# THE EFFECT OF X RAYS ON THE MACROMOLECULAR ORGANIZATION OF ESCHERICHIA COLI[1]

DANIEL BILLEN AND ELLIOT VOLKIN

*Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee*

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Earlier studies of the effects of X irradiation on *Escherichia coli* had shown that adenosinetriphosphate (ATP) as well as other purine and pyrimidine containing materials is released from such exposed cells under certain conditions (Billen *et al.*, 1953*b*; and unpublished data of the authors). Two possible causes of this release of adenosinetriphosphate from exposed cells were postulated. Either an increased permeability of the cellular membrane had resulted from exposure to X rays, or the release of bound adenosinetriphosphate from an organized system within the cytoplasm was responsible for the leakage of adenosinetriphosphate. The investigation reported here was initiated both to test the latter hypothesis of the mechanism of adenosinetriphosphate release and to learn more of the role of macromolecular organization in normal and X-irradiated *E. coli*.

Other investigators have found that cell-free extracts of *E. coli* obtained by grinding show several characteristic boundaries when studied with an analytical ultracentrifuge (Schachman *et al.*, 1952; Siegel *et al.*, 1952). These components possessed sedimentation constants of approximately 40, 29, 20, 8, and 5 S.[2] Separation of the 40 S component from the cell extract revealed that this fraction consisted of particles 20 to 40 mμ in diameter and was composed mainly of ribonucleoprotein. The fraction composed of both the 8 and 5 S components accounted for the major portion of the desoxyribonucleic acid (DNA) of the whole extract (Schachman *et al.*, 1952). The 8 S spike has been reported to consist of free desoxyribonucleic acid (Siegel *et al.*, 1952; Siegel and Singer, 1953). This communication presents the results of studies on the effect of X irradiation both on the structure and the succinoxidase and oxidative phosphorylation activities of the macromolecular components of cell-free extracts. The extracts were analyzed both immediately after exposure of the bacterial suspension and following a period of incubation of the exposed cells in solutions with and without added nutrients.

## MATERIALS AND METHODS

The procedure used for ultracentrifugal analysis and isolation of the macromolecular component fractions of bacterial extracts was similar to that described by Schachman *et al.* (1952). Cells of *E. coli*, strain B/r, were harvested following an 18 hr to 20 hr incubation (under aeration) at 37 C in nutrient broth, and the cells (from 800 ml of culture medium) were washed once by resuspension in 0.05 M NaCl and reharvested. The washed cells then were resuspended in 20 ml of 0.05 M NaCl, and a 10 ml aliquot of this cell suspension containing approximately $5 \times 10^{10}$ to $10^{11}$ viable cells per ml was exposed to 120,000 r of X rays under conditions similar to those described in an earlier communication (Stapleton *et al.*, 1952). This dosage causes a 99.0 to 99.5 per cent decline in the number of cells of the suspension capable of forming visible colonies on nutrient agar plates incubated at 37 C. Both the unexposed and the exposed cells were reharvested, and the pellets obtained were ground then in a chilled mortar for 5 minutes with alumina (Linde A, Union Carbide and Carbon Corporation) as described by McIlwain (1948). The grist was resuspended in 3 to 5 ml of 0.05 M NaCl, and the coarse debris and remaining intact cells were removed by centrifugation of the extract for 15 minutes at an average force of 1,600 times gravity.

Ultracentrifugal analyses of the components in the extracts were carried out as described by Schachman *et al.* (1952) using the Spinco Model E analytical ultracentrifuge at an average force of 130,000 times gravity. Separation of the component fractions was carried out with the Spinco Model L preparative centrifuge.

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[2] Sedimentation constants are reported in Svedbergs: one S is $10^{-13}$ cm per sec per unit field.

The distribution of phosphorus in the centrifugally isolated fractions was determined by a modification of the Schmidt-Thannhauser method (Morse and Carter, 1949) while Kjeldahl nitrogen determinations were carried out for standardization of the whole extracts.

Succinoxidase activity of either whole extract, or of the centrifugally prepared fractions thereof, was determined by manometric measurements of oxygen consumed (described in figure 2). Adenosinetriphosphate analysis was made by the firefly luminous-organ extract method (Strehler and Totter, 1952; Billen *et al.*, 1953*b*) with the exception noted in the text.

