glutinated the sensitized sheep erythrocytes treated with components of horse complement used to demonstrate conglutination (*1*). These sugars were lacto-*N*-tetraose, 2-acetamido-2-deoxy-D-altrose, *N*-acetyl-neuraminic acid, lacto-*N*-fuco-pentaose II, 2-acetamido-2-deoxy-L-fucose, and methyl-3-acetamido-3-deoxy-D-guloside. The mechanism of this agglutination is under investigation.

To determine whether the inhibitors were dissociating the erythrocyte-antibody-complement complex rather than the conglutination reaction, portions of the complex were extracted at 32°C for 15 minutes with either triethanolamine buffered saline solution (TBS) (*3*) or with this solution containing 0.02*M* L-fucose, 0.02*M* *N*-acetyl-D-glucosamine, or 0.02*M* galactose. The complexes were centrifuged, washed with the buffered saline solution, and tested for activity by incubation with various dilutions of purified conglutinin. No difference in activity was observed. Therefore extraction with either inhibitor (*N*-acetyl-D-glucosamine, L-fucose) or a non-inhibitor (D-galactose) had no significant effect on reactivity of the antigen-antibody-complement complex with conglutinin.

Conglutination of aggregated γ-globulin, protein-antiprotein immune aggregates, and polysaccharide-antipolysaccharide immune aggregates was inhibited by *N*-acetyl-D-glucosamine. Furthermore, conglutinated immune aggregates, washed with TBS, released conglutinin on extraction with the buffer solution containing 0.01*M* *N*-acetyl-D-glucosamine. These extracts had a higher ratio of conglutinin activity to total protein than extracts with ethylenediaminetetraacetic acid previously used to prepare purified conglutinin (*1*).

The data indicate that an acetamido sugar with structure I and possibly L-fucose take part in determining the specificity of the conglutination reaction. From the data we cannot discriminate between the possibility that the specific sugar moiety represents a portion of the conglutinin molecule, a portion of a complement component, or even a portion of the antibody molecule which has acquired the appropriate reactive structure after combination with complement.

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Melatonin Synthesis in the Pineal Gland: Effect of Light Mediated by the Sympathetic Nervous System

Abstract. *Exposure to light reduces the ability of the rat pineal gland to synthesize melatonin and decreases the weight of the gland. When the sympathetic nerves to the pineal gland are cut, light no longer has an effect on melatonin synthesis or pineal weight. The response of the gland does not require that the gonads or the pituitary gland be present.*

Melatonin (5-methoxy *N*-acetyl tryptamine) (*1*) is synthesized in the mammalian pineal gland by the *N*-acetylation and subsequent *O*-methylation of serotonin (*2*). It has recently been shown that the activity of hydroxyindole-*O*-methyl transferase (HIOMT), the enzyme responsible for the *O*-methylation step, is influenced by illumination. Rats kept in constant darkness have 3 to 10 times as much melatonin-synthesizing-ability in their pineal glands as littermates kept in continuous light (*3*).

The effect of illumination on the pineal transferase, which is accompanied by smaller but consistent changes in pineal weight (*4*), is demonstrable within 24 hours (*5*) and appears to be specific, in that other enzymes in the pineal gland such as monoamine oxidase are unaltered (*3*). Since evidence has been presented that melatonin is secreted by the mammalian pineal gland (*6*) and inhibits ovary growth and the subsequent incidence of estrus in rats (*7*), it has been suggested that the light-induced inhibition of HIOMT activity may constitute a mechanism of neuroendocrine regulation of gonad function (*3*).

There are several ways in which information about lighting could reach the pineal gland of the rat. (i) Light could penetrate the skull and impinge directly upon the pineal. It has recently been shown that sufficient light penetrates the mammalian skull to activate photoelectric cells implanted within the brain (*8*). (ii) Light could act, through the hypothalamus and pituitary body or other neuroendocrine organs, to alter the level of a circulating hormone, which might in turn influence pineal HIOMT. Although the effect of light upon pineal weight does not require pituitary, gonad, adrenal, or thyroid function (*9*), ovarian hormones and the estrous cycle do influence the phospholipid content of the pineal gland (*10*). (iii) Information about lighting could be transmitted to the pineal via a neural route.

