causing factor that can be partially overcome by feeding ferrous

sulfate orally and completely overcome by boiling the fish or by

administering iron intramuscularly.

In a progress report (Oregon State University Experimental

Fur Farm, 1961), work was reported in which chemical, iron-balance trials were conducted on mink fed rations based on hake or sole. Urine and fecal samples were collected and total iron was chemically determined. It was reported that there was 78 percent more iron in the urine from hake-fed mink and 47 percent less fecal iron excretion. From these results, it was concluded that hake contains an iron-binding factor (probably a chelating agent), which complexes with iron, making it unavailable. The report further stated that the factor-iron complex was thought to be absorbed into the blood and excreted via the kidneys.

Absorption of Ionic Iron

Wiseman (1964) reviewed the topic of iron absorption and found that ferrous iron was usually absorbed better than ferric, when a standard dose was fed. Available evidence suggests that ferric iron in food must be reduced to the ferrous state before it can be absorbed. In most cases, with humans, rats, and mice, the absorption of iron is greater when ferrous iron is fed. However, in some cases where dogs and rats were used, there was no difference in the amount of iron-59 absorbed when test doses of either ferrous or ferric iron were given. Why some animals absorb ferrous and ferric iron equally well was not explained. The classical "mucosal block" concept of iron absorption is no longer

considered valid. However, it is well known that there is a regulatory control and at least three factors influence the amount of iron absorbed at a given time: (1) the level of iron stored in the body, (2) the rate of erythropoiesis and, (3) the components of a given diet.

Absorption of Hemoglobin Iron

It had been widely accepted that iron present in food in the form of heme compounds was not available for absorption (Granick, 1954), although Black and Powell (1942) had some evidence to the contrary. Later, Callender, Mallett, and Smith (1957) studied the absorption of iron following the ingestion of either raw or cooked hemoglobin (5 mg Fe) labeled with five microcuries of ^{59}Fe. Their results indicated that there was appreciable absorption of both forms of hemoglobin iron and that absorption was 11 percent in normal subjects and 22 percent in iron-deficient subjects. This work was further supported by Turnbull, Cleton, and Finch (1962) who found that anemic, human subjects absorbed an average of 15.6 percent of a dose of iron-59 labeled hemoglobin.

Bothwell and Finch (1962) studied the absorption of iron when administered with various types of food and found that humans absorbed liver and hemoglobin iron as well as iron salts added to food, although iron in vegetables and eggs was much less available.

They also found that man absorbs hemoglobin iron five times better than does the rat. Georgi (1964) found that sheep absorb an average of 3.8 percent of a dose of ^{59}Fe hemoglobin and that absorption remained the same whether the animals were anemic or normal. However, absorption of ^{59}FeSO$_4$ was higher in anemic animals (4.9% vs. 16.0%).

Two theories concerning the mechanism of hemoglobin iron absorption are discussed by Callender (1963). One theory presumes that iron from hemoglobin is absorbed as heme with the iron being split off in the mucosal cell. The other contends that iron is "split off" from hemoglobin in the gastrointestinal tract before absorption. There is evidence supporting both theories, but neither is completely conclusive.

Factors Influencing Iron Absorption

The chemical nature of iron occuring in foods is probably of less importance in determining its physiological availability than a variety of other factors that exert favorable or unfavorable influence on its absorption from the gastrointestinal tract. Gubler (1956) has reviewed factors which influence absorption of iron and indicates that form of iron, reducing mechanisms present, size of dose, acidity of gastric juice, levels of phosphate, calcium, and phytic acid, influence amount of iron absorbed. Follis (1948) has

shown that the rate of passage of food through the gastrointestinal
tract affects absorption. When the contents pass rapidly, iron is
absorbed poorly. It has been shown (Gubler, Cartwright, and
Wintrobe, 1949) that during a pyridoxine deficiency the absorption
of iron is increased, and that a copper deficiency decreases iron
absorption (Wintrobe, Cartwright, and Gubler, 1953).

Factors Inhibiting Iron Absorption

Ethylenediaminetetra-acetate (EDTA). Wiseman (1964)
reviewed the effect of feeding the chelate, EDTA, on the absorption
and metabolism of radioactive iron. EDTA, which forms non-
ionized complexes with many metals, caused below normal absorp-
tion of ^{59}Fe from a test dose of ferric chloride when fed at a one
percent level in the diet of rats. Bodily retention was also less
due to loss of absorbed ^{59}Fe via the urine. A rise in the plasma
iron level reflected the non-utilizable EDTA-iron complex, which
was excreted in the urine. When larger amounts of EDTA were
fed (up to 12 percent of the diet), absorption was sufficiently lowered
to deplete body stores.

Pancreatin. Experimental work with animals (Kinney, Kauf-
man, and Klavins, 1955) showed that the pancreas influenced iron
absorption. Later, Davis and Badenoch (1962) found that patients
with chronic pancreatic damage had increased iron absorption.

More recently, Callender (1963) tested iron absorption in humans

using either ^{59}Fe labeled ferrous sulfate or hemoglobin, fed with

and without pancreatin. Pancreatin reduced the absorption of iron

in every case whether fed with inorganic or hemoglobin iron, but

heating the pancreatin abolished the inhibitory effect. She concluded

that the pancreatic secretion contains a heat-labile substance which

normally inhibits iron absorption.

Desferrioxamine B. Desferrioxamine B, a new and potent

specific iron-binding agent, has been studied and reported by

Moeschlin and Schnider (1963). It is a water-soluble substance

featuring three molecules of trihydroxamic acid, having a molecular

weight of 597. This chelating agent under physiological conditions

possesses a remarkably high relative affinity and specificity for

a single cation, namely trivalent iron. Desferrioxamine B is able

to take iron from the relatively stable transferrin and ferritin

molecules since it has a higher stability constant. By intra-

muscular injection of this chelating agent, urinary excretion of

iron can be increased by 300 times. Administered orally, it

reduces iron absorption and increases urinary iron excretion much

the same as EDTA does.

Soybean Protein. Fitch et al. (1964) fed monkeys an ^{59}Fe

labeled diet containing cooked soybean protein and found that iron

absorption was greatly reduced with the soybean diet, as compared

to a casein-containing diet. They concluded that soybean protein

contains a factor that prevents the absorption of iron.

Conalbumin. The specific iron-binding capacity of an egg

white component was first recognized by Schade and Caroline (1944)

and it was identified as conalbumin by Alderton, Ward, and Fevold

(1946). Later, Kirch et al. (1947) showed that egg white prevented

the reduction of ferric iron due to the complexes which were formed.

This made iron totally unabsorbable from the gastrointestinal tract.

Feeney (1964) further described conalbumin and since it is so simi-

lar to the transferrin of blood serum, referred to it as ovotransfer-

rin. Conalbumin binds two atoms of ferric iron per molecule and

the complex is stable at a pH greater than 5.5. The iron complex

is postulated to occur through three hydroxyls of the protein, an

added bicarbonate, at least two nitrogens of the protein, and possi-

bly water.

General Methods and Materials

All animals used for these iron balance trials were housed

in the small animal facility at the Radiation Center at Oregon State

University. Cages were the same as described in Part 1. Animals

were fed either hake or sole based rations (Table 5) on the top wire

of the cage and tap water was continuously supplied by an automatic

system.

Table 5. Ration composition.

| Feed Item | Ration (percent as fed) | |
	Hake Ration	Sole Ration
Pacific hake	50	--
Sole	--	50
Turbot	15	15
Rockfish	10	10
Horsemeat	7	7
Cereal Mixture 49A[*]	18	18

[*] Composition of cereal mixture 49A

Item	Percent
Wheat Germ	25
Alfalfa Meal	13
Skim Milk Powder	8
Meat Meal	18
Soybean Oil Meal	18
Ground Oat Groats	18
D-alpha tocopheryl acetate (50,000 I. U. of vitamin E per pound)	25 g/100# mixture

Since these animals were not exposed to the natural environment, artificial lighting was used. Hours of light and darkness were controlled by using a time clock programmed for a seven day period. To approximate natural conditions, the lights were turned on 15 minutes before sunrise and turned off 15 minutes after sunset according to tables for sunrise and sunset at Corvallis, Oregon (Zimmerman and Cowan, 1965).

After a single dose of iron-59[5] was given, total urine and fecal samples were collected and weighed for a period of seven to ten days or until total excretion for a 24 hour period was less than 0.5 percent of the dose administered. Trays, used for collection of urine and fecal material, were suspended underneath the cages as shown in Figure 4. These trays were constructed so that fecal material was collected on a copper wire screen; whereas, the urine passed through onto vinyl coated paper and was collected in a glass container below. Trays were washed and papers changed after each collection.

