

Absorption of Hemoglobin Iron: The Role of Xanthine Oxidase in the Intestinal Heme-Splitting Reaction

By R. BEN DAWSON, SHEILA RAFAL AND LEWIS R. WEINTRAUB

HEME, A CONSTITUENT OF HEMOGLOBIN AND MYOGLOBIN is a major source of dietary iron in this country. Recent investigations have shown that the iron is not released from heme in the intestinal lumen, but the heme complex is taken up intact by the intestinal epithelial cell.¹⁻⁴ Subsequently in the plasma, the iron appears bound to transferrin. Weintraub and co-workers have demonstrated a substance in intestinal mucosal homogenates of dogs which is capable of releasing iron from a hemoglobin substrate in vitro.⁴ The kinetics of the reaction suggest that it is mediated by an enzyme.

The chemical action of hydrogen peroxide is capable of oxidizing the alpha-methene bridge of the heme ring with the subsequent release of iron.⁵⁻⁷ Mills demonstrated catabolism of hemoglobin by liver homogenates and suggested that this was secondary to the hydrogen peroxide produced by the action of xanthine oxidase on its substrates.⁸ To determine if a similar enzyme system generating H₂O₂ is present in the intestinal mucosa and is responsible for the heme-splitting reaction, the following studies were performed.

METHODS

Preparation of Mucosal Cell Homogenate

The small intestine was removed from fasting mongrel dogs under Nembutal anesthesia. The specimen was opened lengthwise and thoroughly washed with saline at 4°C. The intestine was scraped with a glass slide to remove the mucosa. The mucosa was suspended in 9 volumes of 0.1M tris-HCl buffer, pH 8.3, and homogenized at 4°C in glass containers with a motor driven Teflon pestle and then a Virtis 45 apparatus. The homogenate was centrifuged at 10,000 G or 50,000 G for 10 min. at 4°C, and then supernatants were used as stated in the text.

The following procedure was carried out to concentrate the mucosal homogenate for use in the spectrophotometric analyses. The 50,000 G supernatant was heated in a 60°C

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water bath for 10 min. and then centrifuged at 10,000 G for 10 min. to remove the precipitated proteins. The supernatant was subjected to ammonium sulfate precipitation. Protein with heme-splitting activity was precipitated in the fraction between 0.35 and 0.60 saturation. This resulted in a four-fold increase in heme-splitting activity per mg. of protein when compared to the original 50,000 G supernatant. Protein was determined by the Biuret method.⁹ Human serum albumin was used as a standard.

In Vitro Determination of "Heme-Splitting Activity"^{*}

Hemoglobin labeled with ⁵⁹Fe was prepared in the following manner. Red cells containing ⁵⁹Fe hemoglobin were obtained by cardiac puncture from rabbits at least seven days after the intravenous administration of ⁵⁹Fe citrate. The cells were washed three times in saline and then lysed in distilled water. The stroma was removed by centrifugation at 15,000 μ g. to yield a solution of hemoglobin labeled with ⁵⁹Fe.

The ⁵⁹Fe-hemoglobin substrate, 2 mg. in 2 ml. of normal human serum was incubated with 5 ml. of a preparation of mucosal homogenate in 25 ml. flasks for one hour at 37° or 47°C in a Dubnoff Metabolic Shaker. All reactions were performed in duplicate. At the end of the incubation period, 1.5 Gm. of nonradioactive hemoglobin was added as a carrier to the contents of each flask. Hemin chloride was then crystallized.¹⁰ Heme-splitting activity was determined by comparing the ⁵⁹Fe specific activity of the hemin chloride with that of a control mixture of substrate without homogenate, treated in a similar manner. The mean value of the duplicate determinations is reported in the text followed by the individual values in parenthesis. The details of this procedure have been published.⁴

Spectrophotometric Determination of Xanthine Oxidase Activity

The xanthine oxidase activity of mucosal homogenate preparations was determined by the production of uric acid from xanthine. Each sample contained 0.2 ml. of the homogenate preparation and 4.8 ml. of a 5×10^{-5} M solution of xanthine in 0.1 M tris-HCl buffer, pH 8.3. Samples were placed in 1-cm. quartz cuvettes and the O.D. at 292 $m\mu$. was read in a Beckman DB-G spectrophotometer equipped with a U.V. light source. Uric acid production was determined by the change in O.D. at 292 $m\mu$. Determinations were done at 32° or 37°C.

