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Loss of Hepatic Monoamine Oxidase Activity Resulting from Replacement of its Coenzyme Flavin[1] (40078)

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Three mammalian mitochondrial enzymes are known to be covalently bound to flavin adenine dinucleotide (FAD). Succinic dehydrogenase (SDH) (EC 1.3.99.1) is part of the inner membrane while monoamine oxidase (MAO) (EC 1.4.3.4) is part of the outer membrane. The third enzyme, sarcosine dehydrogenase (EC 1.5.3.1) is present in the soluble or matrix space of the mitochondrion. SDH has been reported to be covalently bound to FAD from N-3 of the imidazole ring of a histidine residue to the 8α methyl group of riboflavin (Fig. 1A) (1). MAO has been shown to involve a bond between the sulfur atom of a cysteine residue in the enzyme and the 8α methyl group of riboflavin (2), while the linkage in sarcosine dehydrogenase is like that in SDH.

The two homologs of riboflavin which possess vitamin-like properties in mammals, 7-ethyl-8-methyl-10-(1′-D-ribityl)isoalloxazine (7-ethyl-8 methylflavin or 7-Et) (Fig. 1B) and 7-methyl-8-ethyl-10-(1′-D-ribityl)isoalloxazine (7-methyl-8-ethylflavin or 8-Et) (Fig. 1C) (3) have been studied for their ability to replace riboflavin in the coenzyme component of SDH. When 7-Et was the sole metabolically active flavin in the tissues of the rat, the activity of the liver enzyme was reduced as much as 90% (4) while when 8-Et was the sole flavin utilized by the tissues, the loss of activity was about 50% (5). It must be appreciated that 7-Et or 8-Et were the only flavins available to these rats; no detectable riboflavin can be found in the tissues. These flavins have, therefore, replaced riboflavin as the coenzyme group in all flavoprotein enzymes required for metabolism in these rats. When the rat was deprived of riboflavin the reduction of SDH activity was about 65% (4). However, rats receiving either of the homologs are indistinguishable with respect to rate of growth, efficiency of food utilization, physical appearance and survival from rats receiving riboflavin (3, 6) while riboflavin-deficient rats show extreme abnormalities with respect to these criteria and invariably die. The indispensability of SDH for life, and the absence of riboflavin in the tissues of the homolog-fed rats, suggests that the two homologs must be covalently linked to the enzyme, forming holoenzyme analogs. The bonds most likely to be formed are illustrated in Fig. 2. We have discussed these structures in earlier publications (5, 7).

7-Et possesses an 8 α methyl group as in riboflavin yet it is not as well utilized by SDH as is 8-Et in which the link is assumed to be at the α carbon of the ethyl group (Fig. 2). A similar covalent bond is likely to be required to produce a functional MAO. If the S of a cysteine residue replaces the N-3 of a histidine residue (Fig. 2) one can visualize the form of bonds required, if the homologs are to function as coenzymes for MAO. These considerations prompted us to study the rate of change of MAO activity during the time when liver mitochondrial riboflavin is being lost due to riboflavin deprivation (Study I), and the rate of change of MAO and SDH during the replacement of riboflavin by one or the other of the homologs (Study II). The results of those investigations suggested a comparison of L-DOPA metabolism by rats utilizing riboflavin with rats utilizing one or the other of the homologs (Study III).

Materials and methods. Weanling female rats of the Wistar strain,[4] initially weighing between 35 and 45 g were used for the several

