SENSITIZING BACTERIAL SPORES TO HEAT BY EXPOSING THEM TO ULTRAVIOLET LIGHT

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Among the many physico-chemical changes produced in proteins by ultraviolet radiation, the changes that affect stability to heat are of particular interest to the biologist. With those rare exceptions noted by Bovie and Woolpert (1924) and Clark (1925) the heat-coagulation temperature of probably all proteins is lowered by the absorption of ultraviolet light. In a study of twelve purified proteins Stedman and Mendel (1926) found that ultraviolet light from a quartz-mercury lamp reduced the temperature of coagulation from 8.5° to 41°C.

Since the killing of living organisms by heat is accompanied by coagulation of the protoplasm it is reasonable to suppose that their heat-resistance would be decreased by ultraviolet radiation. Despite the interesting implications involved, only Bovie and Klein (1919) and Bovie and Daland (1923) have studied the heat-sensitization phenomenon with living microörganisms. Working with Paramecia they observed that sublethal exposures to ultraviolet light, transmitted by fluorite, so affected the organisms that they subsequently died at temperatures which were optimal for the untreated organisms. These striking effects were tacitly assumed to be caused by those rays of very short wave length (Schumann) which are transmitted only by fluorite. While this assumption was undoubtedly correct, these experiments might very naturally lead to the belief that the Schumann rays alone were possessed of heat-sensitizing power. This circumstance, together with certain technical difficulties involved in the generation and transmission of Schumann rays, is perhaps

chiefly responsible for the absence of additional work on this subject.

Consideration of these facts and of the potentialities inherent in the heat-sensitizing principle led us to study the action of ultraviolet rays upon the heat-resistance of bacterial spores. The report which follows presents the results obtained in this investigation.

EXPERIMENTAL

Cultures and Methods

Three cultures (*Bacillus cohaerens*, *Bacillus albolactis* and CC) were used. The first two were obtained from the American Type Culture Collection. CC, an aerobic spore-former belonging to the *Bacillus mesentericus* group, was isolated in our laboratory from evaporated milk. The cultivation and preparation of the test suspensions have been detailed in a previous paper, by Curran and Evans (1937). The stock and test suspensions when not in use were held in the ice chest.

Two sources of ultraviolet light were used. The first was a cold-mercury-in-quartz lamp of the orificial type. Tests by the National Bureau of Standards upon the spectral energy distribution of this lamp have indicated that at least 95 per cent of the total radiation of all wave lengths less than and including the line at 3,130 Å. is contained in the emission line of mercury vapor at 2,537 Å. Four milliliter quantities of the spore suspension at 30°C. were irradiated in a small quartz balloon flask (18 ml. capacity), placed $2\frac{3}{4}$ inches from the burner. The flask was equipped with a quartz stirrer which provided rapid and uniform agitation. After the exposure to ultraviolet light, equal portions of the spore-suspension were mixed on plates with 15 ml. of agar of the desired composition. Colony-counts were made after 48 hours of incubation at the optimum temperature of the organism. A magnifying glass was used in counting and the figures represent the averages of triplicate plates. From time to time the plates were returned to the incubator, and re-examined 5 days later to check the possibility of slowly developing colonies. The intensity of the cold-quartz radiation was

measured by exposing a sensitive thermopile at a fixed distance from the burner and noting the galvanometer deflections. These values were then converted into absolute energy units by reference to a standard of radiation.

The second source of ultraviolet light was a hydrogen-discharge tube (15 by $1\frac{1}{8}$ inches) fitted with ring electrodes of aluminum. In one end of the tube was sealed a fluorite disc (2 mm. thick) and in the other end a quartz disc of the same thickness. The discs were equi-distant from the electrodes. The tube was excited by a 450-watt luminous tube transformer operating on a 110-volt alternating current of 60 cycles. The current passing through the discharge tube was 33 milliamperes and the drop of potential across the tube was 1,570 volts. The intensity of the radiations transmitted by the tube was not measured. Exposures to this light source were made as follows. A small platinum loopful of a heavy aqueous suspension of the spores was spread uniformly over a 15 x 5 mm. area of a sterile coverslip until dry. The dried film was then applied directly to the window of the discharge tube and exposed. The temperature of the discharge tube windows did not rise perceptibly during the exposures recorded. The spores were recovered in 5 ml. of sterile distilled water; the process of loosening the film from the glass was facilitated by the use of a small, sterile, rubber-tipped glass rod. The suspensions were then thoroughly agitated and plated. The count so obtained served thereafter as a guide for the dilution required to equalize the number of viable cells in the several samples.

