Planarian Regeneration

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Introduction

The planarian long has been a subject of scientific inquiry because of its remarkable regenerative ability. As early as 1814, Dalyell observed that planarians were "immortal under the operator's knife." This phenomenon of normal regeneration has been observed, studied and reported by different investigators including Bronsted (1955), Wolff (1961) and Hay (1966). Although there is much information on the morphological pattern of regeneration, the control of regeneration is less well known.

Recent advances in molecular and cell biology suggest a variety of hypotheses. Regeneration involves the formation of new cells and tissues and, as a necessary requisite, the synthesis of new proteins. The sequence of synthesis of a protein can be interrupted at several sites by known inhibitory agents and experiments can be designed to study inhibitor effects on the regeneration pattern. Such experiments are reported in the present paper. To provide a background for these reports, a review of the current concepts of the molecular events in protein synthesis is presented first.

Review of Protein Synthesis

It is generally accepted that protein synthesis is controlled by desoxyribose nucleic acid (DNA) located on the chromosomes in the nucleus. DNA is structured as a helix composed of two chains or strands of nucleotides; each nucleotide is made up of a phosphate, a desoxyribose sugar and a base (adenine, thymine, guanine or cytosine). The sugar and phosphate of the nucleotides are on the outside of each strand and the bases of one strand pair specifically with bases of the opposite strand. Adenine always pairs with thymine, and guanine with cytosine.

The genetic information needed for the proper alignment of amino acids into polypeptide-proteins chains is stored in the DNA strands in the sequence of their 4 constituent nucleotides; it is carried to the cytoplasmic site of protein synthesis, the ribosomes, granules about 150 Angstroms in diameter, which consist of ribose nucleic acid (RNA) combined with protein. RNA is a single stranded molecule consisting of a chain of nucleotides, each composed of a phosphate, a ribose sugar and a base (adenine, uracil, guanine, and cytosine). There are three known types of RNA: ribosomal RNA, messenger RNA, and transfer RNA.

The second type of RNA, the messenger RNA, serves as an intermediary between the DNA of the chromosomes and the protein chains formed on the ribosomes. It is formed on the DNA template in the chromosomes, by complementary pairing of the nucleotide bases: the adenine, thymine, guanine and cytosine of DNA pair specifically with uracil, adenine, cytosine, and guanine of RNA. A sequence of three RNA nucleotides, determined by complementary pairing with three DNA nucleotides, is called a codon. Each codon in the molecule has specific instructions as to which amino acid is added to the protein undergoing synthesis.

A ribosome "reads" these instructions by attaching itself at an active site to the first codon of the messenger RNA (Fig. 1). The first codon specifically determines the first amino acid of the polypeptide-protein chain. The ribosome then moves on to read the next triplet codon and condenses

the second amino acid to the first. The site of the first codon is then available for another ribosome to initiate synthesis of the same type of polypeptide-protein chain. Each ribosome continues reading one codon at a time until it has completed the protein. The amino acids, or components of the protein, are brought to the active site on the ribosome by the third type of RNA, transfer or soluble (s-RNA). There seem to be as many different s-RNA molecules as there are different codons for specific amino acids. Each s-RNA carries at one end an activated amino acid to the ribosome and at its other end a triplet codon to translate the messenger RNA. Several excellent articles and books by currently active researchers may also be consulted for further details; these include Allfrey and Mirsky (1961), Watson (1961), Nirenberg (1963), Rich (1963), Beadle and Beadle (1966).

The relation of the present experiment to these processes involved in protein synthesis is based on the ability of certain substances to interrupt the sequence of events. Two antibiotics, actinomycin D and puromycin, have been shown to inhibit protein synthesis at two different sites in the cell. Actinomycin D prevents synthesis of messenger RNA on the DNA template (Reich, Franklin and Tatum, 1962); it can be used to determine the time of formation and the lifetime of a messenger RNA. Puromycin acts in inhibiting the transfer of amino acids from s-RNA into protein on the ribosome (Yarmolinsky and Haba, 1959), and can be used to study protein synthesis on the ribosomal level.