Spectrophotometric Identification of Pigment Formed in Heme-Splitting Reaction

0.2 ml. of the mucosal homogenate preparation was added to 0.25 mg. of oxyhemoglobin. This was brought to a final volume of 4 ml. with 0.1 M tris-HCl buffer, pH 8.3, which had a final concentration of xanthine of 5×10^{-5} M. An aliquot was then placed in a 1-cm. quartz cuvette at 32°. The entire visible range was observed using a Beckman DB-G scanning spectrophotometer connected to a 10-in. linear recorder. This was repeated one hour later.

Molecular-Weight Estimation of Intestinal "Heme-Splitting" and Xanthine Oxidase Activities

Sephadex G-200 (Lot number 5563 from Pharmacia Fine Chemicals, Piscataway, N. J.) was used for molecular-weight estimation as described by Andrews.^{11,12} The Sephadex column was prepared with a 0.1 M tris-HCl buffer, pH 8.3. The void volume of the column was determined with Blue Dextran (Lot no. 7759)^{*} before or after the elution of each protein. The proteins used as molecular weight marks were: chymotrypsinogen "A" (no. 5575)[†], cytochrome "C" (no. 5071)[†], xanthine oxidase (no. 174)[†], horse spleen ferritin (no. 57831)[‡], and human albumin[§]. Elution of protein was followed with

^{*}Heme-splitting refers to the release of iron from the heme ring as measured by the disappearance of ⁵⁹Fe from hemin chloride crystallized from the original substrate.

Fig. 1.—Effect of allopurinol on “heme-splitting” reaction of intestinal mucosal homogenates. Bar represents mean of duplicate determinations. Results of each individual determination represented by short horizontal line.

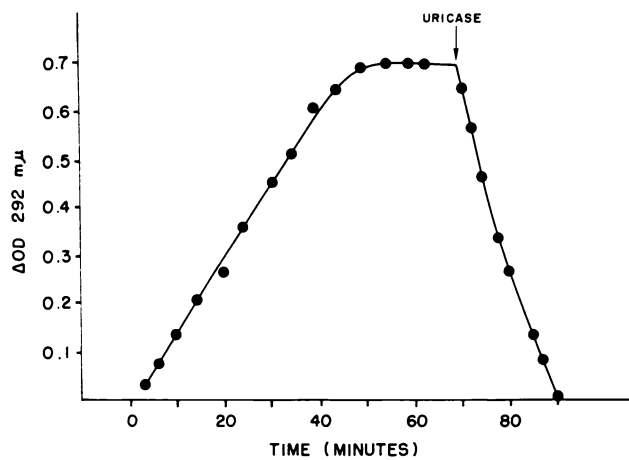
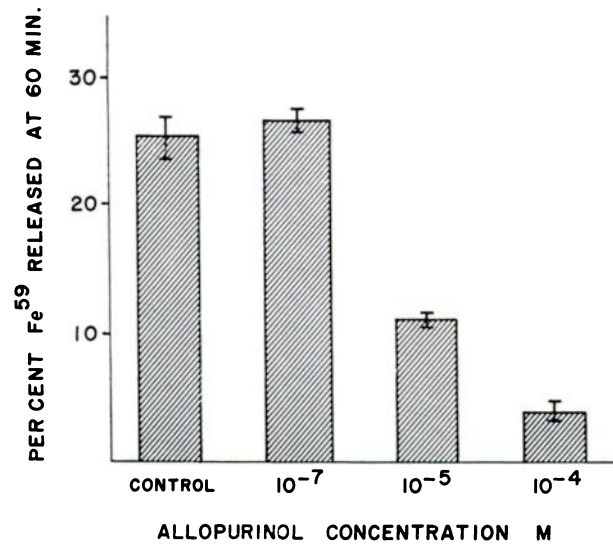


Fig. 2.—Xanthine oxidase activity of intestinal mucosal homogenates measured by uric acid production from xanthine.

a GME-280 monitor interposed between the column and the automatic fraction collector. The procedure was carried out at 4°C. The log of the molecular weight was plotted versus the V_e/V_0 (V_e = elution volume of protein, V_0 = void volume of column) to allow for variations in packing of the gel which occurred during use. A standard curve was established from the V_e/V_0 ratio of the reference proteins of known molecular weight. An aliquot of the 50,000 G supernatant of the homogenate of the intestinal mucosa was applied to the column. Xanthine oxidase and “heme-splitting” activity were measured as previously described, in the elution fractions.