Aliquot portions of the standardized samples were heated at 98°C. for 4 minutes and plated.

The medium used was standard nutrient agar of the following composition: 0.5 per cent peptone (Difco), 0.3 per cent beef extract (Liebig's), 0.5 per cent sodium chloride, 1.5 per cent agar (granulated). This basic medium was enriched with either blood or glucose, depending on the nature of the treatment and the enrichment requirements of the organism (See Curran and Evans, 1937).

When bacteria are exposed to both heat and ultraviolet radia-

tion, of constant intensity for the same period of time, the order in which the two treatments are applied should not appreciably affect the number of survivors, if sensitization to neither heat nor light occurs, or if sensitization to both heat and light occurs to the same extent. However, consistent differences in the number of survivors under these conditions do occur and must be attributed to differences in the sensitizing power of the initial

TABLE 1

The effect of heat (98°C.) and ultraviolet light (21 Ergs/mm²/sec) on viability of bacterial spores, according to the sequence of treatment and time of exposure

KIND OF SPORES, AND TIME EXPOSED TO HEAT AND LIGHT	VIABLE SPORES SURVIVING PER MILLILITER WHEN THE SEQUENCE OF TREATMENT WAS:		SURVIVOR RATIO (H/L : L/H)
	Heat/Light	Light/Heat	
*B. cohaerens:**			
1½ minutes	900,000	843,000	1.0:1.0
3 minutes	205,000	48,000	4.2:1.0
5 minutes	3,460	420	8.2:1.0
7 minutes	32	13	2.4:1.0
CC:†			
2 minutes	400,000	346,000	1.0:1.0
3 minutes	308,000	138,000	2.2:1.0
6 minutes	23,400	4,900	4.7:1.0
9 minutes	392	270	1.0:1.0
B. albolactis:‡			
1 minute	65,000	64,000	1.0:1.0
2 minutes	2,920	1,470	1.9:1.0
3 minutes	556	130	4.2:1.0
3½ minutes	186	30	6.2:1.0

* Untreated control contained 1,900,000 viable spores per ml.
† Untreated control contained 2,100,000 viable spores per ml.
‡ Untreated control contained 350,000 viable spores per ml.

lethal agent. The results presented in Table I were obtained when spores uniformly dispersed in distilled water were exposed to radiation from a quartz-mercury lamp and heat of constant intensity in the order, and for the periods, indicated. Changes in the reaction of the spore suspension during irradiation did not exceed 0.1 pH; hence this factor may be disregarded in the interpretation of results. These data clearly show that the spore-

killing action of any given combination of heat and ultraviolet light is materially influenced by the order of their application.

When the periods of exposure were short and the mortality relatively low, the order of treatment had but little influence upon the number of spores which survived; a slight but distinctly greater mortality resulting when heat succeeded irradiation. With lengthening of the exposure periods and consequent rereduction in number of survivors, the order of treatment became of increasing significance. Comparative differences are indicated by changes in the survivor ratio. Light preceding heat was always more destructive than heat preceding light. As may be noted in table 1, the survivor ratio increased with length of the exposures until maximum sensitization was attained. With some species, this was followed by a reduction in the survivor ratio; with one species such an effect was not observed.

The spores of the three species reacted in essentially the same way to the combined action of irradiation and heat. The sensitizing effect was greatest with the spores of *B. cohaerens* in which the survivor ratio at its maximum was more than 8:1. The increased mortality attributable to sensitization, manifestly represents only a very small part of the total population but it is significant that the spores most easily sensitized are those of high resistance.

The change produced in spores by exposure to ultraviolet light is apparently irreversible since their tolerance to heat was found to be independent of the time which elapsed between the two treatments.

The heat-sensitizing action of ultraviolet light is again brought out in table 2. Under the conditions of the experiment, irradiation for 2 minutes was about equal to heat for 5 minutes in sporocidal action. By making a suitable dilution of the untreated suspension, a control suspension of spores could be obtained in which the number of viable spores was comparable with the number surviving a 5 minute heating period or 2 minutes' irradiation. To the control suspension was added a sufficient amount of heat-killed spores to make the total number of living and dead spores essentially the same in the three samples.