Experiments

The experiments on regenerating planarians were designed to test the effects of inhibitors on protein synthesis and messenger RNA formation during regeneration. However, before the effects could be studied, it was necessary to establish the normal pattern of regeneration. This would serve as a standard from which any deviation from normal would be evaluated. To measure regeneration, an estimate of its extent and an end point of completion of regenerative processes per unit time were made. Thus, the appearance of the pigmented eye cup was used as an index of recovery as had been done by earlier investigators (Bardeen, 1901, 1902; Bron-

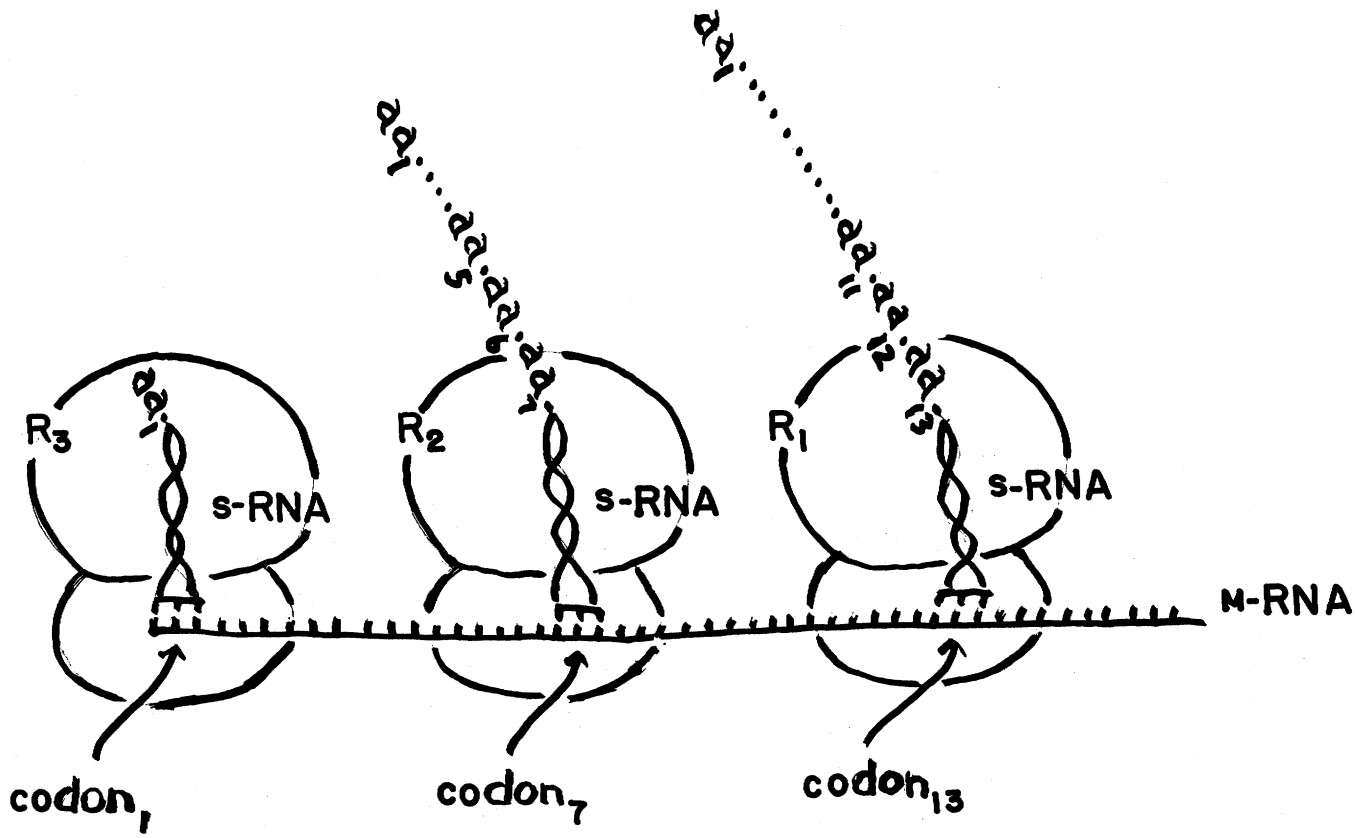

Fig. 1. A ribosome (R_3) is shown with its active site on $codon_1$ at the beginning end (left) of a messenger RNA strand (m-RNA); the first amino acid (aa_1) is brought to the ribosomal site by a transfer RNA molecule (s-RNA). The ribosome presumably will then move along the messenger RNA to $codon_2$, then to $codon_3$, etc. adding appropriate amino acids which are transferred to the active ribosomal site by different transfer RNA molecules. Ribosome R_2 is shown at $codon_7$ and an amino acid (aa_7) is condensed to the existing chain of amino acids forming the polypeptide-protein. Ribosome R_1 is already at $codon_{13}$ having begun the process of protein synthesis before the other 2 ribosomes.

sted, 1955). The photoreceptors of the planarian are easily recognized even under low magnification and were described as early as 1897 by Hesse. Further studies on their structure and function were reported by Taliaferro (1916, 1920). Electron microscopy has elucidated still further the structure of both the visual sensory cells and the surrounding pigment cup cells comprising the photoreceptors (Press, 1959; Rohlich and Torok, 1961; MacRae, 1964).

The experimental animal used was the brown planarian *Dugesia tigrina*.[1] The animals were maintained at room temperature in synthetic pond water (equal parts of boiled filtered tap water and distilled water); all further references to pond water are to this mixture. The animals were fed beef liver twice weekly; however, feeding was discontinued during regeneration experiments. Animals of similar size were decapitated by a razor blade just posterior to the laterally projecting auricles. The posterior parts were transferred to shallow dishes containing 10 ml of pond water; 5 animals were kept per dish. The temperature averaged 20°C. The animals were observed daily for 7 days at 10 X magnification under the dissecting microscope. The shape of the head and the appearance and general size of the pigmented eye cups were observed and recorded.

