ethanol. The ethanol extract was then used for the succeeding chromatograms. Fig. 1 shows the absorption spectra of the materials and of authentic acetyl hydralazine as determined on the Beckman DU spectrophotometer. The extract from the rat liver homogenate was carried through the same purification procedure; however it still exhibited appreciable absorption below 230 mμ. But in the range 240-250 mμ it gave an identical spectrum.

In none of the extracts was free hydralazine detectable. When the extracts were evaporated to dryness, taken up in 1 N HCl and allowed to hydrolyze on the steam bath for an hour, it was possible to demonstrate free hydralazine.

TABLE I. R_f Values of Acetyl Hydralazine and White Fluorescent Zone from Ethyl Acetate Extracts.

Solvent	Acetyl hydralazine	White fluorescent zone from: Guinea pig urine	Pigeon liver	Rat liver
22% isopropyl alcohol	.74	.72	.73	.72
60% *idem*	.85	.86	.84	
Water	.64	.62	.59	.60
2.5 N acetic acid	.81	.81	.82	
10 N *idem*	.86	.85	.89	.89
n-Butanol: 0.6 N NH_4OH (6:1)	.90	.88	.88	.89
20% $CHCl_3$: 80% n-butanol (water saturated)	.00	.00	.00	.00

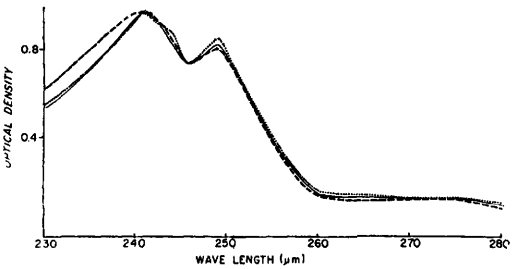

FIG. 1. Ultraviolet absorption spectra of authentic acetyl hydralazine (solid line), white fluorescent material from pigeon liver (dotted line), and guinea pig urine (dashed line).

From the above experiments, and from our previous(1) observations that for appreciable hydralazine disappearance from tissue extracts all the components of the metabolic acetylation system must be present, it seems evident that acetyl hydralazine is a major metabolic product of hydralazine.

Summary. 1-Acetyl-2-phthalazyl hydrazine (acetylhydralazine) has been identified as a metabolic product of hydralazine in the rat, guinea pig, and pigeon.

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Action of Biogenic Amines, Amine Oxidase Inhibitors, and Other Agents on Chromatophores of Squid, *Loligo pealii*.* (24658)

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Regulation of smooth muscle-chromatophore system in the squid involves neurohumoral mechanisms which bear certain points of resemblance to smooth muscle systems in higher forms. Chromatophores of cephalopod molluscs have been extensively studied since the original work of Phisalix(1), but primarily for their own intrinsic value. Particular attention has been given to central neural controls. The purpose of the present experiments was to study local control of smooth muscle-chromatophore system in the squid, *Loligo pealii*. Particularly the action on this system, of biogenic amines known to be effec-

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tive in higher forms as mediators of smooth muscle reactivity. Components of chromatophore system have the advantage of being easily accessible to treatment and observation in living, non-narcotized animals. The data clearly indicate that work with this preparation may shed light on problems of smooth muscle function in general.