Under these conditions, when an irradiated suspension was given a second exposure to light only 100,000 spores survived. When the suspension was first heated and then irradiated, over six times as many spores survived and this number was about equal to the number that survived a single exposure to ultraviolet light when the suspension was neither heated nor irradiated previously. The spores previously heated were, therefore, no more sensitive to irradiation than spores not previously heated or irradiated. When heated spores were given a second heating the mortality was somewhat greater than for the light-heat combination, but the control spores in this instance were consider-

TABLE 2

The effect of ultraviolet light (incident energy 22.2 Ergs/mm²/sec.) and of heat (98°C.) upon spores of B. cohaerens

VIABLE SPORES PER MILLILITER IN UNTREATED SUSPENSION	VIABLE SPORES PER MILLILITER WHEN THE SEQUENCES AND TIMES OF TREATMENT WERE					
	First treatment	Time	Count	Second treatment	Time	Count
		min.			*min.*	
2,700,000	Light	2	770,000	Light	2	100,000
				Heat	5	230,000
	Heat	5	860,000	Light	2	650,000
				Heat	5	180,000
	Dilution		840,000	Light	2	540,000
				Heat	5	450,000

ably more resistant to heat than were the spores that had been irradiated.

It is apparent that spores which survive lethal heat or lethal ultraviolet light are sensitized to that particular form of energy. Although the results are somewhat variable the general relationships were consistently obtained under conditions of adequate irradiation. Thus, where heat and ultraviolet light of about the same sporocidal power are concerned, sensitization to light is best produced by light, and sensitization to heat is best produced by heat. When combinations are involved, light sensitizes to heat less than heat does to heat. Since heated spores are no more sensitive to irradiation than spores that have not been

first heated or irradiated, heat may be regarded as having little or no light-sensitizing action. In the foregoing experiments the spores were exposed in a fused quartz vessel, hence the effects observed were produced by rays of wave lengths longer than 2,000 Å.

Since the effects described by Bovie *et al.* were believed to be caused by ultraviolet rays of very short wave length it seemed of particular interest to compare the heat-sensitizing action of long and very short ultraviolet rays. In order to accomplish this, the spores were dried on sterile coverslips and during exposure pressed tightly against the fluorite and quartz windows of a hydrogen discharge tube. After exposure, the spores were collected and treated as previously described. Ozone effects may be considered negligible owing to the exclusion of air between the discharge tube windows and the spores.

Examination of table 3 shows that spores not previously exposed to ultraviolet light were clearly much more resistant to heat than those previously exposed to light passing through fluorite or quartz. The objection may be made that spores which survive a destructive influence might reasonably be expected to be so weakened that they would die more rapidly when exposed to another destructive influence. However, reasonable as this supposition may seem, it is not universally true. As pointed out in connection with the study of the light-heat sequence in table 2, spores which survived heat were no more sensitive to ultraviolet light than those not previously exposed to heat. We believe, therefore, that irradiated spores are more susceptible to heat because of a specific change in the condition of the cells produced by the ultraviolet rays. The heat-sensitizing effects of the rays passing through fluorite and quartz as shown in the table are not directly comparable because of differences in transparency of the two substances. With equal exposures somewhat more energy would be emitted at the fluorite window.

In an effort to obtain results which would serve as a more satisfactory basis for comparison, the exposures through quartz were lengthened by 5 second increments up to 30 seconds. Ex-

amination of the results obtained shows that the light transmitted by quartz was much less effective in sensitizing the spores to heat even with much longer exposures. These effects are best observed in the percentage reduction values. Only when the exposure through quartz was three times that through fluorite were the heat-sensitizing effects similar. From this it is evident that ultraviolet rays of very short wave length have much greater heat-sensitizing action upon spores than those transmitted by quartz. The sample of fluorite used was transparent to 1,250 Å.

TABLE 3

The influence of ultraviolet light transmitted by quartz and fluorite upon the heat-resistance of B. cohaerens spores

ULTRAVIOLET TREATMENT OF B. COHAERENS IN ORIGINAL SAMPLES	VIABLE SPORES PER MILLILITER		PERCENTAGE REDUCTION BY HEATING
	In original samples after irradiation	In irradiated samples after heating for 4 minutes at 98°C.	
			per cent
No treatment (control)	368	181	50.8
Fluorite, for 10 seconds	445	40	91.0
Quartz:			
For 10 seconds	430	153	64.4
For 15 seconds	389	94	75.8
For 20 seconds	421	77	81.7
For 30 seconds	407	40	90.1
Fluorite, plus quartz for 10 seconds	380	151	60.2
Quartz, plus quartz for 10 seconds	385	151	52.3
Fluorite, for 10 seconds at 5 mm	440	211	52.0
Quartz, for 10 seconds at 5 mm	430	201	53.2

Crystal quartz of the thickness used is transparent to 1,600 Å., according to Lyman (1914), hence the effects observed are to be attributed to the Schumann rays through a spectral range of 350 Å.