Since ^{59}Fe is a gamma emitter and gamma rays pass easily into the counting crystal, sample preparation was relatively simple. After complete homogenization of fecal material, a two to three gram sub-sample was weighed and carefully placed in a plastic

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[5] Obtained from Nuclear Consultants Corporation, Glendale, California.
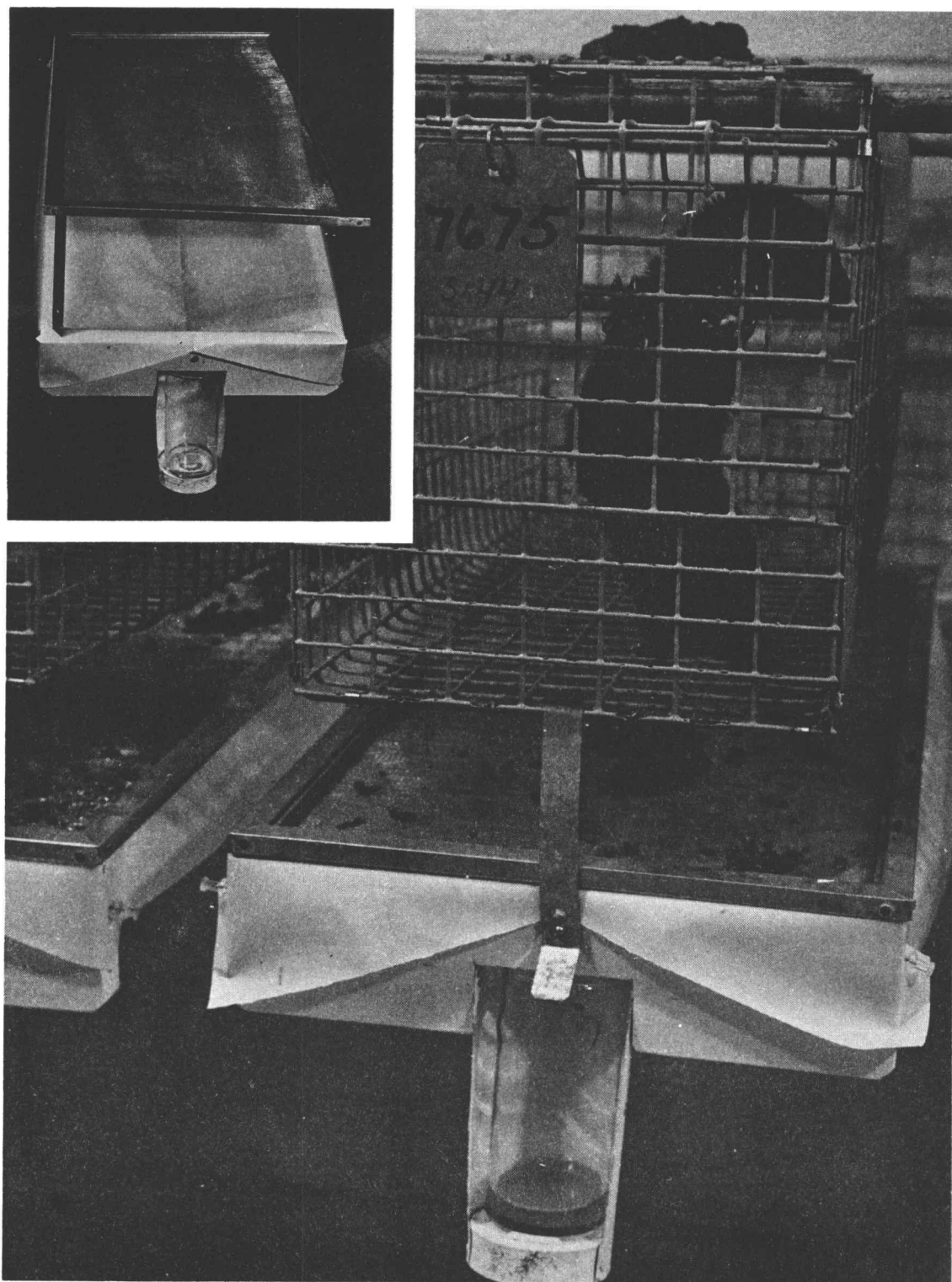
Figure 4. Cage setup showing tray for collection of urine
and feces suspended underneath. Inset (upper
left) shows view of an assembled collection tray.

container and inserted into a plastic counting tube. Each sample was counted to a standard error of one percent. (Wang and Willis, 1965) on an auto-gamma, deep-well, solid scintillation counter (Packard Model 410A). Counter settings and the emission spectrum of ^{59}Fe are given in Appendix I.

Three milliliter blood samples were withdrawn from unanesthetized mink by cardiac puncture and mixed with an anticoagulant prepared as in Part 1. Radioactivity of whole blood was determined by counting a one milliliter sample. Of the remaining two milliliters, the hematocrit was determined and the remainder centrifuged at 5200 rpm for 20 minutes. Plasma was then drawn off, weighed into counting tubes, and counted. From these data, i. e.: activity of whole blood, activity of plasma, and hematocrit; activity of erythrocytes was calculated.

Specific methods and results will be described separately for each trial, as they varied considerably. Animals used, rations fed, amounts and types of ^{59}Fe used, methods of dose administration, and dates of Trials 1 through 8, are listed in Appendix II.

<div align="center">Specific Methods and Results</div>

Trial 1

This was primarily a pilot study to become acquainted with

techniques involved in this type of iron balance trial. Two female

mink approximately eight months old were used. They had been

fed either the hake or sole diets (Table 5) from weaning (June, 1964)

until the termination of this trial (December, 1964). Each animal

received three and one-half microcuries of ferrous citrate-^{59}Fe

with 170 grams of the respective ration. The animals had not been

previously starved and therefore ate very little of the ^{59}Fe containing

feed during the first 10 hours. After allowing access for 24 hours,

the uneaten feed was removed and weighed to determine the amount

of feed actually ingested. Urine and fecal material were collected

for 10 days and blood samples were taken 32 days after dosing.

Assuming the amount of blood to be eight percent of the body

weight, absorption of ^{59}Fe into the body was 0.6 percent of the

administered dose for the anemic, raw hake-fed mink and 13 percent

the non-anemic mink receiving sole.

Trial 2

This was an expanded rerun of Trial 1 with four animals in

each group (two males and two females). Each animal received

10 microcuries of ferrous citrate-^{59}Fe with 140 grams of either

the hake or sole ration. Excrement was collected for 10 days and

blood samples were taken at seven hours and again 10 days after

dosing. In this trial, plasma and washed erythrocytes were

counted instead of whole blood and plasma.

Trials 1 and 2 were basically the same, an objective of both being to determine if hake-fed animals were absorbing iron which was later excreted in the urine. It was found that urinary excretion data were not valid due to cross-contamination between feed, feces, and urine; however, total excretion data are shown in Appendix III. Results of Trial 2 confirmed earlier results, in that anemic hake-fed animals absorbed 1.5 percent of the dose and non-anemic sole-fed animals absorbed 10.7 percent as estimated by ^{59}Fe blood activity. The seven hour blood samples indicated that the radioactive iron was never being absorbed in the case of the mink fed the raw hake ration, since plasma ^{59}Fe levels remained low.

Trial 3

With the ad libitum feeding method, it was impossible to get all animals to eat at once and to know the exact amount ingested by each. It was decided that a gavage method might produce better results. Four mink received a dose of ferrous citrate-^{59}Fe in distilled water. Four others received the ^{59}Fe in raw hake filtrate prepared as follows. The hake was finely ground through a regular kitchen model food grinder. Equal weights of water and ground hake were then homogenized in a Waring Blender, Model 700B.[6] The resulting homogenate was centrifuged at 5200 rpm for

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[6] Waring Products Corporation, Winsted, Connecticut.

30 minutes and the supernatant poured off, filtered several times

through Celite Analytical Filter Aid[7], and finally through a

sterilizing Seitz Filter[8]. Twelve and one-half microcuries of the

^{59}Fe was then added to 25 milliliters of the resultant filtrate and

given by stomach tube to each animal. The animals were starved

12 hours prior to administration of the filtrate-isotope mixture

and were fed two and one-half hours later. Blood samples were

withdrawn at 0.5, 2, 6, 24, and 74 hours and again at 10 days.

Previous work at this station using microbiological assay

with Aerobacter aerogenes and Serratia marcescens indicated that

the iron-inhibiting factor in hake could be extracted in a water

filtrate (Oregon State University Experimental Fur Farm, 1961).

This trial was conducted to determine if the filtrate would have an

effect on the utilization of ^{59}Fe.

Iron absorption as determined by blood data was unlike the

previous trials, in that the absorption of ^{59}Fe was greater (16.3%)

in the presence of the raw hake filtrate than with distilled water

(11.6%). These results suggest that either an aqueous filtrate of

hake does not inhibit the utilization of ferrous iron, or that the

filtrate contains an insufficient amount of the inhibiting factor to

influence iron absorption.

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[7] Johns-Manville Corporation.
[8] Hercules Filter Corporation, Hawthorne, New Jersey.

This was the first trial in which animals were used that had

received a previous dose of ^{59}Fe. To account for this, an initial

blood sample was withdrawn just prior to administration of the dose

of ^{59}Fe. In all trials where animals were used that had received

previous ^{59}Fe, the isotope level in the red cells was assumed to

be constant and taken as the amount of radioactivity present in the

erythrocytes from the initial blood sample. This is assumed valid,

since the ^{59}Fe level in the erythrocytes reaches a constant level

after approximately seven days (Bothwell and Finch, 1962).

