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MECHANISM OF MELANOPHORE DISPERSION—II.
STIMULUS-REACTION-RECOVERY AND RECEPTOR PROBLEM*

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Abstract—In a previous publication[1], similarity was shown to exist between the melanophore movement in the Hyla frog skin and various stimulus—reaction—recovery phenomena, such as muscle contraction, nerve firing etc. The main advantage of the melanophore reaction is that both the darkening and lightening process last about 30 min, while other stimulus reaction models take a fraction of a second. The lightening is, moreover, not necessarily coupled to the darkening.

The various agents inducing darkening and lightening have nothing in common structurally. Experimental evidence points to the important role played by a flavo-protein-regulated ATP-ase system which, according to our hypothesis, is the functioning unit of the "receptor".

METHODS AND MATERIALS

THE skin of the *Hyla arborea* (tree frog) was used in all experiments. The technique applied has been described in our previous publication.[1]

Colour changes were recorded with a reflectometer (Photovolt Corp., N.Y., Model 610), which was adapted to the size of the frog skin by an internal and external iris of 0·7 cm diam. White light was used throughout without any filter, and the international standard furnished by the manufacturer was adapted to our calibration. To increase sensitivity, the reflectometer was calibrated to the threefold scale extension of the international standard, thus 1 RV (reflectance value) used in this paper equals $\frac{1}{3}$ of the international reflectance standard (IRS). For measuring, wet filter paper was put on the external iris of the reflectometer's measuring head, with the frog skin covering it and the whole area of the iris. Reading began 3 min after starting the reflectometer, since it takes a few seconds for the scale reading to become stable.

For measuring ATP, 3 pieces of the back skin from 3 frogs were transferred into 5 ml boiling distilled water, left there for 5 min, homogenised when hot and again left for 5 min in boiling water. The mixture was filtered and its ATP content measured by the luciferin–luciferase method, using a Tri-Carb Liquid Scintillation Counter as quantum counter.[2] Simultaneously, protein was determined in the filtrate by a modified Folin method.[3]

To study the melanophore contraction (lightening), the skin was incubated at room temperature in a 10 mM theophylline solution, until it darkened to a RV of less than 15. To study melanophore dispersion (darkening) the skin was put for 10 min in 37° in frog Ringer solution.

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The agents and enzyme inhibitors studied were the following:

Substances promoting melanophore contraction: Dyphylline, sodium succinate, sodium hydrosulphite, fructose.

Substances promoting melanophore dispersion: MSH, theophylline, sodium ferricyanide, indigo carmine, menadione, sodium 3-hydroxyanthranilate, sodium thiooctate.

Substances inhibiting melanophore contraction: Amobarbital, antimycin, sodium benzoate, sodium fluoride, 2,4-dinitrophenol.

Substances inhibiting melanophore dispersion: 2,4-dinitrophenol.

Substances which did not significantly influence melanophore movement: The sodium salts of D,L-lactate, D,L-β-hydroxybutyrate, ketoglutarate, acetoacetate, acetate, malate, oxaloacetate and isocitrate.

RESULTS

A. Lightening of Frog Skin

1. *Lightening of theophylline darkened skin.* In our previous publication it was shown that dyphylline and noradrenaline at 10 mM concentrations are powerful lightening agents.[1] Sodium succinate of up to 3 mM concentration (with higher concentrations inhibition is observed) and fructose at 10 mM concentration may now be added to the list. Since electron transport inhibitors usually caused some lightening, sodium hydrosulphate at 1 mM was tried, and this caused rapid lightening of the skin. The effect of glucose was found to be erratic and weak.

The following sodium anions were found not to cause lightening at 1–10 mM concentrations: DL-lactate, DL-β-hydroxybutyrate, α-ketoglutarate, α-ketoglutarate + NH_3, acetoacetate, acetate, malate, oxaloacetate, isocitrate (Table 1).

