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IN VITRO PIGMENT FORMATION FROM TRYPTAMINE

ROLE OF INDOLE-3-ACETALDEHYDE

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Abstract—The metabolic significance of indole-3-acetaldehyde in the process of *in vitro* pigment formation from tryptamine in the presence of guinea-pig liver mitochondria was investigated. Among the four type selective MAO inhibitors used, pargyline and deprenyl appear to be more effective in inhibiting pigment formation from tryptamine than serotonin, while in the presence of clorgyline and Lilly 51641, pigment formation from serotonin was preferentially inhibited. Reducing agents like ascorbic acid, cysteine and glutathione were found to block pigment formation significantly. Also, a reduction of pigment formation was noted in the presence of NADH and ethanol but not in the presence of NAD. It was observed that the amount of indole-3-acetaldehyde produced enzymatically from tryptamine under the present experimental conditions is not sufficient to account for the total amount of pigment formed in the standard incubation mixture and the generation of nascent aldehyde has greater contribution in pigment formation than that supplemented to the system exogenously. It appears that indole-3-acetaldehyde, tryptamine and MAO are associated with the process of pigment formation.

Biogenic amines, under certain abnormal physiological conditions, are reported to follow some metabolic pathways leading to the biosynthesis of compounds which are commonly referred to as pigments [1–4]. The formation of pigments during abnormal metabolism of catecholamines comprises of compounds like dopachrome, adrenochrome, adrenolutin etc. and quite a number of studies have been carried out towards this end [5–8]. However, existing knowledge about the phenomenon of pigment formation during indoleamine metabolism is indeed very meagre, although it has long been observed that indoleamines when incubated with tissues rich in monoamine oxidase (MAO, EC 1.4.3.4) activity produce some coloured products [9–11]. In an earlier report we described the development and standardization of a simple method for the quantitative measurement of pigment produced from tryptamine in the presence of guinea-pig liver mitochondria [12]. In an attempt to elucidate further the reactions involved in the biotransformation of indoleamine to pigment-like substances, the effects of certain compounds on pigment formation were studied the results of which are described in this communication.

MATERIALS AND METHODS

The mitochondrial fraction of guinea-pig liver was prepared according to the method of Schneider and Hogeboom [13] and was described earlier [12]. The standard assay system, unless otherwise stated, contained 25 mM sodium phosphate buffer (pH 7.5), mitochondrial suspension containing 1.1–1.5 mg protein, 2 mM tryptamine or 5 mM serotonin in a final volume of 2 ml. Serotonin, whenever used, was always freshly neutralized prior to addition to the incubation medium. The amount of pigment formed was measured quantitatively essentially by the method as described earlier [12]. The system containing all the ingredients including the substrate in presence of head denatured (100°, 10 min) mitochondrial suspension was used as blank.

Preliminary experiments were performed to ensure that the rate of pigment formation was linear with respect to time and protein concentration employed. Protein concentration was determined by the method of Lowry *et al.* [14] using bovine serum albumin as standard. Indole-3-acetaldehyde was generated from its bisulphite salt according to Gray [15]. The experimental results are expressed as mean ± S.D. and the test of significance were analyzed by Student's *t*-test.

Tryptamine, serotonin, indole-3-acetaldehyde, L-cysteine, L-ascorbic acid, reduced glutathione, NAD, NADH and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). Pargyline was obtained from Abbott Laboratories (North Chicago, IL). Deprenyl was kindly supplied by Prof. J. Knoll, Semmelweis University of Medicine, Budapest. Clorgyline and Lilly 51641 were generous gift samples from May and Baker Ltd (Dagenham, U.K.) and Lilly Research Laboratories, Eli Lilly and Co. (Indianapolis, IN), respectively. All other reagents were of analytical grade.

