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A BIOACTIVE PIGMENT ("ALPHA PIGMENT") IN CECAL CONTENTS OF GERM-FREE ANIMALS

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Abstract—From cecal contents of germ-free rats and mice, a pigment fraction was isolated by centrifugation and chromatography ("Alpha Pigment") which: 1) caused vasodilation and stimulated the spontaneous rhythmic motility of intestinal villi in anesthesized dogs, and 2) occasionally inhibited the effects of epinephrine on rat blood pressure. The same pigment fraction isolated from conventional control animals proved inactive. The listed effects of alpha pigment were quite similar to those of ferritin and apoferritin in their vasodepressor (VDM) form. In reduced form, all three agents were active; in oxidized form, all three were inert. Antiferritin serum neutralized these agents. Pretreatment with desferrioxamine, a ferric-iron chelator, failed to inactivate these substances. This is in accordance with previous findings which indicated that ferric iron is not needed for sustaining ferritin's vasodepressor properties. The concurrence of other autakoids or of other contents of the gut in this phenomenon could be excluded either by the nature of their effects or by the use of appropriate blockers. It is indicated that alpha pigment is available for absorption from the intestine and, in its bioactive form, it may be responsible for some of the anomalies observed in germ-free animals (e.g. reduction of metabolic rate and of cardiac output). In conditions of conventional life, alpha pigment is inactivated in the gut, presumably by the intestinal microflora. This is supported by the observation that after treatment with trypsin, by incubation with an inoculum of conventional cecal contents, germ-free alpha pigment, ferritin and apoferritin are inactivated.

WE HAVE recently reported[1] that the contents of the enlarged cecum of germ-free rodents contain substances that affect smooth muscle in a variety of preparations and are toxic to mice on intraperitoneal administration. Cecal contents from comparable conventional control animals showed no such activities or displayed them in a substantially reduced form. On ion-exchange chromatography and charcoal treatment, the agents of germ-free cecal contents could be separated into at least three active components (see Table 1). (1) One substance, which was found in a decolorized fraction, caused contraction of rat uterus and of guinea pig ileum *in vitro* and lowered blood pressure *in vivo*. This agent has been studied in some detail and tentatively identified as a fecal kallikrein.[2] (2) Another substance in a pigment peak (designated as "initial pool" pigment) was found to exert vasoconstrictor effects and caused transient standstill in the spontaneous rhythmic contraction of intestinal villi in the anesthesized dog. In these details the pigment fraction suggested the effects of epinephrine or of epinephrine sensitization. The identity of this agent is unknown.[3]

(3) A third substance in a different pigment peak ("terminal pool", "alpha pigment") exerted vasodilator effects and proved a potent stimulant on the motility of intestinal villi. These characteristics suggested epinephrine inhibitory effects, similar to those of vasodepressor (VDM) ferritin.[4] The purpose of this paper is to report on the progress made in the study of alpha pigment.

METHODS

Source of material

Donors of cecal contents were germ-free and convention alcontrol Fischer 344 rats (Ch.R., CD-F) and HaM/ICR Swiss mice (Ch.R., CD-1) supplied at the age of 28 days by Charles River Breeding Laboratories, Brookline, Mass. At term, the animals' age was 3–6 months; sex distribution was approximately equal; the diet, after arrival in Lexington, was sterilized L-462.[5] In a few parallel groups, Wistar rats and Swiss–Webster mice (kindly donated by Lobund Laboratories, University of Notre Dame), were used. As an alternate diet, sterilized L-356[6] was used in a few groups of Fischer rats. Two groups of young adult Swiss–Webster mice serving as donors of cecal contents were switched 3 weeks prior to sacrifice from L-462 to the liquid (amino acid-type) diet of Greenstein *et al.*[7] All diets were supplied by a commercial source (General Biochemicals, Inc., Chagrin Falls, Ohio; GBI). The germ-free animals were housed in the flexible plastic isolators of Trexler;[8] the conventional controls were held in an open environment. In all details of gnotobiotic and conventional animal rearing, established techniques were followed.[9, 10] The animals were killed by a blow on the head. The freshly harvested cecal contents were collected in batches (contents from 8–12 rats or from 10–50 mice forming a batch). After thorough mixing, the dry matter content of the batch was determined by drying triplicate samples to weight constancy in a vacuum oven at 90°. The batches were then diluted with distilled water to 15 per cent dry weight and stored at −18° over a period of not greater than 3 months.

