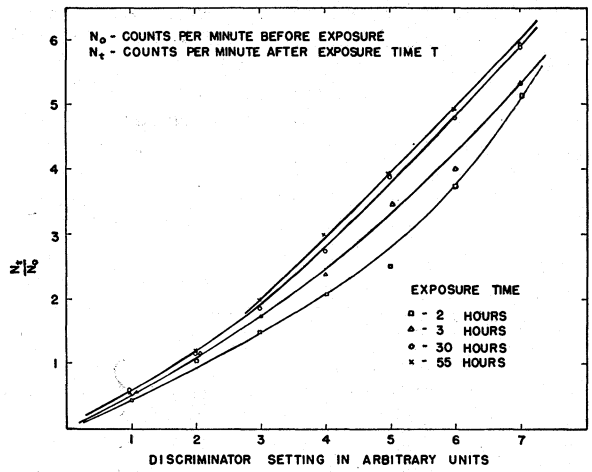

FIG. 2. Differential pulse amplitude distribution and the effect of exposure time on the relative counting rate.

ual increase in activity during the first 24 hr, after which little further increase in activity could be detected. This increase probably represents the gradual approach to equilibrium conditions among the fission products and is due to the extra pulses produced by the recoil of these nuclei and to a lesser extent to the more energetic β-particles associated with them. Similar measurements were carried out with 10% and 1% U_3O_8 samples. For a 10% sample only a small increase in counting rate was observed, whereas no effect was observed for the 1% sample. It appears, therefore, that some useful information concerning the distribution of fission pulses might be obtained by this method, particularly with a stronger source and a more sensitive photomultiplier; for assay purposes, determination of the natural decay radiation is more accurate and convenient.

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Evidence for the Occurrence of Intermediates during Mutation[1]

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A great deal of experimental evidence has been published which demonstrates that environmental factors such as temperature, gas tension, and infrared irradiation can greatly alter the effectiveness of various mutagenic agents (*1, 2*). Much of this evidence suggests that the action of x-ray and ultraviolet radiation on genes and chromosomes is indirect rather than direct and that chemical mutagens are the immediate agents of mutation. Whether the delayed effects of x-ray and ultraviolet radiation depend upon some photochemical product which is produced in the cytoplasm or whether they are due to a slow stabilization of structural alterations in the chromosome induced at the time of irradiation cannot be determined conclusively by supplementary treatments. Previous results concerning the effect of pressure on the mutations induced by nitrogen mustard clearly established the fact that chemical alterations that lead to gene changes are freely reversible for a considerable time after the removal of the mutagenic agent (*3*). These results suggest that a transitory, semiactivated complex is formed which finally decomposes either to the original state or to a new, mutated state. Decomposition to a mutant state apparently proceeds with an increase in volume, since pressure can prevent its occurrence. The results set forth in the present communication also suggest that intermediate activated states are formed by the action of radiation and that it is these activated states that are affected by the supplementary treatments. From the effects of temperature and pressure one must conclude, therefore, that all molecular alterations involved in a change to a mutated form do not necessarily occur simultaneously with the absorption of the radiant energy and that a latent period exists which is affected by temperature or pressure. Swanson and Yost (*4*) have recently published experiments which demonstrate that a similar interpretation can be applied to the effects of infrared irradiation. A theoretical treatment of the subject has been published by McElroy and Swanson (*5*).

