Table II. Adoptive transfer of antinuclear autoimmunity

Antinuclear antibody titre of the donors	8		64		512		1024		1024		1024		1024	
Recipients 1 month old thymectomized at birth	3	4	1	2	5	6	7	8	9	10	11	12	13	14
No. of days after transfer														
8	8	4	2	0	8	8	4	2	4	8	0	8	2	0
15	64	8	2	0	8	64	64	2	8	8	dead	64	2	0
25	2058	8	0	0	8	dead	64	0	4	8		64	0	0
55	4096	8	0	0	dead		64	dead	dead	dead		dead	dead	0
75	4096	0	0	0			dead							0

The spleen was collected from a CF1 animal thymectomized at birth, in which antinuclear antibodies had appeared at the end of several months. The spleen was cut into 4 fragments of equal size. Each of these fragments was implanted into the peritoneal cavity of 1-month-old CF1 mice. Two of these recipient animals were normal and served as controls, the other 2 were mice thymectomized at birth. The antinuclear antibodies were determined in the recipients at the end of 8 days, and then periodically as indicated in Table II. Seven CF1 mice thymectomized at birth with antinuclear antibodies served as donors. 28 mice of 1 month of age, 14 of them thymectomized at birth, were used as recipients.

It is seen (Table II) that 11 of the recipient animals thymectomized at birth had antinuclear antibodies from the 8th day of implantation onwards, sometimes at high titres, whereas none of the 14 controls showed a positive reaction. Adoptive transfer of antinuclear autoimmunization was therefore possible only in animals thymectomized at birth. How can these results be interpreted? The antibodies found in the recipient mice will originate from adoptive transfer and not from autoimmunization of the recipient, which in thymectomized animals can develop only after the third month. It seems that transferred spleen cells can multiply or continue to secrete antinuclear antibodies only if the recipient is thymectomized. Thymus seems to have an inhibiting effect on the autoimmune reactions. Another possibility is that the grafted cells multiply easily in the thymectomized animals by occupying depopulated thymus-dependent zones in the animals thymectomized at birth. This possibility cannot be rejected, but the results of late thymectomy of the CF1 mice combined with those of adoptive transfer experiments appear to favour the hypothesis of a regulatory effect of the thymus on autoimmune reactions.

The high number of deaths among the 14 recipient thymectomized animals may seem surprising and lead us to think that these mice died from a graft-versus-host reaction in relation to a minimum antigen difference in the inbred CF1. In fact, the percentage of deaths among those animals is the same as that we obtained in a previous study[4] with CF1 thymectomized at birth.

Recipient animals died with symptoms of wasting disease, which is not surprising since these mice had been thymectomized at birth and the spleen cells, which had been transfered to them, were of not use to prevent the wasting disease since these cells came from animals thymectomized at birth[10].

Résumé. La thymectomie chez des souris CF1 âgées de 1 mois est suivie comme dans le cas de la thymectomie néonatale de l'apparition d'anticorps antinucléaires. Le transfert adoptif de l'autoimmunité antinucléaire apparu chez des animaux thymectomisés à la naissance n'est possible que si les animaux receveurs sont eux-mêmes thymectomisés. Ces constatations mettent en évidence un rôle possible de contrôle des réactions autoimmunes par le thymus.

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Hygenic Laboratory of the Medical Faculty of Lyon, Lyon 8e (France), 20 November 1969.

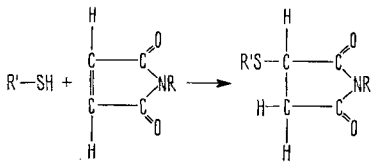
[10] With the technical cooperation of Mrs. D. TILLE.