Centrifugation of cecal contents

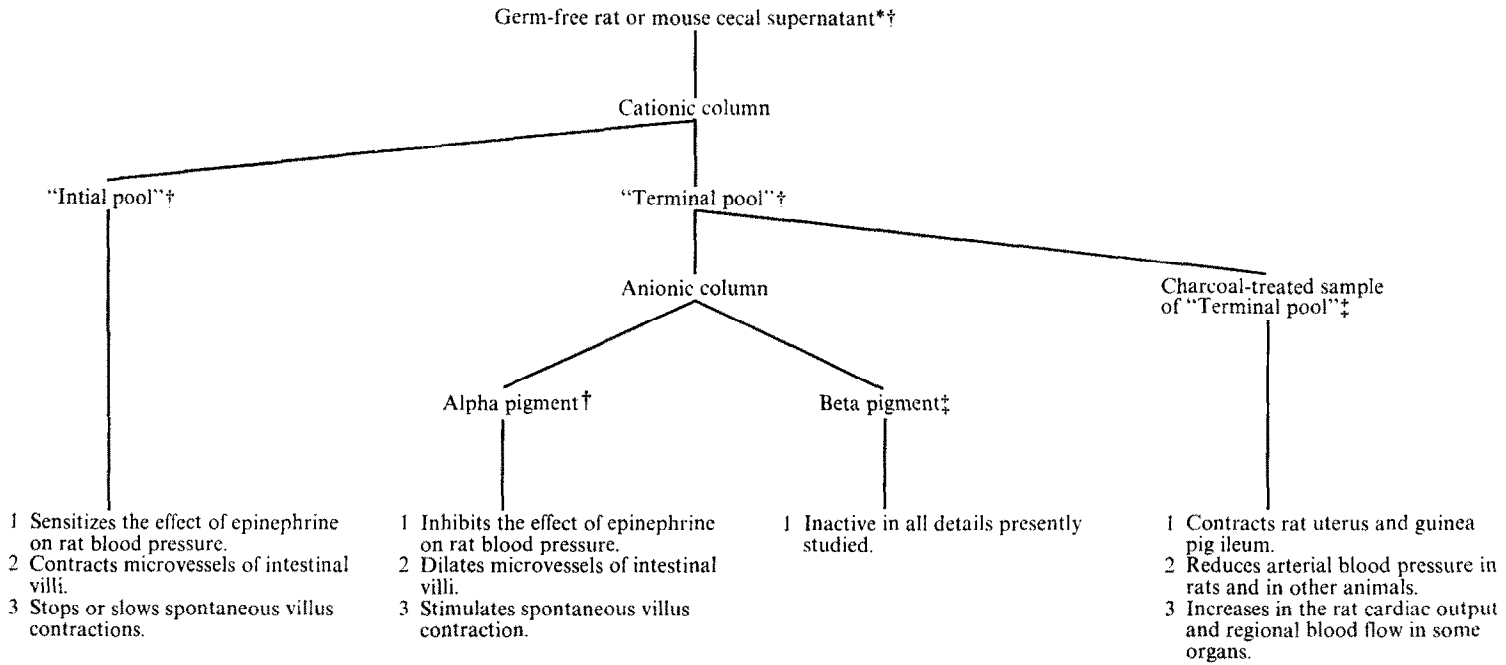
The batches were centrifuged at 97,000 *g* for 30 min. The supernatant was pipetted off and then stored frozen. The sediment, which showed no demonstrable activity, was discarded.

Fractionation of the cecal supernatant (CS, Table 1)

(*A*) *Cationic column.* The first chromatographic separation of CS was on a column of a cation-exchange resin (Bio-Rex 70, 100–200 mesh, Bio-Rad Corp., Richmond, Calif.) cyclized to acidic form. The dimensions of this column were 1·5 × 22 cm. Gradient elution was employed by using 0·01 to 0·25 N NaOH. The flow rate of the eluent was 0·5 ml/min, each fraction being 5 ml. This procedure caused the separation of CS into two pigment fractions. One was present in the initial, the other in the terminal samples of the effluent (initial and terminal pools). A detailed account of this step, together with the results obtained, was given in a previous report.[2] Presently, these fractions were formed by dividing the effluent into two equal parts.

The initial pool pigment fraction was reconstituted to the original volume of the CS input into the column (usually 15 ml) by evaporation under vacuum at 37°. After this, the pH was adjusted to 7·2 and the osmolarity to 0·9% NaCl equivalent (Osmometer, Advanced Instruments Inc., Newton Highlands, Mass.) by using small amounts

TABLE 1. SCHEDULE OF FRACTIONATION



* Identical fractions prepared from cecal supernatant of conventional control animals were either inactive or exerted similar effects in a reduced form (see text).
† Toxic to mice on intraperitoneal administration.
‡ Nontoxic.

of 1 N NaOH and crystalline NaCl respectively. In the previously reported tests, the initial pool pigment was used in this form.[3]

The terminal pool fraction contained pigments as well as the entire amount of kallikrein of CS.[2] Preparatory to further pigment separation, the terminal pool was reconstituted by evaporation to the original volume of the CS input.

(*B*) *Anionic column.* The separation of terminal pool pigments was carried out on an anion-exchange resin column (Bio-Rad AG1-X4, 200–400 mesh, same source as that given for the cationic resin) cyclized to alkaline form. The dimensions of this column were 0·9 × 35 cm. Elution was with 0·01 N HCl. Flow rate of the eluent and the volume of each fraction were the same as in the cationic column. This procedure resulted in a well defined separation of the two pigment peaks, named alpha and beta (see Fig. 1). The fractions derived from these were designated as germ-free and conventional, alpha and beta pigments. These pigment fractions were reconstituted to the volume of the original CS input by evaporation; pH and osmolarity of the samples were adjusted to 7·2 and 0·9% NaCl equivalent respectively. In addition, the samples were heated to 70° for 10 min to destroy the activity of kallikrein.

Quantitation of the pigment content

In order to estimate the quantity of pigments in various fractions, an arbitrary grading scale was adopted. According to this, 1 unit represents the amount of pigment in CS or in some of its fractions which, when taken up in 1 ml Krebs phosphate buffer (pH 7·2), will give an optical density reading of 0·3 (Zeiss PMQ II spectrophotometer; 2-ml quartz cuvettes; light path 1 cm; wavelength 420 mμ). This coincides with the reading given by a 0·25 mg/ml ferritin solution under the same circumstances. Some variation exists from this point of view among batches obtained from our commercial source.