## RESULTS

*Ultracentrifugal analysis of normal and X-irradiated E. coli extracts.* Analysis of the ultracentrifugal pattern obtained from extracts of unexposed *E. coli*, strain B/r, revealed a macromolecular organization essentially similar to that reported by Schachman *et al.* (1952) and Siegel *et al.* (1952) in their studies with this organism. Two major and two minor peaks are noted in the ultracentrifugal pattern shown in figure 1A. The major peaks possess sedimentation constants of 5-8 S and 40 S; the 5-8 S peak consists of two components, one showing a relatively broad boundary (5 S) and the other a sharp spike

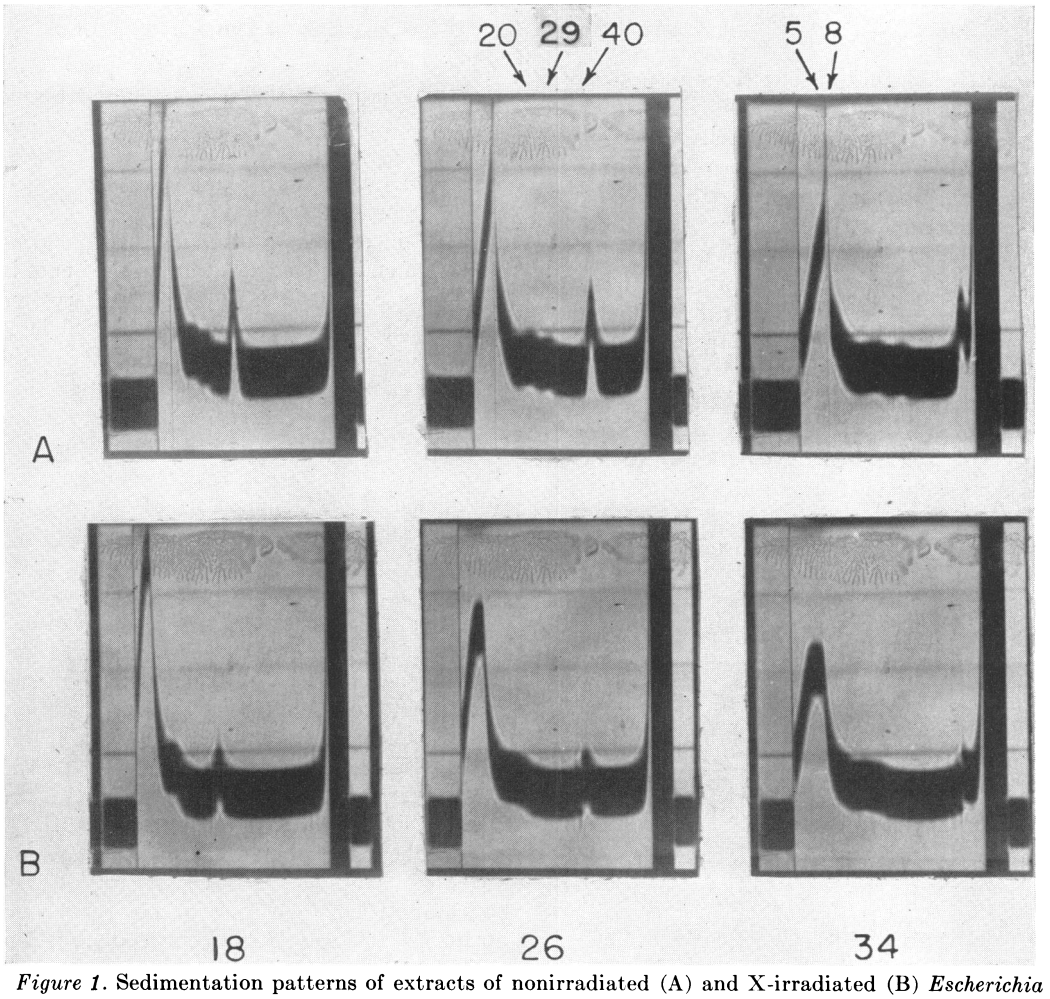


*Figure 1.* Sedimentation patterns of extracts of nonirradiated (A) and X-irradiated (B) *Escherichia coli*. Cells were incubated at 37 C in a phosphate buffer glucose solution for 90 minutes prior to extraction. Sedimentation proceeds to the right. The numbers under the diagrams indicate the time in minutes after the rotor attained full speed, while the numbers over the diagrams refer to the sedimentation rates of the component boundaries pictured.