TABLE 1. MELANOPHORE CONTRACTION OF ISOLATED FROG SKIN BY DIFFERENT AGENTS AFTER DARKENING THE SKIN WITH 10 MM THEOPHYLLINE

Substance	Concentration (mM)	Degree of melanophore contraction in RV*			
		0	10	20	30
			(min)		
Fructose	10	9	20	30	38
Na-succinate	1	10	20	30	40
Na-succinate	3	10	20	36	55
Na-succinate	10	10	16	22	26
Na-hydrosulphite	1	9	70	—	—
Frog Ringer	—	9	15	18	22

* Reflectance values (see Methods)

Lactate, β-hydroxybutyrate, ketoglutarate, ketoglutarate + 1 mM NH_3, acetoacetate, acetate, malate, oxaloacetate and isocitrate (all as sodium salts) did not differ from the controls at concentrations ranging from 1 to 10 mM.

Both succinate and hydrosulphite reduce the flavoproteins of the electron transport system.

2. *Inhibition of lightening.* Table 2 shows the effect of various inhibitors on melanophore concentration. Assays No. 1 and 2 show that the succinate effect (lightening) is inhibited by amobarbital (Amytal) and antimycin, indicating the importance of a

flavoprotein. The inhibition of succinate action by amobarbital is hardly surprising, since amobarbital inhibition persists in fresh mitochondria. It is, however, lost through washing or aging.[4] Moreover, the reduction of flavoprotein by succinate is amobarbital sensitive.[5]

TABLE 2. EFFECT OF INHIBITORS OF MELANOPHORE LIGHTENING AT 37°; EACH ASSAY REPRESENTS THE MEAN OF 3 EXPERIMENTS

No.	Lightening agent	Concentration (mM)	Inhibitor	Concentration (mM)	Inhibition %
1	Succinate	3	Amobarbital	10	80
2	Succinate	3	antimycin	0·02	100
3	Hydrosulphite	1	amobarbital	10	10
4	Dyphylline	10	amobarbital	3	10
5	Dyphylline	10	amobarbital	10	30
6	Dyphylline	10	antimycin	0·02*	10
7	Dyphylline	10	benzoate	10	90
8	Succinate	3	benzoate	10	10
9	Succinate	3	fluoride	10	0
10	Dyphylline	10	fluoride	10	100
11	None	—	fluoride	10	100
12	Dyphylline	10	dinitrophenol	1	0
13	Succinate	3	dinitrophenol	1	100

* This concentration causes only 10 per cent or less inhibition of the darkening action of theophylline.

Hydrosulphite and dyphylline lightening is insensitive to amobarbital (assay No. 3 to 6), which means that both agents affect the flavoprotein from the substrate side. Further justification of this assumption is obtained from assays No. 7 and 8. Benzoate which inhibits CoA in our test system (1), had no effect on succinate lightening, but completely inhibited the action of dyphylline. The same argument applies to assays No. 9 to 11, in which fluoride was used to poison glycolysis.

Hydrosulphite undoubtedly reduces flavoproteins, probably by anoxia,[6] the reduction being caused by the internal substrate. This phenomenon corresponds to the inhibition of the darkening action of MSH and xanthines by anoxia.[7] The fact that sodium benzoate and sodium fluoride inhibit lightening caused by dyphylline but not by succinate shows that the effect of dyphylline is probably due to an increased supply of a pyridine nucleotide reducing substrate, which requires CoA for activation.

The succinate reduction of the flavoprotein dehydrogenase requires ATP.[5] The ATP content of the frog skin averages 3×10^{-9} mol ATP/mg protein (see methods.) It is reduced by 90 per cent after 1 hr immersion in 1 mM dinitrophenol at room temperature. No effect on dyphylline lightening is observed after similar immersion, but succinate is almost totally inhibited (assays No. 12 and 13). Our finding that a 20 min incubation of the frog skin in 3 mM succinate decreases the ATP content by 40 per cent further substantiates the ATP dependence on the succinate action. Similarly, incubation of frog skin for 20 min in 10 mM dyphylline did not change the ATP content significantly.