In Vivo Absorption Studies

Mongrel dogs weighing 14–18 kg. were the subjects in this study. ⁵⁹Fe hemoglobin (0.5 μCi/0.5 mg. of Fe) was mixed with the animal’s daily portion of meat (total iron intake, 6–7 mg./day) during a three day period. All stools were collected and the radioactivity present was counted by placing the specimens in a small animal, whole body, liquid scintillation detector (Armac, Packard Instrument Co., Inc., Downers Grove,

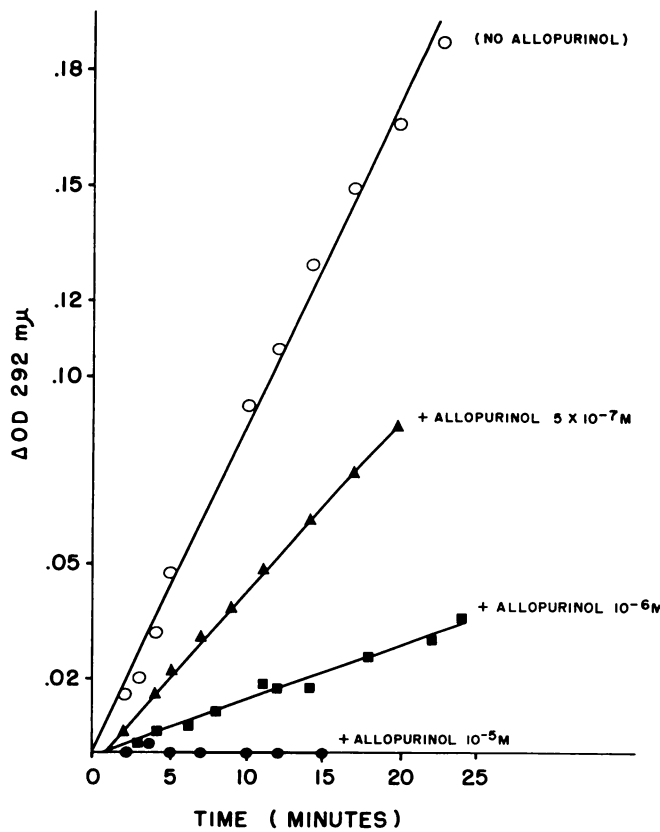


Fig. 3.—Inhibition of xanthine oxidase activity of intestinal mucosal homogenates by allopurinol.

Ill.). The collections were continued 10–14 days until there no longer was any significant radioactivity. Absorption was calculated as the difference between the total radioactivity administered and that recovered from the stools.

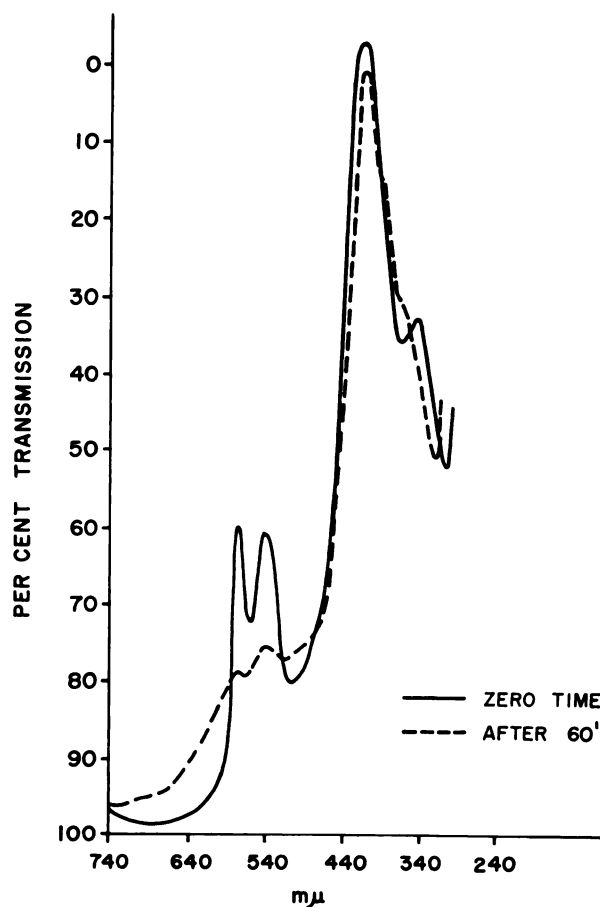
RESULTS

The Effect of Catalase on "Heme-Splitting Activity" of the Mucosal Cell Homogenate

