RADIATION INHIBITION OF AMINO ACID UPTAKE BY *Escherichia coli*

E. S. KEMPNER *and* E. C. POLLARD

From the Biophysics Department, Yale University, New Haven. Dr. Kempner's present address is National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda

ABSTRACT The inhibition of macromolecular synthesis in *Escherichia coli* by ionizing radiation has been investigated. The survival of the ability to incorporate arginine, leucine, isoleucine, histidine, uracil, and glucose after various doses of gamma radiation, deuteron and alpha particle bombardment has been measured. All amino acids are incorporated by processes which show the same radiation sensitivity. The sensitivity of uracil corresponds to a volume which is roughly spherical, of radius about 160A, whereas the amino acids possess sensitive regions which are long and thin in character. The uptake of glucose is concerned with a smaller, roughly spherical unit. The possible identification of the radiation-sensitive targets with cellular constituents is discussed. The long thin character observed for amino acids suggests that the sensitive region affected by radiation is an unfolded form of a ribosome, or alternatively a long nucleic acid molecule. For uracil the sensitive region fits with a 70S ribosome, while for glucose a smaller particle would fit the data.

INTRODUCTION

Studies on the uptake of amino acids by bacterial cells have begun to reveal some of the aspects of the cellular synthetic mechanism. Roberts and coworkers at the Carnegie Institution, in particular, have exploited the method in a sustained series of studies (Roberts *et al.*, 1957). Recently McQuillen, Roberts, and Britten (1959) have shown, by rapid pulse-labeling methods, that fractions of the particulate structure of *Escherichia coli,* the 70S and 85S ribosomes, are the site of first synthesis of protein in the cell. It is of great interest to determine the actual nature of the macromolecular organelle responsible for this process. It is possible that within the cell the ribonucleoprotein particles do not all possess the character of being spherical. Instead they may be extended in some way, or at least might be expected to be a mixed population, some being unfolded and others not. Alternatively, some of the processes of amino acid uptake may be concerned with large RNA molecules themselves.

Preliminary indications regarding these questions can be made by studying the

way ionizing radiation inhibits the uptake of amino acids. Such studies employ a simple statistical analysis of the inhibition, taking advantage of the fact that ionizing radiation produces energy releases which are largely localized, either in small separate clusters occupying a region not more than a few Angstrom units across, or along lines of relatively dense ionization, with ionizations spread apart to a greater or lesser extent depending on the kind of ionization source used. The technique of such irradiation studies has been under development in this laboratory for some time and the validity of some of the necessary assumptions has also been the subject of much research (Pollard *et al.*, 1955). Recent work supporting this method of analysis may be found in the papers of Pollard (1959), Hutchinson (1957), and Pollard and Barrett (1959).

Comparative studies of various kinds of cell damage by radiation have been made and are briefly reviewed by one of the authors (Pollard, 1960). The uptake of amino acids is quite insensitive, by comparison with cell division or uptake of phosphate, and it therefore seems probable that amino acid uptake (and so probably protein synthesis) is thus a measure of ribosomal damage, or at any event, of a process which does not require the whole organization of the cell. On the other hand, ionizing radiation is not disruptive of the entire cellular contents, as is the case for extractive techniques, and therefore the statistical study of inhibition should be informative of the character of the synthetic units.

Preliminary experiments on the incorporation of certain amino acids have been reported (Hutchinson *et al.*, 1957; Kempner and Pollard, 1958); these have now been extended to include four other amino acids, and glucose and uracil for comparative purposes. In this paper we report the extended work and include a summary of all the findings.

MATERIALS AND METHODS

Cultures of *Escherichia coli* B (A.T.C.C. No. 11303) were grown with aeration at 37°C in Roberts' (1957) minimal "C" medium containing five gm of glucose per liter. Growth was measured turbidimetrically in a Bausch and Lomb "spectronix 20" colorimeter at 650 mμ. When the cultures reached a concentration of 3.0 to 5.0 $\times$ 10^8 cells/ml, samples were removed and treated as follows:—

1. Irradiation in a cobalt^{60} source. Twenty ml samples of the bacterial culture were placed in screw-top culture tubes and placed in a 1500 curie cobalt^{60} source. The dose rate was found to be 330,000 r/hr. by ferric sulfate dosimetry. Irradiations were performed at 30°C, and also at dry ice temperatures. For the latter experiments, the bacterial samples were rapidly frozen and then placed in the cooled cobalt source.