A microconidial strain of *Neurospora crassa* (*6*) was used to study the effect of pressure on the mutation rate after exposure to ultraviolet irradiation. Five-day-old conidia were suspended in sterile water and filtered through cotton pads in order to remove mycelial fragments. Samples of the suspension (containing an average of 5×10^6 spores/ml) were placed in a quartz flask, and the latter was attached to a low-speed motor at a distance of 24″ below a Westinghouse sterilamp. During irradiation the suspension was continuously rotated. Immediately after irradiation a sample of the suspension was placed in a sterile rubber balloon, which was then inserted into a pressure bomb, in which the hydrostatic pressure was raised as rapidly as possible to 10,000 psi. From 1 to 2 min always elapsed between the termination of irradiation and the application of pressure. Part of the irradiated suspensions was kept at atmospheric pressure in the sterile quartz flask until the termination of the pressure treatment, which was always for 30 min. At the end of this time both control and pressure-treated suspensions were plated by serial dilution onto a complete medium containing 1.5% *l*-sorbose. After 3–4 days single isolates were transferred to complete slants without sorbose and subsequently scored for morphological mutations. In some experiments the transfers were made to large tubes (16 × 150 mm) containing the complete medium, whereas in others the transfers were made to small tubes (10 × 75 mm). The rate of morphological mutations appears to be somewhat lower on the latter

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TABLE 1

EFFECT OF PRESSURE ON ULTRAVIOLET-INDUCED MUTATIONS IN *Neurospora crassa*. SURVIVAL GREATER THAN 5%

Expt. No.	Treatment	No. isolations	No. morphological mutants	Mutation (%)	Kill (%)
3-18	15′ UV	300	18	6.0	72.1
	15′ UV + 10,000 psi	300	13	4.3	74.4
3-27	15′ UV	289	16	5.5	90.0
	15′ UV + 10,000 psi	299	11	3.7	93.0
3-26	15′ UV	289	17	5.9	84.6
	15′ UV + 10,000 psi	294	10	3.4	90.0
12-15	20′ UV	292	54	18.5	93.3
	20′ UV + 10,000 psi	285	30	10.5	94.1
12-29	20′ UV	300	71	23.7	90.2
	20′ UV + 10,000 psi	200	31	15.5	92.2
2-10	20′ UV	229	42	18.3	94.5
	20′ UV + 10,000 psi	129	22	17.0	95.1

TABLE 2

EFFECT OF PRESSURE ON ULTRAVIOLET-INDUCED MUTATIONS IN *Neurospora crassa*. SURVIVAL LESS THAN 5%

Expt. No.	Treatment	No. isolations	No. morphological mutants	Mutation (%)	Kill (%)
2-1	20′ UV	335	51	15.2	96.3
	20′ UV + 10,000 psi	225	50	22.2	96.5
1-27	20′ UV	200	35	17.5	98.5
	20′ UV + 10,000 psi	200	42	21.0	99.1
11-8	25′ UV	354	78	22.0	98.5
	25′ UV + 10,000 psi	162	49	30.2	99.4
3-6	25′ UV	300	20	6.7	98.9
	25′ UV + 10,000 psi	289	45	15.6	99.5
3-15	25′ UV	294	41	13.9	99.94
	25′ UV + 10,000 psi	153	27	17.7	99.99
3-23	25′ UV	289	12	4.2	99.2
	25′ UV + 10,000 psi	293	21	7.2	99.8
6-24	35′ UV	297	24	8.1	99.8
	35′ UV + 10,000 psi	299	28	9.4	99.9
6-30	35′ UV	306	25	8.2	99.93
	35′ UV + 10,000 psi	300	37	12.3	99.97

slants, since the entire surface of the agar may be covered before certain morphological effects can express themselves.

Preliminary experiments indicated that the effect of high pressure after ultraviolet treatment may lead either to no change, or to an increase or to a decrease in the mutation rate. However, it soon became evident that these changes depend upon the ultraviolet dose as measured by the percentage of survival. The figures presented in Tables 1 and 2 show that at a radiation intensity which results in more than 5% survival (Table 1), pressure reverses the delayed mutagenic action of ultraviolet, whereas at high intensities (less than 5% survival) pressure increases the apparent frequency of mutations. It is significant that the 5% survival level is approximately at the point where the mutation frequency curve begins to decrease with increased ultraviolet dosages.