In this study some exposures were made in which a second quartz disc 2 mm. thick was interposed between the spores and the discharge tube windows. So treated, the heat-sensitizing effects in the two combinations were almost identical and both somewhat less than those obtained with light transmitted by the

single quartz disc. The second quartz disc absorbs completely the shorter rays passing through fluorite and the exclusion of these rays reduces the heat susceptibility of the spores to about 60 per cent.

In order to confirm the belief that the heat-sensitizing action of the rays transmitted by fluorite was caused by the very short rays in the Schumann region the spores were exposed 5 mm. from the window. An air column of this thickness will absorb practically all the Schumann rays as pointed out by Pflüger (1904). Increasing the path of light does, of course, reduce the light intensity, but the decreased sensitization resulting therefrom is obviously much greater than can be explained by the inverse-square law. In calculations based on the inverse-square law the reduction in intensity could not exceed 12 per cent, and inasmuch as this is not a point source, the reduction in intensity is less (of the order of 4 to 6 per cent), whereas the observed reduction in heat susceptibility is from 91 per cent to 52 per cent.

The data recorded in this table represent the averages of 10 separate experiments in which the deviation from the average was approximately $\pm$ 4.5.

DISCUSSION

In this study, sensitization to heat was observed only under conditions which were sporocidal for a considerable number of cells. Sublethal exposures did not measurably decrease the heat-resistance of spores so treated. It is apparent that there is a minimum level of radiant energy below which sensitization does not occur. Where the weaker cells are concerned this threshold value may exceed their ultraviolet light tolerance; this we believe to be the most plausible explanation for the low sensitization which accompanies low mortality.

The exact mechanism by which ultraviolet light renders bacteria more susceptible to heat is a matter for conjecture. A satisfactory explanation may be found in the reactions known to occur in simple proteins under the influence of ultraviolet radiation.

Clark (1935) has demonstrated that the coagulation of iso-

electric egg albumin solutions by ultraviolet radiation involves three distinct processes; first, a physical process, independent of temperature and largely independent of hydrogen-ion concentration; secondly, a chemical reaction between the light-denatured molecule and water, which is similar in some respects to denaturation by heat, and finally flocculation of the previously-altered molecules. The significant fact observed by Clark was that heat-denaturation, the precursor of flocculation, occurs at a lower temperature after light-denaturation; in the latter state, therefore, the protein must be in a chemically active condition.

By analogous reasoning, bacterial protoplasm is converted by the absorption of ultraviolet energy into a more reactive and therefore less resistant state, in consequence of which the subsequent processes of heat-denaturation and flocculation occur at lower energy levels.

The permanent nature of light-denaturation is in accord with our observation previously described in which heat sensitization effects were found to be independent of the time elapsing between irradiation and heat treatment. The irreversibility of the reaction with Paramecia appears to be open to question since Bovie and Klein (1918) observed complete recovery from the effects of Schumann rays when sufficient time elapsed before heat was applied.

SUMMARY

Many spores which survive lethal heat or ultraviolet radiation are thereby sensitized to that particular influence and in consequence are more easily killed by further applications of the same treatment than are untreated or control spores.

Many spores which survive lethal ultraviolet radiation are thereby sensitized to heat. The number of spores sensitized to heat by ultraviolet radiation is less than the number sensitized to light by ultraviolet radiation; similarly the number of spores sensitized to heat by ultraviolet radiation is less than the number sensitized to heat by heat.

Apparently not all spores are susceptible to sensitization by

heat; those moderately and highly resistant to light and heat are most affected.

When spores are exposed to the combined action of heat and ultraviolet radiation the order of treatment materially affects the number which will survive. Under conditions of adequate irradiation, the light-heat sequence is always the more destructive.

Heat has no appreciable light-sensitizing action upon spores.

Ultraviolet rays of wave lengths longer than 2,000 Å., under suitable conditions of exposure, sensitize spores to heat. Ultraviolet rays in that portion of the Schumann region between 1,250 and 1,600 Å. are more effective in sensitizing spores to heat than those transmitted by quartz.

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