ISOLATION AND IDENTIFICATION OF THE XANTHINE OXIDASE FACTOR AS MOLYBDENUM*

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The establishment and maintenance of normal levels of xanthine oxidase (XO) in the liver and intestine of weanling rats were previously shown (1–3) to be dependent upon an adequate diet. With a purified synthetic ration containing adequate riboflavin, two dietary factors were shown to be essential for this response: (1) protein, and (2) a hitherto unidentified substance referred to as the "liver residue factor" or "xanthine oxidase factor" (XOF). The XOF could not be identified with any established vitamin or any other factor known to be required in animal nutrition. Both the protein and XOF had to be present in the diet simultaneously. Protein was the dominant nutritional factor as far as liver XO was concerned; in the absence of an adequate protein intake, nearly all of the XO was lost from the liver, irrespective of the presence or absence of XOF from the diet. With adequate protein in the diet, a deficiency of XOF resulted in a loss of about 25 per cent of the normal amount of liver XO. Protein did not dominate the intestinal response, and a deficiency of either factor alone caused a loss of most of this enzyme from the intestine. By maintaining an adequate protein intake, the amount of xanthine oxidase found in the intestine was utilized as a bioassay for XOF in the diet (4).

The active material present in soy flour was isolated (5) by following its purification with bioassays for XOF. Although the purification procedure was not specifically designed to yield inorganic material, nevertheless the active products obtained were molybdate salts, and all of the biological activity could be accounted for by the molybdenum present. De Renzo et al. (6) have also found molybdenum to be active in the bioassay for the xanthine oxidase factor.

EXPERIMENTAL

Various fractions obtained during the purification procedure were assayed by adding them to a purified 24 per cent Labco casein diet (5). This diet contained 4 per cent Phillips and Hart salt mixture (7) which had been modified by increasing the manganese concentration 5-fold. Wean-

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ling male rats were fed the control diet alone for 6 to 10 days; the test diet was then fed for 7 days before the xanthine oxidase activity of the intestine was determined (3). Activity was related to a standard response curve obtained with varying amounts of liver residue in the diet (4).

Molybdenum was determined colorimetrically as the ether-soluble thio-
cyanate according to the procedure described by Marmoy (8), except that the color was allowed to develop for 5 minutes and its intensity was measured with a Coleman spectrophotometer at 470 m\(\mu\) (9).

Isolation of XOF

Soy flour\(^1\) was hydrolyzed in 750 gm. batches by refluxing it for 2 hours with 10 volumes of 1 \(N\) HCl. About one-third of the flour remained undissolved, and was filtered off, washed with 1.8 liters of water, and discarded. This insoluble residue was as active as the original soy flour; this and other evidence indicated a relatively firm binding of molybdenum with some component of the flour. The filtrate contained as much activity as was present in the original starting material. The increase in total biological activity upon hydrolysis was due to the unavailability of some of the molybdenum in the soy flour for the assay response.

The filtrates from 4.5 kilos of soy flour were combined and stirred with 1.4 kilos of Nuchar\(^2\) for 1 hour. The charcoal was then filtered off, washed with 6 liters of water, and resuspended in 7 liters of 1 \(N\) \(NH_4\)OH. After stirring for 1 hour and filtering and washing the charcoal with 6 liters of 0.5 \(N\) \(NH_4\)OH, the elution process was repeated once more with 0.5 \(N\) \(NH_4\)OH. The combined eluates (containing 36 gm. of solids per kilo of original soy flour) from 9 kilos of soy flour were acidified to Congo red with concentrated HCl and stirred for 1 hour with 600 gm. of Nuchar. The charcoal was filtered, washed with 6 liters of water, and eluted with 3 liters of 1 \(N\) \(NH_4\)OH. After filtering and washing with 1.8 liters of 0.5 \(N\) \(NH_4\)OH, the elution was repeated with 0.5 \(N\) \(NH_4\)OH. The combined eluates (5.3 gm. per kilo of soy flour) from 18 kilos of soy flour were acidified, stirred with 270 gm. of Nuchar, and filtered. The charcoal was washed with 2.5 liters of water, eluted twice with 1.3 liters of 1 \(N\) and 0.5 \(N\) \(NH_4\)OH, and washed after each elution with 0.8 liter of the same strength \(NH_4\)OH. This eluate contained 1.7 gm. of solids per kilo of original soy flour and contained from 40 to 50 per cent of the original activity. Most of the lost activity was present in the filtrate from the initial char-

\(^1\) Extracted soy flour from the A. E. Staley Manufacturing Company, Decatur, Illinois. 2.6 \(\gamma\) of Mo per gm. In the assay procedure soy flour was about 25 per cent more active than liver residue.

\(^2\) Nuchar C, West Virginia Pulp and Paper Company, Industrial Chemical Sales Division, New York.
coal adsorption, and the subsequent charcoal adsorption-elutions were made without significant losses.