Catalase was added to the 2 mg. hemoglobin substrate incubated with 5 ml. of the 50,000 G supernatant at 47°C. A final catalase concentrate of 3×10^{-7} M reduced the heme-splitting activity to 16.7 (15–17.5) per cent from a control value of 30.1 (29.6–30.7) per cent. When the catalase was increased to 3×10^{-6} M, heme-splitting was decreased to 4.8 (4.5–5.0) per cent. Sodium azide (NaN_3) 10^{-3} M, an inhibitor of catalase, was added to the reaction. This resulted in an increase in heme-splitting to 63 (60–66) per cent compared to a control value of 24 (23–25) per cent.

One tenth of an ml. of a 1 per cent solution of H_2O_2 added to the hemoglobin substrate and buffer alone resulted in 11 per cent of the ^{59}Fe released from the hemoglobin. This was increased to 26 per cent by the addition of 0.1 ml. of a 30 per cent H_2O_2 solution.

Fig. 4.—Change in spectrum of oxyhemoglobin after addition of intestinal mucosal homogenate and xanthine.



The Effect of Allopurinol on "Heme-Splitting" Activity

Allopurinol was added in varying concentrations to a 10,000 G mucosal supernatant and hemoglobin substrate at 37° (Fig. 1). A final concentration of allopurinol of 10^{-4} M reduced the amount of ^{59}Fe split from the substrate to four per cent compared to a control value of 25 per cent.

Xanthine Oxidase Activity of Mucosal Homogenate

An increase in O.D. at 292 $m\mu$, occurred upon adding xanthine, 5×10^{-5} M, to the mucosal homogenate (Fig. 2). The subsequent addition of uricase resulted in a fall in the O.D. The production of uric acid by the intestinal homogenate was inhibited by allopurinol (Fig. 3).

Identification of Pigment Formed in the "Heme-Splitting" Reaction

The addition of the concentrated 50,000 G supernatant of the mucosal homogenate to a mixture of oxyhemoglobin and xanthine resulted in a significant reduction in the oxyhemoglobin peaks at 578 and 542 $m\mu$. after one hour (Fig. 4). In addition, there was an increase in O.D. at 630–50 and 375–80 $m\mu$.

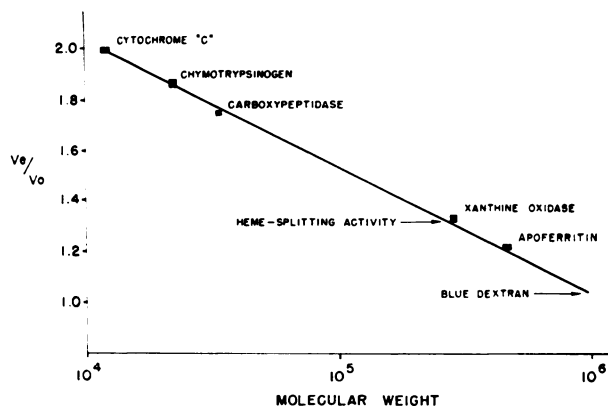


Fig. 5.—Molecular weight determination on Sephadex G-200 column.

This may represent the formation of choleglobin and biliverdin with absorption peaks at 630–50 and 375–80 $m\mu$., respectively.

Molecular-Weight Estimation of Intestinal “Heme-Splitting” and Xanthine Oxidase Activities

The elution volumes of “heme-splitting” and xanthine oxidase activities of the mucosal homogenate were found to be similar. The V_e/V_o of the protein fraction with heme-splitting and xanthine oxidase activities from the intestinal homogenate was plotted on the graph constructed from applying proteins of known molecular weight to the column (Fig. 5). The molecular weight of the protein is 280–290,000. This is similar to the known molecular weight of xanthine oxidase and was also in the range obtained when several commercial samples of xanthine oxidase were applied to the column as markers.

Effect of Allopurinol on the In Vivo Absorption of Hemoglobin Iron

Absorption of hemoglobin labelled with ^{59}Fe was measured in three dogs. They were then fed 600 mg. of allopurinol per day for 10 days and the absorption study repeated. The dose of allopurinol was continued through the period of the absorption study. Following this, the dose of allopurinol was increased to 1200 mg. per day for 10 days and a final absorption study was performed. There was no significant reduction in the absorption of hemoglobin iron with either dose of allopurinol (Table 1).