(8 S). The two minor peaks have sedimentation constants of approximately 20 and 29 S. Ultracentrifugal analysis of irradiated cell extracts obtained immediately after exposure revealed no alteration in the component pattern. However, if the cells were resuspended in either nutrient broth supplemented with one per cent glucose or in 0.067 M phosphate buffer (pH 6.8) plus one per cent glucose and incubated for 90 minutes subsequent to X-ray exposure, marked changes were observed to have occurred in the macromolecular composition of these cells as compared to no change in similarly treated control cells. A typical example is shown in figure 1B. It is seen that incubation of X-irradiated cells in buffer-glucose medium at 37 C for 90 minutes caused a disappearance of the sharp spike attributed to the 8 S component as well as a marked decrease in the 40 S component. The 29 S component, which is present normally in a much smaller relative amount, appears to have disappeared completely. Quantitative area measurements of the components observed in the experiment illustrated in figure 1 reveal that, though there is no significant change in the relative amount of 5 S component, a 65 per cent decrease is evident in the 40 S material in extracts derived from exposed and incubated cells as compared to no change in the control cell extracts. The quantitative change in the 40 S component of extracts from exposed cells in five similar experiments ranged from a 60 per cent decrease to its complete disappearance. Though the relative area of the 5-8 S component of irradiated incubated cells remains essentially unchanged, it should be noted that in all cases the sharp desoxyribonucleic acid spike has disappeared, probably indicating depolymerization of this component. Further evidence of the secondary nature of the observed destruction of macromolecular components in irradiated cells was the observation that if exposed cells were incubated at 37 C for 90 minutes in buffer alone without added metabolite (such as glucose), there was no observable change in component characteristics found in the extract of the exposed cells as compared to the extract obtained from the control cells.

*Release of particulate ribonucleic acid in exposed bacterial cells.* Confirmation of the disintegration of the 40 S particulate component in exposed *E. coli* following incubation in a buffer-glucose solution was obtained by differentially centrifuging the extracts into several fractions and assaying each fraction for chemical composition. Three fractions were obtained as follows: Particulate fraction A consisted of the sediment obtained by centrifugation of the cell-free extract at an average force of 41,200 times gravity for 15 minutes. The supernatant remaining following removal of particulate fraction A was recentrifuged at an average force of 105,400 times gravity for 90 minutes. The sediment obtained (20, 29, and 40 S components) was called particulate fraction B. Electron micrograph studies of particulate fraction B revealed particles 20 to 50 mμ in diameter and were similar to those observed by Schachman *et al.* (1952). The supernatant remaining following removal of particulate fraction B was designated fraction C and included the 5 and 8 S components. Chemical analysis of phosphorus distribution in these fractions revealed, when compared to similar fractions from unexposed cells, that about 50 per cent of the particle ribonucleic acid (RNA) (fraction B) was converted to material now found in fraction C as acid soluble material (both organic and inorganic phosphate) (table 1). The desoxyribonucleic acid in fraction C, although ostensibly depolymerized as a result of irradiation and incubation, remained essentially as acid insoluble material.