Most of the ammonia was removed from the above eluate by aeration, and the solution was then acidified to pH 1 with concentrated HCl. A small amount of insoluble precipitate was removed by filtration. The clear filtrate from 58 kilos of soy flour was poured through a column containing 150 gm. of alumina, and the column was then washed with distilled water until the effluent was colorless. The active material was eluted from the column with 1 N NH₄OH. The first colorless and then yellow effluents were discarded, and collection of the active eluate was begun with the appearance of a dark brown pigment. 800 ml. of eluate were collected and concentrated in vacuo to 150 to 200 ml. This solution (0.12 gm. of solids per kilo of soy flour) was acidified to pH 1 with concentrated HCl and filtered. The filtrate was adsorbed a second time on 60 gm. of alumina in a 17 mm. column, and the activity was again eluted with 400 ml. of 1 N NH₄OH. This fraction contained 0.04 gm. of solids per kilo of soy flour, and both alumina purification steps were carried out without any significant loss of activity.

Excess ammonia was removed from the above solution by distillation, and the solution was then poured through a 16 mm. column containing 35 gm. of Amberlite IRA-410 in the hydroxyl form. All of the activity was retained on the column. Some impurities were washed from the column with 2 liters of 0.2 N HCl. The active material was removed from the resin by eluting alternately with 400 ml. of 1 N NaOH and 200 ml. of 1 N HCl; the alkaline and acid solutions were used four and three times respectively. The eluates were combined, acidified to Congo red, and the active material was recovered from the solution by stirring with 50 gm. of Nuchar. The charcoal was filtered, washed with water, and eluted three times with 250 ml. portions of 1 N NH₄OH. The combined eluates were concentrated to 10 ml. by distillation in vacuo, and then centrifuged to remove an inactive precipitate. All of the activity was precipitated from the supernatant fluid by the addition of 5 ml. of 5 per cent barium chloride and 80 ml. of 95 per cent ethanol. The precipitate was collected by centrifugation and dried in a desiccator. The yield from 58 kilos of soy

³ Alumina, activated, catalyst grade, Harshaw Chemical Company, Cleveland, Ohio. The alumina was pretreated by suspending it in water, and adding as much HCl as required to maintain a pH of 4 for several days. The alumina was filtered, washed, and oven-dried. The column was prepared by suspending the alumina in 0.2 N HCl and pouring the slurry into a 25 mm. tube.

⁴ The Amberlite resin was obtained from Rohm and Haas, Philadelphia 5, Pennsylvania. It was converted to the hydroxyl form by treatment with 4 per cent sodium hydroxide, and the column was then washed with distilled water until the washings were neutral.
flour was approximately 400 mg. (6.8 mg. per kilo of soy flour), and the over-all recovery of original biological activity as well as of molybdenum was 25 to 30 per cent.

1.1 gm. of the barium salt were leached one time with 50 ml. and three times more with 25 ml. portions of hot water. On cooling the combined supernatant solutions, a precipitate formed and was combined with the hot water-insoluble fraction to give 487 mg. of material containing 12.5 per cent Mo (Fraction 832). The supernatant fluid was evaporated in vacuo to 15 ml. and cooled, and 226 mg. of precipitate containing 15.5 per cent Mo were deposited (Fraction 833). The solids in the filtrate (Fraction 834) contained only 5.7 per cent Mo. Since the middle fraction was most active biologically, it was refractionated. 209 mg. of the precipitate were leached three times with 25 ml. portions of hot water to give 43 mg. of material which did not dissolve (Fraction 833B) and 66 mg. of material which precipitated in seemingly crystalline form when the solution was concentrated (Fraction 833C). The 43 mg. of insoluble material were essentially inorganic, since it lost only 7.9 per cent of its weight when ashed at 460° for 20 hours; it contained 44.4 per cent Ba and 24.2 per cent Mo. Barium and molybdenum were the only two elements that could be detected spectrographically in concentrations greater than 0.1 per cent. The 66 mg. of soluble salt contained 17.3 per cent Mo and some unidentified organic material since it lost 30.6 per cent of its weight on ashing.