Materials and other preparative procedures

(*A*) *Materials.* In the following, the names and the sources of various materials which were used in this study are listed. The doses applied in our tests were uniformly 0·1 ml of solutions whose concentration in weight or other units (U) per ml are given for each material in our list. The solvent used was modified Krebs phosphate buffer at pH 7·2. For effects on blood pressure, the administration was i.v.; for actions on intestinal villi, the application was topical.

(1) Alpha pigment, from CS terminal pool of germ-free and conventional rats and mice; 0·4–3·0 pigment U.

(2) Beta pigment, same source; 15–20 pigment U.

(3) Ferritin, twice crystallized, Cd-free, horse spleen, GBI: 0·1–0·2 mg.

(4) Apoferritin, twice crystallized, horse spleen, GBI; 0·1–0·2 mg.

(5) Villikinin, canine intestinal mucosa, charcoal-treated, desalted and lyophilized; prepared by a modification of the method of Kokas and Johnston;[11] 1–10 mg.

(6) Bradykinin, synthetic, Sandoz Pharmaceuticals, Hanover, N.J.; 1 μg.

(7) Carboxypeptidase B, Worthington Biochemical Corp., Freehold, N.J.; 250 U.

(8) Serotonin, creatinine sulfate complex, GBI; 10 μg.

(9) *d*-Lysergic acid diethylamide, Sandoz; 10 μg.

(10) Histamine dihydrochloride, Mann Research Laboratories, Inc., New York, N.Y.; 10 μg.

(11) Tripelenamine hydrochloride, CIBA Pharmaceutical Co., Summit, N.J.; 1 mg.

(12) Acetylcholine chloride, Merck and Co., Rahway, N.J.; 1 μg.

(13) Atropine sulfate. H_2O, U.S.P., Mann; 1 mg.
(14) *l*-Epinephrine (adrenaline chloride), Parke-Davis & Co., Detroit, Mich.; 0·1–100 μg.
(15) Norepinephrine (levarterenol bitartrate), Winthrop Laboratories, New York, N.Y.; 0·1–100 μg.
(16) Phentolamine methnesulfonate, CIBA; 0·5 μg.
(17) Antiferritin serum, rabbit, Pentex Inc., Kankakee, Ill.; titer 1:3200.
(18) Desferrioxamine (Desferal urethane sulfonate), CIBA; 10 mg.
(19) Trypsin, twice crystallized, salt-free (9500 U/mg), GBI; 2–50 μg.
(20) Sodium taurocholate, Nutritional Biochemicals Corp., Cleveland, Ohio; 1 mg.
(21) Bile, dog, freshly harvested from animal; undiluted.
(22) Krebs phosphate buffer, modified (salts are given in grams to 1000 ml distilled water); *pH* 7·2: NaCl, 7·8; KCl, 0·354; $MgSO_4 \cdot 7\ H_2O$, 0·267; Na_2HPO_4 anhydrous, 0·886; $NaH_2PO_4 \cdot H_2O$, 0·207, *pH* 8·3: same salt mix, except Na_2HPO_4, 1·053; NaH_2PO_4, 0·078.

(*B*) *Pretreatment of experimental animals.* The mode of administration of blockers and of similar agents was the same as in the case of the other materials. They preceded the administration of the material to be tested by 3–5 min.

(*C*) *Activation and inactivation of materials.* The samples of alpha pigments, ferritin, apoferritin and of other agents listed in Table 4 were oxygenated and nitrogenated by bubbling these gases through the solutions for 2 min and then they were stoppered tightly in their containers. At the midpoint of the incubation periods, the same procedure was repeated. Incubation of the samples with conventional rat liver slices was performed by the method of Mazur and Shorr.[12]

Inactivation studies with antiferritin serum were carried out by mixing 0·2 ml of the undiluted serum with 0·8 ml of an alpha pigment solution containing 0·8 units or with ferritin or apoferritin solutions, each containing 0·2 mg of these agents. The incubation period was 2 hr at room temperature.

Treatment with desferrioxamine was performed by adding 10 mg of this substance to solutions of alpha pigment, ferritin or apoferritin of the same volume and concentration as those given in the previous paragraph. The incubation was the same as with antiferritin serum. Trypsin digestion was carried out in solutions where the substrate:enzyme ratio was calculated to be 20:1 (by weight). The actual concentration of alpha pigment in the incubation mixture was 0·4 unit/ml; for ferritin and apoferritin it was 0·1 mg. The solvent used was pH 8·3 Krebs buffer. Trypsin digestion was carried out over a period of 12 hr at 37°. In executing this, the conditions stipulated by Matioli and Baker[13] for trypsin digestion of ferritin were considered. Incubation with cecal microflora was performed by adding a loopful of freshly harvested conventional rat cecal contents (fed L-462 diet) to each ml of the sample. The incubation period was 48 hr at 37°.

Testing actions on blood pressure

Conventional control male rats, weighing approximately 300 g and kept under pentobarbital sodium anesthesia (35 mg/kg body wt.), were used for this purpose. The carotid was cannulated with polyethylene tubing and the blood pressure was recorded on a polygraph via a pressure transducer. The materials were injected into the saphenous vein.

