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On the Metabolism of Tartrazine in the Rat

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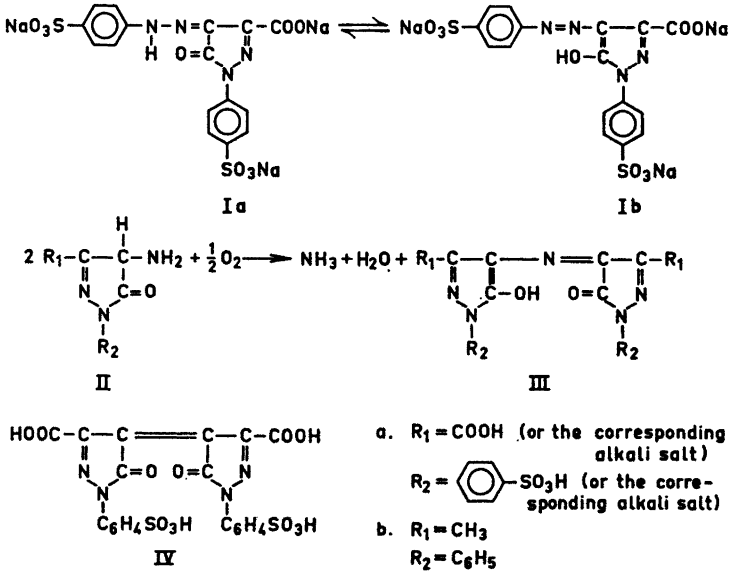
When tartrazine (I) is given to rats, 4-amino-5-oxo-1-(*p*-sulphophenyl)-2-pyrazoline-3-carboxylic acid (II a) is excreted in the feces. In contact with air it is oxidized to a violet compound, III a, related to rubazonic acid (III b).

Tartrazine is one of the five yellow colours which are allowed to be added to food products in Sweden. As it is widely used, its metabolism and excretion are of especial interest.

In 1962 there appeared two interesting papers on the metabolism of tartrazine (I) by Daniel [1] and by Ryan and Wright.[2] Daniel, who gave tartrazine to rabbits by stomach tube, showed by paper chromatography and colour reactions that the part of the tartrazine molecule originating from



sulphanilic acid was excreted in the urine as sulphanilic acid (74 %) and N-acetylsulphanilic acid (22 %). This demonstrates that a reductive fission of the tartrazine molecule has taken place. Ryan and Wright gave small amounts of tartrazine intraperitoneally to rats and stated that in this case no amines corresponding to a reductive cleavage of the tartrazine molecule could be found in the urine. In 1964 Jones, Ryan and Wright [3] further penetrated the excretion of sulphanilic acid (free or conjugated) by animals and man given tartrazine. None of these authors has described any metabolite emanating from the pyrazolone part of the tartrazine molecule, which subject is studied in the present investigation.

Feces from rats given tartrazine by gastric intubation or subcutaneously (0.03—0.3 g/kg body weight) showed a normal fecal colour when excreted, but after some minutes in contact with air they acquired a violet tinge. When the rats were killed 4—16 h after the administration of the tartrazine, the contents in and — when the time had not been too short — also below the caecum likewise changed to violet when exposed to air.* The contents in the small intestines, however, did not yield any violet product, when air was admitted.

Extraction with water at room temperature of the violet feces from rats given tartrazine by gastric intubation gave a violet to red solution, from which a violet product has been isolated by precipitation followed by partition chromatography. Furthermore, three yellow products have been isolated by chromatography of the mother liquor from the precipitation.

The violet substance is a mixture of alkali salts with the light absorption curve shown in Fig. 1. It is decomposed at high or low pH, but at pH 7.0—8.5 it is rather stable. The instability of the compound makes purification difficult. The corresponding acid could not be isolated in a pure form on account of its rapid decomposition.

Essentially the same wave lenghts of light absorption maxima and minima (Fig. 1) and the same stability at pH 7.0—8.5 and instability at high or low pH that are shown by the violet product from the feces are also shown by a compound obtained when 4-amino-5-oxo-1-(*p*-sulphophenyl)-2-pyrazoline-3-carboxylic acid (II a) is dissolved in water and a small amount of alkali (pH = 7 — 8) at room temperature in contact with air. In a nitrogen atmosphere no violet compound is formed from II a.