Trial 4

In Trials 1 and 2, utilization of iron was poor in the animals

receiving raw hake; however, it was not known whether the ^{59}Fe

was absorbed and excreted in the urine or if absorption was blocked.

In this trial, the objective was to determine if iron is absorbed into

the blood and later excreted in the urine or if the blockage is at

the absorptive level. To determine this, blood samples were

taken more often and ^{59}Fe activity in blood plasma was specifically

studied.

Four anemic mink were used (one male and one female in

each group) with one group fed the raw hake ration shown previously,

and the other a ration containing cooked hake. This was prepared

by heating ground hake one and one-half hours at 95 degrees

Centigrade in a water bath. Ferrous citrate-^{59}Fe was included

in the ration as in Trials 1 and 2, but the animals were starved 24

hours prior to being fed the labeled feed, to encourage rapid intake.

Another point of contrast is that the labeled iron was mixed with the

feed a few minutes before it was offered to the animals. Each male

received 75 grams of ration and 18 microcuries of ^{59}Fe, and each

female 50 grams and 12 microcuries. All animals began to eat as

soon as the test meal was offered and although it was allowed to

remain on the cage for five hours, all feed was not consumed. The

remainder was removed and weighed back so that consumption could

be determined. Blood was withdrawn at 1, 3, 6.5, and 23 hours and

again 14 days after dosing.

Absorption values, as calculated from blood and plasma ^{59}Fe

activity, indicated no difference in absorption due to type of ration

fed. Raw hake-fed mink absorbed 7.5 percent and cooked hake-fed

mink absorbed 6.7 percent of the administered dose. Blood levels

of ^{59}Fe indicated that nearly all of the iron absorbed was utilized

for hemoglobin formation.

Results were unexplained in view of previous trials (Trials

1 and 2) utilizing complete rations. Since the ^{59}Fe was mixed with

the feed just prior to feeding and the mink began eating immediately,

it is possible that insufficient time was available for a postulated

reaction between hake and iron to occur.

Trial 5

The method of dose administration was again changed in an
attempt to more accurately measure iron absorption and determine
factors influencing this absorption. $^{59}FeSO_4$ was added to either
a cooked or raw hake suspension and administered by stomach tube.
The suspension was prepared by taking either cooked (prepared as
in Trial 4) or raw hake, adding an equal weight of distilled water
and blending in a Waring Blender until homogeneous. A dose con-
sisted of 25 milliliters of suspension and 10 microcuries of ^{59}Fe
per kilogram of body weight. Two anemic animals received the
cooked hake suspension and three others, also anemic, received
the raw hake suspension with ^{59}Fe added just prior to administra-
tion; still another animal (anemic) received a raw hake suspension
which had the iron added 36 hours previously.

It is possible that, by adding the ^{59}Fe immediately, the hake
iron-binding factor does not have a chance to complex with the iron
before it is absorbed. For this reason ^{59}Fe was added to the raw
hake suspension 36 hours before administration to one animal.
Animals were bled at 1, 2, 4, 6, and 22 hours and again 14 days
after dosing.

Iron absorption as calculated from blood data was 23.2 percent
from the cooked hake suspension, as compared to 15.9 percent ab-
sorption from incubated raw hake and somewhat less (12.3%) from

raw hake with iron added immediately before administration. These

results indicate that an incubation period before administration had

little effect, if any, on absorption of ferrous iron under the conditions

of this trial.

Trial 6

Since most of the iron in mink rations is in the form of hemo-

globin, this trial studied the effect of raw hake on the absorption

of hemoglobin iron. Labeled rat hemoglobin was prepared from

three albino rats by withdrawing four to five milliliters of blood from

each and intraperitoneally injecting with 10 microcuries of $^{59}FeSO_4$.

After three days, each rat was anesthetized and four to seven

milliliters of blood were withdrawn by cardiac puncture. The blood

was centrifuged, the red cells washed three times in a physiological

saline solution, and then lysed with distilled water. The resulting

hemoglobin solution was added to suspensions of hake, as described

in Trial 5. Two anemic mink were used; one received 0.25 micro-

curies in 22.1 grams of cooked hake suspension, the other received

0.29 microcuries in 30.6 grams of raw hake suspension. The

bleeding schedule was 1, 4, 8, and 24 hours and again seven days

after dosing.

Absorption of iron was stopped completely in the animal fed

raw hake; whereas, absorption was only lowered in the case of

the animal fed cooked hake (2.7%). A problem encountered with

this trial was the low activity of the hemoglobin used (approximately 0.25 microcuries per animal) which made measurement difficult.

Trial 7

This trial was similar to Trial 6, except that labeled hemoglobin prepared from mink was used instead of rat hemoglobin. Two anemic mink received ^{59}Fe labeled hemoglobin in a cooked hake suspension and two others (anemic) received labeled hemoglobin in a raw hake suspension, by stomach tube, in doses of approximately 2.5 microcuries per animal. Blood samples were taken from each mink at 1, 4, 8, and 24 hours and again seven days after dosing.

The radioactive mink hemoglobin was prepared by injecting two mink, weighing 1140 grams and 690 grams, with 50 and 37.5 microcuries respectively. Five days after injection of the ^{59}FeCl$_3$, blood was withdrawn and hemoglobin prepared as in Trial 6.

Results of this trial substantiate those of Trial 6. Using ^{59}Fe activity as the criterion of absorption, there was no absorption of iron by the mink receiving raw hake, but 6.5 percent of the administered dose was absorbed by the mink receiving the cooked hake suspension.

<u>Trial 8</u>

Since hemoglobin iron was not absorbed by the animals re-ceiving the raw hake suspension, this further trial was conducted to determine if the raw hake suspension would affect absorption of ferric iron. $^{59}FeCl_3$ was administered in hake suspensions as described previously. The suspensions, prepared and allowed to incubate with the iron 14 hours before administration, were given by stomach tube. Each animal (all anemic) received 40 milliliters of suspension and 30 microcuries of ^{59}Fe per kilogram of body weight. Feces and urine were collected for 48 hours and blood samples were taken at 1, 4, 8, and 24 hours and 16 days after dosing.

Results indicate that absorption of ferric iron was reduced in the presence of raw hake (2.9%) but not completely stopped as was the absorption of hemoglobin iron. Iron absorption for mink receiving cooked hake was 16.6 percent.

<div align="center">General Results and Discussion</div>

<u>Iron Absorption</u>

Summarized ^{59}Fe absorption data for all mink used in the eight iron balance trials are given in Table 6. There are several points evident concerning the absorption of ferrous iron. First,

neither the raw hake filtrate nor suspension had any apparent effect on iron absorption, and incubating ^{59}Fe with the suspension before administration had no effect. When ferrous iron was fed with the complete ration containing raw hake the length of incubation time appeared to be the major factor involved in its absorption. Assuming that ferrous iron is easily oxidized to ferric and that ferric is the form of iron that is inhibited from being absorbed, it probably takes a certain period of time for this oxidation which then allows inhibition of absorption.

Iron absorption data presented in Table 7 are from animals which received all three types of iron. These results are probably more meaningful than those given in Table 6, as Bothwell and Finch (1962) have shown, there is a great deal of individual variability in the absorption of iron. All means shown in Table 7 are significantly different ($P < 0.05$), except between ferrous and ferric groups given cooked hake.

From these latter data, one might suspect that mink normally absorb ferric and ferrous iron equally well, as absorption of ferric iron was essentially the same (no statistical difference, $P < 0.05$) as that of ferrous in the presence of cooked hake suspension. Mink could be similar to dogs in this respect. Moore et al. (1944) found that dogs absorbed ferric iron from feed as well as ferrous; whereas, the absorption of ferrous iron by human subjects was up to 15 times

Table 6. Absorption (percent) of Iron-59 as influenced by the presence of raw Pacific hake. Composite results of all animals and methods used. (± values are standard deviations..)

Method of Administration	Form of Iron			Ferric Suspension (Gavage)	Hemoglobin Suspension (Gavage)	Mean
	Ferrous					
	Filtrate and Susp. (Gavage)	Fed (Time) ad libitum	Fed (Immediate) ad libitum			
Trial No.	3 and 5	1 and 2	4	8	6 and 7	
Raw Hake						
Present	14.3 (8)*	1.4 (5)	7.5 (2)	2.7 (2)	0 (3)	7.1±2.1 (20)
Absent	13.9 (5)	11.2 (5)**	6.7 (2)	16.6 (2)	5.2 (3)	11.0±2.2 (17)
Mean	14.1±1.9(13)	6.3±5.8(10)	7.1±1.3(4)	9.7±8.8(4)	2.6±2.3(6)	8.9±1.8 (37)

* Numbers in parenthsis indicate number of animals involved.
** Non-anemic animals used.

Table 7. Absorption (percent) of Iron-59, by animals receiving all three types of iron. (± Values are standard deviations.)