Comparative Effects of Sulfhydryl Inhibitors on Melanosome Movements within Vertebrate Melanophores

Sulfhydryl groups have been implicated in the regulation of both melanosome (melanin granule) movements[1,2] and the process of melanogenesis[3,4]. The sulfhydryl inhibitors mersalyl[2,5] and N-ethyl maleimide[2] cause in vitro darkening of the skin of the lizard[2] and the frog[5] by dispersing melanosomes within dermal melanophores. Other sulfhydryl inhibitors such as parachloromercuribenzoate, iodoacetamide, and mercuhydrin also are said to darken frog skins and, in addition, are said to inhibit MSH activity[1]. Because these agents appear to mimic the action of melanophore-stimulating hormone (MSH) on these pigment cell organelles it has been suggested that sulfhydryl groups may play an important role in the mechanism of action of MSH[2]. We have further investigated the effects of sulfhydryl inhibition on both melanosome dispersion and aggregation. In the present communication we compare the effects of a number of closely related sulfhydryl inhibitors and clarify the structural requirements necessary for such inhibition.

Methods and materials. The effects of sulfhydryl inhibitors on melanophore responses of the frog, *Rana pipiens*, and the lizard, *Anolis carolinensis*, were studied,

in vitro, utilizing the photoreflectance method as originally described for the frog skin bioassay for melanophore-stimulating hormone (MSH)[6]. In this assay the movement of melanosomes within dermal melanophores in response to hormonal or pharmacological stimulation results in a



lightening (melanosome aggregation) or darkening (melanosome dispersion) of skin which can be measured, in vitro, as reflectance changes from the outer epidermal surface of the skin. Details of our experimental procedures have been described previously for the frog[7] and the lizard[8].

The following sulfhydryl agents were obtained from Nutritional Biochemicals Corp.: N-methyl maleimide, N-ethyl maleimide, N-butyl maleimide, N-phenyl maleimide, N-cyclo hexyl maleimide. Maleimide and succinimide were obtained from Aldrich Chemical Co. and maleic acid was obtained from Sigma Chemical Co. The porcine α-MSH and β-MSH were obtained from Dr. Aaron B. Lerner; this MSH was lyophilized with lactose as carrier[9]. All concentrations of experimental agents are expressed as the final concentration after addition to the skins. All agents were put into solution immediately prior to their intended use in an experiment.

Results. In the first experiment using the lizard, *Anolis carolinensis* skins were incubated for 30 min in Ringer solution containing maleimide or other N-substituted maleimides. One group of skins was maintained in Ringer solution as a control and 2 other groups of skins were placed in Ringer containing either succinimide or maleic acid. After 30 min, super-maximal concentration (5×10^{-9} g/ml) of α-MSH was added to the Ringer control skins to determine their maximal darkening response to this hormone. The darkening response of the skins to the experimental agents (solid black bars) is expressed as a percentage of the darkening induced by MSH which is considered as 100%, and the standard errors are indicated (Figure 1). Although all the maleimides caused a profound darkening of the skins, neither succinimide nor maleic acid had such an effect. The darkening of *A. carolinensis* skin in response to the maleimides was irreversible as they did not relighten when rinsed a number of times in Ringer solution whereas the MSH-darkened skins did relighten to their pre-darkened level (lined bars in Figure 1) when returned to Ringer solution in the absence of MSH.

In the second experiment we used skins from the frog, *Rana pipiens*. The skins were subjected to the same experimental agents as in the last experiment (except maleic acid) for a 60-min time period, as the response of frog skin melanophores to hormonal or pharmacological agents is slower than that of the lizard[7]. All of the maleimides caused some slight darkening of the skins which was quite minimal compared to that induced by MSH (Figure 2). Succinimide had no darkening effect on the skins. All skins were then rinsed for another 60 min with several changes of Ringer solution. None of the skins darkened by the maleimides relightened, but the MSH-darkened skins returned to a near pre-MSH reflectance value. The addition of MSH (5×10^{-9} g/ml) redarkened the control skins to their previous MSH-darkened value by 60 min. In contrast, the skins previously immersed in the maleimide solutions failed to respond to MSH. The skins previously immersed in succinimide, however, darkened to an even greater degree than did the MSH controls.