Parallel experiments were conducted in which various dosages of the two inhibitors (actinomycin D and puromycin) were added to the pond water in which the animals were regenerating. These animals were also observed daily for 7 days. Such experiments established the dosage level which would result in an observable effect on regeneration of the head and eyes. Excessive dosages caused the animals to die, while minimal dosages produced no observable effect. After establishment of the effective dosages, animals were exposed to the inhibitors for various lengths of time after decapitation.

Some animals were sacrificed at the time of the experiments by fixation in 5% glutaraldehyde in O.1M phosphate buffer at pH 7.2. Other standard fixatives could be used and the animals should be placed under a cover slip during addition of the fixative to prevent curling. Fixation was followed by dehydration, paraffin embedding, sectioning, and staining. After staining, the sections were examined microscopically to verify the gross observations made on the whole animals and to determine whether the photoreceptors appeared normal.

The inhibitor actinomycin D was generously supplied as "Lyovac Cosmogen" powder by Merck, Sharp and Dohme Research Laboratories. Stock actinomycin D solutions of 10 ml and 12.5 μg per ml were made up in pond water. Concentrations of 2, 5, 8, 10 and 12.5 μg/ml were used for the various experiments with actinomycin D.

Puromycin hydrochloride powder was purchased from Nutritional Biochemical Co., and a stock solution of 200 μg/ml was made with pond water. This was neutralized to pH 7.0 with 0.5 M KOH and then used in making concentrations of 20, 33, 40, 66 and 100 μg/ml to which the decapitated animals were exposed.

The variables in the experiments were dosage of inhibitor, duration of exposure, and the time after decapitation that the animals were exposed to the drug. Controls, without inhibitors in the water, were established for each experiment.

Observations

The observations are summarized by the drawings in Fig. 2. The photoreceptors and heads represented in the top row are typical of the normal pattern of regeneration; this pattern was used as a standard of comparison with the drug treated animals. The appearance at the time of decapitation is represented by 0 hours. By 24 hours, the closure of the wound is completed; by 48 hours, a mass of undifferentiated tissue (the blastema) is evident. At 72 hours, pigmented eye cups are visible at low magnification in the region where the less pigmented blastema borders the posterior epidermis. By 96 hours, the adult shape of the cephalic region is established, although the adult size is not yet achieved. Lateral auricular projections are evident at this time and the eyes are more fully developed. At 128 hours after decapitation, the auricles are motile, and by 168 hours (seven days) the adult appearance of the head and eyes is completely restored.