2. Cyclotron irradiations. Samples of 1 $\times$ 10^8 or 1 $\times$ 10^9 cells were drawn through "HA" millipore filters. The excess liquid was removed, and the filters carrying the bacteria were kept moist with a porous backing containing minimal medium with no glucose added. The samples were irradiated in the Yale cyclotron at 2°C as described elsewhere (Kempner and Pollard, 1958; Pollard *et al.*, 1955). After irradiation, the bacteria were resuspended in minimal C medium and equilibrated to 37°C.

3. Incubation with isotopically labeled compounds. Irradiated bacterial suspensions were added to an equal volume of minimal medium containing glucose and 0.1 μc of carbon per 20 ml of incubation medium.

The isotopically labeled compounds used in these studies and their specific activities are listed below. All were commercially available and checked for chromatographic purity.

L—Arginine—C^{14}	13.1 mc/millimole
L—Histidine—2 (ring)-C^{14}	0.284 mc/millimole
L—Isoleucine—C^{14}	12.6 mc/millimole
L—Leucine—C^{14}	5.13 mc/millimole
Uracil—2—C^{14}	2.94 mc/millimole
Glucose—C^{14}	1.0 mc/millimole

Studies on the incorporation of glucose were performed in the same medium without the addition of carrier (C-12) glucose. Incubation was conducted with aeration in a 37°C water bath. During an incubation period of 15 minutes, 2.0 ml samples were withdrawn after various time intervals. Half of the samples were drawn through individual collodion membrane filters with an average pore size of 0.85μ. The filters were washed with 2.0 ml of minimal medium and dried in air. These constituted the "intact cell" samples. The remaining samples were added to 2.0 ml of cold 10 per cent trichloroacetic acid (TCA) and placed at 2°C for 1 hour. These were then drawn through membrane filters, washed with 2.0 ml cold 5 per cent (TCA), and air-dried. These are referred to as the (TCA) insoluble samples.

The dried filters were counted under a thin-window Geiger tube on an automatic sample changer (Kempner and Bisbee, 1958).

RESULTS

After various doses of radiation, cultures of *E. coli* all showed increases in optical density during a 90 minute growth period in minimal glucose medium. As an example, after 360,000 r the optical density rose from 0.35 to 0.40. At the doses used in these experiments there is essentially no colony-forming ability left.

To see whether any great redistribution of activity among fractions took place, the proportion of radioactive label in the cold TCA-insoluble material was further extracted with 75 per cent ethanol, ethanol-ether, and hot TCA by the method of Roberts *et al.* (1957). This method of study did not reveal any differential effect in amino acid or uracil uptake.

In Fig. 1 we show the incorporation of C^{14}-leucine as a function of time for unirradiated cells and cells which had received various doses of gamma radiation. The control cells show a normal uptake behavior, with a small difference (which we will refer to as the pool) between whole cell and (cold) TCA-insoluble fractions. The tracer quantity of exogenous label is soon exhaused and the activity of each fraction reaches a plateau. After 665,000 r the cellular uptake has been depressed markedly and similarly the labeled TCA-soluble fraction is lower. The pool size is also decreased. After still greater radiation doses (1,110,000 r) the pool size is unmeasurable.

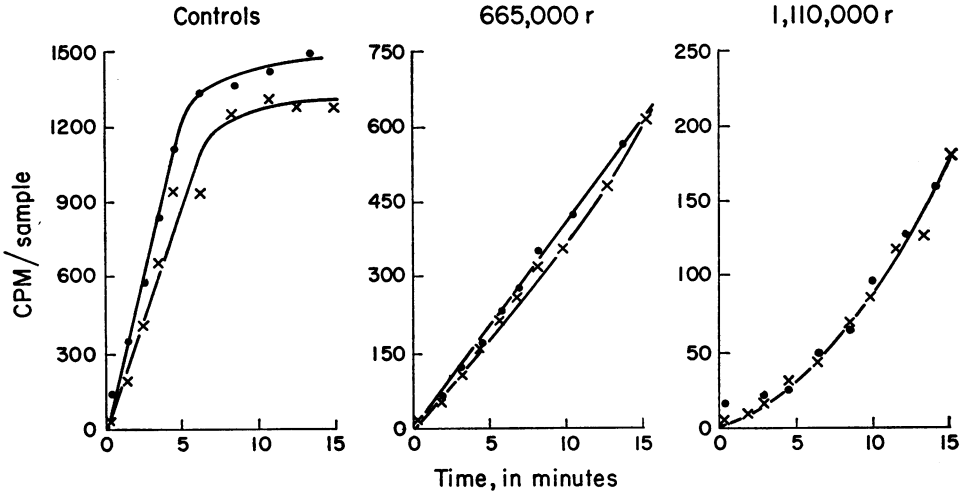

FIGURE 1 Incorporation of L-leucine into the whole cell (upper) and TCA-insoluble (lower curve) fractions as affected by various doses of Co^{60} gamma radiation. There is always a steady increase in the TCA-insoluble fraction even after massive radiation dosage.