	Type of Iron			
	Ferrous	Ferric	Hemoglobin	Mean
Cooked Hake Suspension	16.8 (4)*	16.6 (2)	5.2 (3)	12.8±2.1(9)
Raw Hake Suspension	8.2 (4)	2.7 (2)	0 (3)	3.6±1.6(9)
Mean	12.5±2.5(8)	9.7±8.8(4)	2.6±2.3(6)	8.2±1.8(18)

* Numbers in parenthesis indicate number of animals involved.

greater than absorption of ferric.

Both ferric and hemoglobin iron were absorbed and utilized to a greater extent when given with a suspension of cooked hake than when given with raw. As expected, hemoglobin iron was absorbed less well than either ferrous or ferric, and these limited data suggest that raw hake completely blocks absorption of hemoglobin iron and reduces the absorption of ferric iron.

Most of the iron present in a conventional mink ration consisting of raw meat and fish would be in the form of hemoglobin and myoglobin. Since raw hake appears to block absorption of this form of iron, this accounts for the severe iron-deficiency noted when animals receive a ration of raw hake. In such a ration very little iron would be present in the more utilizable ferrous state.

The small amount of ferric iron absorbed in the presence of the raw hake suspension (Trial 8) could be due to the limited amount of hake present. The suspensions were prepared using 50 percent water. Hake contains 80 percent water, so the suspensions contained about 10 percent dry matter. Each dose amounted to approximately 30 grams of suspension, hence, three grams of dry matter. In such a small amount of hake, there might not have been enough of the presumed factor present to completely bind the amount of iron given.

Blood Activity and Incorporation of ^{59}Fe into Erythrocytes

Figures 5-11 show the level of ^{59}Fe present in whole blood, plasma, and erythrocytes for Trials 3-8, in which the activity of blood components was studied. Results are given as the percentage of ^{59}Fe present in blood components versus length of time after dose administration. In every trial where iron utilization was impaired by the presence of raw hake, plasma ^{59}Fe levels indicate that iron does not gain access to the body. Therefore, one must conclude that the inhibition is at the absorptive level. Erythropoiesis is apparently not inhibited; as shown by incorporation of ^{59}Fe into the erythrocyte, and mink seem to be capable of utilizing iron once it gains systemic access. This is further illustrated by the fact that mink injected intramuscularly with iron utilize it immediately for hemoglobin formation (Stout, Oldfield, and Adair, 1960b). Such was also the case with the two mink in Trial 7 that were injected with radioactive iron for the production of ^{59}Fe-hemoglobin. Five days after injection, 66 percent of the injected dose was found in the erythrocytes, and 74 percent at 14 days. During this short period, average hematocrit value increased from 28 to 47 percent and the average increase in weight was 75 grams.

Comparing the rate of absorption of labeled ferrous and ferric iron, one can see that they are absorbed at the same rate in the

Figure 5. Absorption (percent) of ^{59}Fe (ferrous) given by stomach tube with cooked hake suspension (Trial 5).
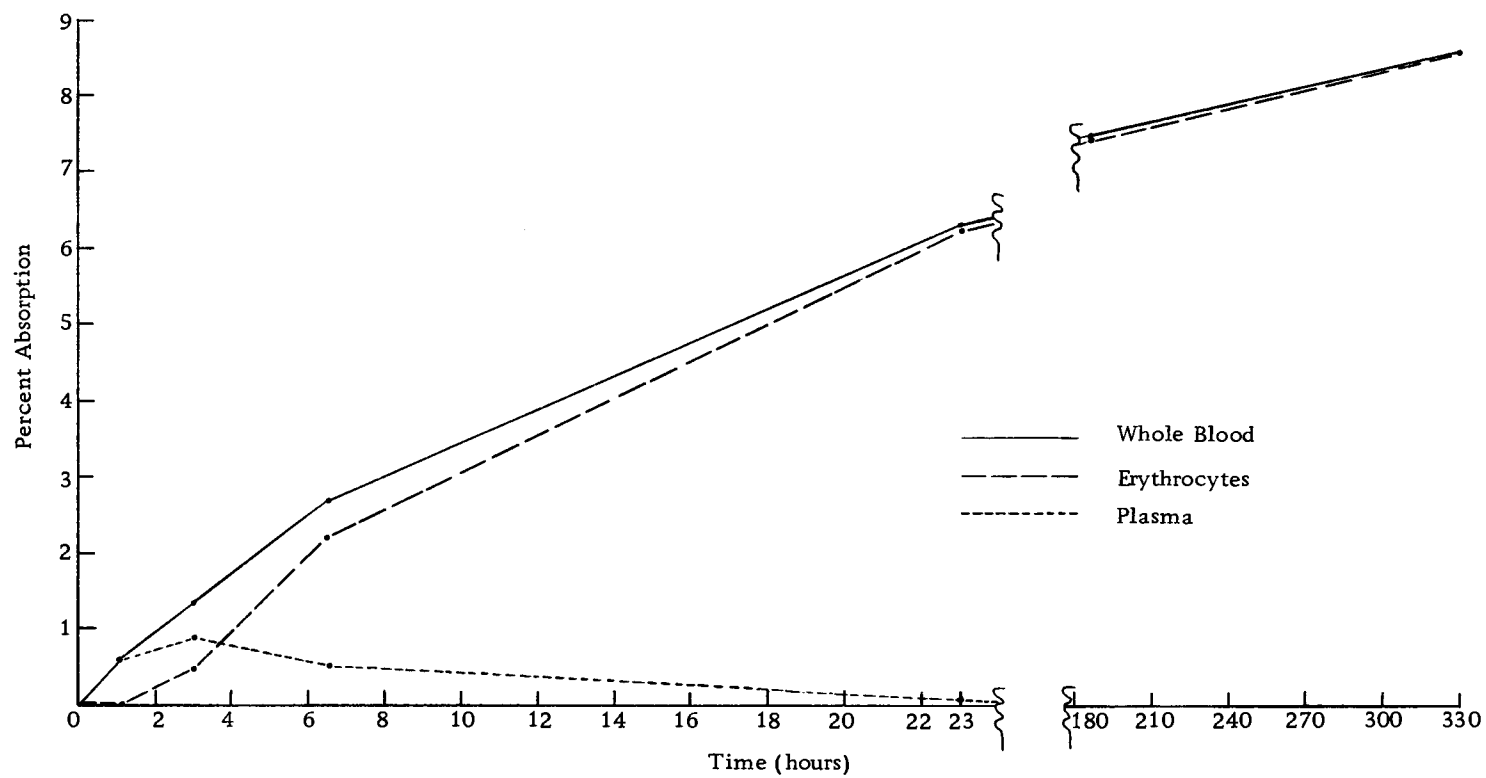
Figure 6. Absorption (percent) of ^{59}Fe (ferrous) given _ad libitum_ in the whole raw hake ration (Trial 4).

Figure 7. Absorption (percent) of ^{59}Fe (ferrous) given by stomach tube
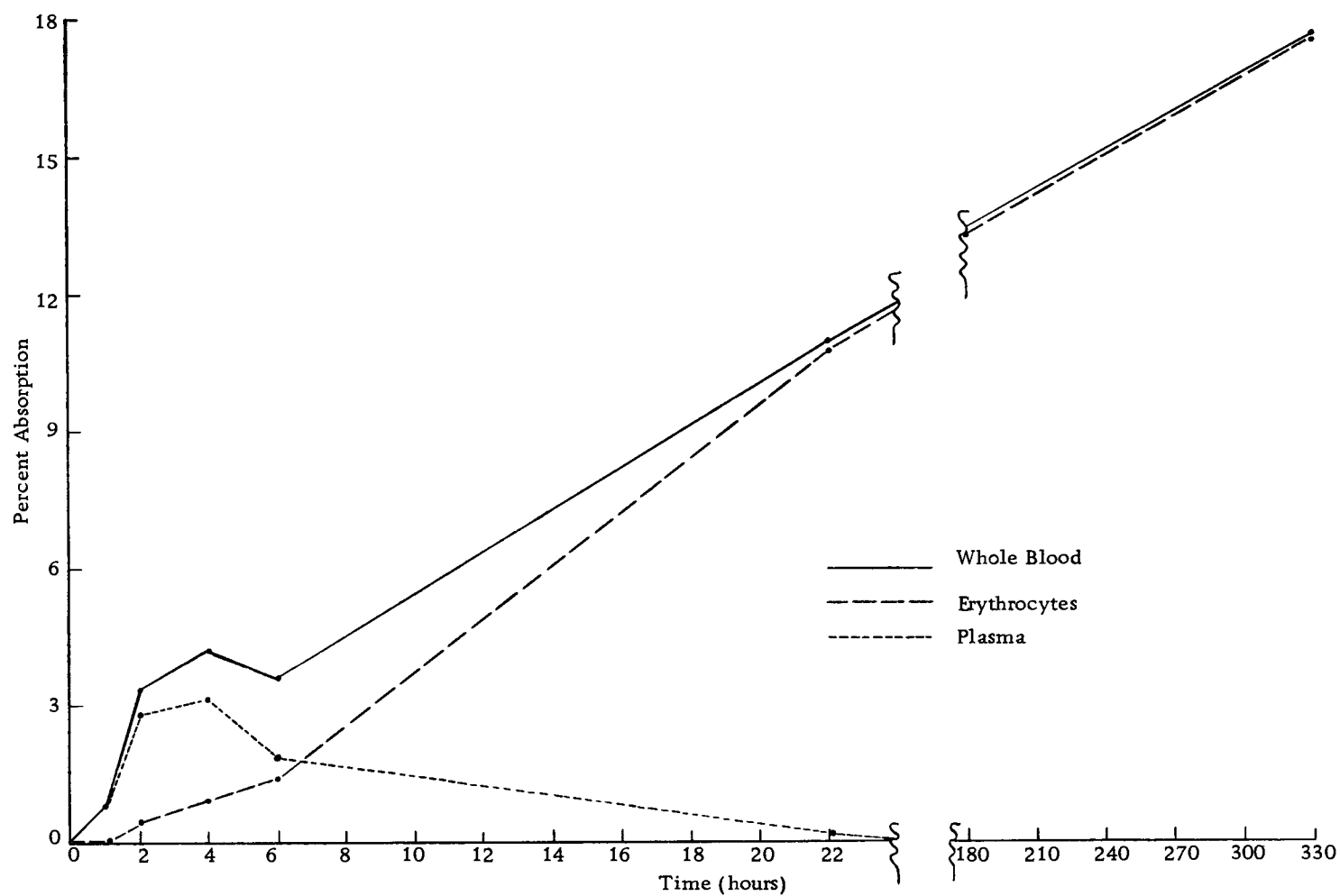with raw hake filtrate (Trial 3).