It has recently been demonstrated that the major, if not the only, innervation of the rat pineal gland is sympathetic, and consists of fibers whose cell bodies are in the superior cervical ganglia (*11*). It will be shown that the pathway by which information about lighting reaches the pineal gland involves the eyes and the sympathetic nervous system, and is independent of the pituitary body or gonads. The experiments described here will also demonstrate that it is possible to trace

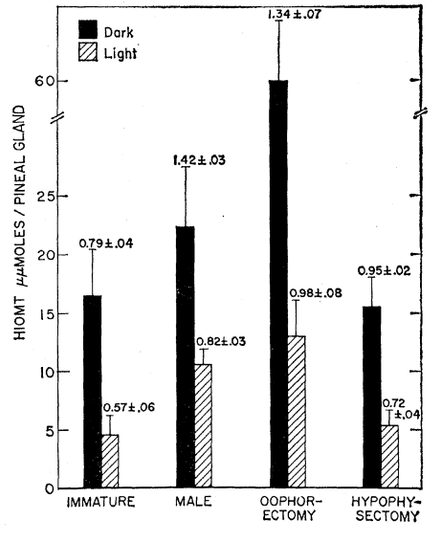

Fig. 1. The effect of constant light or darkness on the weight of the pineal gland and HIOMT activity in the rat. Immature 21-day-old female rats, and bilaterally-oophorectomized or hypophysectomized adult females were maintained under experimental lighting conditions for 6 days. Adult males were kept in light or darkness for 42 days. The HIOMT activity is expressed as micromicromoles of C^{14}-melatonin formed per whole pineal gland per hour. Average pineal weights ± standard error are given over the appropriate bars.

Table 1. The effect of bilateral orbital enucleation or superior cervical ganglionectomy on the response of the rat pineal gland to constant light or darkness. Groups of 36 mature female animals were placed under experimental lighting conditions for 11 days, beginning 1 day after surgery.

Condition	Pineal weight (mg)	HIOMT activity ($\mu\mu$mole/gland)
	Sham-operated	
Dark	1.20 ± 0.02	13.8 ± 1.3
Light	0.95 ± 0.03*	4.8 ± 2.1*
	Enucleated	
Dark	1.17 ± 0.07	9.7 ± 1.0
Light	1.15 ± 0.08	11.6 ± 2.3
	Ganglionectomy	
Dark	1.02 ± 0.06	6.1 ± 2.0
Light	1.03 ± 0.05	7.1 ± 1.0

* $p < .001$

a neuroanatomic tract by using an appropriate enzymatic indicator.

To determine whether a given organ was participating in mediating the effect of light upon pineal weight and HIOMT activity, the organ was removed and the subsequent capacity of the pineal gland to respond to light was examined. Sprague-Dawley rats weighing 160 to 180 g were subjected to various surgical procedures under ether anesthesia. One or two days later, groups of 8 to 36 animals were placed in constant-light or constant-dark rooms equipped with double-door light baffles and air conditioning. After 6 to 11 days, animals were killed by neck fracture while still in light or darkness. Pineal glands were quickly removed, weighed, and assayed for HIOMT activity (*12*). All animals were killed between 9:00 and 10:00 A.M.

Removal of both eyes resulted in a complete loss of the capacity of the pineal gland to respond to altered illumination with the accompanying changes in weight or HIOMT activity (Table 1). This indicates that the action of light upon the rat pineal gland is not direct, but is mediated by retinal receptors.

To determine whether pituitary or ovarian hormones are required for light to influence the HIOMT activity of the pineal gland, the effects of continuous illumination or darkness were examined in adult males, immature females (21 days old), adult females in which both ovaries had been removed, and hypophysectomized adult female rats (*13*). In all cases, light produced its characteristic inhibitory effect on pineal weight and melatonin-synthesizing ability (Fig. 1). In rats exposed to constant light, the pineal glands weighed significantly less ($p < .001$), and there was significantly less HIOMT activity per gland ($p < .001$ for all groups except males: $p < .01$) than those of littermates exposed to constant darkness. These observations show that although ovarian and pituitary hormones may influence the pineal gland, their presence is not required to mediate the effect of light upon HIOMT activity in the pineal gland.