Material and methods. Chromatophores of cephalopod molluscs(2,3) are pigment containing cells controlled by smooth muscles, which in turn are under the influence of animal's nervous system. When smooth muscle fibers contract, the cells expand and the animal darkens. Relaxation of muscle allows the cells to return to their initial, tiny, spherical shape, and the animal acquires a blanched appearance. *Loligo* has red, yellow, and brown chromatophores, the latter predominating(3). The skin is attached to the underlying mantel muscle by a dense adhesive layer. Injections made under the skin actually deposit the fluid between this adhesive layer and the mantel muscle. The injected material forms a circumscribed bleb which remains localized for a considerable time. Although the chromatophore bearing layer alone has been referred to as "skin," we shall refer to such injections as "subcutaneous." Sereni(4) has shown that chromatophores of cephalopods are controlled by separate excitatory and inhibitory centers in the ganglia in the head of the animal. These centers were found to exercise their control *via* nerves to the skin. Bozler(3) brought forth evidence for double innervation of muscle fibers, one innervation being excitatory (tetanic) and the other inhibiting tonus. He also presents microscopic evidence(5) that muscle fibers contain 2 types of fibrils. He assigns to one set of fibrils the function of ordinary contraction, and to the other the function of maintaining the fiber in a protracted contraction or tonus. In the present experiments, the squid were placed in confining glass bowl, slightly smaller than their fully extended length. The bowl was filled with sea water to a depth sufficient for animal to breathe, but exposing its convex dorsal surface to the air. The temperature had previously been dropped to about 10°C by placing the bowl in an ice bath. While experiments progressed, the water usually cooled further, to a minimum of about 5°C. Under these conditions, chromatophores of the squid generally dilate of their own accord, a condition essential for study of constricting substances. Test substances were dissolved in filtered sea water. The factor of pH will be discussed later. Four to 6 injections were administered subcutaneously to local areas along one side of exposed dorsum of animal, using 1 ml syringe and 24 gauge needle. A total volume of 0.2 ml was injected at each spot. Control injections of filtered sea water were made at locations symmetrical with those of experimental spots. Serotonin, as creatinine sulfate, was obtained from Sandoz Pharmaceutical Co. Iproniazid and Ro-5-0700 were kindly supplied by Hoffmann-La Roche Inc. and PIH by Lakeside Labs.

Results. Table I indicates substances injected into the squid, dose range used, and effects of injection. It should be kept in mind that closure of chromatophores represents relaxation of muscle and opening of chromatophore contraction of the muscle.

In vivo. A. *Agents which can open chromatophores.* Since it is well known that electrical stimulation of the skin, and acetylcholine (Ach) topically applied(3,4,5) cause chromatophores to open, these agents were used to test reactivity of the preparation. A 3 volt current with duration of 5 milliseconds, and with frequency of 60/sec. kept chromatophores of an area approximately one inch in diameter in open state during period of stimulation. The acetylcholine effect was most striking on a blanched squid, blanching being best obtained by cutting off the head of the squid. Injections should follow rapidly thereafter. Sea water alone causes a mild dilation in 27 out of 57 cases. The percentage of acetylcholine injections resulting in dilation is, however, much greater (48 out of 64 cases). In addition, dilation was considerably more intense with Ach than with sea water. The data are statistically significant, well within the 1% level of confidence when chi square test of significance is applied, using results from control injections as the expected results for the experimental series. Acetylcholine was effective in doses of 0.2 μg/ml or

higher. These doses are at least 1000 times greater than values reported by other investigators. The different results may be accounted for by factors such as makeup of diluent medium, method of application, and particularly order differences. It may be significant that this order of cephalopod (especially the species used here) is unusually sensitive to trauma as contrasted with the octopus.

B. *Agents which close chromatophores.* (1) *Amines.* Kahr(6) reported that in the octopus, topical application of 5-hydroxytryptamine (serotonin) closed the chromatophores. This amine is known to have varied effects upon nerve-smooth muscle systems in general (7). In *Loligo,* serotonin causes blanching of skin when administered subcutaneously. However, the effective dose range was approximately 1000 times greater than that reported by Kahr(6). Sereni(4) reported that tyramine, administered to the octopus intravenously, caused opening of chromatophores, presumably by an effect on ganglionic centers controlling chromatophore activity. In our experiments, tyramine HCl, when injected subcutaneously, closes the chromatophores, an action diametrically opposite to its reported central effect. Tryptamine is reported by Woolley and Shaw(8) to have a serotonin-like effect in other systems. This was substantiated in the present preparation where it was found to close the chromatophores.