Figure 8. Absorption (percent) of ^{59}Fe (ferrous) given by stomach tube with a raw hake suspension (Trial 5).

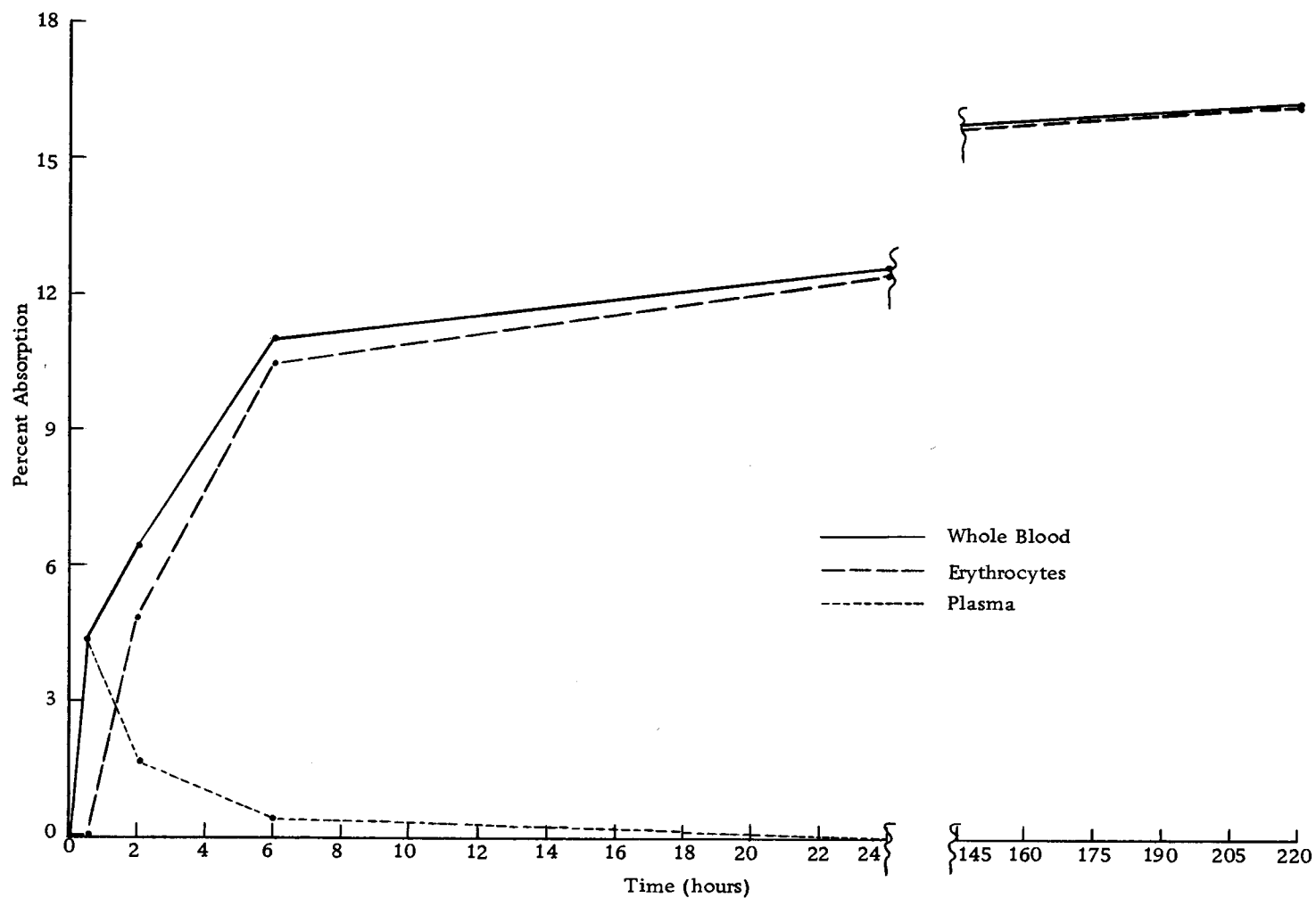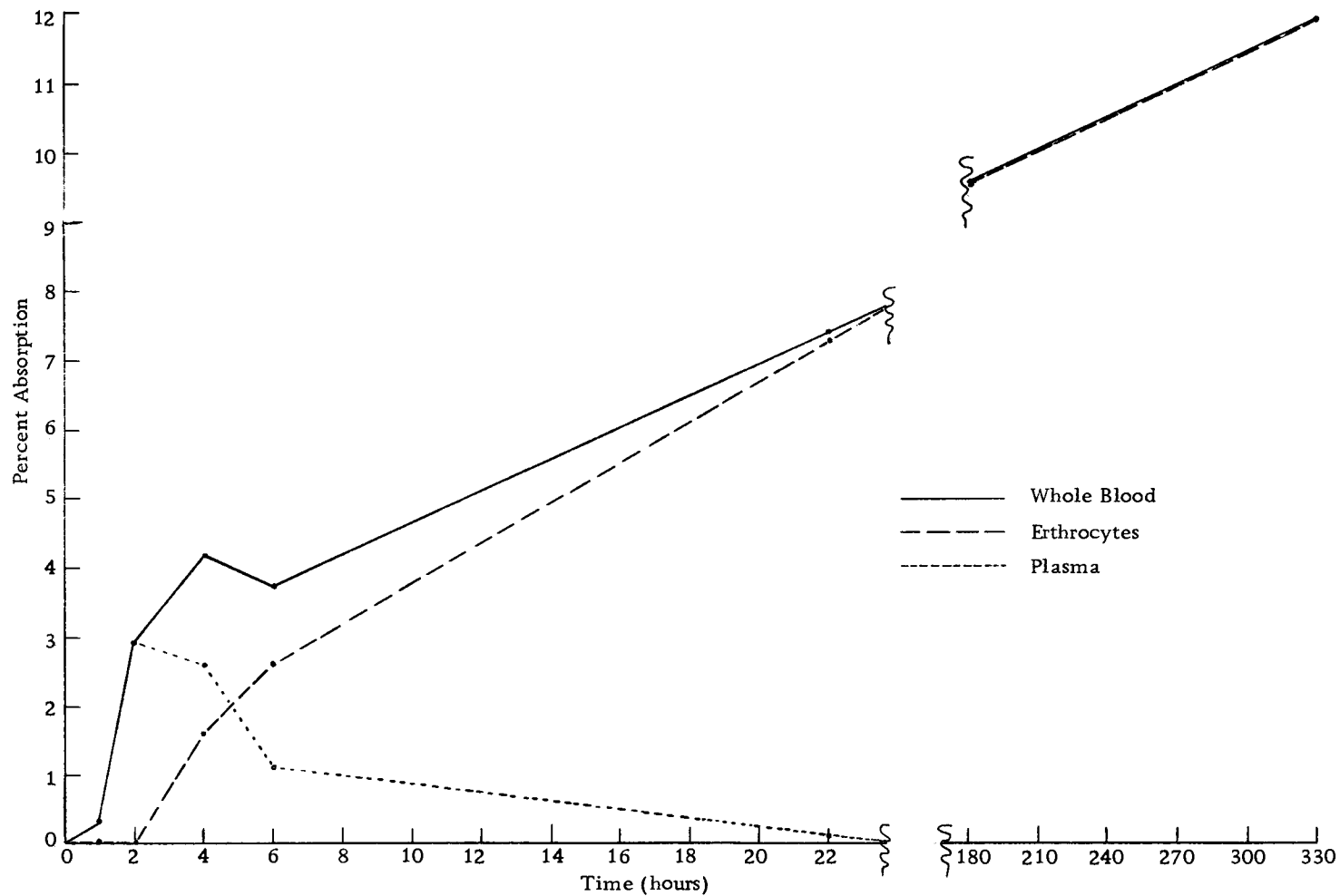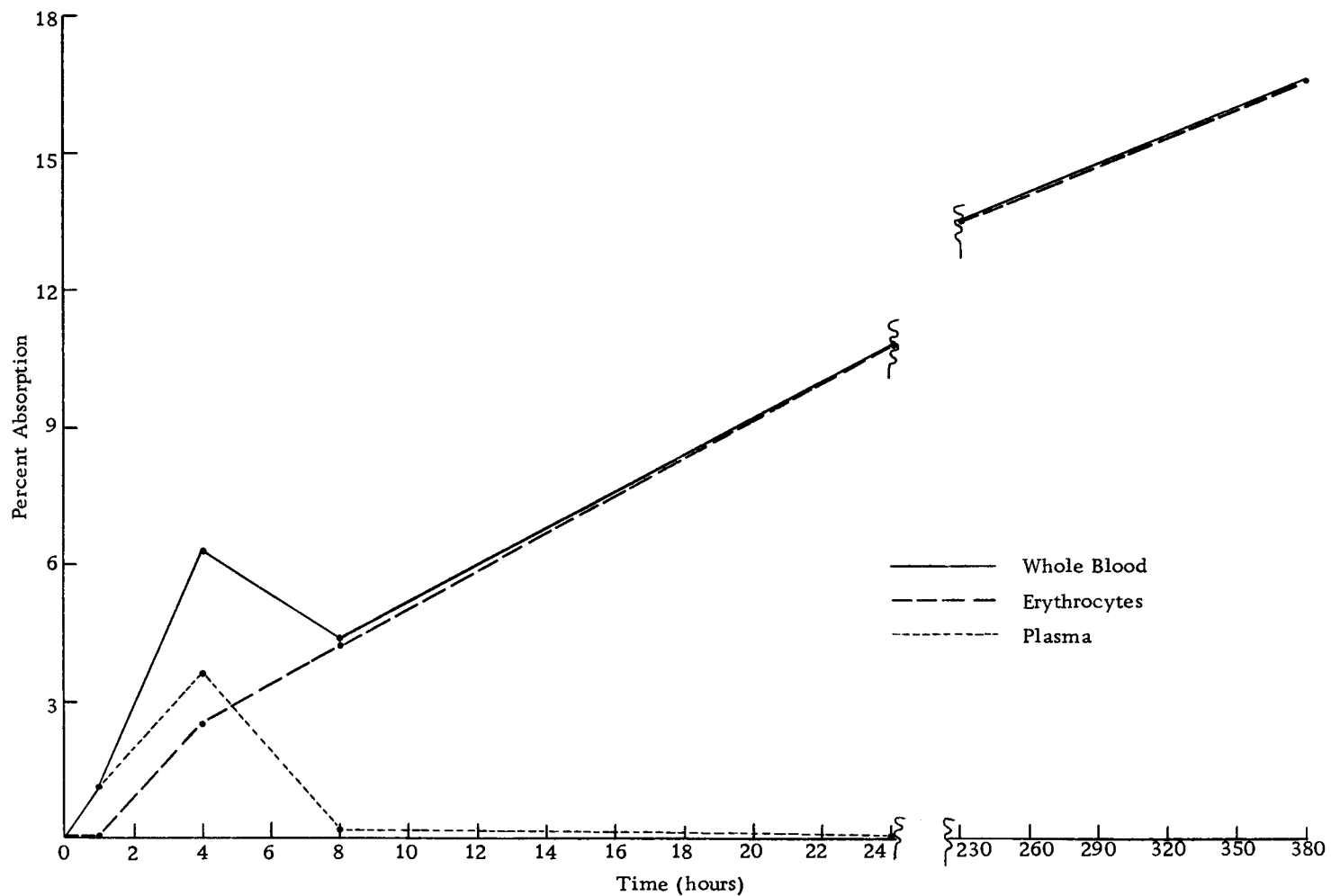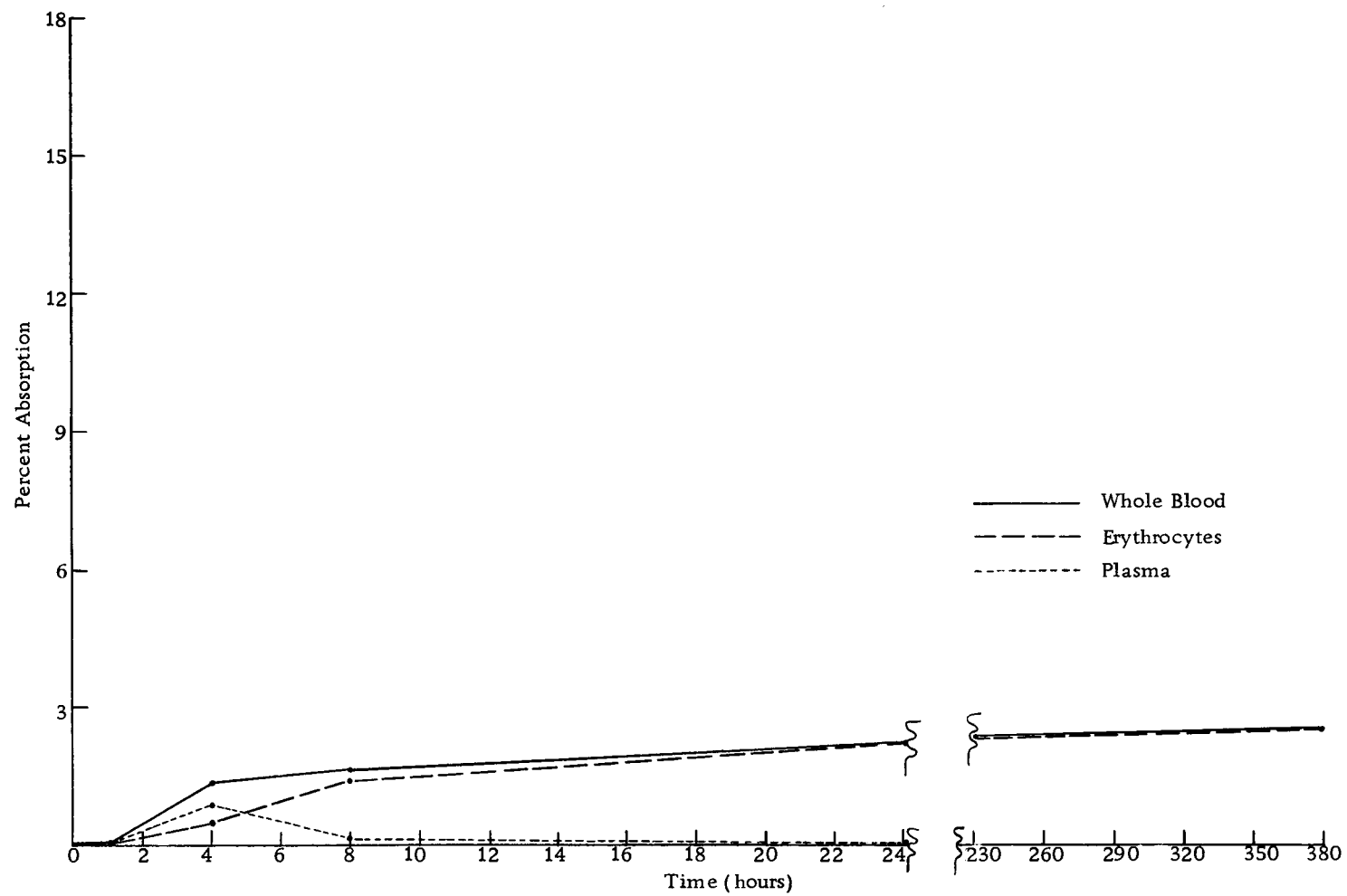Figure 9. Absorption (percent) of $^{59}FeCl_3$ given by stomach tube with cooked hake suspension (Trial 8).

Figure 10. Absorption (percent) of $^{59}FeCl_3$ given by stomach tube with raw hake suspension (Trial 8).