The violet product must have the structure III a, for when it is formed from II a oxygen is taken up, and ammonia is expelled. The light absorption curve (Fig. 1) of the violet product supports a structure like III a with a system of conjugated double bounds involving two pyrazolone groups. The only possible alternative, the pyrazole blue relative, IV, should be formed from II a without any uptake of oxygen and is thus excluded. Besides, the formation of III a from II a is analogous to the formation of rubazonic acid (III b) from 1-phenyl-3-methyl-4-amino-2-pyrazolin-5-one (II b) already described by Knorr.[4]

* Even a small amount of the violet product was easily visible, when a drop of an aqueous extract of the feces was put on a filter paper, followed by a drop of water one minute later. If the violet compound was present, it moved rapidly to form a border around the coloured area.

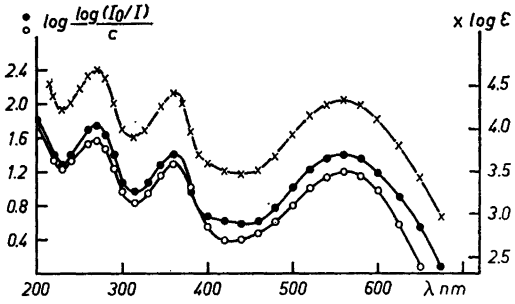


Fig. 1. O Light absorption curve of violet product, III a, from feces of rat given tartrazine (c = mg of violet product per ml of water solution).
● Light absorption curve of III a prepared from II a in ammonium hydroxide solution (c = mg of ammonium salt of III a per ml of water solution).
× Light absorption curve of III a prepared from II a in a buffer solution (pH 7.95). The extinction was measured directly in the diluted reaction solution, and log ε was calculated with the presumption of 100 % yield.

When III a is transformed into the acid form in a water solution, it is rapidly decomposed. A yellow solution is formed from which two yellow products have been isolated. Judging from their light absorption curves these decomposition products are identical to two of the yellow products found in the fecal extracts. As these products have probably been formed after the feces left the animal, they have not been further studied.

The third yellow compound isolated from the fecal extract is tartrazine, identified by its light absorption curve.

From the above it is seen that on oral administration of tartrazine one fecal metabolite is 4-amino-5-oxo-1-(*p*-sulphophenyl)-2-pyrazoline-3-carboxylic acid (II a), which in contact with air is oxidized to III a. This verifies the findings of Daniel [1] and Jones *et al.*[3] that a reductive cleavage of tartrazine takes place in the rat. This cleavage occurs not only when the tartrazine is given orally, but also when it is administered subcutaneously in larger doses. As no II a was found in the small intestines either after gastric intubation or subcutaneous administration of tartrazine, it is probable that the cleavage of I is caused by the bacterial flora in the caecum. When tartrazine is administered subcutaneously in large amounts (0.3 g/kg), part of it is evidently transferred into the intestines and is thus exposed to bacterial action resulting in the formation of II a. A control experiment was performed with the contents of the caecum of an untreated rat. When these contents were allowed to react with tartrazine anaerobically at 37° *in vitro*, the same cleavage with formation of II a and, when air was admitted, of III a was observed.

As II a is only slightly soluble in most solvents, it is very difficult to isolate from the feces for determination of the yield. Instead attempts have been made to estimate the amounts of II a excreted in the feces from the extinction at 560 nm of the violet product III a formed from it.

When III a is prepared from II a in a buffered solution, the yield is dependent on the concentration of II a, especially when this concentration is small,

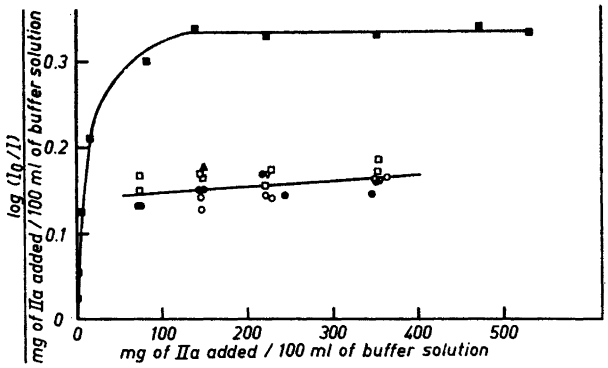


Fig. 2. The extinction of III a (formed from II a) per mg of II a added per 100 ml of buffer solution as a function of the initial concentration of II a.

1. ■ No feces added
2. ○ 3.5 g of feces added /10 ml of buffer solution
3. ● 3.0 g » » » /10 » » » »
4. □ 2.3 g » » » /10 » » » »
5. △ 1.1 g » » » /10 » » » »

At the curves 2—5 a 33 % ethanolic buffer solution with pH = 8.1 was used, at the curve 1 an aqueous buffer solution with pH = 7.95.

as seen in Fig. 2. If feces are added to the solutions, the yields are decreased (Fig. 2).