These experiments make the hypothesis of flavoprotein reduction by succinate very probable and at the same time indicate that the action of dyphylline is not mediated through succinate.

B. Darkening of Frog Skin

3. *Classification of the active agents.* The darkening agents may be grouped into three categories:

(*a*) *Oxidising agents*

(*b*) *Substances which change the adenine nucleotide system coupling*—MSH, the xanthines, thiooctic acid, 3-hydroxyanthranilic acid and menadione (partially).

(*c*) *Enzyme poisons*, such as arsenate and fluoride could also be listed here, but they mainly inhibit lightening and have only a slight darkening effect.

The effect of materials in groups (*a*) and (*b*) is summarised in Table 3.

TABLE 3. DARKENING BY VARIOUS AGENTS AND THE EFFECT ON ATP CONTENT OF THE FROG SKIN. EACH ASSAY REPRESENTS THE MEAN OF 3 EXPERIMENTS

No.	Substance	Concentration (mM)	Darkening in 1 hr (RV)	Change in ATP content (%)†
1	Indigo carmine	1	20	0
2	Ferricyanide	1	15	−10
3	Menadione	0·1	30*	−40
4	Theophylline	10	50	+15
5	MSH	2 units‡	50	+60
6	Thiooctate	10	40	0
7	3-hydroxyanthranilate	1	30*	−60

* After 1 hr—no further darkening observed.

† Reliable within 10 per cent.

‡ See Ref. 7.

It appears that even oxidising agents with negative redox potential (assay No. 1) are active darkening agents; i.e. their site of action cannot be higher than at the flavoprotein level. Ferricyanide (assay No. 2) and menadione (assay No. 3) also pick up electrons from flavoproteins (8). Results again point to the regulatory role of a flavoprotein in the receptor.

More difficult to explain is the action of xanthines, MSH and thiooctate (assays No. 4, 5, 6). It is known from our previous work,[1] that their action is not influenced by sodium benzoate, which means that their action is not connected with substrate supply (see previous section). The following experiments further substantiate this hypothesis and at the same time point to the regulatory role of a phosphokinase. The effect of 3-hydroxyanthranilate (assay No. 7) will be discussed in section 6.

4. *Effect of sodium fluoride on darkening of frog skin.* Table 4 shows that 10 mM NaF has no marked influence on the darkening effect of the substances studied before (Table 3). This substantiates the independence of darkening from glycolysis.

5. *Effect of amobarbital on darkening of frog skin.* The effect of 10 mM amobarbital on various darkening agents is shown in table 5.

It will be seen that the effect of amobarbital on oxidants depends on where they pick up the electrons. Amobarbital acts as a blocker between the flavoprotein and the ubiquinones, so that the inhibition of menadione and ferricyanide can be expected (assays No. 1 and 2) (8). Assays No. 3 to 6, i.e. 3-hydroxyanthranilic acid, theophylline, MSH and thiooctic acid, are not influenced by amobarbital; probably because their effect is not mediated through the oxidised moiety of the flavoprotein.

Chapters 4 and 5, together with lack of inhibition by benzoate, as described in our earlier paper,[1] prove that the action of the group b materials mentioned in section 3 (b) as substances which change the adenine nucleotide system coupling, is not directly connected with flavoprotein.

6. *Effect of dinitrophenol on the darkening of the frog skin.* Incubation in 1 mM dinitrophenol for 1 hr irreversibly inhibits the action of all darkening agents, probably

TABLE 4. EFFECT OF 10 MM FLUORIDE ON MELANOPHORE DARKENING AT 25°. EACH ASSAY REPRESENTS THE MEAN OF 3 EXPERIMENTS

No.	Darkening Agent	Concentration	% Inhibition by 10 mMNaF
1	Ferricyanide	1 mM	30
2	Menadione	0·1 mM	10
3	3-Hydroxyanthranilate	10 mM	0
4	Theophylline	10 mM	0
5	Thiooctate	10 mM	0
6	MSH	2 units	0