The finding of a relatively large quantity of ribonucleic acid, primarily of the ribose type, in particulate fraction B (table 1) is in good agreement with the observations of Schachman *et al.*

TABLE 1

*Distribution of organic phosphates in cell-free extracts obtained from normal and X-irradiated Escherichia coli*

| CHEMICAL FRACTION ISOLATED | PHOSPHATE IN COMPONENT FRACTIONS* (μG/MG N OF EXTRACT) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | | | | Irradiated | | | |
| | A | B | C | Total | A | B | C | Total |
| Acid soluble | 1.9 | 3.5 | 21.1 | 26.5 | 1.5 | 2.7 | 35.6 | 39.8 |
| Phospholipids | 3.1 | 2.1 | 1.5 | 6.7 | 3.1 | 2.5 | 1.6 | 7.2 |
| Ribonucleic acid | 4.9 | 20.7 | 54.3 | 79.9 | 5.0 | 10.5 | 49.5 | 65.0 |
| Desoxyribonucleic acid | 2.5 | 1.5 | 15.9 | 19.9 | 2.6 | 1.4 | 16.2 | 20.2 |
| Protein | 1.4 | 0.9 | 2.0 | 4.3 | 1.2 | 0.2 | 1.7 | 3.1 |

* Fractions obtained as described in text.

(1952). However, ribonucleic acid is present also in a relatively large quantity in fraction C, an observation that differs from that of Schachman and investigators. In their analysis of a similar fraction, they found the nucleic acid present to be mainly of the desoxyribose type.

*Succinoxidase and oxidative phosphorylative activity of the macromolecular components from unexposed and X-irradiated cells.* A study of both succinoxidase activity and adenosinetriphosphate synthesis during succinate oxidation by the cell extract obtained immediately following X irradiation of *E. coli* showed that exposure of the cells to 120,000 r had no observable effect on these systems. Both oxygen uptake and net adenosinetriphosphate formation in the presence of succinate were normal in the extract from exposed cells (table 2*a*). However, extracts obtained from exposed cells that had been incubated subsequently in the buffer-glucose solution showed a marked decrease in ability to show a net adenosinetriphosphate gain (table 2*b*). Since some stimulation of succinoxidase activity is seen, the over-all effect resembles that obtained with such chemical agents as azide and dinitrophenol that uncouple oxidative phosphorylations.

The finding that the cell-free extracts, as obtained in these experiments, possessed the ability to couple oxidation of the succinate with phosphorylation with a resultant net increase in adenosinetriphosphate made it desirable to attempt to localize the site of adenosinetriphosphate formation in the macromolecular components. Extracts obtained from nonexposed cells were

TABLE 2

*Succinoxidase and oxidative phosphorylation activity of cell-free extracts obtained from normal and X-irradiated Escherichia coli*

| X-RAY DOSE | SUCCINOXIDASE ACTIVITY | | NET ATP SYNTHESIS | |
|---|---|---|---|---|
| | $Q^0_{N_2}$ | Relative activity experimental/ control | μg ATP/ mg N/hr | Related activity experimental/ control |
| | *a** | | | |
| r | | | | |
| 0 | 83 | — | 12.1 | — |
| 60,000 | 85 | 1.02 | 12.5 | 1.03 |
| | *b*† | | | |
| 0 | 82 | — | 11.16 | — |
| 60,000 | 106 | 1.29 | 2.34 | 0.21 |

* Extracts obtained immediately following X irradiation.

† Extracts obtained following both X irradiation and subsequent incubation at 37 C for 90 minutes in a buffer-glucose solution.