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studies. The basic diet of the rats (feeding started at time "0" in all studies) consisted of the riboflavin-deficient diet previously described (8); this diet was used for the deficient group (Group Def) in Studies I and III. To this basic diet was added 20 mg/kg of riboflavin for Group Rb only (all Studies), 20.8 mg/kg of 7-Et for Group 7-Et only (Studies II and III) and 20.8 mg/kg of 8-Et for Group 8-Et (Studies II and III). The animals had free access to food and water and they were caged and maintained as described before (9). Study I: Groups D and Rb each consisted of 36 weanling rats. At time "0" and days 1 and 2, 2 rats were taken from each group and the liver of each rat assayed independently. The same procedure was carried out on days 13, 14, 15; 27, 28, 29; 41, 42, 43 and 55, 56, 57. Thus 6 independent values were obtained for each Group for each time interval (Table I). Study II: Group Rb consisted of 36 and each of Groups 7-Et and 8-Et of 33 rats. The precision of the MAO assay using kynuramine as substrate used in Study I and our experience with the assay for SDH permitted the use of fewer assays than were made for Study I. At the start of day 1, equal portions of the liver of three rats from each Group were pooled by Group and duplicate assays performed on each such pooled sample. The same procedure was performed on days 14, 28, 42 and 56. On days 28 and 56, three additional rats from each Group were taken for MAO assays using 1-[^{14}C]-tyramine as substrate. On days 57, 58 and 59, three rats from each Group were taken for MAO assays using 1-[^{14}C]-dopamine, 2-[^{14}C]-serotonin and 2-[^{14}C]-tryptamine, respectively, as substrates (Table I). Study III: Groups Rb, D, 7-Et and 8-Et consisted of 44, 41, 34 and 36 rats, respectively. At time "0", 10 rats from Group Rb were given interperitoneal injections of L-DOPA (320 mg/kg of body weight) (10). The same procedure was used for 10–16 rats from each Group on days 14, 28 and 56. Rats able to metabolize L-DOPA and its products survived while those unable to do so, died within 4 hr from the time of injection (Table III).

Prior to the assays for MAO and SDH, the rats were fasted for 16 hrs, then lightly anesthetized and killed by decapitation. The livers were removed and placed in cold 0.25 *M* sucrose. For Study I, tissue (2 g) was taken from the right median lobe from each rat of Group Rb, but because of their small size for all rats at time "0" and days 1 and 2, and all those from Group D at later times as well, portions from more than one lobe were required to obtain 2 g. For Study II, approximately 0.7 g portions taken from each of the three rats of a Group were pooled (2 g total).

Isolation of mitochondria. In all cases a 10% homogenate in 0.25 *M* sucrose was prepared by using 10 slow passes of the Teflon pestle

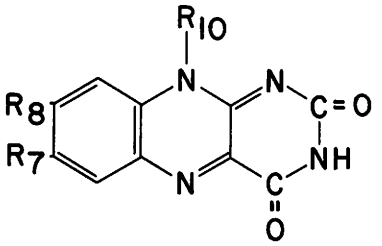
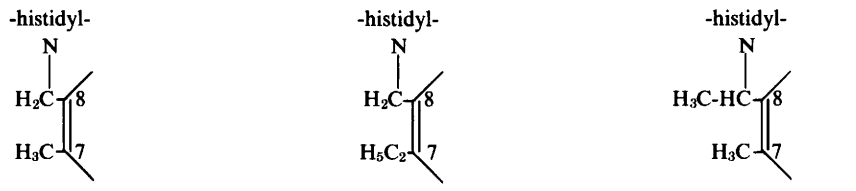


FIG. 1. Basic Flavin Structure:

Flavin	R_7	R_8	Trivial Name
A	CH_3—	CH_3—	Riboflavin
B	C_2H_5—	CH_3—	7-Ethyl-8-methylflavin or 7-Et
C	CH_3—	C_2H_5—	7-Methyl-8-ethylflavin or 8-Et



Riboflavin (Rb) | 7-Ethyl-8-methylflavin (7-Et) | 7-Methyl-8-ethylflavin (8-Et)

FIG. 2. Forms of the covalent bond between riboflavin and the homologs and SDH. The structure of the bond between 7-methyl-8-ethylflavin and histidine as shown is hypothesized on the basis of the rule of hyperconjugation which identifies the most active hydrogen atoms as those *alpha* to a double bond or other electron-deficient unit (19).