It is evident that after ultraviolet irradiation a considerable time must elapse in order for the mutagenic effectiveness of this agent to be completely expressed. There are at least two ways in which these facts can be interpreted. The first is that some chemical mutagen is produced which persists in the cytoplasm for a considerable period after the application of the mutagenic agent. If one assumes that ultraviolet light produces some chemical mutagen, then it must be concluded that at low doses of irradiation the concentration of this mutagen results in a high ratio of mutants to the percentage of lethality, whereas at high doses the ratio is lowered, presumably because of increased sensitivity of the mutated types to higher concentrations of the mutagen. Pressure, by preventing the action of the mutagen in the processes leading to mutation and lethality, would affect the mutation-lethality ratio in opposite directions after low and high doses of ultraviolet, respectively. The fact that the difference in response to pressure occurs at the UV dose level where the mutation rate begins to decrease renders the above interpretation plausible. The second interpretation of the above results is based upon the possibility that changes are being induced directly in the chromosome by the absorbed energy, a situation which with time would lead to a mutation or, if the alterations were sufficiently great, to a decrease in viability. It is these alterations in the chromosomes which we refer to as a transitory semiactivated state, a normal intermediate in the mutation process. If the second interpretation is correct, it suggests that the processes leading to mutation and lethality are essentially the same, with the exception that the latter is accompanied by more extensive molecular rearrangements and that the suppression of the latter effect results in an apparent increase in the mutation rate.

An effort has been made, therefore, to obtain evidence that more than one sort of change can occur in the chromosome prior to the mutation event. An experiment was performed to determine whether pressure would have a differential effect on high and low concentrations of nitrogen mustard as mutagenic agents and as supplementary agents in combination with ultraviolet. *Aspergillus terreus* was used, and the procedures were essentially the same as those described for *Neurospora*. In the experiments described, the spore suspension was divided into two parts, one of which was treated for 15 min with ultraviolet and the other with 0.1% nitrogen mustard for 30 min at 25° C. At the end of the two treatments samples were taken from each suspension, diluted, and plated onto complete medium. The remaining mustard-treated sample was centrifuged and washed twice with sterile water and finally divided into two samples. One sample was placed under 10,000 psi for 30 min and the second was maintained at room temperature and pressure for the same period of time. At the end of 30

min, both samples received a 15-min dose of ultraviolet. The samples were then diluted and plated onto complete medium. Isolates from single colonies were subsequently transferred to slants, and after 4 days of growth the morphological mutants were scored. A similar experiment with a higher concentration of nitrogen mustard but without the ultraviolet supplementary treatment was also performed. The results presented in Table 3 clearly indicate that the mutations induced by nitrogen mustard require a lapse of time before they are completely stabilized. Furthermore, the intermediate which is formed apparently decomposes and returns to the original phenotypic state under the influence of high hydrostatic pressure (*cf.* 1, 5, and 6, Table 3). An important point is that this intermediate evidently cannot re-form upon the release of pressure, a fact indicating that the initiating agent is no longer present. Also of interest, however, is the fact that hydrostatic pressure does not eliminate the potentiating effects of the nitrogen mustard upon the ultraviolet-induced mutations. On the contrary, there appears to be a slight stimulatory effect of pressure, such as might be expected for bimolecular reactions (*cf.* 3 and 4, Table 3). The results of this series of experiments suggest that the nitrogen mustard first combines with the chromosome in a reaction which is not depressed by pressure, and that this reaction alters the structure of the chromosome or gene in some way so that it becomes more sensitive to supplementary mutagenic agents such as ultraviolet radiation. If so, this altered structure of the chromosome can itself eventually react in a manner which leads to a considerable molecular rearrangement, resulting in a volume increase and finally decomposing either to the original state or to a mutated state. It is the later reaction which is pressure-sensitive and which decomposes to the original state under high hydrostatic pressure.