All of the active constricting agents (amines, inhibitors, indoles, etc.), except serotonin, cause the skin site to blanch to a white or near white color, in contrast to surrounding brown area. Although in general the area of blanching decreases in size with lower doses, minimal effective doses occasionally cause disproportionately large, pale spots. Occasionally, several minutes after constriction, scattered chromatophores open in the otherwise constricted area.

The responses to serotonin seemed qualitatively different from the others, in that the areas were uniformly pale but not white. The same response was obtained irrespective of whether the dose was 20 μg/ml or 2000 μg/ml of serotonin. Other agents cause skin areas to become white, due to closure of all chromatophores in the area. Such a reaction develops only occasionally with serotonin. More commonly, after serotonin, the red and brown chromatophores remained open, but the spaces between them paled considerably, losing their normal, yellowish cast. This yellow color is due to yellow chromatophores which are difficult to see as distinct entities in *Loligo,* since they are quite small. It is probable that the characteristic pallor following serotonin treatment, is due to a constriction chiefly of the yellow chromatophores.

(2) *Amine oxidase inhibitors.* The above experiments suggest that an amine might be the local mediator affecting closure of chromatophores. Assuming that such an amine was continually being destroyed by an amine oxidase, administration of an amine oxidase inhibitor should bring about the same effect as addition of the amine. Three inhibitors of monamine oxidase were used: PIH, iproniazid, and Ro-5-0700. All 3, as anticipated, cause chromatophores to close down. As can be seen in Table I, iproniazid was the least effective in this respect. This corresponds with the reported monamine oxidase inhibitory activities of these 3 compounds(9).

(3) *Indoles.* In addition to being an amine, serotonin contains an indole ring. LSD 25 is one of the indoles extensively studied with respect to serotonin-mediated systems. The brom derivative of this compound was tried by Kahr(6) on octopus chromatophores and he states that " . . . brom LSD opposes the effect of serotonin . . ." We found in the squid that LSD 25 produces the same effect as serotonin—*i.e.,* it closes chromatophores. However, LSD 25, although active, required much higher concentrations than serotonin.

(4) *Competitors for choline esterase.* Eserine sulfate was injected and had an effect only in large doses. Under such circumstances, instead of producing an Ach-like effect it closes the chromatophores.

(5) *Autonomic blockers.* Since amines and Ach are both believed to be important mediators of the autonomic nervous system in mammals, it was of interest to observe the effects of an autonomic blocking agent, chlorpromazine, on squid chromatophores. The drug closed the chromatophores.

TABLE I.

Agent	No. of exp.	Dose range* (μg/ml)	Effect
In vivo			
Electrical stimulation	20	5 volts	opens
Cholinergic			
Acetylcholine chloride	64	.2–20	"
Competitors for acetylcholine esterase			
Eserine sulfate	33	1000–2000	closes
Autonomic blockers			
Chlorpromazine	17	250	"
Nerve blockers			
Procaine HCl	8	1%–4%	"
Monamines			
5-OH-tryptamine (serotonin)	40	.2–2000	"
Tryptamine HCl	21	200–2000	"
Tyramine HCl	20	2500–4500	"
Catechol amines			
Epinephrine	10	100	*none*
Norepinephrine	23	100–200	"
Inhibitors of monamine oxidase			
1-isopropyl-2-isonicotinyl hydrazine (iproniazid)	36	1000–2000	closes
para-isopropyl hydrazine (PIH)	26	200–4500	"
2-benzyl-1-picolinyl hydrazine (Ro-5-0700)†	20	600	"
Amino acids			
L-lysine	6	2000	*none*
L-alanine	8	4000	"
Serotonin precursor			
5-OH-tryptophan	15	20–2000	"
Indoles			
LSD 25	15	100–2000	closes
Diamines			
Histamine dihydrochloride	5	.0002–20	*none*
In vitro			
Electrical stimulation	20	5 volts	opens
Acetylcholine	10	20	*none*
Histamine	6	20	"
Serotonin	15	.2–20	closes

* Low end of range represents dose of minimal effectiveness. Only 0.2 ml is inj. in single location.