The sympathetic innervation of the pineal gland was interrupted by removal of both superior cervical ganglia. After this procedure, rats placed in continuous light no longer responded with decreased pineal weight or HIOMT activity (Table 1). Thus the transmission of information about illumination to the pineal gland, or the capacity to respond to such information, requires the presence of an intact sympathetic innervation.

After superior cervical ganglionectomy, rats develop some degree of ptosis; this sign may be used to assay the success of the operative procedure. To rule out the possibility that ptosis blocked the passage of light into the eyes, and thereby rendered ganglionectomized animals unresponsive to illumination, two experiments were performed. In the first, it was found that removal of one eye, which reduced the orbital surface by an amount approximately equal to that covered in ptosis, did not interfere with the effects of light on pineal weight and HIOMT activity (Table 2). Next, it was demonstrated that prevention of ptosis in ganglionectomized rats by removal of the eyelids did not restore the capacity of the pineal gland to respond to light (Table 2).

These observations indicate that the sympathetic nervous system is directly involved in the regulation of melatonin synthesis in the rat pineal gland. Furthermore, the actions of the sympathetic nervous system on the pineal gland are influenced by environmental lighting. Changes in illumination could alter the rate of release of a transmitter substance from the sympathetic nerve endings in the pineal gland, and this neurotransmitter could then influence the activity of the melatonin-synthesizing enzyme. It is well established that sympathetic nerves release norepinephrine (*14*). Recently it has been

Table 2. The effect of unilateral orbital enucleation or bilateral superior cervical ganglionectomy with the removal of the eyelids on the response of the rat pineal gland to constant light or darkness. Groups of seven littermate mature female animals were kept under experimental lighting conditions for 11 days, beginning 1 day after surgery.

Condition	Pineal weight (mg)	HIOMT activity ($\mu\mu$mole/gland)
	Unilateral enucleation	
Dark	1.64 ± 0.13	25.5 ± 0.7
Light	1.02 ± 0.05*	9.3 ± 0.8*
	Ganglionectomy and eyelid removal	
Dark	1.03 ± 0.05	17.3 ± 2.2
Light	0.99 ± 0.06	14.6 ± 1.1

* $p < .001$

shown that sympathetic nerves in the pineal gland also contain serotonin (*15*). It is possible that the liberation of these amines could affect HIOMT activity. If one way by which light influences the rat estrous cycle (*16*) is by controlling the synthesis and secretion of pineal melatonin (*3*), it would be expected that removal of the sympathetic innervation of the pineal gland might alter the effect of light upon the gonads of the rat. Further experiments are in progress to test both of these hypotheses.

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N-acetylserotonin, and 0.1 μc of C^{14}-methyl-*S*-adenosylmethionine (3.7 mμmole, 10,000 count/min) were incubated at 37°C for 1 hour. The C^{14}-melatonin formed was extracted with 8 ml of chloroform, which was washed twice with borate buffer, *p*H 10.0. A 6-ml portion of the chloroform extract was evaporated to dryness, and counted in a liquid scintillation spectrophotometer after the addition of ethanol and phosphor.

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28 January 1964

Differences in the Amino Acid Composition of a Third Rabbit Antibody

Abstract. *Antibody directed against a neutral hapten,* p-*amino-β-phenyl lactoside, has been purified, and its amino acid composition has been determined. When the results were compared with the previous analyses of two rabbit antibodies, small but significant differences were found. The demonstration of a third unique antibody composition supports the hypothesis that the amino acid differences are related to specificity rather than to the heterogeneity of γ-globulin.*

In recent studies (*1*) a few small but significant differences have been found in the amino acid compositions of two rabbit antibodies. The antibody directed against a negatively charged haptenic group, *p*-aminobenzenearsonic acid, had a higher arginine and isoleucine content whereas the one directed against a positively charged hapten, *p*-aminophenyltrimethylammonium ion, had a higher aspartic acid and leucine content. The remaining amino acid contents were strikingly similar.

One interpretation of these results is that antibody synthesis is under genetic control, and the differences represent changes in the amino acid residues at or near the active site. An alternative interpretation is that the differences represent pools of differently charged γ-globulin molecules in which the antibody activities are segregated. The latter possibility was minimized by isolating both antibodies from individual rabbits which were homozygous with respect to their γ-globulin production and by finding the same differences in amino acid composition (*1*). Further evidence to resolve these alternative hypotheses would be obtained by the analyses of other rabbit antibodies, particularly those prepared against uncharged haptenic groups. The first of such studies, a comparison between the amino acid compositions of antibody to *p*-azo-β-phenyl lactoside and antibody to *p*-azobenzenearsonic acid is given in this report.