The data of Fig. 1 are from a single experiment of five different radiation doses. We generally completed at least two or three such experiments and drew conclusions from the average of all.

In Fig. 2, the incorporation of uracil by *E. coli* after irradiation by cobalt^{60} is shown. The depression of cellular activity is similar to that shown with leucine in Fig. 1, except that although the pool-forming ability is diminished by radiation, it is not completely destroyed.

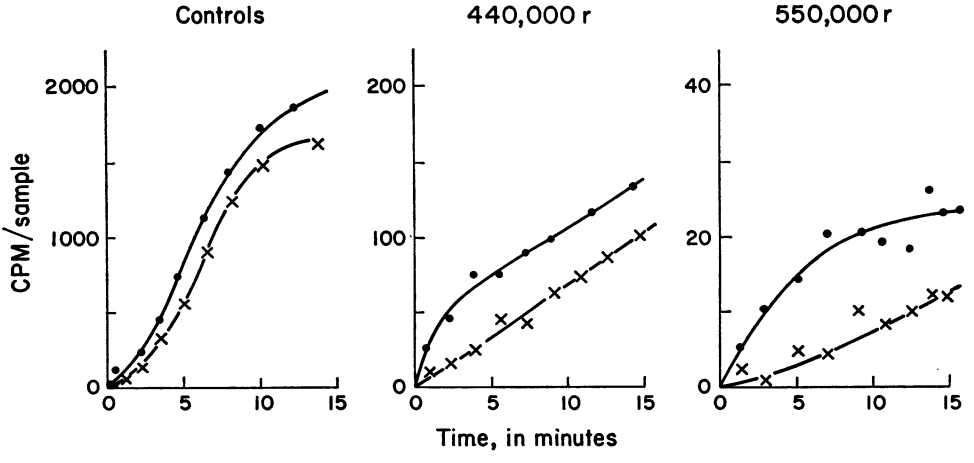

FIGURE 2 Incorporation of C^{14}-uracil into the whole cell (upper) and TCA-insoluble (lower curve) fractions as affected by various doses of Co^{60} gamma radiation.

In order to have some estimate of the relative effects of different doses, we adopted the procedure of plotting the ratio of uptake to that of the control for several different doses, choosing also several different times. Such a set of points for 7 minutes' incorporation of leucine and uracil into the TCA-insoluble fraction is shown in Fig. 3.