Actinomycin D used on decapitated animals was found to be effective in dosages of 10 μg/ml. The second row in Fig. 2 shows the extent of regeneration of the head and its parts at 168 hours (seven days) after exposure of the animals to 10 μg/ml for 3 varying lengths of time (24, 48 or 72 hours) after decapitation. These variations in exposure time to the inhibitor agent were performed to determine when the messenger RNA would be made during regeneration. An exposure to actinomycin D of only 24 hours duration immediately after decapitation with later development taking place in pond water produced no deviation from the controls. However, exposure to actinomycin D directly after decapitation for 48 or 72 hours duration with subsequent regeneration occurring in pond water, resulted in the failure to produce normal heads and the development of eyes by 7 days regeneration time. When eye cups did form after actinomycin D exposure, histological examination revealed an irregular eye cup of scattered pigment.

The effects of exposure to puromycin are shown

[1]Other planarian species such as the black planarian *Dugesia dorotocephala* could also be used.

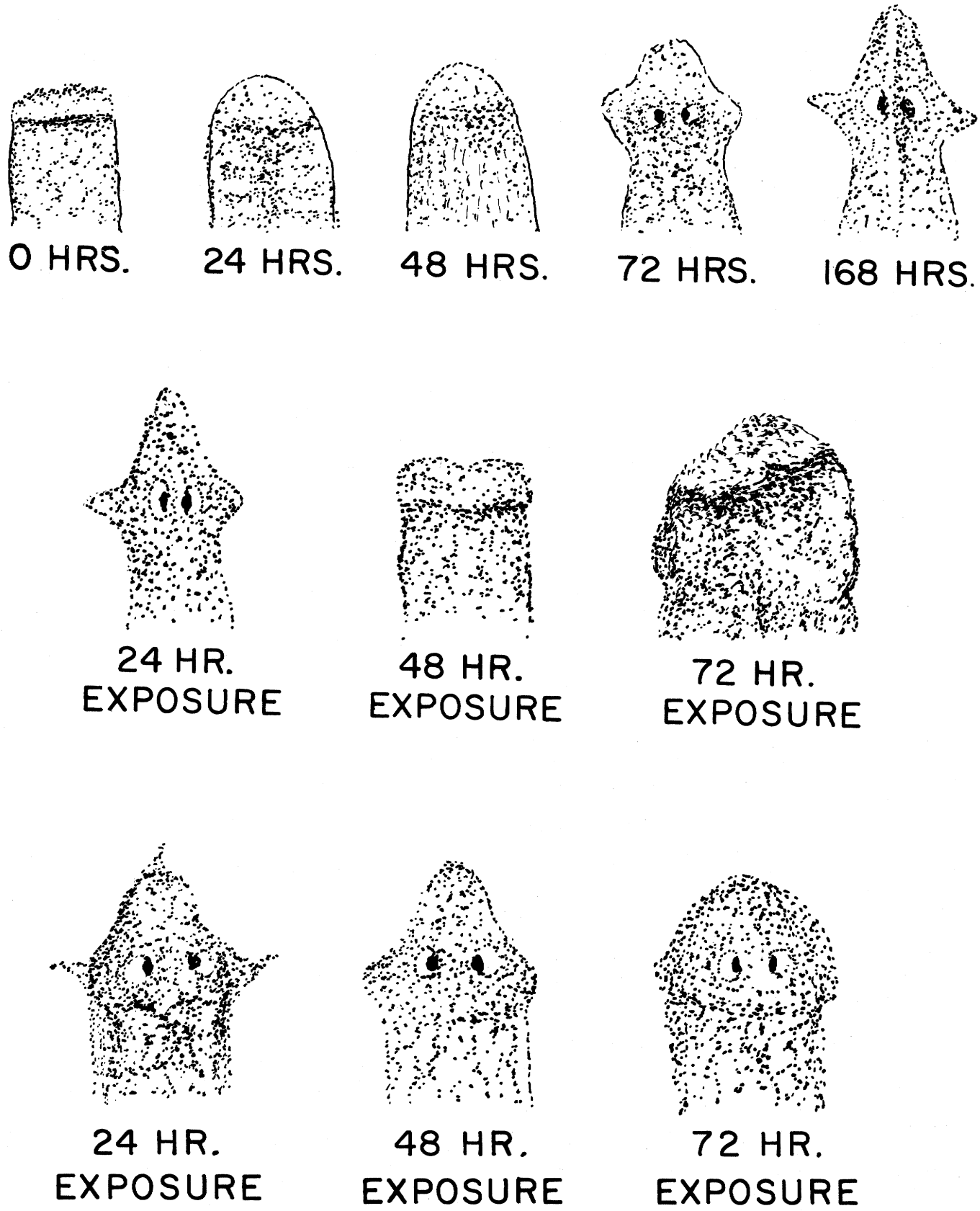

Fig. 2. The top row shows normal regeneration of the planarian head at various times after decapitation (0 hours). The second row shows the extent of regeneration of the head at 168 hours (7 days) after exposure of the animals to actinomycin D (10 μg/ml) for 24, 48 or 72 hours duration after decapitation. The third row shows the extent of regeneration of the head at 168 hours after exposure of the animals to puromycin (40 μg/ml) for 24, 48 or 72 hours duration after decapitation.

in the bottom row of Fig. 2. The effective dosage of puromycin was found to be 40 μg/ml. Animals which were exposed to the puromycin for 24 hours and allowed to complete regeneration in pond water developed normal heads and eyes by 7 days, although the eyes were smaller than controls. Animals exposed to puromycin for 48 or 72 hours duration after decapitation, and allowed to complete regeneration in pond water, developed normal eyes after a delay proportional to the duration of the initial exposure to puromycin. The histological examination of the eyes found after puromycin exposure showed them to have the appearance of a normal eye cup.

Discussion