† Dissolved in propylene glycol and administered as one part propylene glycol to 9 parts sea water.

(6) *Miscellaneous biogenic substances.* The amines mentioned above are all monamines, and the inhibitors were inhibitors of monamine oxidase. Adrenaline and noradrenaline are monamines with no effect, the former in doses up to 100 μg/ml. A diamine, histamine dihydrochloride, was tested in doses similar to those of the active compounds, without any effect on the chromatophores. A precursor of serotonin, 5-hydroxy-tryptophan(7) was also administered in comparable dose ranges and had no effect. Two monamines, L-lysine and L-alanine, not known to affect nerve-muscle preparations, were used to rule out a non-specific property of amines in general, or osmotic phenomena, and were without effect on the chromatophore system.

(7) *Interaction of oppositely acting agents on chromatophores.* Chromatophores opened by acetylcholine could be closed by subsequent subcutaneous administration of serotonin. The best results were obtained when equivalent doses of both agents were used. On the other hand, it was found that an electric stimulus of 5 volts, with duration of 5 msec. and frequency of 60/sec, failed to open chromatophores that had been closed with the above agents. The only possible exception to this finding occurs in serotonin-treated areas. As previously indicated, serotonin affected chiefly the yellow color. The chromatophores in such areas dilated upon electrical stimulation, to the extent that spaces between them disappeared. The resulting color is a rich brown, presumably due to combined effect of open red and brown chromatophores. This circumstance obscures any yellow color that might actually reappear upon such stimulation. To interpret the failure of treated areas to respond to electrical stimulation, it was necessary to determine whether electric stimulation normally dilates chromatophores because of an effect on muscle fibers directly, or because of stimulation of peripheral nerves. Bozler(3) reported that the effects of such stimulation are due to excitation of peripheral nerves. We attempted to corroborate this idea by using procaine, an agent known to block nervous conduction. Procaine HCl (1-4%) was injected into the skin in the usual manner and caused chromatophores to close. The chromatophores were then unreactive to electric stimulation.

(8) *Effects of pH.* Inasmuch as most substances used in our study were acidic in nature, it was necessary to rule out the effects of

pH *per se* on the reactions observed. The pH of sea water varies from 7.6 to 7.8. The different substances were tested, not only at pH they naturally assumed in sea water, but also after pH was brought to that of sea water (7.6-7.8), by adding small amounts of 0.1 *N* NaOH. The results at pH 7.6-7.8 were the same as those at lower pH values. The effects of pH alone were tested by injecting sea water acidified with HCl. Chromatophores are not sensitive to pH changes over a wide range(5-8). Lowering the pH on the acid side as far as 4, has no visible effect. Below pH 4, injections begin to cause constriction though this is not consistent until a pH of 3 is reached. These observations agree with those of Hill(10) who measured photoelectrically rapidity of response to a dilating electric stimulus. He showed no slowing of muscular response until pH was lowered below 5.

Chlorpromazine, LSD 25, or iproniazid could not be neutralized by this method without precipitate formation. Chlorpromazine solutions were at an unadjusted pH of 6.7 and the LSD was at an unadjusted pH of 6.5. Since this range of pH by itself had no effect, and since raising the pH of other agents did not alter their effects, we believe that the responses to LSD 25 and to chlorpromazine are probably not pH-dependent.

Injections of HCl ranging from pH 7 to 4 had no effect on chromatophores. Many of the active chemicals were administered as hydrochloride salts and therefore lowered the pH of seawater. Only one (iproniazid) lowered it below 6. Iproniazid lowered the pH to 3.5. Although sea water at pH 3.5 constricts chromatophores, iproniazid constricted them in 33 out of 36 occasions, whereas acidified sea water was positive in only 5 out of 32 occasions. Whenever possible, solutions of active substances were also adjusted back to pH of sea water by adding small amounts of .1 *N* NaOH. In no case did the findings differ from those made with solutions whose pH had not been adjusted.