Testing actions of intestinal villus motility

Mongrel dogs of both sexes weighing 15–18 kg were anesthesized with pentobarbital sodium (same dose as that given to rats). An approximately 10 cm^2 area of jejunal

mucosa was exposed after laparotomy and after an incision on the intestine's free margin. With a suitable stage, while using vertical illumination, the exteriorized mucosa was held in the focal plane of a stereomicroscope (magnification, 60×). The rate of spontaneous contraction of villi per field of vision was counted before and after the topical application of the sample. After application, the first count was taken at 30 sec and from then on it was taken at 2-min intervals, each counting period lasting for 30 sec. This was continued until the count returned to the basal value. Dissipation of the sample from the area of the count was in part prevented by a shallow cylindrical "collar" made of thin cardboard, whose height was 2 mm, its diameter was 0·7 cm, and it weighed 30 mg. Previous tests have indicated that the presence of this device on the mucosa did not influence the rate of contraction of the villi. From the count per minute at the height of the effect (cpm) and from the basal count (B), the contraction index (CI) was calculated [CI = (cpm − B)/B]. In all other details of this procedure, the routine described by Kokas and Johnston[13] was followed.

Preparation of diet extracts

In addition to CS, batches of diet extracts were prepared. Sterilized L-462 diet was mixed with an equal quantity of distilled water and allowed to stand overnight at 37°. After the procedure used for the extraction of alpha pigment, a fraction was prepared from this mixture (designated as "diet alpha pigment"). It was tested on the motility of intestinal villi. In addition, another comparable fraction was prepared from the liquid diet of Greenstein *et al.*[7]

Miscellaneous observations

In alpha pigments, preliminary determinations of ferritin-iron (water-soluble non-heme iron) were carried out by the method of Kaldor,[14] as modified by Reddy *et al.*[15] Nitrogen determination on the same material was made by the method of Lanni *et al.*[16]

RESULTS

Pigment separation

Table 2 shows the quantitative distribution of pigment in various fractions separated according to our schedule in Table 1. The results obtained in rats and mice from this point of view essentially coincided. The CS of germ-free animals contained approximately twice as much pigment per unit volume as that of conventional controls. In either case, the pigment ratio between initial and terminal pools was about 6:4. The total recovery of pigment (i.e. of the combined initial and terminal pools) from the cationic column was consistently over 90 per cent of the CS pigment input. Fig. 1 illustrates the separation of the terminal pool fraction into alpha and beta pigments on the anionic column. In essence, the qualitative aspects of separation into alpha and beta pigments of this fraction were similar for both the germ-free and the conventional groups. To the naked eye, alpha pigment always appears as a distinctly reddish fraction in comparison to the darker brown of beta pigment. The germ-free alpha peak occasionally showed a slight spur on the ascending leg, which is not shown on the chromatogram. Since it represented only a very small fraction of the pigment, its effect on our biologic models could not be evaluated. A more frequently occurring secondary peak was shown on the descending leg of the germ-free beta pigment. In view of the inactivity of beta pigment, the role of this peak was not considered. As

shown in Table 2, the total recovery of pigment (i.e. of alpha and beta pigments) from the anionic column was approximately 25 per cent of the terminal pool pigment input. The rest remained irreversibly attached to the resin by this procedure. Considering the quantity of alpha pigment per ml of CS in germ-free and conventional animals, little or no difference could be found, as indicated in Table 2. However,

TABLE 2. AVERAGE AMOUNT OF PIGMENT UNITS* FOUND IN UNFRACTIONATED AND FRACTIONATED RAT CECAL CONTENTS AND IN DIET EXTRACT (DIET: L-462 STERILIZED)

			Fractions			
			Cationic column		Anionic column (Terminal pool only)	
		Unfractionated	Initial pool	Terminal pool	Alpha	Beta
Pigment U/ml found in cecal supernatant	Germ-free	210	117	75	2·7	15·7
	Conventional	102	51	41	2·4	8·8
Pigment U/rat calculated for total cecal contents†	Germ-free	4820	2680	1720	62	360
	Conventional	230	115	92	5	20
Pigment U/ml found in diet extract		7	3	2	< 0·10	0·12

* Pigment unit (U) is an arbitrarily selected amount of pigment found in cecal supernatant, which when taken up in 1 ml Krebs phosphate buffer (pH 7·2) will give an optical density reading of 0·3 (Zeiss PMQ II spectrophotometer; light path 1 cm; wavelength 420 mμ). The values scattered approximately 25 per cent from batch to batch (4–12 batches per group).

† Calculation was made on basis of average values found in 300 g, 3–4 months old, male Fischer rats. In germ-free rats, total wet cecal contents = 9 g/100 g body wt. and water content of same = 85 per cent, in conventional rats; total wet cecal contents = 1 g/100 g body wt. and water content of same = 75 per cent.

when the alpha pigment content was expressed per animal, i.e. referred to the entire cecal pool, the germ-free rats showed greatly elevated values, reflecting their considerably increased cecal contents in comparison to conventional controls. When diet extracts were chromatographed in the same fashion as CS, at the end of this procedure two very faint pigment peaks resulted, which coincided with those of alpha and beta pigments. The pigment concentration in various fractions prepared from sterilized L-462 diet are also illustrated in Table 2.

Preliminary observations kindly made by Dr. B. S. Reddy at Lobund Laboratories, University of Notre Dame, and at our laboratory indicated that the ratio in non-heme, soluble iron content between color-matched solutions of germ-free alpha pigment (L-462 diet) and ferritin (GBI) at pH 7·2 and 420 mμ was approximately 1:3. For nitrogen, the ratio between the same alpha pigment and ferritin solutions was 4:1.