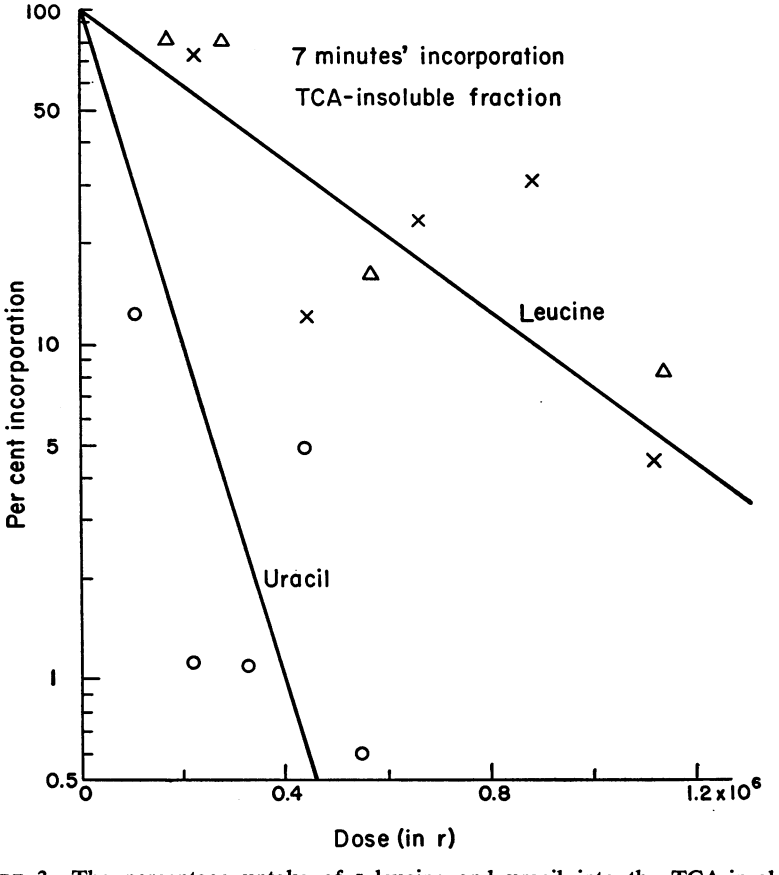

FIGURE 3 The percentage uptake of L-leucine and uracil into the TCA-insoluble fraction at 7 minutes, as a function of gamma radiation dose. Leucine data (×) from experiment shown in Fig. 1. Triangles from a duplicate experiment. The plot of the per cent is on a logarithmic scale and it can be seen that if n/n_o is the ratio of uptake to original uptake, then the relation $\ln n/n_o = \text{constant} \times \text{dose}$ is obeyed.

The ordinate in this graph is plotted logarithmically, and it can be seen that although there is considerable scatter in the points, there is a plausible relationship between the logarithm of the ratio, so plotted, and the dose. If different times are chosen, essentially the same slope is found. The agreement between different times and duplicate experiments was usually 20 per cent or better.

In some experiments the uptake was carried out in the presence of all exogenous

TABLE I

SUMMARY OF RADIATION TARGETS ASSOCIATED WITH THE INCORPORATION OF METABOLITES INTO THE TCA-INSOLUBLE MATERIAL OF *E. coli*

Metabolite	Sensitive volume (V) cm³	Corrected maximum cross-section (S_o) cm²	Length (A)*	Radius (A)*
L-Arginine	5.3×10^{-18}	32×10^{-12}	16,000	10
L-Isoleucine	6.9×10^{-18}	25×10^{-12}	7800	18
L-Leucine	5.6×10^{-18}	25×10^{-12}	9000	14
L-Histidine	5.6×10^{-18}	25×10^{-12}	9000	14
L-Proline	4.3×10^{-18}	30×10^{-12}	17,000	9
L-Cystine	4.3×10^{-18}	Not available		
L-Methionine	10.0×10^{-18}	10.0×10^{-12}	Spherical	150
Uracil	15.1×10^{-18}	10.0×10^{-12}	Spherical	160
D-Glucose	5.9×10^{-18}	1.5×10^{-12}	Spherical	90

* The calculation of the length l and the radius r was made by equating $\pi r^2 l$ to V and $2rl$ to S_o.

unlabeled amino acids, as well as the labeled one under study. Within 10 per cent, there was no effect on the slope of the line. Nor was there any difference between the effects of radiation at dry ice temperatures and at the normal temperature of the cobalt source within the same limit of error. This is significant, in that it implies that indirect inactivation due to migrating radicals of short half-life must be at a very low value.

Data very similar to the results with leucine were obtained for arginine, isoleucine, cystine, and proline. The radiation-sensitive volumes calculated from these data are shown in Table I.

Bacterial cells were also irradiated in the Yale cyclotron with deuterons at two different energies and also with alpha particles. Subsequent to this treatment, the

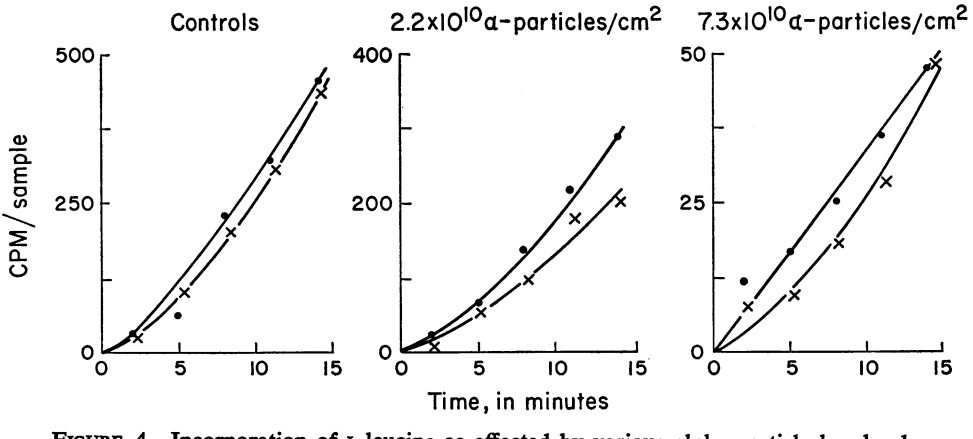

FIGURE 4 Incorporation of L-leucine as affected by various alpha particle bombardments.