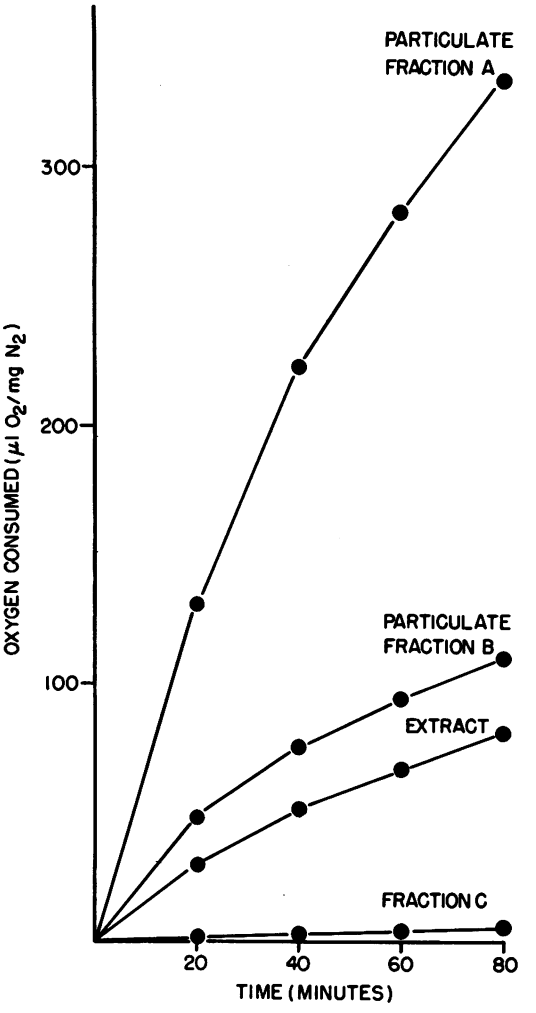


*Figure 2.* The succinoxidase activity of cell-free fractions obtained from *Escherichia coli*. To the main compartment of each manometer cup were added 0.5 ml of cell-free extract or fraction thereof, 0.1 ml of adenylic acid (500 mg per ml), and 0.5 ml of 0.067 M phosphate buffer at pH 6.8; 0.2 ml of 0.1 M succinic acid was added to the side arm and 0.2 ml of 20 per cent KOH added to the center well. The final total volume was made to 3 ml with distilled $H_2O$. The succinic acid was tipped in after equilibration of the contents of the cups in a water bath at 37 C.

fractionated by ultracentrifugation as already described. It was found that particulate fractions A and B both possessed succinoxidase activity, the former being approximately 3 times more active than the latter (figure 2). However, the more interesting finding was that only particulate fraction B was able to effect a net synthesis of adenosinetriphosphate (table 3). Thus, the ribonucleic acid containing particulate component of particulate fraction B, found to break down in *E. coli* incubated in a buffer-glucose solution subsequent to exposure, apparently is the sole site of coupled oxidative phosphorylation in the cell extract under the conditions imposed for these studies.

It was observed also that, whereas boiling of intact cells was a necessary step prior to adenosinetriphosphate assay in order to make adenosinetriphosphate available for the firefly luminescence system, boiling of either the whole cell extract or particulate fraction B did not increase the assayable adenosinetriphosphate. Apparently then, the adenosinetriphosphate initially present, or formed subsequently during oxidative phosphorylation by the particulate material, was either released from the particle or bound in such a manner that it was available to the added firefly system. Since earlier observations had shown that adenosinetriphosphate was released by the exposed intact cells, it was a matter of some importance to determine the disposition of the adenosinetriphosphate formed during oxidative phosphorylation by particulate fraction B. This was done as follows: Particulate fraction B obtained from nonexposed cells was incubated in the presence of succinic acid for 60 minutes at 37 C, and oxygen consumption measured manometrically. Aliquots of the mixture then were placed in Visking Casing dialyzing membranes and dialyzed against 0.05 M NaCl at 2 C. At the end of 4 hours the dialyzate was tested for adenosinetriphosphate content. It was found that ca 75 per cent of the adenosinetriphosphate that had been formed during oxidation of the succinate appeared in the dialyzate at the end of this 4 hour period. Studies with particulate fraction B prepared from X-irradiated cells produced similar results in that the same portion of adenosinetriphosphate formed during aerobic phosphorylation appeared in the dialyzate after the same treatment.

TABLE 3

*Locus of ATP formation in cell-free extracts of Escherichia coli*

| FRACTION | ATP SYNTHESIS (μG/MG N) | |
|---|---|---|
| | Initial | Final* |
| Whole extract | 0.89 | 6.1 |
| A | 0.06 | 0.09 |
| B | 0.13 | 35.7 |
| C | 0.14 | 0.22 |

* Seventy minutes after addition of succinic acid.

## DISCUSSION

Since information concerning bacterial cytology and physiology is still in the early formative state, it would be rather presumptive to ascribe any fundamental relation between the disorganization of the macromolecular components of the bacterium and the loss of its ability to reproduce. However, it has been shown, both in this report and elsewhere (Billen *et al.*, 1953*a*,*b*), that several rather important changes are induced in the character of the bacterial cell as a result of X irradiation, and that these changes appear to be due to delayed and indirect effects of the exposure. Although the observations recorded here may well represent changes that are several steps removed from the initial site or locus of damage, the possibility remains that such drastic changes as have been observed in this investigation are associated closely both with the X-ray induced changes in the metabolic character of the cell and its ultimate death.