Except on the concentration of II a, the yield of III a is dependent on the pH (Table 1) and the oxygen supply. It is difficult to get exactly the same conditions in all these respects in the experiments with the II a excreted in the feces as in the model experiments.

If the feces are passed into the buffer solution when excreted, the concentration of II a will be small and vary in an unknown way, and the horisontal parts of the curves in Fig. 2 can not be used. If instead the formation of III a takes place mainly in the feces before the addition of buffer solution, the pH and the oxygen supply might be rather different from the conditions in the model experiments.

Collection of the feces on solid carbon dioxide for 24 h and addition of buffer solution at room temperature when the collection was finished resulted in low yields of III a, probably because of side reactions. Therefore it has instead been tried to collect the feces in air at room temperature, allowing part of the reaction to take place before addition of buffer solution. A weighed amount of II a was added to part of the feces to make a control of the reliability of the results possible.

So much tartrazine (0.3 g/kg) was given to the rats by gastric intubation that the amount of II a excreted in the feces should have been large enough for the yield of the III a formed from it to be almost independent of the concentration of the II a. When the approximate yields of the II a formed in the animals were calculated from the extinctions of III a in buffered ethanol-water (1:2) extracts of feces and the curves in Fig. 2, the result was found

that 38 — 67 % (mean value 55 %) of II a formed from I were excreted in the feces from the rats during the first 24 h after the administration of the tartrazine. During the next 24 h no II a or only minute amounts were excreted.

EXPERIMENTAL

Purification of tartrazine (I). Tartrazine was recrystallized 6 times alternately from 60 % ethanol and water. After the purification partition chromatography of the corresponding acid (obtained by passage of a solution through a cation exchange resin) on kieselguhr (4.5 g) with phases obtained by equilibrating 110 ml of isobutanol, 10 ml of ethanol and 30 ml of water (3 ml of stationary water phase were used) gave one single yellow band.

Administration of tartrazine to rats. Unanesthetized, adult, white, female rats weighing about 300 g were given 1.0 ml of a 0.83 % or 5.0 ml of an 1.77 % water solution of tartrazine (8.3 or 89 mg) by gastric intubation or subcutaneously. Before and during the experiments the usual food and water were given *ad libitum*. The percentage of tartrazine in the stock solution was determined by titration with titanium trichloride.[5]

Isolation of a violet substance, III a, its decomposition products and of tartrazine from the feces. The feces from the first 24 h after the administration by gastric intubation of 89 mg of tartrazine were extracted with water at room temperature. A violet to red solution was obtained. It was centrifuged, filtered and evaporated to dryness under reduced pressure at room temperature. The residue was dissolved in a few ml of water, filtered and precipitated with anhydrous ethanol. A violet precipitate and a yellow solution were obtained. The precipitation was repeated once.

Part of the violet product was purified by chromatography at 25.0° on kieselguhr (4.5 g) with phases obtained by equilibrating 110 ml of isobutanol, 45 ml of ethanol and 110 ml of water. When the eluate containing the violet product was cooled, two phases were formed. The small water phase contained the violet compound. It was evaporated to dryness under reduced pressure as soon as possible on account of the instability of the compound. The light absorption curve of the violet substance is seen in Fig. 1.

After passage of the mother liquor from the precipitation of the violet compound through a cation exchange resin in the acid form, three yellow products could be isolated by chromatography of a small amount of the dry substance as described above. One of them was tartrazine, after purification by repeated chromatography identified by its light absorption curve.

When a solution of the violet compound was passed through the acid form of a cation exchange resin, the corresponding violet acid obtained was rapidly decolorized. The

Table 1.

pH	log I_0/I of solutions of III a after							
	0 min	4 min	30 min	60 min	29 h	51 h	98 h	220 h
1.0		0.740	0.033	0.002				
5.9	0.825				0.808	0.735	0.640	0.510
6.5	0.890				0.890	0.850	0.840	0.745
7.0	1.200				1.200	1.180	1.157	1.151
7.5	1.020				1.020	0.995	0.993	0.960
7.9	1.000				1.000	0.990	0.985	0.950
8.5	0.970				0.970	0.960	0.950	0.910
9.3	0.212				0.213	0.205	0.193	0.173
~13		0.908	0.630	0.413	0.000			

decomposition products were chromatographed with the system described above. Two yellow products were isolated. They showed essentially the same light absorption curves as were found for the two unidentified yellow products in the extracts of the feces.