Actions of alpha and beta pigments

(*A*) *Effects on blood pressure.* Fig. 2 illustrates the epinephrine inhibitory effect of germ-free alpha pigment on rat blood pressure. Generally it was indicated that 1–2 units of germ-free alpha pigment reduced by approximately 50 per cent the hypertensive effect of 1 μg epinephrine in a 300 g conventional rat. Variation in the intensity of this pigment from batch to batch was observed. Administration of germ-free alpha

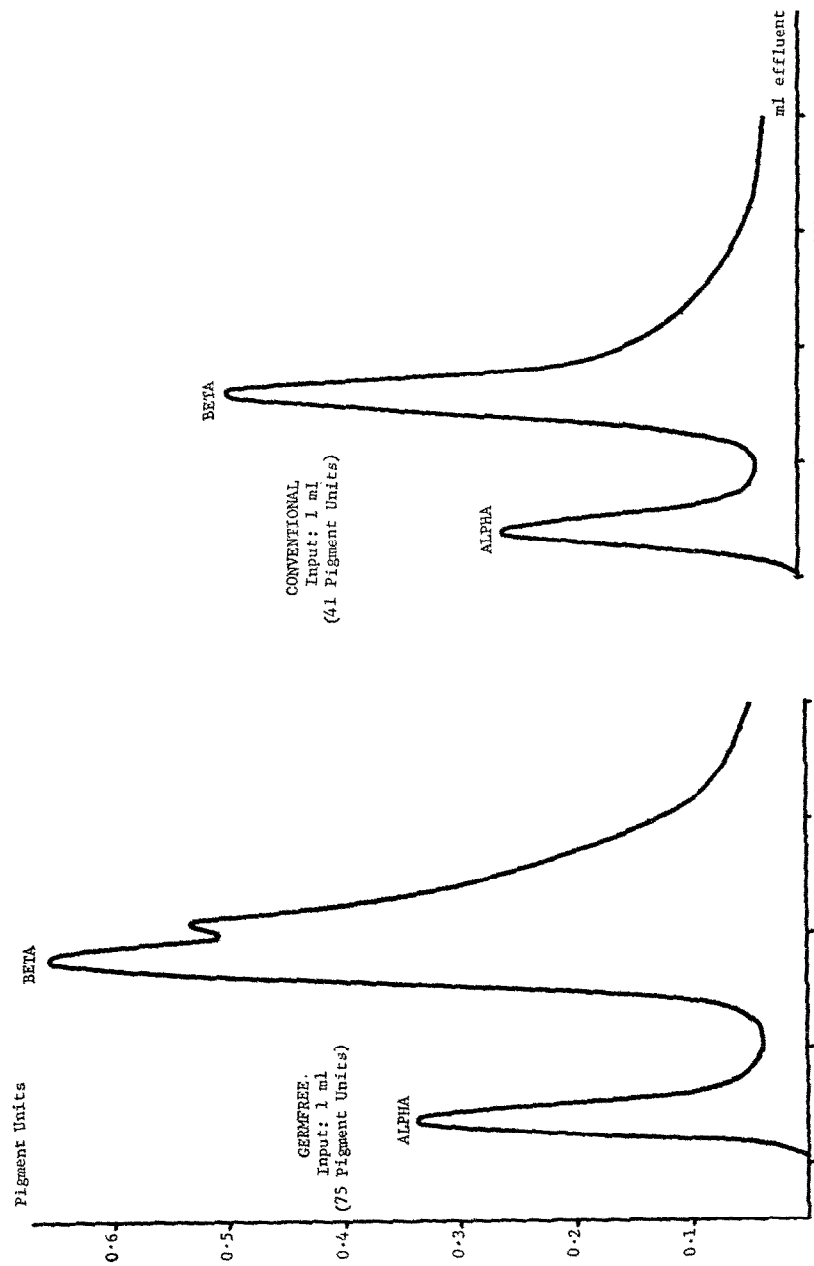


FIG 1. Separation of germ-free and conventional cecal supernatant, terminal pool, originating from rats fed sterilized L-462 diet, on a column of the anionic resin Bio-Rad AG1-X4. The dimensions of this column were 0·9 × 35 cm; elution was with 0·01 N HCl. Ordinate, pigment units (for definition, see Methods); abscissa, ml of effluent.

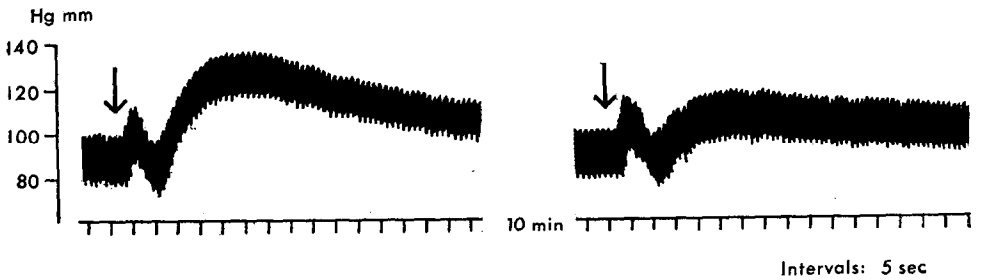


FIG. 2. Epinephrine-inhibiting effect of germ-free rat cecal supernatant, terminal pool, alpha pigment. Actions of 1 μg epinephrine on the carotid blood pressure of a conventional rat ($\downarrow$). All agents were given intravenously. Left, pretreated with Krebs buffer (0·5 ml) 2 min prior to the injection of epinephrine; right, pretreated with 1·5 pigment units of germ-free rat, terminal pool, alpha pigment (in 0·5 ml buffer) 2 min prior to the injection of epinephrine.

pigment by itself had no appreciable effect on blood pressure. The alpha pigment derived from conventional control animals exerted no epinephrine inhibitory action.