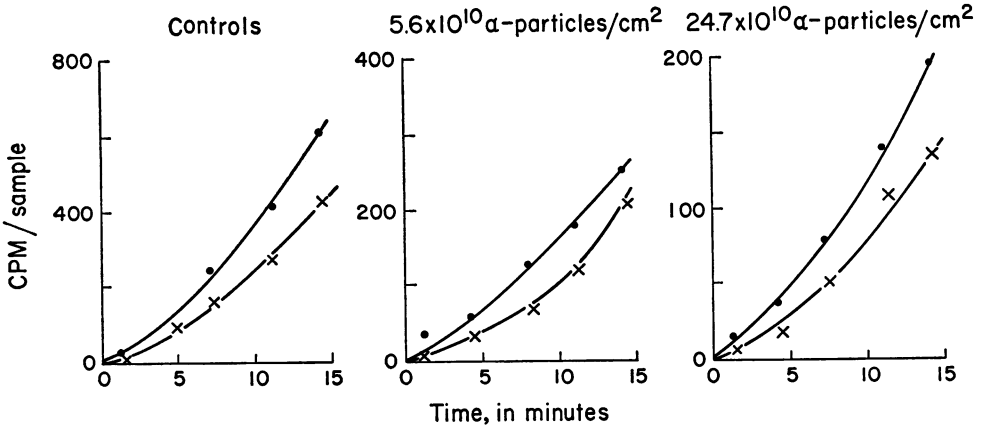

FIGURE 5 Incorporation of uracil as affected by various alpha particle bombardments.

incorporation of the labeled compounds was studied as in the case of gamma ray irradiation. Figs. 4 and 5 show the incorporation of leucine and uracil, respectively, after alpha particle bombardment of *E. coli*. The inhibition of cellular utilization appears to be similar to the results previously shown for gamma ray irradiations. However, the survival curve of the TCA-insoluble fraction, shown in Fig. 6, clearly indicates a distinct difference. The greater sensitivity to gamma rays of cellular incorporation of uracil over leucine is reversed, and the alpha particle radiation shows 37 per cent dose for leucine incorporation to be some 5 times smaller than for uracil. Other amino acids (arginine, isoleucine, and histidine) show the same sensitivity as leucine. Glucose incorporation into a TCA-precipitable form is extremely insensitive to alpha particle bombardment, with a D_{37} some 40 times greater than that of the amino acids.

ANALYSIS

An ionization within, or very near a macromolecule of protein or nucleic acid causes it to lose its function (Pollard, 1959). In material of the density of a bacterial cell (1.05), the number of primary ionizations per cm^3 per roentgen is 5.0×10^{11}. Since ionization is an "all-or-none" process, one very simple basis for analysis is to inquire as to the probability that a macromolecule of volume V can wholly escape an ionization when these are distributed randomly such that there is a number of ionizations I per unit volume. Since VI is the average number per macromolecule, the application of Poisson's equation yields the probability of no ionization, by purely random occurrence, as e^{-VI}. Thus we can suggest that the fraction left active, which is an experimental measure of the probability of escape, can be equated to

e^{-VI}. If we denote by n the number still active and by n_o the number initially, before radiation, we obtain

$$\frac{n}{n_o} = e^{-VI}$$

or equivalently,

$$\ln \frac{n}{n_o} = -VI$$

The fact that a plausible fit to this relation holds, makes it possible to calculate V within rather large limits of error. It will be seen that the limits of error are still not