Stability of III a at different pH. III a was prepared from 7.9—8.4 mg portions of II a, dissolved in 5 ml of buffer solutions (pH = 5.9—9.3). After 23 h at room temperature 2 ml of the solutions were diluted to 100 ml with the same buffer solutions, and the extinctions were measured at 560 nm immediately (0 min) and after various intervals. The extinction decrease of solutions of III a at pH 1.0 and ~ 13 was also studied. The results are seen in Table 1. It is seen that solutions of III a show a good stability at pH 7.0—8.5 and a reasonably good stability down to pH 6.5 and up to pH 9.

Yield of III a in the feces. III a was synthesized *in vitro* by solution of different amounts of II a in a phosphate-citrate buffer solution, pH 7.95. After 22 h at room temperature the extinctions of the solutions were measured at 560 nm in 1 cm cuvettes after proper dilution with buffer solution. The results are seen in Fig. 2. Addition of feces to the solutions at the beginning of the reaction decreases the yields of III a (Fig. 2). In this case a slightly different buffer solution (pH 8.1) obtained by addition of ethanol (1:2) to the initial buffer was used.

Feces from rats given 5.0 ml of a water solution containing 17.74 mg of tartrazine (I) per ml (gastric intubation at 9 o'clock in the morning) were collected during 24 h. Each bit of feces when excreted was pressed to a thin layer in order to facilitate the uptake of oxygen. 24 h after the administration of the tartrazine the feces from each rat were weighed, homogenized with 5.0 ml of water (rats Nos. 1 — 5) or with a few ml of a phosphate-citrate buffer solution (33 % ethanol, pH 8.1) and divided into two equal parts by weight. To one of the parts were added 10 — 11 mg of II a, which were well mixed with the feces (at 11 — 12 o'clock). At 12 — 13 o'clock 10.0 ml of phosphate-citrate buffer solution (33 % ethanol, pH 8.1) were added to each beaker and thoroughly mixed with the contents. After 21 — 24 h the samples were transferred to 100 ml volumetric flasks and diluted to 100 ml with buffer solution. The suspensions obtained were centrifuged, and 4.00 ml of the clear solutions were diluted with buffer solution to 10.00 ml. The light absorptions were then measured at 560 nm in 1 cm cuvettes. Another 4.00 ml of the centrifuged, fecal extracts were transferred into 10 ml volumetric flasks, and 0.5 ml of 5 N hydrochloric acid was added. In about half an hour the violet product was completely destroyed by the acid, which was then neutralized with 0.5 ml of 5 N sodium hydroxide solution. The flasks were filled to the mark with the buffer solution, and the light absorptions were again read at 560 nm in 1 cm cuvettes. The extinction values obtained are seen in Table 2.

Table 2.

Rat No.	mg of II a added to beaker 2	g of feces in each beaker	pH after addition of 10 ml of buffer solution		$\log I_0/I$ beaker 1		$\log I_0/I$ beaker 2	
			beaker 1	beaker 2	before decomposition of III a	after decomposition of III a	before decomposition of III a	after decomposition of III a
1	10.57	1.8	6.5	6.4	0.672	0.072	1.370	0.105
2	10.25	2.3	6.65	6.4	1.314	0.182	1.686	0.175
3	10.49	1.7	6.9	6.75	0.880	0.106	1.578	0.116
4	10.41	0.9	7.45	7.25	1.168	0.075	1.522	0.081
5	10.45	0.3	7.2	7.1	1.145	0.075	1.788	0.073
6	11.00	1.8	7.2	7.15	1.130	0.087	1.750	0.118
7	10.07	2.2	7.65	7.3	1.095	0.087	1.880	0.122
8	10.95	0.9	7.45	7.35	1.448	0.186	—	—
9	11.11	2.0	7.3	7.2	0.858	0.130	1.748	0.165
10	10.11	1.9	7.2	7.05	0.708	0.075	1.730	0.135

The approximate yields of II a from I in the rats have been calculated in three different ways from the amounts of III a found in the fecal extracts.

e_1 = extinction of diluted solution emanating from beaker 1
$e_{1,0}$ = » » » » » » » »
after destruction of III a
e_2 = extinction of diluted solution emanating from beaker 2
$e_{2,0}$ = » » » » » » » »
after destruction of III a
b_2 = weight of II a added to beaker 2

I.

$$\frac{2 \times 100 \times b_2 \times (e_1 - e_{1,0})}{[(e_2 - e_{2,0}) - (e_1 - e_{1,0})] \times 49.7} = \% \text{ of II a formed from I in the rat } (= x)$$

This calculation has been performed without the aid of Fig. 2, presuming only that the fecal suspension was homogeneous when it was divided into two parts and that the percentage yield of III a from II a was the same in the two beakers (including the yield of the III a formed from the II a added to the beaker 2). If these presumptions are not correct the percentage errors of x may be large, because a small percentage error in $e_2 - e_{2,0}$ or in $e_1 - e_{1,0}$ will both give larger percentage errors in x, and if both $e_2 - e_{2,0}$ and $e_1 - e_{1,0}$ possess small percentage errors, but in different directions, x will be strongly affected. Thus, if this method of calculation leads to results which are in accordance with the results obtained by the other calculations, this is an indication that the results are reliable.