TABLE 5. EFFECT OF AMOBARBITAL ON MELANOPHORE DARKENING AT 25° EACH ASSAY REPRESENTS THE MEAN OF 3 EXPERIMENTS

No.	Substance	Concentration	Inhibition by 10 mM amobarbital (%)
1	Ferricyanide	1	70
2	Menadione	0·1	50
3	3-Hydroxyanthranilate	10	0
4	Theophylline	10	0
5	MSH	2 units	0
6	Thiooctate	10	0

due to a lack of ATP (see section 2). In our view the difference between the uncoupling effect of 3-hydroxyanthranilic acid and DNP lies in the fact that the former influences ATP-ase more strongly or rapidly than it uncouples. In the presence of 10 mM hydrosulphite or 10 mM dinitrophenol slowly darkens the skin[9] but at aerobic conditions no colour change is observed.

DISCUSSION

Available data on the biochemistry of the melanophore reaction indicate that the drugs effecting melanophore contraction or dispersion have nothing structurally in common. Thus in our case, as in pharmacology in general, the structure-activity relationship holds good only for a limited number of drugs; having a particular pharmacological effect. The number of drugs having both a common structure and function may form a smaller or larger percentage of the drugs having that common pharmacological function, but will never constitute 100 per cent.

This being the case, a physiological chain of functions effected by all agents influencing melanophore movements, has to be sought. Since both adenine nucleotide and flavoprotein participation is strongly suggested, the following hypothesis is offered:—

Hypothesis for melanophore movement

The system responsible for melanophore movement is an ATP-ase structural protein complex of the membrane, controlled by the redox state of a flavoprotein (see Fig. 1 and its subunits).

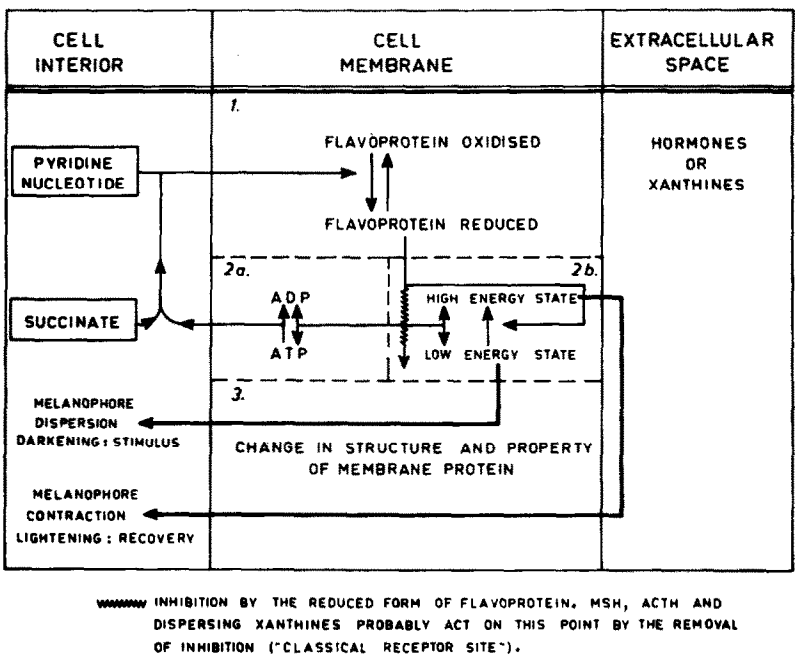

FIG. 1. Subdivisions of the hypothetical melanophore receptor system in the cell membrane. 1. Flavoprotein; 2. Energy storage; 3. Nucleotide subsystems.

The following mechanism is proposed for the activation of the darkening and lightening steps: Darkening occurs because of the loss of a high energy intermediate (unit 2 b) connected with the ATP system (unit 2a) and the structural protein (unit 3). Oxidation of the flavin (unit 1) thus induces darkening by removing the coupling inhibition of the phosphate potential (unit 2 b). Reduction of the flavoprotein (unit 1), on the other hand, decouples the phosphate potential[10] and at the same time restores the intermediate to its original high energy state (unit 2 b) resulting in lightening. According to our hypothesis darkening is thus an energy downhill process while lightening is an energy uphill process.[1] In general, stimulation is defined as a reaction which causes disappearance of high energy intermediates, and recovery as the resynthesis of the same intermediates.