(*B*) *Effects on intestinal villi.* Table 3 shows that germ-free alpha pigment exerted a stimulatory effect on the spontaneous contraction of intestinal villi. Bioactivity was

TABLE 3. EFFECTS* OF GERM-FREE AND CONVENTIONAL CECAL SUPERNATANT, TERMINAL POOL. ALPHA AND BETA PIGMENTS† AND OF OTHER AGENTS ON THE CONTRACTION INDEX OF INTESTINAL VILLI OF DOGS

	No pretreatment	Pretreatment with:‡ Carboxypeptidase B[1] *d*-Lysergic acid[2] Tripelenamine[3] Atropine[4] Phentolamine[5]
Alpha pigment, germ-free rat	++	+, ++[1, 2, 3, 4]
Alpha pigment, germ-free mouse	++	•
Alpha pigment, conventional rat	−, 0	•
Beta pigment, germ-free rat	0	•
Beta pigment, germ-free mouse	0	•
Beta pigment, conventional rat	−, 0	•
Ferritin	+, ++	+, ++[2, 3, 4]
Apoferritin	+, ++	+, ++[2, 4]
Villikinin	++	0[1]
Bradykinin	+	0[1]
Serotonin	+	0[2]
Histamine	+	0[3]
Acetylcholine	+	0[4]
Epinephrine	−	0, +[5]
Norepinephrine	−−	0[5]

* Contraction index: ++ = > + 1·0; + = + 0·3 to + 1·0; 0 = − 0·2 to + 0·3; − = − 0·5 to − 0·2; −− = − 1·0 to − 0·5; • = no observation.

† These pigments were derived from animals fed L-462 or L-356 sterile diets. The nature of the diets did not affect qualitatively or quantitatively the actions of alpha or beta pigments.

‡ Superscripts (1–5) in this heading and column cross identify the listed blocking agents with which the villi were pretreated in the different tests described in this table.

demonstrated in alpha pigment derived from germ-free rats fed L-462 and L-356 diets and similarly from germ-free mice fed L-462 and the liquid diets. This effect was quite pronounced and was consistently observed over the 3-yr period of this experiment. Alpha pigment from conventional controls always proved inactive on the villus preparation. Among various autakoids, which are present or possibly present in the intestine, several exerted stimulatory effects on the villi under similar experimental circumstances. In this group only ferritin and apoferritin showed parallelism of action with germ-free alpha pigment and, along with this pigment, resisted the effect of the variety of blockers. The concurrence of villikinin, bradykinin, acetylcholine, serotonin, histamine, epinephrine and norepinephrine in this phenomenon could be excluded either by the use of appropriate blockers or by the nature of their effects. Other agents that are normally present in intestinal contents (tested were trypsin, bilirubin, sodium taurocholate and whole bile) proved inactive from this point of view. A 2 days' incubate of conventional rat cecal inoculum in pH 7·2 Krebs buffer or thioglycollate medium was either inactive or mildly depressant on villus motility.

Table 4 indicates that germ-free alpha pigment, as well as ferritin or apoferritin, could be activated by incubation in a nitrogen atmosphere in the presence or absence

TABLE 4. FACTORS GOVERNING THE ACTIVATION AND INACTIVATION OF ALPHA PIGMENTS, FERRITIN AND APOFERRITIN; EFFECTS ON CONTRACTION INDEX OF INTESTINAL VILLI (dog)*

	Alpha pigment (germ-free rat)	Alpha pigment (conventional rat)	Ferritin	Apoferritin
No pretreatment	++	—, 0	+, ++	+, ++
O_2 atm., 6-hr incub.	0	•	0	0
O_2 atm., liver slices, 2-hr incub.	0	•	0	0
N_2 atm., 6-hr incub.	++	0	+, ++	++
N_2 atm., liver slices, 2-hr incub.	++	•	++	+, ++
Antiferritin serum, rabbit, 2-hr incub., N_2 atm.	0	•	—, 0	—, +
Cecal flora, rat, 48-hr incub., N_2 atm.	0	•	++	++
Trypsin, 12-hr incub., N_2 atm.	++	•	+	++
Trypsin, 12-hr incub., then cecal flora 48-hr incub. in N_2 atm.	0	•	0	0
Desferrioxamine B, 2-hr incub., N_2 atm.	+	•	+	+

* All pigments were derived from animals fed L-462 diet. Incubation was at 37°. See legend to Table 3 for definition of symbols; atm. = atmosphere; incub. = incubation.

of liver slices in the solution. Incubation in an oxygen atmosphere under similar circumstances inactivated all three agents. Antiferritin serum neutralized the effects of these substances in spite of holding the samples in nitrogen atmosphere. Incubation with cecal flora *in vitro* inactivated germ-free alpha pigment, but not native ferritin or apoferritin. Predigestion with trypsin, however, rendered also ferritin and apoferritin susceptible to microbial inactivation. Desferrioxamine slightly reduced (but in no case suppressed) the activity of germ-free alpha pigment, ferritin or apoferritin.