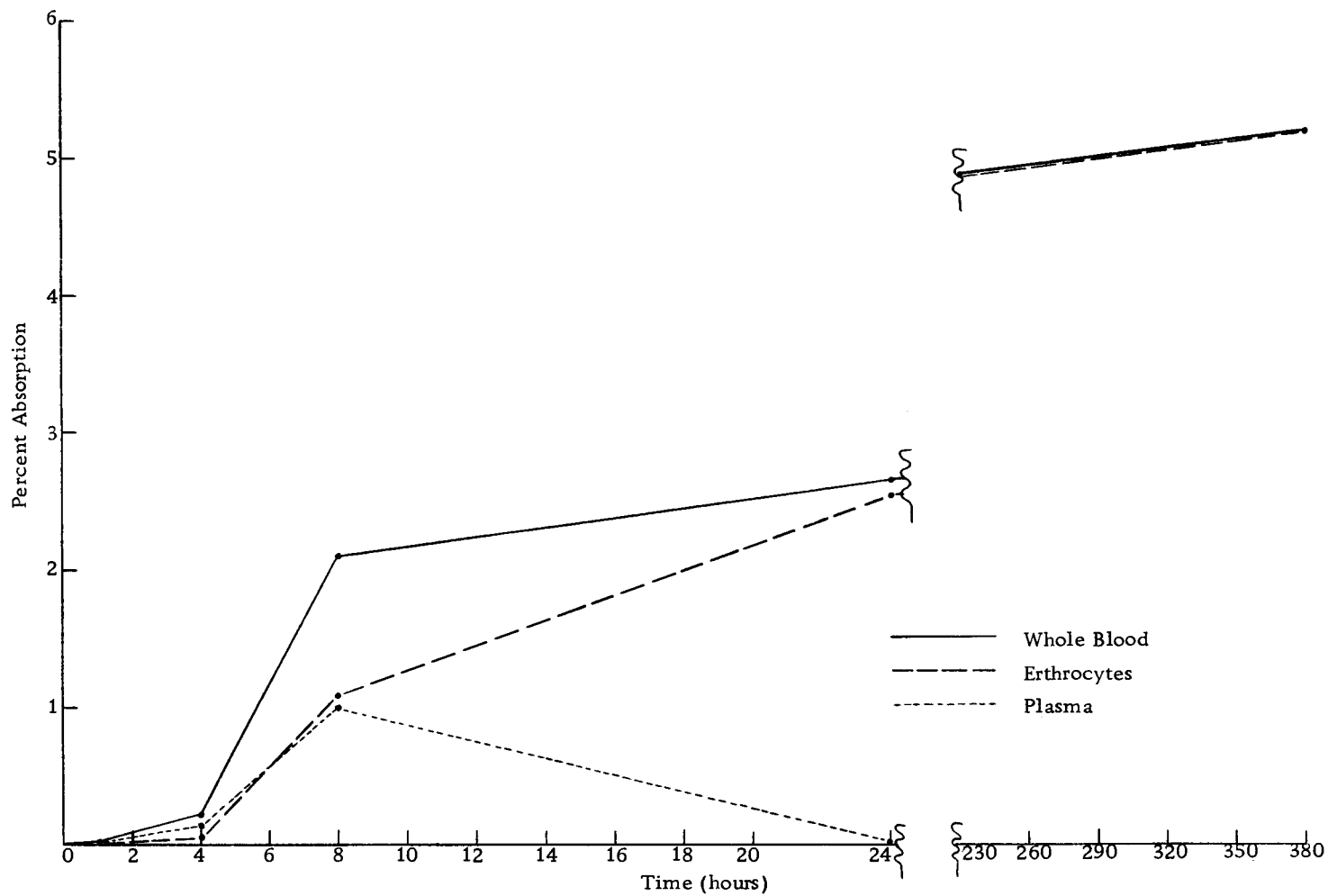Figure 11. Absorption (percent) of ^{59}Fe- hemoglobin given by stomach tube with cooked hake suspension (Trials 6 and 7). When ^{59}Fe-hemoglobin was given with raw hake suspension, there was no detectable absorption.

presence of cooked hake (Figures 5 and 9) but not when raw hake

is present (Figures 6, 7, 8, and 10). Hemoglobin iron is absorbed

when in combination with cooked hake at a slower rate than either

ferrous or ferric iron (Figure 11). The peak ^{59}Fe activity in the

plasma was at four hours with both ferrous and ferric iron; however,

this plasma peak did not occur until eight hours after administration

of labeled hemoglobin.

Method of administration had more effect on the rate of

ferrous iron absorption when given with raw hake than with cooked.

Rate of iron absorption was fastest when mink were stomach tubed

with raw hake filtrate (Figure 7) and slowest when fed (Figure 6).

Iron-59 plasma activity remained high over a longer period of time

when the radioisotope was given in the feed because the animal

did not receive one immediate dose, but ate intermittently over a

five hour period. Since curves are the same general shape, a

comparison of Figures 9 and 10 in which all animals received

labeled ferric iron with either raw or cooked hake, indicates that

the magnitude of absorption and not rate of absorption was effected

by the presence of raw hake.

The Iron-Binding Factor

The CF-causing factor present in Pacific hake, whiting, and

coal fish inhibits the utilization of dietary iron by the animal, and

is heat-labile (Stout, Oldfield, and Adair, 1960b; Helgebostad and Martinsons, 1958). Results of Trials 3-5 indicate that raw hake has little or no effect on absorption and utilization of ferrous iron whether given in the whole ration, water filtrate, or suspension. However, when ferric iron was given with raw hake in Trial 8, absorption was greatly reduced. These data strongly suggest that, in addition to the above mentioned characteristics, the factor present in raw hake specifically interferes with the utilization of ferric iron.

A similar factor may be present in fish that Alaskan Eskimos consume. Scott, Wright, and Hanan (1955) have reported the occurrence of anemia among Alaskan Eskimos, especially during winter months when their diet was partially composed of raw whole fish (whiting). Observed anemia was severe among adults and especially women, who did not have regular meals, but ate as they pleased during the day. School children who normally had a hot breakfast of cooked oatmeal had hemoglobin values approaching the normal.

There are several other substances that inhibit the utilization of iron much the same as the factor found in Pacific hake, namely: EDTA (Wiseman, 1964), Pancreatin (Callender, 1963), Desferrioxamine B (Moeschlin and Schnider, 1963), Soybean Protein (Fitch et al., 1964), and conalbumin (Feeney, 1964). They all block absorption of iron when given orally; however, when Desferrioxamine B and EDTA are injected intramuscularly, urinary iron excretion

is greatly increased.

The mechanism whereby iron metabolism is blocked by the hake factor has not been completely established. However, in view of results reported herein, this factor acts much the same as these other substances by greatly decreasing the absorption of iron when given orally.

Whether mink consuming hake rations excrete more iron in their urine than normal mink has not been answered unequivocally by this study. Urinary ^{59}Fe activity is not necessarily of urinary origin, due to contamination of urine from feces and feed. With the type of collection trays used, it is almost impossible to collect urine samples without fecal contamination due to the liquid nature of feces from mink consuming raw hake rations, unless urine is collected before defecation. In several cases, urine samples were collected prior to defecation, and in no case was the ^{59}Fe activity higher in the urine of hake-fed mink than in that of controls.

Previous work at this station using chemically-determined iron balance data indicated that mink fed raw hake excreted significantly more iron in the urine than control animals fed sole (Oregon State University Experimental Fur Farm, 1961). Since the same type of collection trays were used in that study as were used in the present one, the validity of their results is questionable.

The ^{59}Fe balance trials reported hereinbefore, indicate that

several factors including form of iron, presence of other feedstuffs,

and length of incubation prior to administration, modify iron utiliza-

tion as affected by raw Pacific hake. The phase of research dealing

with the urinary excretion of iron by raw hake-fed mink was not

completely answered, and there is a need for further research in

this area; however, methods have been established which will ulti-

mately lead to the solution of this problem.

Time of Passage of Feed

Wood (1956) studied the time of passage of feed in mink. He

reported mean passage times of 106, 123, and 109 minutes for a

normal diet marked with Sudan III, charcoal, and chromium oxide,

respectively. With unmarked feed, the mean time of passage was

found to be 89 minutes. Neseni and Piatkowski (1958) fed five

different rations marked with fuchsin-stained straw to groups of

10 mink. They observed the first recovery of the marker in the

feces within a range of two to four hours and a mean of three hours.

They contrasted this with the fox, which has a digestive tract of

similar length but a time of passage of eight hours.

Using mink, Sibbald et al. (1962) reported a mean time of

passage of 142 minutes and a range of 62 to 215 minutes. They

used nine different carmine-dyed diets and made a total of 502

observations. They found no statistical difference in rate of

passage associated with either diet or sex of the animals involved.

Since ^{59}Fe can be used as a marker, the rate of passage of

feed through the intestinal tract can be determined and is reported

herein. The overall mean for the total of 37 observations indicates

a time of passage of 185$\pm$22 (mean $\pm$ standard deviation) minutes

with a range from 59 minutes to 360 minutes. However, the results

in all cases may not be comparable since the length of fasting prior

to, and after dose administration varied among trials.

Most of the observed times fall in the ranges reported (Neseni

and Piatkowski, 1958 and Sibbald et al., 1962). However, the mean

time of 185 minutes is somewhat longer than the mean times reported

by Wood (1956). This difference, if real, might be the result of

differences in rations fed, ages of the animals used, or of the

temporary fasting period before dose administration.

Initially, urine and fecal material were collected for a 10

day period. In no case was more than one percent of the adminis-

tered dose excreted after the initial 48 hour period, or more than

0.5 percent excreted in any 24 hour period after the initial 48 hours.

Iron-59 balance trial excretion data are shown in Appendix III.