It seems fairly well established, both from the data reported here and from the work of Schachman *et al.* (1952), that a particulate component of the order of 20 to 50 mμ is present in *E. coli* cells and that these particles are the sites of a number of important enzyme systems, i.e., succinodehydrogenase, catalase, apyrase, formic dehydrogenase, ribonuclease (Schachman *et al.*, 1952), and as reported here, succinoxidase, and aerobic phosphorylation activity. The disruption of these particles in irradiated cells has been studied by ultracentrifugal and chemical analyses of the cell-free extracts obtained from such cells. Since these particles appear to be the sole site of adenosinetriphosphate formation in the cell-free extracts, their disruption could account for the decrease in adenosinetriphosphate forming ca-

pacity exhibited by extracts obtained from irradiated cells which were incubated subsequently in the phosphate-glucose medium (table 2). It should be noted that extracts obtained from cells *immediately* following their exposure to X rays show neither an altered ability to produce adenosinetriphosphate nor a change in the particulate component pattern as studied with the ultracentrifuge.

Since the release of adenosinetriphosphate by irradiated cells (Billen *et al.*, 1953*b*) occurs under conditions similar to those causing the breakdown of the particulate components of fraction B, it is possible that the release of adenosinetriphosphate by intact irradiated cells was a consequence of this destruction in that this component also served as a site of adenosinetriphosphate formation. The finding that the adenosinetriphosphate as formed apparently is not bound strongly, if at all, to the particles would seem to negate such a possibility. However, it is conceivable that under *in vivo* conditions the major portion of the adenosinetriphosphate may be bound firmly. At the moment, it would seem likely that the release of adenosinetriphosphate from intact irradiated cells is a result of some change in membrane permeability. The depolymerization of both the 40 S component of particulate fraction B consisting primarily of ribonucleic acid and the 8 S component, reported to consist of free desoxyribonucleic acid (Siegel and Singer, 1953), could account for at least a portion of the 260 mμ wavelength absorbing material other than adenosinetriphosphate that is found to be released by irradiated cells (unpublished data of the authors).

The nature of the mechanism of the disruption of the particulate component found in fraction B is yet unknown. Preliminary studies have shown that a very active ribonuclease system is present in the particles obtained from both irradiated and nonirradiated cells. It has been reported that, in the intact normal *E. coli* cell, ribonuclease activity appears to be nil (Manson, 1953). Thus, it is possible that X irradiation brings about an activation of the latent ribonuclease system or a destruction of some ribonuclease inhibitor, which in turn could cause a depolymerization of the ribonucleic acid of the particulate material. The dependence of particulate disruption in the intact irradiated cell on the presence of a substrate which can serve as a metabolite, i.e., glucose, makes this a rather unlikely explanation. The role of added substances under conditions in which they can be metabolized actively in augmenting observable radiation induced damage is unknown. It may be that actively metabolizing cells, previously exposed to lethal doses of X rays, no longer are able to maintain the integrity of the important particulate components due to an inability to replace by synthesis some substance necessary for the maintenance of the complex materials of which the particulate matter is composed, i.e., nucleic acid and proteins. This hypothesis would be based on the assumption that in the normal actively metabolizing cell, whether in a proliferating or nonproliferating state, there is a constant turnover of material at the sites of enzyme action in particulate systems. Although the nature of the disruption of cellular macromolecules is unknown, the data emphasize the dependence for expression of the secondary effects on the catabolic and/or anabolic activities of the irradiated cells.

The finding in cell-free extracts of *E. coli* of two particulate fractions with relatively large differences in size but both showing succinoxidase activity merits some comment. It would seem, on the basis of the limited information available, that the smaller particles of fraction B capable of synthesizing adenosinetriphosphate during succinate oxidation more closely resemble the mitochondria of animal cells than do the larger particulate materials (observed to be heterogeneously composed of cellular debris, i.e., cell fragments) found in fraction A. It would be most interesting to determine what relation, if any, may exist between these particulate fractions obtained by ultracentrifugation of cell-free extracts and the mitochondria identified by vital staining of *E. coli* in the investigations of Mudd *et al.* (1951).