TABLE 3

COMBINED EFFECTS OF NITROGEN MUSTARD, ULTRAVIOLET, AND PRESSURE ON MORPHOLOGICAL MUTATIONS IN *Aspergillus terreus*

No.	Treatment	No. isolations	No. mutants	Mutation (%)	No. Expts.
1	0.1% N_2 mustard	2173	74	3.4	14
2	Ultraviolet	1383	225	16.2	14
3	0.1% Mustard + UV	3815	879	23.1	14
4	0.1% Mustard + pressure + UV	4056	1075	26.5	14
5	0.3% Mustard	656	67	10.2	3
6	0.3% Mustard + pressure	827	33	4.0	3

The results cited above would suggest that the changes observed with ultraviolet radiation and high pressure must be due primarily to the action of the pressure on changes that have already occurred in the chromosome at the time of pressure treatment, rather than that the latter prevents the reaction of a chemical mutagen with the chromosome. In other words, the delayed effects of ultraviolet irradiation are apparently not due to the production of a chemical mutagen which remains in the cytoplasm for as long as 20 min. Rather, the results suggest that the delayed effects are due primarily to semistable intermediates in the mutation process which require time for stabilization. The probability is still very great, however, that the initiation of a large number of these changes is due to the production of a chemical mutagen which rapidly reacts with the chromosomes as well as with other cellular components.

Additional evidence has been obtained concerning the characteristics of the semistable intermediate which occurs during and after treatment with a mutagenic agent. If such an intermediate were indeed formed, its decomposition would presumably be affected by temperature. Several experiments have been performed to test this, using both *Neurospora* and *Aspergillus,* and similar results have been obtained with both organisms. The results with *Aspergillus* are reported below. After the spores were treated for 30 min with 0.3% nitrogen mustard, the suspension was rapidly cooled and centrifuged. The spores were resuspended in sterile water and the suspension was divided into two fractions. One of the fractions was placed at 10° C and the other at 25° C. After 30 min at the respective temperatures, a sample of each was placed under pressure (10,000 psi) for 30 min. At the end of the pressure treatment all samples were plated, and individual colonies were subsequently isolated onto complete agar slants. At the end of 4 days the isolates were classified according to morphological characteristics. The results of several experiments are summarized in Table 4. It is evident that when the treated suspension is kept at 25° C (lines 3 and 4), pressure has no influence on the number of mutations when its application begins 30 min after the treatment with the mutagenic agent. On the other hand, if the treated suspension is kept at 10° C during this 30-min period (lines 1 and 2), pressure considerably lowers the number of mutants. The results suggest that some intermediate state is formed in the mutation process and that it thereafter decomposes to the final mutant state, the rate of decompositon de-

TABLE 4

EFFECT OF TEMPERATURE AND PRESSURE ON THE ACTIVATED STATES INDUCED BY NITROGEN MUSTARD IN *Aspergillus terreus*

No.	Treatment	Temperature 30 min (°C)	No. isolations	No. mutants	Mutation (%)	No. Expts.
1	0.3% N_2 Mustard	10	1755	213	12.2	5
2	0.3% Mustard + pressure	10	1465	107	7.3	5
3	0.3% N_2 mustard	25	1864	221	11.8	6
4	0.3% Mustard + pressure	25	1590	182	11.4	6

pending on the temperature. In addition, the significant effect of high pressure confirms the previous observations that these changes proceed with an increase in volume. The latter observations are of some importance, since they indicate that with pressure treatment the intermediate decomposes to the original, normal state, which cannot revert to the intermediate after the pressure is removed. In this respect, the results are quite similar to those obtained with ultraviolet treatment followed by high pressure.[2]

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[2] I would like to acknowledge the capable assistance of Mrs. R. Metcalf and Miss M. Ono in performing the experiments described in the present report.