Fig. 3 illustrates the parallelism in stimulation caused by germ-free alpha pigment, ferritin and apoferritin, and the slight depression caused by conventional alpha pigment on villus motility. Table 5 shows that, though scattering was considerable in these observations, the arithmetic means of the contraction indices of germ-free

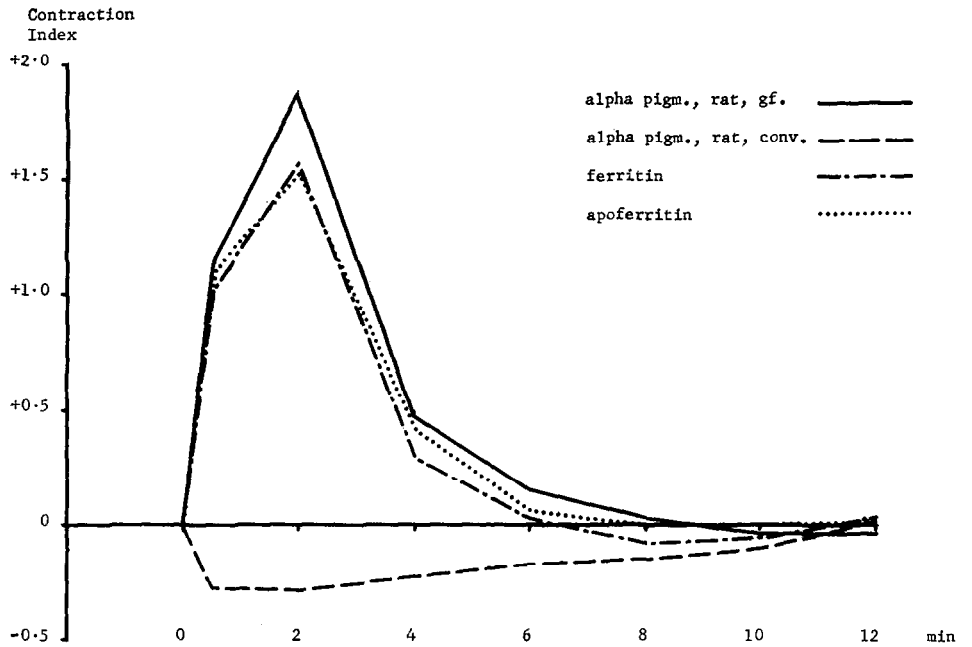


FIG. 3. Time sequence of the effects of alpha pigments, ferritin and apoferritin on the contraction index (for definition, see Methods) of intestinal villi. Arithmetic means of 5–21 counts taken per points in the curves. Ordinate, contraction index; abscissa, time (counts were taken at 30 sec and at 2, 4, 6, 8, 10 and 12 min). Conv. = conventional; gf. = germ-free.

alpha pigment, ferritin and apoferritin were closely aligned. The differences among these values were statistically nonsignificant. The arithmetic mean of conventional alpha pigment, on the other hand, was separated from those of the former group by statistically significant differences. The statistical P values for some of these comparisons are shown in Table 5. By testing dose–response relationships for germ-free alpha pigment, ferritin and apoferritin on villus motility, comparable threshold concentrations were found for these agents.

Microscopic observation showed that germ-free alpha pigment, ferritin and apoferritin caused considerable transient dilatation of the blood vessels of the intestinal villi. Conventional alpha pigment was inactive from this point of view.

The alpha fraction prepared from sterilized L-462 diet had a mild and somewhat inconsistent stimulatory effect on the contractility of intestinal villi (among five tested batches, three caused an approximately 50 per cent increment of the contraction index and two were inactive). This effect, when present, persisted after nitrogenation and disappeared after oxygenation or treatment with antiferritin serum. Similar alpha fractions prepared from the liquid diet of Greenstein *et al.*[7] proved consistently inactive.

TABLE 5. PEAK EFFECTS OF ALPHA PIGMENTS, FERRITIN AND APOFERRITIN ON THE CONTRACTION INDEX OF INTESTINAL VILLI OF DOGS*

	Alpha pigment													
	Germ-free						Conventional Rat			Ferritin			Apoferritin		
	Rat			Mouse										
	N	M	S.D.	N	M	S.D.	N	M	S.D.	N	M	S.D.	N	M	S.D.
	14	+1·88	±0·71	5	+1·80	± 0·77	5	−0·28	± 0·43	21	+1·56	± 1·06	7	+1·54	± 0·37
Statistical P between M of alpha pigment, germ-free rat, and					0·86			< 0·01			0·37			0·25	

* All pigments were derived from animals fed L-462 diet. The solutions of alpha pigments, ferritin and apoferritin were incubated in N_2 atmosphere prior to the experiment, as listed in Table 4. N = number of observations (in a total of 20 dogs); M = arithmetic mean; S.D. = standard deviation. Dosage range (in concentration/ml): alpha pigments, 0·25–2·5 pigment units; ferritin, 0·1–2·0 mg; apoferritin, 0·1–0·2 mg. All were topical applications of 0·1 ml solution.

DISCUSSION

Our study strongly suggests that alpha pigment isolated from the cecal contents of germ-free rats and mice is a congener of vasoactive ferritin. Additional work of purification, chemical analysis and biological testing on alpha pigment is needed to prove the correctness of this indication.

The relation between germ-free alpha pigment and ferritin was first suggested by the somewhat inconsistent epinephrine inhibitory effect which germ-free alpha pigment exerted on rat blood pressure. The vasodilation which was observed in the microcirculation of the intestinal villi after the topical application of this agent and of ferritin could be interpreted on the same basis. The detailed study of the stimulatory effects of germ-free alpha pigment and ferritin on the spontaneous contraction of intestinal villi supported the original assumption. In this model, epinephrine inhibitory substances generally enhance villus contraction; epinephrine itself (particularly norepinephrine) or epinephrine-potentiating substances slow down or stop villus contraction.