Final isolation of the molybdenum in pure crystalline form was effected as the benzoinoxime derivative. 425 mg. of Fraction 832 (containing 53 mg. of Mo) were dissolved in 100 ml. of hot 5 per cent HCl, 20 ml. of 5 per cent H₂SO₄ were added, and the BaSO₄ was removed by filtration. The filtrate was cooled to 5° and treated with 13.5 ml. of 2 per cent benzoinoxime dissolved in 95 per cent ethanol. After 15 minutes, the precipitate was filtered, washed with cold water, and dried in a desiccator. About 25 mg. of the dried material were redissolved in 2 ml. of 1 N NaOH, and diluted with water and concentrated HCl to give 50 ml. of 5 per cent HCl. The reprecipitated complex was filtered, washed with cold water, and dried in a desiccator for analysis. A solution of sodium molybdate was treated identically for comparative purposes.

\[ (C_4H_4N_2O_2)MoO_4. \] Calculated. Mo 15.57, N 4.54

Found for isolated material. " 15.54, " 4.51

" " Na₂MoO₄ derivative. " 15.74, " 4.33

Assay Response to Sodium Molybdate

Fig. 1 shows the intestinal xanthine oxidase response to increasing amounts of molybdenum in the diet. Less than 0.1 mg. of Mo per

We are indebted to Dr. George Oplinger, Solvay Process Company, Syracuse, New York, for this analysis.
kilo of diet was required to produce "saturation" levels of this enzyme in the intestine. Although intestinal values above 30 (c.mm. of O₂ per 20 minutes per flask) increased in accordance with the molybdenum content of the diet in Fig. 1, the results in this range were sometimes erratic, and assays giving such high values were repeated at lower molybdenum concentrations. When tested in the range of 20 to 60 γ of Mo per kilo of diet, this assay proved to be a surprisingly accurate measure of inorganic molyb-

![Graph showing bioassay response of intestinal xanthine oxidase to increasing amounts of molybdenum in the diet.](image)

**Fig. 1.** The bioassay response of intestinal xanthine oxidase to increasing amounts of molybdenum (as sodium molybdate) in the diet. Each point represents the average of eight rats. Intestinal X0 values above thirty are sometimes erratic and not strictly proportional to the molybdenum content of the diet, and are therefore considered "saturation" levels.

Phosphomolybdic acid or MoO₃, dissolved in dilute HCl, had the same activity as sodium molybdate when assayed at a level of 50 γ of Mo per kilo of diet. Sodium vanadate and vanadium sulfate were inactive at 425 γ of vanadium per kilo of diet.

**Identification of XOF with Mo**

With molybdenum identified as a major constituent of the active material isolated from soy flour, and the XOF activity of sodium molybdate demonstrated, it remained to be determined whether all of the biological activity of the isolated products could be accounted for on the basis of
their molybdenum contents. Such was found to be the case by (1) correlating biological activity with molybdenum content, (2) ashing the isolated fractions without loss of biological activity, and (3) removing the biological activity along with the molybdenum when the latter was extracted as an ether-soluble thiocyanate or was precipitated with benzo-inoxime.

The assays of the isolated barium salts were used to estimate their Mo contents by reference to Fig. 1, and such bioassay values for Mo were compared with the chemical determinations. The barium salts were dissolved in warm 1 N HCl and the barium precipitated with H2SO4 before incorporation in the diet for bioassay or before the chemical determination of molybdenum. The results are shown in Table I and leave no doubt that the biological activity of these fractions was due entirely to their molybdenum content. A dozen other fractions were compared in the same manner and in no case was the discrepancy between the chemical determination and bioassay value greater than 25 per cent; the average of all values agreed perfectly. Ashing Fractions 833B and 833C did not cause any loss of molybdenum or any decrease in biological activity (Table I). This is further evidence that the organic component of the latter fraction did not influence its biological activity.

**Table I**

**Correlation of Biological Activity and Molybdenum Content of Various Fractions Obtained from Soy Flour**

<table>
<thead>
<tr>
<th>Fraction No. (Ba salt)</th>
<th>Weight of fraction added to diet</th>
<th>Mo content of fraction</th>
<th>Mo content of diet, γ Mo per kg. diet</th>
<th>Chemical analysis</th>
<th>Bioassay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>γ per kg.</td>
<td>per cent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>832</td>
<td>547</td>
<td>12.5</td>
<td>68</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>834</td>
<td>560</td>
<td>5.7</td>
<td>32</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>833B</td>
<td>116</td>
<td>24.2</td>
<td>28</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>193</td>
<td>47</td>
<td></td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>833C</td>
<td>232</td>
<td>17.3</td>
<td>40</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>833C (ash)</td>
<td>286</td>
<td>24.1</td>
<td>50</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>204</td>
<td>49</td>
<td></td>
<td>48</td>
<td></td>
</tr>
</tbody>
</table>

**Extraction As Thiocyanate**—0.747 mg. of Fraction 833C (containing 120 γ of Mo) was divided into three separatory funnels, and the molybdenum was converted to the thiocyanate by treating it in acid solution with KCNS and SnCl2, as described in the procedure for its determination. The colored molybdenum thiocyanate was extracted with ether, and the combined ether-soluble residue was neutralized with NaOH before being incorpo-
rated in the diet for bioassay. Essentially all of the activity was found in the ether-soluble fraction; less than 7 per cent was recovered from the aqueous solution by adsorbing the activity onto charcoal and eluting with NH₄OH. In a separate experiment with the untreated fraction it was demonstrated that the charcoal adsorption procedure would have recovered small amounts of biological activity from the aqueous layer if it had been there.