TABLE I. MONOAMINE OXIDASE ACTIVITY OF HEPATIC MITOCHONDRIAL PREPARATIONS FROM RATS RECEIVING RIBOFLAVIN (Rb), NO RIBOFLAVIN (Def), 7-ETHYL-8-METHYLFLAVIN (7-Et) OR 7-METHYL-8-ETHYLFLAVIN (8-Et). (STUDIES I AND II).

Group / Substrate	Days 0	14	28	42	56
Riboflavin (Rb)					
kynuramine	100[a]	100	100	100	100
1-[^{14}C]-tyramine			100[c]		100
1-[^{14}C]-dopamine					100
2-[^{14}C]-serotonin					100
2-[^{14}C]-tryptamine					100
Riboflavin deficient (Def)					
kynuramine	100[b]	49	29	16	16
7-Ethyl-8-methylflavin (7-Et)					
kynuramine	100	34	20	19	18
1-[^{14}C]-tyramine			39[c]		22
1-[^{14}C]-dopamine					29
2-[^{14}C]-serotonin					35
2-[^{14}C]-tryptamine					30
7-Methyl-8-ethylflavin (8-Et)					
kynuramine	100	15	6	4	3
1-[^{14}C]-tyramine			16[c]		11
1-[^{14}C]-dopamine					21
2-[^{14}C]-serotonin					23
2-[^{14}C]-tryptamine					7

[a] All assay values determined as n moles substrate oxidized/min/mg protein. The normal values for the enzyme activities in Group Rb were: time "0", 4.2 ± 0.12; day 14, 4.4 ± 0.14; day 28, 4.0 ± 0.11; day 42, 4.2 ± 0.20; day 56, 4.2 ± 0.20. All values in the table are expressed as percentage of the value for Group Rb done in parallel with the experimental Groups.

[b] The assay values for the enzyme activity for Group Def were: time "0", 4.2 ± 0.12; day 14, 1.7 ± 0.12; day 28, 1.1 ± 0.02; day 42. 0.67 ± 0.02; day 56, 0.66 ± 0.04. The "P" value for the difference between Group Rb and Group Def for each time interval is less than 0.0001.

[c] The assay values for the enzyme activity for this time interval were: day 28, Group Rb, 8.80 ± 0.36 m U/mg; for Group 7-Et, 3.41 ± 0.18 m U/mg, and for Group 8-Et, 1.38 ± 0.06 m U/mg, where one m U = 1 nmole/min. The other assays by the radiometric procedure were of comparable precision.

TABLE II. SUCCINIC ACID DEHYDROGENASE ACTIVITY OF HEPATIC MITOCHONDRIAL PREPARATIONS FROM RATS RECEIVING RIBOFLAVIN (Rb), 7-ETHYL-8-METHYLFLAVIN (7-Et) OR 7-METHYL-8-ETHYLFLAVIN (8-Et). (Study II).

	Days 0	14	28	42	56
Riboflavin	100[a]	100	100	100	100
7-Et	100	67	34	24	19
8-Et	100	71	64	58	49

[a] Assay values determined as μmoles succinate oxidized/min/mg protein. The normal value for hepatic mitochondrial preparations found in this study was 0.397 ± 0.026 μmoles/min/mg protein. All values are expressed as percentage of the value for the Rb Group done in parallel with the experimental Groups.

TABLE III. SURVIVAL OF RATS RECEIVING RIBOFLAVIN (Rb), NO RIBOFLAVIN (Def), 7-ETHYL-8-METHYLFLAVIN (7-Et) OR 7-METHYL-8-ETHYLFLAVIN (8-Et) FOLLOWING THE AMINISTRATION OF L-DOPA.[a] (Study III).

	Days 0	14	28	56
Rb	0/10[b]	0/10	1/10[c]	0/14
Def		0/10	0/11	1/20[d]
7-Et		0/10	3/10	1/14
8-Et		3/10	10/10	16/16

[a] L-3,4-Dihydroxyphenylalanine administered IP, 320 mg/kg body weight. All deaths occurred in 4 hr or less except as indicated (see d below). No hyperactivity observed.

[b] Number of rats that died over number used.