In other experiments using both *A. carolinensis* and *R. pipiens* skins we have found that both the rate and the degree of melanosome dispersion and, hence, the darkening of the skins, depends upon the concentration of the sulfhydryl agent used. In *R. pipiens* we can sometimes induce a greater degree of darkening with N-ethyl maleimide by decreasing the concentration of this agent. We can block MSH darkening in *A. carolinensis* by using lower concentrations of N-ethyl maleimide which itself

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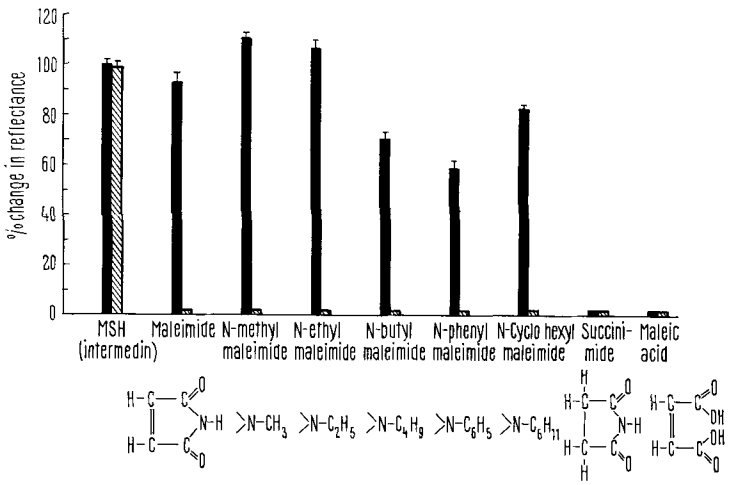

Fig. 1. Darkening response of lizard, *Anolis carolinensis*, skins to maleimide, other N-substituted maleimides, succinimide, and maleic acid, compared to the darkening of skins to α-MSH (5×10^{-9} g/ml) considered as 100%. All experimental agents at a $10^{-3}M$ concentration. Solid bars represent the darkening of skins whereas the lined bars represent the percent relightening of the darkening response. Structures of the various experimental agents are given. Results are the means of the reflectance measurements from the 8 skins which comprise each experimental group. Vertical lines represent the standard error of the means.

only slightly darkens the skins. We have obtained quite similar results as those described above using the sulfhydryl agent, mersalyl (Salyrgan). Although mersalyl, like N-ethyl maleimide, will cause a maximal and irreversible darkening of *A. carolinensis* skins[2] when used at low concentrations that barely darken the skins, this agent will totally block the melanosome dispersing action of MSH (Figure 3). In addition, mersalyl causes a rapid, maximal, and permanent reversal (lightening) of MSH-darkened skins (Figure 3).

Discussion and conclusions. The results clearly reveal that sulfhydryl inhibitors are potent melanosome dispersing agents and in that respect mimic the action of MSH. Of additional importance is the observation that these agents block the action of MSH. In addition, mersalyl can cause a rapid and complete reversal of maximally-darkened MSH-treated skins. Although HOROWITZ[2] noted that mersalyl and N-ethyl maleimide darkened *A. carolinensis* skins, he did not attempt to block MSH action. Although other sulfhydryl agents such as parachloromercuribenzoate, iodoacetamide and mercuhydrin have been reported[1] to darken frog skins and to inhibit MSH activity, no experimental data was provided to support such a suggestion.

The present study is the first to clearly define the structural requirements for sulfhydryl effects of maleimides on melanophores. It would appear that the nature of the N-substitution is unimportant for sulfhydryl inhibition in that all N-substituted maleimides darkened the skins and also blocked MSH action. Because succinimide differs from maleimide only by the absence of the C=C bond, it would appear that this bond is essential for establishing blockade. It has been suggested[10] that the unsaturated imides react rapidly with thiol groups under physiological conditions and that the C=C bond is involved in the following reaction:

It was also suggested[10] that a demonstration of a failure by succinimide to duplicate any of the actions of maleimide would insure that the effects of maleimide were related to its specific ability to react with thiols. Such a demonstration has been provided in our experiments as reported here. The present experiments have clearly implicated a reaction between maleimide and its N-substituted analogs and thiol groups associated with melanophores of both the frog and the lizard. The failure of maleic acid, considered to be a sulfhydryl inhibitor[10], to mimic the maleimides would suggest that the integrity of the 5-membered ring in combination with the C=C bond may be of importance and responsible for the instability and consequent reactivity of the C=C bond.