RESULTS AND DISCUSSION

Selective MAO inhibitors like pargyline, deprenyl, clorgyline, Lilly 51641 when tested for their effects on pigment formation from tryptamine and serotonin in presence of guinea-pig liver mitochondria showed remarkable inhibition of pigment formation (Table 1). Pargyline and deprenyl appear to be more effective in inhibiting pigment formation from tryptamine in comparison to that of serotonin. Again, preferential inhibition of pigment formation in the presence of serotonin was observed with clorgyline and Lilly 51641. However, clorgyline appears to be much

Table 1. Effects of MAO inhibitors on pigment formation in guinea-pig liver mitochondria using tryptamine and serotonin as substrates

Inhibitor	Concentration (μM)	Pigment formed (μg/mg protein/hr)		% Inhibition	
		Tryptamine	Serotonin	Tryptamine	Serotonin
None	—	269.5 ± 10.3	376.6 ± 25.8	—	—
Pargyline	10	52.5 ± 5.0	150.0 ± 14.1	80	60
	1.0	175.0 ± 5.8	295.0 ± 12.9	35	22
Deprenyl	10	12.5 ± 5.0	75.0 ± 9.2	95	80
	1.0	130.0 ± 14.1	278.6 ± 5.8	52	26
Clorgyline	0.1	112.5 ± 17.1	70.0 ± 14.1	58	81
	0.01	185.0 ± 20.8	120.0 ± 18.3	31	68
Lilly 51641	100	41.0 ± 3.5	16.1 ± 6.8	85	96
	10	189.3 ± 14.9	185.7 ± 13.0	30	51

The inhibitors were incubated with the otherwise complete reaction mixture for 15 min prior to addition of tryptamine (2 mM) or serotonin (5 mM) and the assays were carried out under the standard conditions described in the text. The values represent mean ± S.D. of six (controls) or four (inhibitors) separate determinations carried out in different mitochondrial fractions. Per cent inhibitions are significant to the level of $P < 0.001$ when compared to their corresponding control values (Student's *t*-test). Other details are given in the text.

more potent than Lilly 51641. In fact, among the four inhibitors, clorgyline exhibited highest potency in inhibiting pigment formation from both the substrates. As regards the possible involvement of MAO types in pigment formation, considering the classification of MAO types on the basis of substrate specificity and inhibitor sensitivity [16, 17], it may be stated that the higher degree of inhibition of pigment formation from serotonin by type A specific inhibitors and from tryptamine by type B inhibitors apparently indicate that pigment formation from serotonin and tryptamine are possibly carried out by type A and type B MAO, respectively. However, it has previously been demonstrated that guinea-pig liver mitochondrial MAO is predominantly composed of type A enzyme, and the oxidative deamination of tryptamine by this tissue preparation is to some extent anomalous in nature in respect of its sensitivity towards different selective MAO inhibitors [18]. Similar anomalous inhibition profile is again reflected here while measuring the pigment formation in presence of type selective MAO inhibitors. This is borne out from the fact that the concentrations of deprenyl and pargyline needed to produce any significant inhibition of pigment formation in presence of both the substrates are relatively high which indicates the involvement of MAO-A in pigment formation. On the other hand, the pigment formation was inhibited only by high concentration of Lilly 51641 suggesting the participation of MAO-B in pigment formation. Nevertheless, it seems likely from the above considerations that the pigment formation from sero-

Table 2. Effects of cysteine, ascorbic acid and reduced glutathione on pigment formation from tryptamine in guinea-pig liver mitochondria

Compound	Concentration (mM)	Pigment formed (μg/mg protein/hr)	% Inhibition
None	—	274.6 ± 12.9	—
L-Cysteine	10	21.4 ± 6.5	92
	1.0	200.4 ± 28.7	27
	0.1	273.6 ± 4.3	0*
L-Ascorbic acid	10	122.1 ± 9.1	56
	1.0	201.4 ± 9.9	27
	0.1	247.5 ± 5.0	10
Glutathione	10	126.1 ± 7.9	54
	1.0	211.4 ± 21.4	23
	0.1	246.8 ± 11.7	10*

The solutions of the above compounds were adjusted to pH of neutrality prior to use and added along with tryptamine (2 mM) to the otherwise complete reaction mixture already preincubated for 10 min and the assays were carried out under the standard conditions as described in the text. The values represent mean ± S.D. of eight (control) of four (inhibitors) separate determinations carried out in different mitochondrial fractions. Per cent inhibitions are significant to the level of $P < 0.001$ when compared to control value (excepting *). Other details are given in the text.