In vitro. Experiments were also conducted with skin, excised from back of squid, and with pieces of tentacle. When lightly colored pieces of skin were stimulated electrically, the chromatophores opened. Pieces of skin were also placed in solutions of either acetylcholine, serotonin, or histamine. Both light and dark pieces were used. Only serotonin has an effect, lightening the dark tissue. The effective dose range was the same as for *in vivo* work. Kahr(6) reported findings in isolated skin similar to those above for serotonin and histamine, but in contrast to our study, he obtained dilation of octopus chromatophores with acetylcholine.

Discussion. The data on amines and amine oxidase inhibitors suggest that chromatophore control at the local level is achieved by local production or release of an amine, and its continual destruction by amine oxidase. Such an amine would relax smooth muscle and constrict the chromatophore. Another agent, such as acetylcholine, would also be produced locally and expand the chromatophore by contracting the muscle elements. The balance between these opposing agents could be upset by any one of several contingencies, adding an active amine, an amine oxidase inhibitor, acetylcholine, or autonomic blocking agent.

What are the agents concerned at the level of regulation? Of amines investigated, only serotonin acted in a dose range low enough to make it a reasonable possibility. It is of interest that Florey and Florey(11) found both serotonin and acetylcholine in the stellate ganglion of the squid, *Sepia*. Other adrenergic mediators, epinephrine, and norepinephrine, seem to have no local influence, although Sereni(4) found that systemic injections produce an expansion of chromatophores apparently through stimulation of the central ganglia. We must not overlook the possibility that permeability factors are of major importance here. Agents relatively ineffective by subcutaneous route may be much more effective by other routes.

The evidence obtained with amine oxidase inhibitors lend further support for local presence of an amine serving to relax muscle and thereby as an agent closing the chromatophores. Blaschko and Himms(12) have reported presence of monamine oxidase in different tissues of 2 species of squid. Apparently, diamines such as histamine are not concerned with funtcional responses of this nerve-muscle unit.

LSD 25 contains an indole nucleus, as does serotonin. It is known(13) to oppose serotonin in some systems and to mimic its action in others. Some biological properties of serotonin may be due to its indole nucleus, rather than its amine configuration. This would explain why another indole containing compound, LSD 25, mimics its action in the chromatophore system. The fact that minimal effective dose of LSD 25 is much higher than that for serotonin points to the highly specific nature of the receptor system in the chromatophore.

In our experiments, threshold concentration of serotonin was 1000 times greater than that reported by Kahr(6) for the octopus *Eledone*. He also reports in this cephalopod mollusc that brom LSD opposes the action of serotonin. However, as Page(7) indicates actions of LSD and its brom derivative are not always the same. The discrepancy in serotonin threshold may be due to differences in the order of mollusc used, or in method of application of the drug. The apparent differences in effectiveness of serotonin, as compared to other amines, may be due to 2 or more types of receptors. Woolley and Shaw(8), for example, have described different receptors for tryptamine and serotonin in the carotid artery of the dog. Sereni(4) showed that certain amines are carried by blood of the octopus and apparently act on ganglionic centers controlling chromatophores. Of the agents used by Sereni, tyramine, in the present study, was also found active locally. When injected intravenously, tyramine caused opening of chromatophores, while we found that its local administration causes contraction. Sereni felt that tyramine acted on ganglionic centers controlling chromatophores. He also found that acetylcholine, though causing expansion of chromatophores when locally administered, causes chromatophores to close when administered intravenously. Again he assumes intravenously administered compound to act upon ganglionic centers. The dual behavior of a single compound is analogous to the situation in mammals, where agents such as acetylcholine may have a dilating effect on end organs, when they come in contact with peripheral receptors, such as myoneural junctions, but exert a constricting effect when they contact more central receptors, such as synapse between pre- and post-ganglionic fibers. It is also possible, if, as Bozler(5) reports, chromatophore muscles are doubly innervated, that locally active amines may act preferentially on one or the other set of nerves.