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Localization of Protein-bound Radioactive Iodine by Filter Paper Electrophoresis

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The serum of an euthyroid patient with a Hurthle cell carcinoma of the thyroid with metastases was analyzed by filter paper electrophoresis, using a modification of the method of Kunkel and Tiselius (*1*), 1 hr after a dose of 40 mc of I^{131} and at daily intervals thereafter for 10 days. One tenth ml of serum was placed on two thicknesses of Whatman #3 filter paper strips held between glass plates. The ends of the paper strips were placed in veronal buffer, pH 8.6 and ionic strength 0.05. A current of 1 ma/strip was passed through at a potential of 300 v. At the conclusion of the electrophoresis, the bottom strip was stained with bromphenol blue, dried, washed with acetic acid, and cut into numbered strips. With the dye elution method the protein fractions were localized and their quantities determined. The top strip was cut into numbered segments, and the relative radioactivity determined in a bell-type Geiger counter.

The total protein-bound I^{131} after electrophoresis was compared with the total raidoactivity of a comparable amount of serum before electrophoresis. These values were identical after 3 days. The radioactivity was only 2% protein-bound after 1 hr and was freely distributed among all the serum proteins. At the end of 30 hr the radioactivity was 33% protein-bound, and there was definite evidence of concentration in the albumin and in the α-2 globulin. At 48 and 72 hr, the concentration at the latter site was more clearly evident. Over 80% of the activity was concentrated in and just beyond the α-2 globulin area at 72 hr and thereafter.

A typical radioelectrophoretogram of the 7-day serum is shown in Fig. 1, together with a graph of the

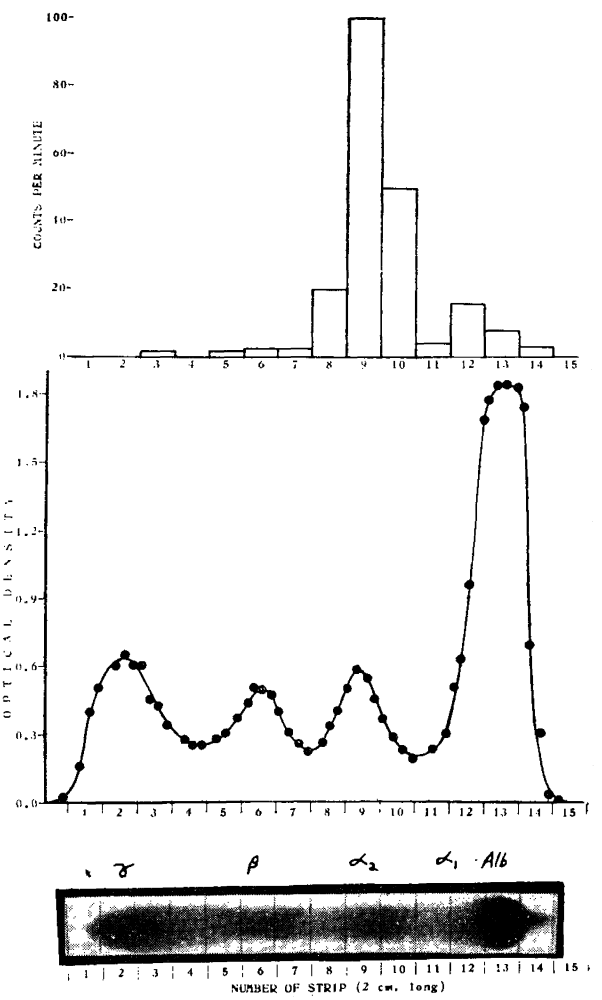

FIG. 1. Top graph shows the radioactivity of the 7-day serum on the segments of filter paper. Center graph shows the relative quantities of protein on similar segments. Photograph of the corresponding stained strip is shown at the bottom.

quantities of protein fractions as determined by the bromphenol blue elution method, and a photograph of the stained strip. The standard electrophoretic pattern of the same serum is shown in Fig. 2.

This method is reported as a new approach to the study of the nature of the circulating thyroid hormone as well as other substances which can be conveniently traced. It is not assumed that this is conclusive evidence of the behavior of normal thyroid hormone, since the subject under investigation had a carcinoma of the thyroid which was possibly in the functioning category. Some evidence that this serum was not completely normal can be seen in the standard electrophoresis in which a small abnormal peak is seen just beyond the α-2 area, especially in the ascending limb.