The immunizing antigens were synthesized by coupling at *p*H 9.0 (i) the diazonium salt of *p*-aminobenzenearsonic acid to bovine γ-globulin and (ii) the diazonium salt of *p*-amino-β-phenyl lactoside (*2*) to either bovine γ-globulin or bovine serum albumin. The amounts of diazotized hapten added were twice the molar concentration of tyrosine present in the protein carrier. After dialysis to remove excess reagent, equal quantities of each antigen were precipitated with alum (*3*), and the mixture was injected into New Zealand white rabbits in increasing doses over a period of 4 weeks.

Each animal received a total of 120 mg of antigen and was killed 5 days after the last injection. One-half of the animals received the two haptens conjugated to the same protein carrier while the other half received the two haptens conjugated to different protein carriers. Five of the rabbits used were γ-globulin homozygotes kindly provided by the National Institutes of Health; the remaining rabbits were heterozygotes at the "*a*" locus.

The isolation of purified arsonic antibody has been described fully (*4*, *5*). Similar methods were employed in the purification of the lac antibody (*4*). First, antibody formed against the protein component of the azoantigen and complement were removed from the antiserum by the addition of 20 μg of bovine serum albumin nitrogen per milliliter. The antihapten antibody remaining in the supernatant was then precipitated with concentrations of antigen ranging from 8 to 12 μg of nitrogen per milliliter. The antigen used consisted of carboxymethylated human fibrinogen, of which 10 μmole were coupled to 6 mmole of diazotized β-phenyl lactoside. The washed immune precipitate was dissolved in 0.05*M* lactose and the resulting solution was applied to a diethylaminoethyl-cellulose column equilibrated with 0.02*M* phosphate buffer, *p*H 7.2. The azofibrinogen was retained on the resin because of the increase in net negative charge achieved by the preliminary carboxymethylation. The antibody, mixed with hapten, appeared in the eluate as a single peak at column volume. The hapten was then removed by dialysis against 0.075*M* phosphate buffer, *p*H 7.2 and 0.1*M* in NaCl.

The efficiency of the separation procedure was assayed by use of radioactive reagents. When the azofibrinogen was labeled with I^{131} (*6*), only negligible amounts of radioactivity could be detected in the eluted antibody solution. The antigen contaminant was calculated to be less than 0.02 percent on a molar basis. When the hapten concentration was measured with C^{14} lactose (*7*), it was found that 0.5 to 0.7 mole remained per mole of antibody after the usual 48 hours of dialysis. A further threefold reduction was achieved in the trichloroacetic acid precipitation of the antibody prior to hydrolysis. This amount of lactose contaminant did not have a significant effect on the amino acid recoveries since identical values were obtained when the lactose present in the sample was deliberately increased by a factor of five.

Amino acid analyses were carried out on nine pairs of antibodies isolated from

Table 1. Average amino acid recoveries after acid hydrolysis of two purified antibodies (arsonic and lac) from the same rabbit. Hydrolysis time, 20 hours.

Amino acid *	Antibody			
	Residues per 160,000*g*		Standard error of a single determination	
	Arsonic †	Lac †	Arsonic	Lac
Lysine	70.0	70.8	0.41	0.84
Histidine	16.5	16.9	0.45	0.29
Arginine	44.6	44.6	0.53	0.82
Aspartic acid	105	112	1.41	1.37
Threonine	161	163	2.55	1.77
Serine	148	143	1.90	1.94
Glutamic acid	122	121	2.24	2.35
Proline	110	110	1.27	1.77
Glycine	109	109	1.06	2.03
Alanine	79.8	77.3	1.46	2.21
Valine	128	129	1.00	1.73
Methionine	13.5	13.6	0.21	0.30
Isoleucine ‡	48.1	47.2	0.80	1.17
Leucine	89	89		
Tyrosine	56.3	50.9	0.49	0.55
Phenylalanine	44.2	44.5	0.35	0.61

* Tryptophan + ½ cystine residues were not determined. † Average value from the analyses of 9 different preparations. ‡ Sum of isoleucine and alloisoleucine.