A. Szent-Gyorgyi(14) has recently emphasized that substances able to transfer electrons may be of prime importance in processes leading to or synonymous with muscle contraction. Isenberg and Szent-Gyorgyi(15) have shown that tryptophan, serotonin, tryptamine, and LSD donate an electron to riboflavin and combine with it to form a stable free radical complex. It is of interest that 3 of the agents (serotonin, tryptamine, and LSD) relax smooth muscle in the squid chromatophore system. With the help of Dr. Isenberg, we have shown that PIH, tyramine, and eserine also form a red solution with riboflavin. In terms of basic mechanism involved in smooth muscle reactions, it is therefore suggested that some substances which relax the musculature of chromatophores in *Loligo* may act by virtue of ability to donate electrons to other molecules.

It has been assumed that substances interacting with enzyme systems (*e.g.*, eserine and PIH) have a demonstrable biologic effect because of this particular interaction. The present data suggest that the effect may be due to some other physico-chemical property, such as ability to donate electrons.

Summary and conclusions. (1) The multicellular system that controls chromatophores of *Loligo pealii* has been a useful tool for study of smooth muscle function in non-narcotized living animals. (2) Acetylcholine opened chromatophores. The following compounds closed them: Monamine-serotonin, tryptamine HCl, tyramine HCl; indoles-LSD 25; monamine oxidase inhibitors-iproniazid, PIH, Ro-5-0700; autonomic blockers-chlorpromazine; competitors for choline esterase-eserine sulfate. (3) Diamine, histamine dihydrochloride, had no effect. Other monamines, L-lysine and L-alanine, had no effect in comparable dosage. Catechol amines epinephrine and norepinephrine were ineffective. A precursor of sero-

tonin, 5-hydroxytryptophan, had no effect. (4) Closure of chromatophores by an active substance was not reversible by electrical stimulation. (5) Attention is called to the fact that constricting agents all share a common property, that of resonance, and that a number of them are capable of electron donation. (6) Results obtained are consonant with theory that chromatophore control is achieved by an amine-amine oxidase system (closure), opposed by an acetylcholine-choline esterase system (expansion).

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Effect of Tolbutamide on Glucose and Nitrogen Metabolism in Totally Depancreatized Dog. (24659)

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The manner in which sulfonylureas lower blood sugar has not been fully elucidated. There is considerable evidence that they may stimulate release of insulin from the pancreas when that organ is present and functioning (1-6), and it is well established that the drugs are ineffective when pancreas is absent(1,7,8). We and others have demonstrated, however, that the sulfonylurea, tolbutamide, diminishes blood and urinary glucose in totally depancreatized dog maintained with suboptimal doses of insulin(9-13). Clearly, then, the pancreas is not essential for the action of this compound provided small amounts of insulin from other sources are available. The question now arises, whether, under these circumstances, tolbutamide potentiates the injected insulin or whether, alternatively, its action takes place primarily in tissues where insulin plays only a permissive role. In our experiments, to answer this question, changes in urinary nitrogen excretion of the inadequately treated diabetic animal were used as criterion of insulin potentiation.

Methods. Mongrel dogs approximately 10 kg were subjected to total pancreatectomy. Completeness of operation was verified at autopsy. The animals were kept in metabolism cages and given constant diet (Rival Dog Food) in 2 daily feedings with measured amounts of pancreatin. Acidified 24-hour specimens of urine were collected daily and combined in 3-day pools for later analysis. Feces were collected quantitatively over brief periods from 2 dogs. Total nitrogen was determined in food, pancreatin and feces after acid digestion, and also in urine, using steam distillation(14a) and titration with boric acid (14b). Pooled urine was analyzed quantitatively for glucose(15). Fasting blood sugar (16) was determined several times a week. Dogs were weighed periodically. Animals were maintained on constant suboptimal amount of regular insulin (2 to 6 units twice