Table 3. Effects of NAD, NADH and ethanol on pigment formation from tryptamine in guinea-pig liver mitochondria

System	Pigment formed (μg/mg protein/hr)	% of the control
Control	256.7 ± 14.5	100
Control + NAD (0.5 mM)	245.1 ± 14.5	96*
Control + NADH (0.5 mM)	170.2 ± 11.0	66†
Control + ethanol (1%)	167.3 ± 11.5	65†

After preincubating the otherwise complete reaction mixture for 10 min, NAD, NADH and ethanol were added along with tryptamine (2 mM) and the assays were carried out under the standard condition as described in the text. Each value represents mean ± S.D. of four separate determinations carried out in different mitochondrial fractions. Other details are given in the text.

* $P > 0.2$ when compared to control.

† $P < 0.001$ when compared to control.

tonin is carried out by type A MAO while either or both types of MAO are perhaps involved in pigment formation when tryptamine is the substrate.

Reducing agents like cysteine, ascorbic acid and glutathione were found to cause considerable inhibition of pigment formation from tryptamine (Table 2). At 10 mM concentration, cysteine was found to inhibit the pigment formation almost completely, whereas inhibitions produced by ascorbic acid and glutathione were around 55%. However, at 1 mM concentration, all these compounds have nearly similar inhibition patterns (25%). It is reported that these reducing agents are capable of interacting with the aldehydes derived from biogenic amines thereby making the aldehyde unavailable for binding with protein macromolecules as well as participation towards other metabolic pathways [19, 20]. At the concentrations employed in the present study, these reagents were reported to be ineffective in inhibiting MAO [19] still possessing the capacity to interact with aldehyde molecules. Therefore, the inhibition of pigment formation in presence of these reducing agents indicates that aldehyde generated through the action of MAO is actively involved in the process of pigment formation.

Table 3 shows the effects of NAD, NADH and ethanol on pigment formation from tryptamine. It was found that NAD exerted insignificant effect on pigment formation while almost 35% reduction of pigment formation was observed in presence of both NADH and ethanol. The observed decrease in pigment formation in presence of either NADH or ethanol could perhaps be explained on the basis of the fact that in presence of both these agents, at least a part of indole-3-acetaldehyde, produced by the action of MAO on tryptamine, is utilized towards the formation of tryptophol [21, 22] thereby reducing the available pool of the aldehyde required for the formation of pigment. This is more evident because thin-layer chromatographic analysis of the ether extracted reaction products indicated that in control system tryptamine gives rise only to the cor-

Table 4. Pigment formation from tryptamine and indole-3-acetaldehyde under different experimental conditions

Systems	Substrate	Pigment formed (μg/mg protein/hr)
1. Complete system with enzyme	Amine	272.8 ± 13.6
2. Complete system with boiled enzyme	Aldehyde	95.3 ± 9.6*
3. Complete system with enzyme	Aldehyde	103.5 ± 7.5†
4. Complete system with boiled enzyme	Amine + aldehyde	133.5 ± 11.4‡
5. Complete system with enzyme	Amine + aldehyde	345.9 ± 12.2
6. Complete system with enzyme + clorgyline (0.1 mM)	Amine + aldehyde	130.8 ± 12.2

The complete system consisted of 25 mM phosphate buffer (pH 7.5), mitochondrial suspension containing 1.1 mg protein either boiled or as such, 2 mM tryptamine and/or free indole-3-acetaldehyde (~800–900 μmoles) in a final volume of 2 ml. After preincubating the otherwise complete reaction mixture for 10 min, the substrates were added as indicated above to initiate the reaction. The system containing boiled enzyme and tryptamine served as blank. Each value represents mean ± S.D. of six separate determinations carried out in different mitochondrial fractions. Other details are given in the text.

* $P > 0.1$ compared to system 3.

† $P < 0.01$ compared to system 6.

‡ $P > 0.7$ compared to system 6.

All other values are significantly different from each other ($P < 0.001$).

responding aldehyde, i.e. indole-3-acetaldehyde, whereas in the presence of both ethanol and NADH, tryptophol is produced (unpublished observation). Therefore, the observation that the reduction of aldehyde pool means reduction in pigment formation is another indication that aldehyde has an essential requirement in the process of pigment formation.