The ability to activate the villus stimulatory effect of germ-free alpha pigment in a nitrogen atmosphere and in the presence of liver tissue conforms with the findings of Mazur and Shorr,[12] who have shown this to be the case for the activation of the vasodepressor property of ferritin. In later publications from the same laboratory, Mazur *et al.*[17] and Baez *et al.*[18] have indicated that nitrogen atmosphere, in combination with reducing agents (e.g. glutathione from the liver or uric acid), acting on the surface of the molecule, will convert vasoinert ferric-disulfide-ferritin to active ferrous-sulfhydryl-ferritin. The present observation indicating that germ-free alpha pigment may be activated by exposure to nitrogen without the presence of liver tissue suggests that a reducing agent is present in our pigment fraction. This, among others, may be uric acid, which was found in substantial concentration in the cecum of germ-free mice, while that of conventional controls held none.[19, 20] Also, blood levels of uric acid were found to be consistently higher in germ-free mice in comparison

to conventional controls (approximately 2×).[20] The activation of ferritin observed in this series by exposure to nitrogen without liver slices remains an open question. Similarly, our findings on the inactivation of germ-free alpha pigment and ferritin in oxygen atmosphere, with or without liver slices, are in partial agreement with the previously quoted work.[12] Apoferritin, the protein moiety of ferritin which is responsible for this compound's biologic effect,[12] plausibly acted in unison with germ-free alpha pigment in the present comparison. Other evidence supporting the correlation between germ-free alpha pigment and ferritin was the ability to neutralize their activity with antiferritin rabbit serum. Since the presence of ferric iron in the molecule is not considered necessary for the presently discussed effect of ferritin (only a small amount of ferrous iron seems to be needed), the ineffectiveness of the ferric iron chelator, desferrixoamine,[21] on active germ-free alpha pigment, ferritin or apoferritin also conforms with our premise. The interference of other villus active autakoids or of other intestinal components with this phenomenon could be excluded.

Apoferritin, the carrier protein in the mucosal transport of iron within the columnar epithelium of the intestine, is known to reach the lumen on exfoliation of these cells in considerable quantities (Crosby[22] and Conrad *et al.*[23]). It is at present impossible to correlate the concentration of apoferritin in the gut lumen anticipated on this basis with the actual amount of alpha pigment found there, because some of the needed background information is missing (apoferritin concentration in the epithelium, passage time of the intestinal contents in these animals, etc.). It is assumed that, after the breakup of epithelial cells, a mixture of ferritin and apoferritin is freed into the lumen, the ratio of these substances depending on the availability of iron for absorption at the time of desquamation. This speculation is supported by the observation that alpha pigment is richer in nitrogen (though some of it may come from impurities) and poorer in soluble non-heme iron than ferritin. It is further anticipated that in the gut lumen the large ferritin molecule (mol. wt., approximately 480,000) is degraded into smaller components by digestive processes. In favor of this assumption are preliminary data which indicate that the molecular weight of alpha pigment is substantially less than that of ferritin (< 50,000 by gel filtration method). In apparent contradiction to this speculation is the known resistance of apoferritin to trypsinization *in vitro*.[24] However, this may not be the case *in vivo*. Indeed, it would be interesting to know whether the molecular weight of alpha pigment approaches that of the apoferritin "subunits" (mol. wt., 25,000) which Harrison[25] has identified with X-ray crystallographic and digestive methods. The maintained bioactivity of alpha pigment in germ-free animals and its apparently irreversible loss in conventional controls by interaction with the intestinal microflora (including the loss of activity of ferritin and apoferritin by trypsinization and bacterial exposure) remain an unexplained phenomenon. The absorption spectra of these and related substances are given in a separate report.[26]

The slight amount of alpha pigment that could be isolated from the sterilized L-462 diet extract may have originated from the 2 per cent desiccated liver which is included in the formula of this diet. Since the liver is known to contain ferritin, some of which may be in the bioactive form, this component may account for the slight stimulatory effect of this diet extract on villus motility. This speculation is indirectly supported by the observation that no villus stimulatory effect could be demonstrated in the chromatographic effluent corresponding to the alpha pigment fraction (or in any other fractions) prepared from the liquid diet of Greenstein *et al.*[7] The fact that the

alpha pigment fraction of L-462 originated from a steam-sterilized diet implies that its bioactivity must have resisted the prolonged exposure to heat. This question was not further explored.

The results suggest that alpha pigment may be absorbed from the intestine and that, from the large cecal pool of germ-free animals, sizable quantities of active alpha pigment may reach the circulation. This is supported by the observation of Reddy *et al.*,[15] who found substantially elevated levels of ferritin iron in the liver of germ-free rats. Local effects of active alpha pigment are indicated by slower blood flow and hyporesponsiveness to topically applied epinephrine in some elements of the vascular bed in the mesocecum of germ-free rats (Baez and Gordon[27]). General manifestations that may result from excessive amounts of epinephrine inhibitory substances in the circulation of germ-free animals are: substantially lower metabolic rate[28] and cardiac output,[29] reduced regional blood flow in the gastrointestinal tract and in the liver.[30] The assumption that these anomalies are caused by substances absorbed from the large cecal pool is supported by the observation that surgical removal of the cecum performed at a young age will restore the metabolic rate[31] and cardiac output[30, 31] to normal values in adult germ-free rats.

There is no reason to assume that epinephrine inhibitory substances like alpha pigment are implicated in the reduced muscle tone and in the distention of the cecum observed in germ-free rodents.[32] The excessive amounts of fecal kallikrein or the hypotensive kinins released by this enzyme,[2, 33] as well as the epinephrine-sensitizing agents indicated in the initial pool pigment fraction of cecal contents, mentioned in the introduction of this paper, may be involved in this phenomenon.

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