The thiocyanate experiment was repeated with an aliquot of Fraction 833C containing 30 γ of Mo and with 30 γ of Mo as sodium molybdate. The ether-soluble material was assayed at a level of 50 γ of Mo per kilo of diet. The assay results indicated values of 44 γ of Mo per kilo of diet for both substances, and the same assay results were obtained when the molybdenum thiocyanate was added to the diet without prior neutralization.

**Precipitation with Benzoinoxime**—An aliquot of Fraction 833C containing 100 γ of Mo and a corresponding aliquot of sodium molybdate were each dissolved in 50 ml. of 5 per cent HCl and cooled to 5°. 2 ml. of 2 per cent alcoholic benzoinoxime and 2 drops of bromine water were added (10). In one experiment the precipitate was filtered in the cold after standing for 15 minutes in order to obtain a quantitative precipitation of the molybdenum. In a second experiment the precipitate was filtered at room temperature after standing 1 hour, and the precipitate was washed with 5 per cent HCl. These latter conditions are known (11) to give incomplete precipitation of the molybdenum. The precipitates were dissolved in 1 ml. of cold concentrated H₂SO₄ and the funnels were rinsed with concentrated HNO₃ and hot water. The samples were digested over a flame with the aid of a few drops of 30 per cent H₂O₂ to obtain final clearing. Aliquots of the digest were removed for colorimetric determination of molybdenum and for bioassay. One-fifth of the filtrate was similarly digested and an-
analyzed for molybdenum, while the remaining four-fifths was treated with charcoal to adsorb any remaining activity; the charcoal was eluted with NH₄OH, and the eluate was added to the diet. The results in Table II show that, when the molybdenum was precipitated quantitatively, biological activity was found only in the precipitate; when some of the molybdenum escaped into the filtrate, a corresponding amount of biological activity was also present in the filtrate.

**DISCUSSION**

Molybdenum is known to be required by some plants and microorganisms, but this is the first demonstration of a rôle for molybdenum in animal nutrition and is the first association of molybdenum with an enzyme. It appears to be a part of the enzyme molecule since purified preparations of milk xanthine oxidase contain XOF (4) or molybdenum. Xanthine oxidase, prepared from cream by Ball's procedure (12) and dialyzed, had a molybdenum content of 0.03 per cent when the Q₉₀ was 900 at 37°. It can be calculated (13) that this degree of purity is equivalent to a Q₁₂ of 360 at 20°, and therefore compares favorably with the purification previously achieved. This molybdenum concentration is relatively low when compared with the iron or copper content of analogous substances. While it is possible that molybdenum is present in the enzyme preparation as an impurity, it is significant that it is involved in such diverse situations as the intestinal xanthine oxidase response and the purified milk enzyme.

It was impossible to restore the xanthine oxidase activity of a depleted intestinal homogenate by the addition of sodium molybdate *in vitro*. The addition of 0.1 to 100 γ of Mo as sodium molybdate to the Warburg flask in the presence or absence of methylene blue had no stimulatory or inhibitory effect on the xanthine oxidase activity of intestinal homogenates with low or high initial activities. This cannot be accepted as indicating the absence of the apoenzyme from molybdenum-depleted intestines, since it is not yet known that sodium molybdate is capable of restoring the activity of the apoenzyme. On the other hand, a disappearance of the protein portion of the enzyme as a result of a molybdenum deficiency would be analogous to the iron-ferritin relationship in the intestine (14).

It was pointed out previously (5) that the reduction of nitrates by plants, molds, etc., is probably effected by a molybdenum-containing enzyme which is similar to but not necessarily identical with xanthine oxidase.

**SUMMARY**

The dietary factor required for the deposition and maintenance of normal levels of rat intestinal xanthine oxidase was isolated from soy flour and identified as a molybdate salt. Sodium molybdate gave saturation
levels of intestinal xanthine oxidase when the diet contained less than 0.1 mg. of molybdenum per kilo; the best assay range was 20 to 60 γ of Mo per kilo of diet.

All of the biological activity of the isolated material could be accounted for by the molybdenum present, since (1) all the fractions had the same biological activity as sodium molybdate when tested on the basis of their respective molybdenum content, (2) the molybdenum content and biological activity were not decreased by ashing, (3) a removal of molybdenum by extracting it as an ether-soluble thiocyanate also extracted the biological activity quantitatively, and (4) precipitation of molybdenum with benzoinoxime also precipitated the biological activity to the same extent.

Purified milk xanthine oxidase contained 0.03 per cent molybdenum.

BIBLIOGRAPHY