In vitro xanthine oxidase activity was determined at 37°C in intestinal mucosal homogenates obtained from two dogs fed 1200 mg. of allopurinol per day and two control animals. The mean Δ O.D. 292 $m\mu$./min. with the homogenates from the allopurinol-fed dogs was 86.7 per cent of the control value.

Table 1.—Effect of Allopurinol on Absorption of Hemoglobin ^{59}Fe in the Dog

	Normal	Allopurinol	
		60 mg./day	1200 mg./day
Dog 1	19.4 Per Cent	13 Per Cent	10 Per Cent
Dog 2	20.6 Per Cent	24.8 Per Cent	31 Per Cent
Dog 3	29.4 Per Cent	35 Per Cent	40 Per Cent
Mean	23.1 Per Cent	24.3 Per Cent	27 Per Cent

Table 2.—Effect of In Vivo Administration of Allopurinol on In Vitro “Heme-Splitting” Activity of Intestinal Mucosal Homogenate*

	Normal		Allopurinol 1200 mg./day	
	Dog 1	Dog 2	Dog 3	Dog 4
Per cent ⁵⁹ Fe released	21.9 Per Cent	13.6 Per Cent	21.6 Per Cent	18.6 Per Cent
Per cent ⁵⁹ Fe released with addition of catalase 10 ⁻⁷ M	17.4 Per Cent	10.5 Per Cent	15.0 Per Cent	12.0 Per Cent
Per cent reduction of initial value by catalase	20.5 Per Cent	22.8 Per Cent	30.6 Per Cent	35.5 Per Cent

* 5 ml. of 50,000 G homogenate incubated with 2 mg. hemoglobin substrate for one hour at 37°C.

There was no significant difference in the *in vitro* “heme-splitting” activity between the allopurinol-fed and the control dogs (Table 2). However, the addition of catalase in a final concentration of 10⁻⁷ M to the reaction produced a 1/3 reduction of the initial heme-splitting activity from the allopurinol-fed dogs, compared to only a 1/5 reduction in the controls.

DISCUSSION

The role of H₂O₂ in the heme-splitting reaction of the intestinal homogenate was demonstrated by the significant reduction in “heme-splitting” activity with the addition of catalase. Furthermore, heme-splitting was increased by sodium azide. Since catalase destroys peroxides, its inhibition by sodium azide allows peroxide to persist and split heme.

Xanthine oxidase is present in significant quantities in the small intestine as well as in the liver.¹³ That this enzyme is responsible for the generation of H₂O₂ in the intestinal “heme-splitting” reaction is supported by the inhibition of “heme-splitting” activity after addition of allopurinol, a xanthine oxidase inhibitor. The addition of a final concentration of 10⁻⁵ M allopurinol completely inhibited the xanthine oxidase activity *in vitro* of the intestinal homogenate as measured by the production of uric acid. By contrast, the same final concentration of allopurinol only reduced the “heme-splitting activity” to 40 per cent of the control value. This difference may be explained by the presence of a significant concentration of peroxides in the tissue homogenate generated prior to the addition of the allopurinol. Separation of the intestinal homogenate proteins by Sephadex Column chromatography revealed the “heme-splitting” activity and xanthine oxidase activity to be in the same fraction.

The early work of Lemberg and co-workers⁵⁻⁷ suggested that H₂O₂ oxidizes the alpha-methene bridge of the heme ring of hemoglobin to form the green pigment, choleglobin. Subsequently, the iron is released, the globin denatured, and the tetrapyrrole chain transforms to biliverdin. Spectrophotometric changes indicated the production of choleglobin and biliverdin when preparations of the intestinal mucosal homogenate were incubated with a hemoglobin substrate.

Hemoglobin, because of its solubility, was used in all our experiments as the substrate to demonstrate the ability of intestinal homogenate to release iron

from the heme ring. During actual digestion and absorption this is not the form in which heme exists. Conrad et al. demonstrated the ability of luminal contents of the small intestine to split the globin from heme.¹⁶ The heme is then taken into the epithelial cell. Preliminary work in our laboratory suggests that the intracellular heme becomes bound to protein and the total complex has a molecular weight in the range of 100–200,000. This compound most likely is the substrate for heme-splitting reaction within the intestinal epithelial cell.