The alteration in state of the cell membrane's structural protein induces permeability changes which in turn trigger the movement of the melanophore particles. This

scheme is thus very similar to the hypotheses put forward to explain mitochondrial volume changes.[9, 11]

We now propose to see how the available data on melanophore movement fit into this hypothesis.

Melanophore dispersion

We have shown that oxidising agents darken the frog skin, i.e. oxidise the flavin which in turn induces ATP synthesis; and, *vice versa*, drugs inducing ATP synthesis induce oxidation of the pyridine nucleotide flavoprotein system.[12, 13] Borrowing the terminology from mitochondria—where different stages are distinguished according to the availabliity of the substrate, ADP and oxygen—we can say that darkening agents induce oxidation similar to transition from stage 1 to stage 2, or from stage 4 to stage 3.[9, 14]

It is significant that neither the rate nor the degree of melanophore dispersion is greatly affected by temperature. In the presence of 10 mM NaF darkening by theophylline is the same both at 10° and 37°. Such behaviour is to be expected from the induction of an equilibrium shift from a high energy to a low energy compound, or a shift from the reduced form to the oxidised form. It is known that by lowering the temperatures, the equilibrium of lactic dehydrogenase is displaced in favour of the reverse reaction.[15]

Melanophore contraction

We have seen that agents inducing flavoprotein reduction induce lightening. This can be achieved by reverse electron transport induced by succinate, or by an increased supply of cytoplasmic-reduced pyridine nucleotide. In mitochondrial terms, this is a transition into stage 4.

Effect of cations on melanophore reaction

The last point requiring explanation is: where the lightening effect of K^+ and the requirement of Na^{+}[16] or Ca^{++}[1] for darkening fits into our scheme. It is most probable that the above mentioned ions influence the use of the high energy intermediate as was shown for the mitochondria.[9, 17]

Melanophore reaction and receptor theory

We shall now attempt to fit this hypothesis to the general problem of receptors.

The melanophore movement is only a particular instance of the stimulus-reaction-recovery sequence. Other reactions of this type, at least as well documented, are: the swelling of mitochondria,[8, 9] photosynthesis,[18] the contracture of muscle,[19] and spike production in nerves.[19] It is highly probably that glandular secretion is a similar phenomenon.[20]

Oxidising agents induce swelling of the mitochondria,[8] just as they induce melanophore dispersion. Muscle contracture and spike production are usually much less affected by lowering the temperature than are relaxation or positive after-potentials.[21, 19] This is similar to our findings on the melanophore movement. Rapid spectro-photometric measure of intact muscle and nerve during activity shows 'mitochondrial' state 4 to state 3 transition in accord with our hypothesis (see discussion on melanophore dispersion) [22].

A highly significant clue is that the systems seen in Fig. 1 are dependent for their activity on the presence of phospholipids.[23] It has been shown that the activity of serotonin is dependent on gangliosides[24] and we have found that acetone extracted muscles can be fully resensitised to the stimulative action of acetylcholine and noradrenaline by short incubation with phospholipids.[25] In this connection it should be noted that in the cases cited, the phospholipids fulfilled a functional task and did not act merely as a barrier.[23, 25]

The question now is whether the pharmacological term "receptor" may be supplemented by a biochemical expression, e.g. flavoprotein regulated ATP-ase-structural protein complex. If so, it may be asked how we can account for the well known specificity of the receptors.

The phospholipid microclimate could easily account for the dependence of drug activity on the hydrophilic-lipophilic balance.[26] It is significant that different primary dehydrogenases have been shown to have a different hydrophilic-lipophilic balance.[27, 28] Different flavoproteins may, therefore, constitute the differentr eceptors. On the other hand, it is also possible that agonists interact with the receptors in more than one way, and that different patterns of interaction represent different agonists and antagonists.