The normal physiological dispersal and perinuclear aggregation of melanosomes within *A. carolinensis* melanophores in response to endocrine stimulation are very rapid events. The ability of sulfhydryl inhibitors such as the maleimides and mersalyl to similarly evoke such rapid responses, either melanin granule dispersion or aggregation, might suggest that these agents are directly affecting the mechanisms by which MSH mediates its action. The irreversibility of the effects of the sulfhydryl inhibitors, which is characteristic of such agents[10], suggests that an MSH-sulfhydryl interaction is involved in melanophore regulation, as originally suggested by HOROWITZ[2] for *A. carolinensis*[11].

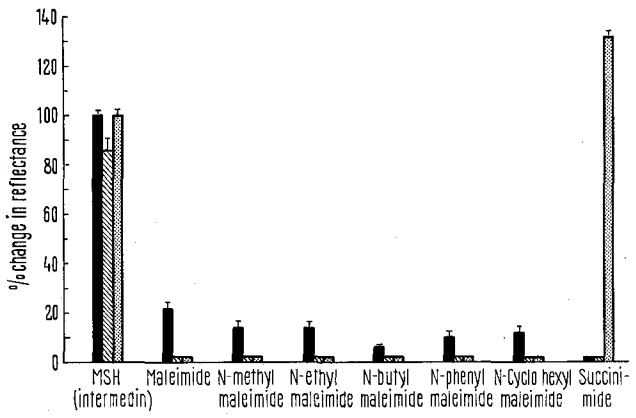

Fig. 2. Response of frog, *Rana pipiens*, skins to sulfhydryl inhibitors, as in Figure 1. (Maleic acid was not used in this experiment.) All experimental agents at a $10^{-3}M$ concentration. This experiment illustrates the blockade of MSH (5×10^{-9} g/ml) darkening by the thiol inhibitors. Note failure of succinimide to block such a response. For each agent, the solid bar on the left represents the darkening response relative to MSH darkening taken as 100%. The lined bars represent the percent relightening of the previous darkening response. The open bar on the right in each group represents the second darkening response after all skins were washed for 1 h after previous exposure to experimental agents and then restimulated by MSH.

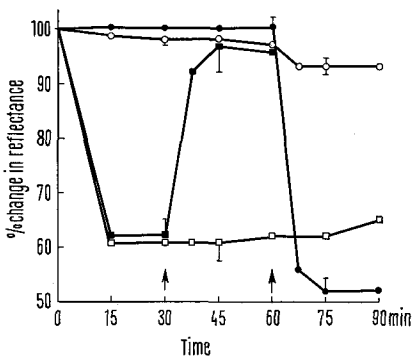

Fig. 3. Reversal and inhibition of MSH action by mersalyl on *Anolis* skins. 2 groups of skins were darkened by β-MSH (2×10^{-9} g/ml) for 30 min and then mersalyl ($10^{-4}M$ final concentration) was added (arrow) to the skins of one of these darkened groups (■) and the other (□) was allowed to remain as a control. 1 group of skins (○) was incubated in the same concentration of mersalyl for 60 min and 1 group of skins (●) was maintained as a Ringer control for the same time period. At 60 min (arrow) β-MSH (2×10^{-9} g/ml) was added to the skins in each of these groups (● and ○). Each point on the graph is the mean of 8 measurements of reflectance. Vertical lines the represent the standard error of the means.

Résumé. Dans les mélanophores des grenouilles et des lézards examinés in vitro, les inhibiteurs du groupe sulfhydryl (thiol) tels que le mérsalyle et les maléimides N-substitués bloquent irrévocablement la dispersion des mélanosomes soumis à l'action de la MSH et provoquent même leur retrait. On étudie les conditions structurales de l'action inhibitrice d'un certain nombre de maléimides N-substitués, étroitement apparentés.

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[11] This study was supported by grant No. GB-8347 from the National Science Foundation.