[c] This rat was found to have bled into the abdomen, probably as a result of an injury during injection.

[d] Died between 20 and 24 h following injection.

in a Thomas "tissue grinder" (size C) at 450 rpm, with the homogenizer immersed in ice and water. The tissue preparation was cooled at all steps of the procedure. The homogenate was filtered through four layers of No. 80 gauge cheese cloth. The filtrate was centrifuged at 600*g* for 10 min, the supernatant suspension was saved and the pellet resus-

pended in 18 ml of 0.25 *M* sucrose in the original homogenizer by gentle hand rotation of the pestle. This suspension was centrifuged as above and the combined supernatant suspensions centrifuged at 9000*g* for 10 min. The supernatant solution was discarded and the pellet transferred to a 15 ml Dounce homogenizer[5] with 20 ml of 0.25 *M* sucrose. Six slow passes were made with the tight-fitting pestle (pestle B) and the suspension centrifuged at 9000*g* for 10 min. The pellet obtained from the last centrifugation was resuspended in 10 ml of 0.25 *M* sucrose in a 7 ml Dounce homogenizer as described before. This mitochondrial preparation was used undiluted for MAO assay and was diluted 1–20 with 0.25 *M* sucrose for SDH assay. MAO activities were determined using either kynuramine as substrate for a spectrophotometric assay (11) or [^{14}C]-labeled amines for a radiometric procedure (12). The SDH activities were assayed by the use of phenazine methosulfate and dichlorophenolindophenol as the electron acceptor system (13). Protein concentrations of the mitochondrial preparations were determined by the procedure of Lowry *et al.* (14).

Results and discussion. The rate of change in SDH activity when the rats consumed diets supplemented with one or the other homolog (Table II) was essentially identical with our earlier findings (4, 5). We here report the assay of hepatic mitochondrial preparations using a spectrophotometric procedure; previously we assayed liver homogenates by a manometric procedure. The importance of this confirmatory information can be appreciated when it is realized that the homolog 7-Et which yields the least enzyme function for SDH, is the one in which the covalent bond at the 8 α carbon could be established as with FAD-containing SDH. The homolog 8-Et which provides a far greater coenzyme function for SDH, is the one in which the covalent bond at the 8α carbon position would need to be formed by using the α carbon of an ethyl group, a structure which must be considered abnormal in relation to the normal FAD bond but mandatory in a physicochemical sense (Fig. 2). Further it was important to confirm the validity of our earlier observations because of the obvious normal appearance of the rats receiving 7-Et, even when they had received the homolog for hundreds of days at which time the SDH activity did not exceed 9% of the normal value.

The rate of change in MAO activity when rats consumed the flavin-free diet or the flavin-free diet supplemented with one or the other of the homologs (Table I), showed the activity was severely depressed when no flavin was available or when 7-Et was the sole metabolically active flavin, and to be vertically abolished (depending on the substrate used) when 8-Et was utilized. The reduction of hepatic MAO activity during the development of riboflavin deficiency had been studied by others (15) but the rate of fall was not as rapid as we have observed. The MAO activities when kynuramine or tryptamine (and possibly tyramine) was used as substrate, appear to reflect the degree of normalcy of the structure of the covalent bond at the pseudo-FAD junction. However, it is difficult to draw a distinction between the homologs when dopamine or serotonin is used as substrate, suggesting that the observed changes in hepatic MAO may not be true of brain MAO. These findings might also be interpreted as providing some supportive evidence for the existence of more than one form of hepatic MAO. However, the multiplicity of species of MAO is not accepted by all workers in the field (16) and for this reason it would be inappropriate to attach too much significance to variations shown among the substrates. Most investigators of MAO believe that the use of substrates to distinguish between species of MAO is not dependable while some differences appear to be demonstrable by the use of specific inhibitors such as clorgyline and deprenyl.