II. This method of calculation only requires that the yield of III a formed from II a was the same in the model experiments reported in Fig. 2 (an average value of log I_0/I per mg of II a/100 ml of solution = 0.16 has been used in the calculations) as in the beakers 1 and 2 (including the yield of the III a formed from the II a added to the beaker 2).

$$\frac{\left[\frac{(e_1 - e_{1,0} + e_2 - e_{2,0}) \times 2.5}{0.16} - b_2\right] \times 100}{49.7} = \% \text{ of II a formed from I in the rat}$$

III. This calculation does not involve the II a added, but requires that the fecal suspension was homogeneous when divided into two parts and that the reaction in beaker 1 gave the same yield as the reactions in the model experiments (an average value of log I_0/I per mg of II a/100 ml of solution = 0.16 has been used in the calculations).

$$\frac{(e_1 - e_{1,0}) \times 2 \times 2.5 \times 100}{0.16 \times 49.7} = \% \text{ of II a formed from I in the rat}$$

The results obtained from the different calculations are seen in Table 3.

By comparison of the results, it is evident that for the rats 2, 4, and 10 the reaction conditions have not been similar in the different parts of the experiments as the results in column I for these rats differ greatly from the results in the other columns. But if the mean values for the yields are calculated only for the rats Nos. 1, 3, 5, 6, 7, and 9 in column I, the result hardly differs at all from the results obtained from all rats as calculated in columns II and III. The latter are less sensitive to differences in reaction condition than are the results in column I.

*Preparation of 4-amino-5-oxo-1-(*p*-sulphophenyl)-2-pyrazoline-3-carboxylic acid (II a).* To boiling, concentrated hydrochloric acid (25 ml) stannous chloride ($SnCl_2,2H_2O$) (5.0 g) was added and then immediately with stirring a tartrazine solution (2.0 g of an 93.8 % product in 5.0 ml of water). The solution was boiled for 2 min, when it was decolorized, and was then cooled in ice-water. The crystals formed were filtered by suction, washed once with 10 N hydrochloric acid, with water, ethanol and ether. The product was recrystallized from 10 N hydrochloric acid (170 ml of solvent per g of product) and

Table 3.

Rat No.	II a formed from I and found in the feces, %		
	calculation I	calculation II	calculation III
1	38	37	38
2	(125)	(63)	(71)
3	47	49	49
4	(132)	(59)	(69)
5	70	67	67
6	78	62	66
7	55	67	63
8	—	—	(79)
9	38	50	46
10	(27)	(50)	(40)
Mean value	68	56	59
Mean value for 1, 3, 5, 6, 7, 9	54	55	55

dried *in vacuo*. *Yield* 0.79 g (75 %). (Found: C 40.3; H 3.05; S 10.4. Calc. for $C_{10}H_9N_3O_6S$: C 40.1; H 3.03; S 10.7).

*Preparation of III a (ammonium salt) from 4-amino-5-oxo-1-(*p*-sulphophenyl)-2-pyrazoline-3-carboxylic acid (II a).* 0.153 g of II a was dissolved in 7.8 ml of 0.144 N ammonium hydroxide solution at room temperature. The solution turned violet rapidly but was kept at room temperature until the next day (21 h). It was then evaporated to dryness under reduced pressure, and the residue was dissolved in the smallest possible amount of water and immediately precipitated by addition of 99.6 % ethanol. The precipitate was filtered and washed with small amounts of ethanol and ether. The purification was repeated once. The light absorption curve of the product obtained is seen in Fig. 1.

The uptake of oxygen and expulsion of ammonia in the formation of III a from II a. When II a was dissolved in a buffer solution (pH 7.5) in a nitrogen atmosphere no reaction took place, but as soon as air was admitted, the violet III a was formed rapidly. When 0.5 millimole of II a (0.147 g) was dissolved in 0.106 N sodium hydroxide solution (7.0 ml) and exposed to air for about 20 h, III a was formed. At the same time half a mole of ammonia was split off per mole of II a. This was shown by passing an aliquot (2/5) of the violet solution through an anion exchange resin in the chloride form in order to remove III a and other possible N-containing anions, adding sodium hydroxide solution to the eluate, distilling and titrating the ammonia formed (0.1 millimole).

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