## SUMMARY

The effects of X irradiation on the structure of the macromolecular components of *Escherichia coli*, strain B/r, have been investigated with the analytical ultracentrifuge and by chemical analysis. Five boundaries were observed with sedimentation rates of approximately 5, 8, 20, 29, and 40 S. Exposure to doses of X rays sufficient to inactivate more than 99 per cent of the cells did not change the component pattern observed in cell-free extracts if the cells were analyzed

immediately following exposure. However, if the cells were incubated in a phosphate buffer-glucose solution at 37 C for 90 minutes subsequent to irradiation, a marked decrease in several of the components of the cell-free extract was noted. In the absence of glucose this decrease was not evident. The most striking changes were the disruption and disappearance of the ribonucleic acid containing particulate 40 S component, and an apparent depolymerization of the 8 S component (reported to be free desoxyribonucleic acid) as evidenced by the disappearance of the sharp spike.

It was found also that a particulate fraction consisting primarily of the 40 S component possessed succinoxidase activity and was the sole site of formation of adenosinetriphosphate in cell-free extracts of *E. coli*. In a more rapidly sedimenting fraction with relatively greater succinoxidase activity, there was no evidence for the synthesis of adenosinetriphosphate. Cell-free extracts obtained immediately following X irradiation of *E. coli* showed normal succinoxidase and aerobic phosphorylation activity. However, extracts obtained from irradiated cells subsequently incubated in buffer-glucose at 37 C gave slightly increased succinoxidase activity and a much reduced capacity to form adenosinetriphosphate.

The significance of the X-ray induced destruction of the 40 S particles to previously reported physiological changes in exposed cells is discussed.

## REFERENCES


Billen, D., Stapleton, G. E., and Hollaender, A. 1953*a* The effect of X radiation on the respiration of *Escherichia coli*. J. Bact., **65,** 131–135.

Billen, D., Strehler, B. L., Stapleton, G. E., and Brigham, E. 1953*b* Postirradiation release of adenosine triphosphate from *Escherichia coli* B/r. Arch. Biochem. and Biophys., **43,** 1–10.

Manson, L. A. 1953 Metabolism of ribonucleic acid in normal and bacteriophage-infected *Escherichia coli*. Federation Proc., **12,** 242–243.

McIlwain, H. 1948 Preparation of cell-free bacterial extracts with powdered alumina. J. Gen. Microbiol., **2,** 288–291.

Morse, M. L., and Carter, C. E. 1949 The synthesis of nucleic acids in cultures of *Escherichia coli*, strains B and B/r. J. Bact., **58,** 317–326.

Mudd, S., Brodie, A. F., Winterscheid, L. C., Hartman, P. E., Beutner, E. H., and McLean, R. A. 1951 Further evidence of the existence of mitochondria in bacteria. J. Bact., **62,** 729–739.

Schachman, H. K., Pardee, A. B., and Stanier, R. Y. 1952 Studies on the macromolecular organization of microbial cells. Arch. Biochem. and Biophys., **38,** 245–260.

Siegel, A., Singer, S. J., and Wildman, S. G. 1952 A preliminary study of the high-molecular-weight components of normal and virus-infected *Escherichia coli*. Arch. Biochem. and Biophys., **41,** 278–293.

Siegel, A., and Singer, S. J. 1953 The preparation and properties of desoxypentosenucleic acid of bacteriophage T2. Biochim. et Biophys. Acta, **10,** 311–319.

Stapleton, G. E., Billen, D., and Hollaender, A. 1952 The role of enzymatic oxygen removal in chemical protection against X-ray inactivation of bacteria. J. Bact., **63,** 805–811.

Strehler, B. L., and Totter, J. R. 1952 Firefly luminescence in the study of energy transfer mechanisms. I. Substrate and enzyme determination. Arch. Biochem. and Biophys., **40,** 28–41.