The results of this investigation show that actinomycin D and puromycin both affect the normal regeneration pattern; their effect is consistent with the accepted mode of action of these drugs from evidence on other cells and tissues. The effect of a 48 or 72 hour exposure to actinomycin D after decapitation appears irreversible. Such an effect would be consistent with the idea that DNA-dependent messenger RNA formation is inhibited. Cells of the blastema presumably migrate from the more posterior parts of the body to form the anterior mass of developing tissues. Under the influence of actinomycin D these cells appear incapable of directing differentiation of the head parts, including photoreceptors, auricles, etc. Either the formation of messenger RNA molecules for these structures was inhibited directly, or actinomycin D inhibited the formation of a messenger RNA which may have controlled a sequence of regeneration events. The data suggest that actinomycin D affects the messenger RNA formed after the initial 24 hours of regeneration. The lack of effect of actinomycin D during the first 24 hours of exposure suggests that necessary messenger RNA is not made during that time, since regenerating animals restored to pond water, after 24 hours in actinomycin D, eventually developed normally. Actinomycin D in these dosages appears to be rapidly metabolized in the body; when the animals are removed from its influence after only 24 hours, it appears to have no later toxic effect.

The effects of puromycin are quite different from those of actinomycin D. Puromycin in small but effective dosages appears to be reversible in effect and is not related to events at a specific time during regeneration. This conclusion is consistent with the reported mechanism of inhibition by puromycin. The structure of puromycin mimics the end of the transfer RNA with its attached amino acid. The growing protein chain is thus transferred to the puromycin molecule and its attachment to the ribosome is destroyed by puromycin, resulting in small "junk" proteins (Nathans and Neidle, 1963). When, in this manner, puromycin is eventually used up, normal protein synthesis is resumed after the delay. The results suggest that the size of the regenerated head and eyes at a particular time is inversely proportional to the duration of the exposure to puromycin. Thus, the amount of protein made was less than normal.