Our findings show that in contrast to observations with SDH, rats utilizing 8-Et for their flavoprotein coenzymes lost hepatic MAO activity. This led us to consider means by which we could demonstrate this loss of activity in the whole animal. A direct test appeared to be an evaluation of the ability of the rats to metabolize the metabolic products (presumably principally dopamine) from a dose of L-DOPA that can be tolerated by normal rats (10). When the sole flavin available to the rat has been Rb, 7-Et or 8-Et for a period of only 28 days, the animal's weight will have increased to four times the initial weight and the mitochondrial turn-over will

[5] Kontes Glass Co., Vineland, N.J.

have completed at least two half lives (4). By this time, the rats receiving 7-Et and 8-Et will have had all ionically bound Rb forms displaced form their tissues and the mitochondrial covalently bound FAD cannot exceed 3% of its original concentration. When the homologs have been fed for 56 days, riboflavin is no longer detectable in the tissues. In contrast to this, rats maintained on a riboflavin-deficient diet usually do not double their weight with the end result that after 28 days the residual total body Rb is present at approximately 55% of its original concentration (17). When the flavin available to the rats for 28 days has been 8-Et, they could not survive the administration of L-DOPA, while the rats receiving riboflavin did, and further, those receiving no flavin or 7-Et also survived in spite of the severe depression of MAO activity in the latter two cases. This raises an interesting paradox. When dopamine was used as substrate, the MAO activities of rats fed either of the two homologs appeared to be approximately the same (Table I). Yet the product of the metabolism of L-DOPA (presumably dopamine) could not be tolerated by the rats utilizing 8-Et but could be tolerated by those utilizing 7-Et. These observations warrant an investigation of the influence of these homologs on brain MAO activity. However, other amine oxidizing systems may be involved in these differences. These findings also suggest that hepatic MAO activity is not essential for the survival of the unstressed laboratory rat.

Our observations that 7-Et supports moderate MAO activity while 8-Et supports virtually none when kynuramine and possibly tryptamine and tyramine are used as substrates is in sharp contrast to our findings relative to SDH. In the latter case, 7-Et supports only very low SDH activity while 8-Et supports appreciable activity. There can be no doubt that the homologs are bound to SDH since the rats utilizing them are, as cited before, indistinguishable from those using riboflavin. These findings might be interpreted as suggestive of the possibility for the 8α methyl binding to the N-3 of histidine and the -S- of cysteine while the α-carbon of the ethyl group can bind to the N-3 on histidine only. Yeasts also possess SDH. Based on the use of samples of the homologs provided by us, it has been reported that the homologs are bound to the SDH of the mitochondria of a riboflavinless mutant of *Saccharomyces cerevisiae* (18). However, the apparent nonessentiality of hepatic MAO activity for the laboratory rat leaves some uncertainty concerning the binding of 8-Et to the hepatic enzyme. Clearly 7-Et is covalently linked to MAO, however, the loss of MAO activity when 8-Et is utilized might be due to failure of the homolog to bind or, if the bond is established, failure of the holoenzyme to be catalytically active.

The binding of these two homologs to SDH and MAO and the influence of these homologs on brain mitochondria are under investigation in our laboratory at present.

Summary. When 7-methyl-8-ethyl-10-(1′-D-ribityl)isoalloxazine (7-methyl-8-ethylflavin or 8-Et) is utilized in place of riboflavin in rat tissues, its coenzyme function for hepatic MAO is so low that enzyme activity is virtually eliminated. The present evidence suggests that 8-Et cannot be covalently bound to the enzyme. The absence of hepatic MAO activity when 8-Et is utilized is confirmed by the inability of rats using this flavin to survive the stress of administered L-DOPA. The use of 7-ethyl-8-methyl-10-(1′-D-ribityl)isoalloxazine (7-ethyl-8-methylflavin or 7-Et) causes a severe decrease in hepatic MAO, however, the activity of the enzyme is great enough to provide evidence that 7-Et is covalently bound to the enzyme. Rats utilizing 7-Et as well as riboflavin-deficient rats are able to survive the stress of administered L-DOPA. Hepatic MAO may not be an essential enzyme for survival of the laboratory rat.

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