Further, we believe that similar mechanisms are responsible for active transport as well, and that the transport ATP-ase, so much in the fore at present, which is connected with the structural protein,[29] is intimately connected with a redox system.[30–32]

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REFERENCES

1. S. DIKSTEIN and F. G. SULMAN, *Biochem. Pharmac.* **13**, 819 (1964).
2. E. TAL, S. DIKSTEIN and F. G. SULMAN, *Experentia*, **20**, 652 (1964).
3. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALE, *J. biol. Chem.* **193**, 265 (1951).
4. A. M. PUMPHREY and E. R. REDFARN, *Biochem. biophys. Res. Commun.*, **8**, 92 (1962).
5. L. ERNSTER, *Fifth Int. Congr. Biochem. Moscow*, **5**, 115 (1961).
6. B. CHANCE, *J. biol. Chem.* **236**, 1544 (1961).
7. A. B. LERNER and Y. TAKAHASHI, *Rec. Progr. in Horm. Res.*, **12**, 303 (1956).
8. F. E. HUNTER, Jr. *Fifth Int. Congr. Biochem. Moscow*, **5**, 287 (1961).
9. L. PACKER and A. L. TAPPEL, *J. biol. Chem.*, **235**, 525 (1960).
10. CH. L. WADKINS, *Sixth Intern. Congr. Biochem., New York*, X-S.14 (1964).
11. J. C. ARCOS and M. F. ARGUS, *Sixth Intern. Congr. Biochem. New York*, VIII-2 (1964).
12. M. KLINGENBERG and P. SCHOLLMEYER, *Fifth Intern. Congr. Biochem., Moscow*, **5**, 46 (1961).
13. B. CHANCE and M. BALTSCHEFFSKY, *Biochem. J.*, **68**, 283 (1958).
14. B. CHANCE and G. R. WILLIAMS, *Adv. Enzymol.* **17**, 65 (1956).
15. M. T. HAKALA, A. J. GLAID and G. SCHWERT, *J. biol. Chem.* **221**, 191 (1956).
16. R. R. NOVALES, *Am. Zool.* **2**, 337 (1962).
17. B. C. PRESSMAN, *Bichem. biophys. Res. Commun.* **15**, 562 (1964).
18. D. I. ARNON, *Fifth. Int. Congr. Biochem. Moscow*, **6**, 201 (1961).
19. A. M. SHANES, *Pharmac. Rev.* **10**, 59, 165 (1958).
20. G. W. KIDDERN, P. F. CURRAN and W. S. REHM, *Fed. Proc.* **23**, 114 (1964).
21. H. LORKOVIC, *Arch. Int. Pharmacodyn.*, **146**, 266 (1963).
22. B. CHANCE and C. M. CONNELLY, *Nature, Lond.* **179**, 1235 (1957).
23. S. FLEISCHER, *Sixth Intern. Congr. Biochem., New York*, VIII-S 2 (1964).
24. D. W. WOOLLEY and B. W. GOMMI, *Nature, Lond.* **202**, 1074 (1964).
25. S. DIKSTEIN and F. G. SULMAN, *Biochem. Pharmac.*, in press.
26. S. DIKSTEIN in *Quantitative Methods in Pharmacology*, North Holland Publ. Co., p. 312 (1961).

27. H. C. Henker, *Biochim. biophys. Acta*, **81**, 9 (1964).
28. H. Baltscheffsky and M. Baltscheffsky, *Sixth Int. Congr. Biochem., New York*, X-4 (1964).
29. R. Whittam, K. P. Wheeler and A. Blake, *Nature, Lond.* **203**, 720 (1964).
30. M. V. Riley and A. L. Lehninger, *J. biol. Chem.* **239**, 2083 (1964).
31. H. Passow, A. Rothstein and B. Loewenstein, *J. gen. Physiol.* **43**, 97 (1959).
32. S. Dibella, *Acta Vitamin. (Milano)* **18**, 49 (1964).