Consequently, it is of interest to assess how far the aldehyde can account for the total amount of pigment formed in the incubation medium. The formation of pigment from tryptamine and its immediate MAO catalysed oxidation product, i.e. indole-3-acetaldehyde either alone or in combination in presence of boiled or untreated guinea-pig liver mitochondria was studied and the observations are depicted in Table 4. In this experiment, calculated amount of aldehyde (~800–900 μmoles), generated from tryptamine during a 2-hr incubation at 37° under the present experimental conditions, was employed. The amount of pigment produced enzymatically from tryptamine is 272.8 ± 13.6 μg (system 1). When calculated amount of aldehyde was employed as substrate, the amount of pigment produced in presence of boiled tissue is 95.3 ± 9.6 μg (system 2) which increases only to 103.5 ± 7.5 μg when active tissue is used (system 3, $P > 0.1$ compared to system 2). Again, in presence of boiled tissue, the amount of pigment formed employing both the substrates, tryptamine and indole-3-acetaldehyde, was slightly but significantly higher than when aldehyde alone was the substrate (133.5 ± 11.4 μg, system 4 compared to 95.3 ± 9.6 μg, system 2; $P < 0.001$). However, in presence of active tissue, the respective amounts of pigments that were produced from aldehyde in presence and in absence of amine were 345.9 ± 12.2 μg and 103.5 ± 7.5 μg respectively under the present experimental conditions (systems 5 and 3). Again, the system which contained both the aldehyde and the amine, when supplemented with 0.1 mM clorgyline to block the enzymatic generation of any fresh aldehyde in the incubation medium, produced 130.8 ± 12.2 μg pigment (system 6) which is almost the same amount (133.5 ± 11.4 μg) that was obtained from a system containing both the substrates in presence of boiled tissue (systems 4, $P > 0.7$ compared to system 6). It appears, therefore, that the aldehyde produced enzymatically from tryptamine during a 2-hr incubation is not sufficient to account for the total amount of pigment formed in the standard incubation system. However, this value (system 2) although increases only slightly in presence of active tissue material (system 3), a marked increase in pigment formation was observed when the system contains both the substrates, tryptamine and indole-3-acetaldehyde and active enzyme (compare systems 2 and 3, $P > 0.1$ and systems 2 and 5, $P < 0.001$). The rate of pigment formation decreases almost in parallel when in the system (system 5) either clorgyline is supplemented (system 6) or active enzyme is replaced by boiled tissue (system 4). It is interesting to note that the sum of the amount of pigment formed in system 1 and 2 almost equals the amount produced in system 5, whereas neither the amount of pigment formed in system 2 nor the amount produced in system 4 can alone account for the amount of pigment produced either in system 5 or even in system 1. This indicates that generation of nascent aldehyde has perhaps greater contribution in pigment formation than that supplied to the system exogenously. Therefore, it may be concluded that indole-3-acetaldehyde, tryptamine and an active source of the enzyme MAO—all are intimately connected with the process of pigment formation.

However, pigment(s) formed in different experimental systems employing different starting materials may be chemically different in nature. Moreover, in presence of air, tissue materials, exposure to light at 37° for as long as 2 hr, it is very unlikely that indole-3-acetaldehyde, itself being highly unstable in nature [15] would remain as such in the system. Udenfreind *et al.* [23] and Nakai [11] considered a two step process of pigment formation, first being the conversion of amine to aldehyde while the second step consists of condensation or polymerization of aldehyde. However, a Pictet–Spengler type condensation between the aldehyde and the precursor amine molecule in the process of pigment formation is another possibility which, to the best of our knowledge, has thus far not been adequately explored. This is more because condensation of small aldehyde molecules like formaldehyde and acetaldehyde with indoleamines is reported to be feasible in biological systems [24, 25]. In fact, one of the components in pigments has been considered to be a β-carboline precursor type of compound [26]. Moreover, liver mitochondria themselves contain various oxidisable substrates and dehydrogenases and also addition of exogenous amine may help in the generation of excited state of O_2 which in its excited states as a singlet species or superoxide ion can produce further transformation of the reaction products [8, 27–29]. Other MAO catalyzed reaction products like hydrogen peroxide and ammonia may also have some influence on this process. Further work is in progress to characterize these steps in the process of pigment formation.

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