SUMMARY

1. Feeding iron-deficient and iron-adequate purified diets to mink establishes iron as a chromotrichial nutrient.

2. Twenty ppm of iron in purified diets of mink appears to be adequate for body growth, fur growth, pigmentation, and maintenance of normal blood values.

3. Limited data indicate that 70 percent of the labeled iron in hair was contained in the melanin granules. Possible enzymatic and structural roles of iron in the synthesis of melanin are discussed.

4. Iron-59 balance trials with mink in the presence and absence of dietary Pacific hake indicate that several factors, including form of iron, presence of other feedstuffs, and length of incubation, modify iron utilization as it is affected by hake.

5. Plasma and erythrocyte iron-59 activity for all trials indicate that where iron utilization is impaired by the presence of raw hake, interference is at the absorptive level.

6. Mean time of passage of feed through the gastrointestinal tract for the mink used was 185 ± 22 minutes ($\pm$ value is standard deviation).

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APPENDICES

APPENDIX I

Part A.   Counter settings for Packard Model 410A,
Auto-gamma Spectrometer.

1.   Counter efficiency: 10 percent.

2.   Off-On-H. V. switch: H. V.

3.   Operating Mode switch: Automatic.

4.   Analysis Mode switch: Wide.

5.   Lower or Baseline Control: 460.

6.   Upper or Width Control: 610.

7.   Attenuator switch: 1.

8.   High Voltage Tap switch: 2.

9.   High Voltage Fine Control: 850.

10.   High Voltage Indicating Meter: 850.
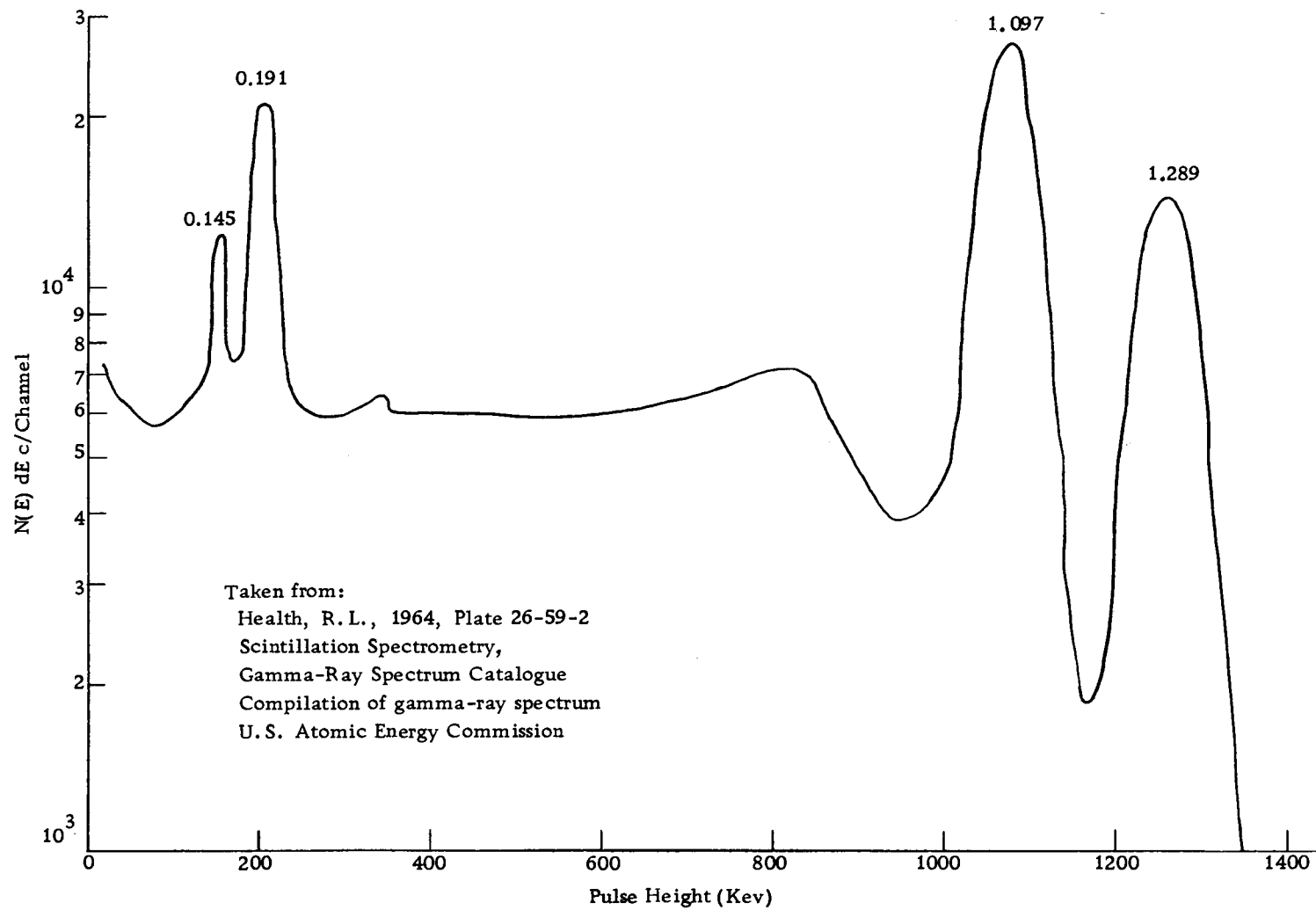
Part B. Emission spectrum of Iron-59.

APPENDIX II

Animals and groups used in ^{59}Fe balance trials (Part 2).

Trial #	Animals Used	Type of Ration	Method of ^{59}Fe Admin.	Amount of ^{59}Fe Used	Type of ^{59}Fe
1	7118	Hake	Feed	3.5 µc	^{59}Fe^{++}-Citrate
"	7139*	Sole	"	"	"
2	7113	Hake	Feed	10 µc	"
"	7115	"	"	"	"
"	7119	"	"	"	"
"	7122	"	"	"	"
"	7123*	Sole	"	"	"
"	7124*	"	"	"	"
"	7125*	"	"	"	"
"	7126*	"	"	"	"
3	7123	Hake	H_2O	12.5 µc	"
"	7694	"	"	"	"
"	7695	"	Raw Hake Filtrate	"	"
"	7697	"	"	"	"
"	7669	Cooked Hake	H_2O	"	"
"	7102	"	"	"	"
"	7103	Raw Hake	Hake	"	"
"	7104	"	"	"	"
4	7104	Cooked	Feed	18 µc	"
"	7675	"	"	12 µc	"
"	7102	Raw	"	18 µc	"
"	7676	"	"	12 µc	"
5	7670	Cooked	Suspension	10 µc	^{59}FeSo$_4$
"	7694	"	"	"	"
"	7126	Raw	"	"	"
"	7675	"	"	"	"
"	7676	"	"	"	"
6	7102	Cooked	Suspension	0.25 µc	^{59}Fe-Hb.
"	7104	Raw	"	0.29 µc	"
7	7104	Cooked	Suspension	2.5 µc	"
"	7694	"	"	"	"
"	7102	Raw	"	"	"
"	7675	"	"	"	"
8	7104	Raw	"	30 µc	^{59}FeCl$_3$
"	7694	"	"	"	"
"	7102	Cooked	"	"	"
"	7675	"	"	"	"

* Non-anemic mink used.

APPENDIX III

Iron-59 balance trial excretion data

(Figures represent average (percent) cumulative fecal and urine ^{59}Fe excretion values by treatment within individual trials)

Type of ^{59}Fe	No. of Animals	Time (hours)						Total Excretion	Range
		2	4	8	12	24	48		
Trial 1 and 2									
Raw hake $^{59}Fe^{++}$-Citrate	5	0	9.2	14.3	54.1	96.8	97.8	98.3	88-99
Sole "	5	0	9.0	11.1	37.1	74.6	84.2	84.8	63-92
Trial 3									
Distilled water $^{59}Fe^{++}$-Citrate	4	35.4	37.2	38.4	61.5	62.8	63.8	64.2	60-69
Raw hake filtrate "	4	15.5	32.5	37.4	51.1	52.8	61.6	61.5	47-68
Trial 4									
Raw hake $^{59}Fe^{++}$-Citrate	2	11.1	11.1	43.6	43.6	62.4	63.9	64.0	42-86
Cooked hake "	2	19.3	19.3	33.1	33.1	47.6	57.9	58.2	41-75
Trial 5									
Raw hake suspension $^{59}FeSO_4$	3	0	71.0	75.2	82.5	82.5	87.9	88.3	76-93
Cooked hake suspension "	2	2.7	12.4	45.4	45.4	60.8	63.9	64.4	56-72
Trial 6 and 7									
Raw hake suspension ^{59}Fe-Hb.	3	0	62.0	85.9	93.5	96.0	96.5	96.5	89-99
Cooked hake suspension "	3	0	56.7	84.9	86.3	86.9	87.2	87.2	84-91
Trial 8									
Raw hake suspension $^{59}FeCl_3$	2	0	45.1	66.8	67.0	67.2	67.5	67.5	64-71
Cooked hake suspension "	2	0	46.8	77.7	77.9	78.3	79.0	79.0	62-96