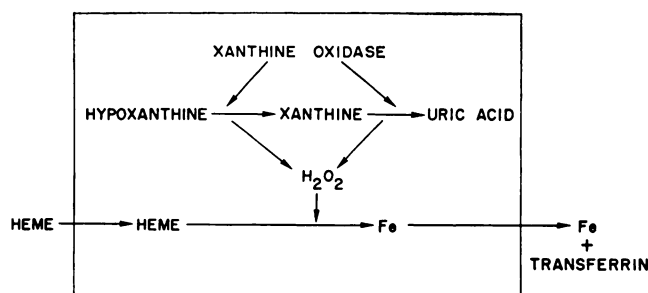
Keilin and Hartree found that the H₂O₂ produced by the glucose dehydrogenase enzyme of *penicillium notatum*, was capable of forming choleglobin from hemoglobin.¹⁴ Later, Mills suggested that the production of H₂O₂ by xanthine oxidase in liver preparations was capable of catabolizing hemoglobin.⁸ A similar system appears to be present in the small intestine. It is likely that the oxidation of hypoxanthine and xanthine by xanthine oxidase results in the formation of tissue peroxides, and not free H₂O₂.

Feeding allopurinol does not inhibit the intestinal absorption of hemoglobin iron or “heme-splitting” activity in vitro in normal dogs. Awai and Brown were also unable to demonstrate a decreased absorption of hemoglobin iron in normal rats following parenteral allopurinol.¹⁵ However, iron deficient rats given allopurinol did have a decreased absorption.

These findings do not negate the physiologic role of xanthine oxidase in the absorption of hemoglobin iron. In our studies there was only a 13 per cent reduction in xanthine oxidase activity in the intestinal mucosa of allopurinol-fed dogs. It appears that the enzymatic reaction of xanthine oxidase produces a greater amount of tissue peroxides in the mucosa than is needed for the “heme-splitting” reaction. Feeding the dogs allopurinol does reduce peroxide production in the intestinal epithelial cell but not enough to alter the “heme-splitting” reaction. Supporting these hypotheses is the observation that the in vitro addition of 10⁻⁷ M catalase causes a greater inhibition of “heme-splitting” activity by mucosal homogenates from allopurinol-fed dogs than from normal dogs.

The gastrointestinal absorption of dietary hemoglobin iron can be summarized in the following sequence (Fig. 6). The heme ring is separated from the globin within the lumen of the small intestine and the heme is taken up intact in the mucosal cell.^{4,16} Within the cell, the heme ring is split by peroxides generated by the action of xanthine oxidase on its substrates. The iron is

Fig. 6.—Role of xanthine oxidase in intestinal “heme-splitting” reaction.



subsequently released. That iron which is needed by the body is transferred to the plasma and bound to transferrin. The remaining iron is incorporated into ferritin and lost from the body when the intestinal epithelial cell is sloughed from the tip of the villus.¹⁷

SUMMARY

Heme from ingested hemoglobin—⁵⁹Fe is taken into the epithelial cell of the small intestinal mucosa of the dog and the ⁵⁹Fe subsequently appears in the plasma bound to transferrin. A substance was demonstrated in homogenates of the mucosa which releases iron from a hemoglobin substrate in vitro. Thus: (1) The addition of catalase to the mucosal homogenate reduces the "heme-splitting" reaction. In contrast, sodium azide, a catalase inhibitor, potentiates the reaction. This suggests that a peroxide generating system participates in the "heme-splitting" reaction. (2) Xanthine oxidase, an enzyme present in the intestinal epithelial cell, produces H₂O₂ by oxidation of its substrate. The addition of allopurinol, a xanthine oxidase inhibitor, to the intestinal mucosal homogenate diminishes the "heme-splitting" reaction. (3) Fractionation of the 50,000 Gm. supernatant of the mucosal homogenate on a G-200 Sephadex column shows the "heme-splitting" activity to have the same elution volume as xanthine oxidase, indicating a similar molecular weight. (4) The addition of a mucosal homogenate to a xanthine substrate results in the production of uric acid. These data suggest that xanthine oxidase in the intestinal epithelial cell is important in the release of iron from absorbed heme. The enzyme mediates the "heme-splitting" reaction by the generation of peroxides which, in turn, oxidize the alpha-methene bridge of the heme ring releasing iron and forming biliverdin.

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