These experiments throw no light on specific molecules of proteins or messenger RNA, but they do support the view that the formation of messenger RNA and protein are integral factors of regeneration. These experiments can be extended further to obtain more precise information on the time of messenger RNA formation. By varying the initial time and duration of exposure to actinomycin D, data may be obtained about the dependence of certain sequential events in regeneration. Such experiments can be performed in the teaching laboratory with minimal equipment and chemicals, and it is hoped that students of biology might be challenged to continue studies along these lines of investigation.

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Note

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SUGGESTIONS FOR LSD TREATMENT

Suggestions on treating the rising number of victims of LSD and other hallucinogenic drugs are offered the nation's physicians in the *Journal* of the American Medical Association. Comprehensive treatment, however, goes far beyond routine, first-step measures, warns a statement by the AMA's Council on Mental Health and Committee on Alcoholism and Drug Dependence.

The problems of drug dependence are so complex that the physician may find he needs all the help he can get. He may need to call on the services of psychiatrists, other medical specialists, and the community's other professional resources, such as clergymen, educators, social service agencies, and attorneys. "Hallucinogen-induced dependence is psychological," the statement said. "The drugs have a particular attraction for adolescents and young adults who are socially maladjusted or emotionally inhibited and who constantly seek new 'experiences' and 'insights'." Drug dependence "is almost universally symptomatic of underlying personality problems, severe neurotic conflicts, or psychotic reactions," according to the AMA statement.

At least three types of users of hallucinogenic drugs have been noted:

One consists of narcotic drug-dependent persons who take LSD or other hallucinogens infrequently after a "fix" with narcotics.

Another group includes users of other drugs, especially marijuana, who relish the more intense effects of hallucinogens.

A third group is composed of persons who repeatedly take LSD only or in combination with other hallucinogens over a sustained period of two weeks to a month. Unlike those in the second group, they take the drugs not for social purposes, but to achieve "some personal, esoteric goal."

A variety of complications have been reported, but three appear to be most prevalent.

1. Reappearance of the hallucinated, disorganized state without drug-taking. This has occurred within two months after a series of relatively few exposures to hallucinogenic drugs. It also occurred more than 12 months after one person took the drugs more than 200 times over a period of years.

2. Panic is a frequent complication. "Hospitalization may be sought by the user or his companion, neither of whom can cope with the sense of terror."

3. A third, relatively common, complication is an extended period of psychosis, sometimes after a single exposure to drugs, and usually involving a person who was "prepsychotic" or had a history of psychosis.

"There is no available evidence to suggest that the massive, disorganizing experience resulting from the taking of hallucinogens has been therapeutic for any psychotic patient. Quite the contrary!" said the statement.

In addition to LSD, the hallucinogens include some kind of morning glory seeds, mescaline, a drug present in the buttons of a small cactus; psilocybin, a drug found in a mushroom, and DMT, a synthetic drug also found in the seeds of a South American plant.

Tolerance to hallucinogens develops rapidly, but usually is lost in two or three days. Further, there is cross-tolerance among LSD, psilocybin, and mescaline, although tolerance to mescaline develops more slowly. Some users report a state in increased sensitivity to LSD once they have lost their tolerance.

DMT, a relatively new synthetic with a somewhat shorter and more "harsh" action than LSD, also has appeared on the illicit drug market. An LSD "trip" usually lasts about 12 hours and the onset is said to be fairly "gentle." A DMT trip lasts about two hours and has a sudden or "rough" onset.

AIR POLLUTION

Free single copies of air pollution leaflets and pamphlets are available from the Public Inquiries Branch, Public Health Series, U. S. Department of Health, Education, and Welfare, Washington, D. C., 20201: *Air Pollution and Respiratory Disease* (PHSP-1257), *Let's Clean the Air* (PHSP-1238), *Scientific Statesmanship in Air Pollution Control* (PHSP-1239), *The Air Around Us* (OM-1806), *How Polluted is the Air Around Us?* (OM-1733), *With Every Breath You Take* (OM-1847), *A Woman's View of Air Pollution* (PHSP-974), *Air Pollution Films* (PHSP-1264), *Clean Air Act* (*P.L.* 88-206) (OM-1732), and *The Clean Air Act Amendments and Solid Waste Disposal Act of 1965* (*P.L.* 89-272) (OM-1846).