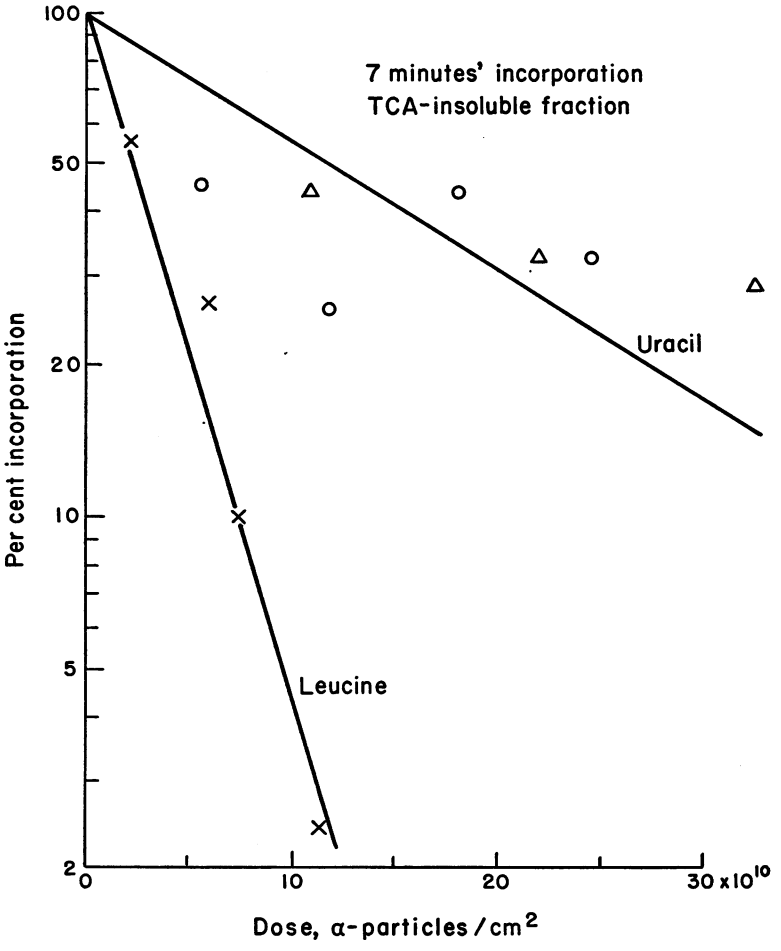

FIGURE 6 The percentage of uptake of L-leucine and uracil into the TCA-insoluble fraction at 7 minutes, as a function of alpha particle dose. Uracil data (o) from experiment shown in Fig. 5. Triangles from a duplicate experiment. The relative sensitivity is seen to be the reverse of that shown in Fig. 3 for gamma radiation.

so great as to preclude interesting deductions, and in fact, the power of the radiation analysis lies in the truth of this last statement.

Before calculating the values of V we can turn to the case of heavy particle radiation. The heavy charged particle cuts a narrow swath of ionization along its path. The swath is, however, accompanied by secondary ionization by ejected electrons, known as delta rays (Pollard *et al.*, 1955). If, for the moment, we ignore these delta rays, we can approach the "escape probability" by reasoning in terms of the idea that if one of densely ionizing tracks passes through the target, an inactivation will result. If we denote the sensitive area by S and the number of particles per unit area by D, then the average number passing through S is SD, and the same reasoning leads to the probability of there being a complete escape as e^{-SD}, so that we obtain

$$\frac{n}{n_o} = e^{-SD}$$

or equivalently,

$$\ln \frac{n}{n_o} = -SD$$

The same kind of logarithmic plot shows that this relation also plausibly holds. Thus a value of S can be calculated from the experimental data.

The value of S is found to vary with the ionization density. There are two major reasons for the variation. The first is the effect of delta rays; the second is the possibility of "straddling" a thin target, by which we mean that sometimes, even though a charged particle has gone through the target, the target is so thin that it may have failed to receive an ionization within it. The first can be corrected for by a simple method described by Pollard and Barrett (1959). Straddling effects have been discussed by Ore (1957) and his corrections have also been used in our analysis. After such corrections we can make an estimate of V from colbalt irradiation, S the area, and t the thickness, from heavy particle data. These estimates are only rough, but they are informative.

In Fig. 7 we show the results of plotting the experimentally found (uncorrected) values of S, which we call the cross-section, *versus* the rate of energy loss for the bombarding particles, for the uptake into the TCA-insoluble fraction of arginine, histidine, isoleucine, leucine, uracil, and glucose, as well as proline and methionine (Kempner and Pollard, 1958). The slopes of the lines near the origin are fixed by the values of V found from cobalt irradiation as described by Pollard *et al.* (1955). Even without any corrections it is quite apparent that there are three groupings. The cellular "targets" for arginine, histidine, isoleucine, leucine, and proline incorporation show a steady increase in radiation cross-section with the rate of energy loss, while the targets for methionine and uracil do not. The case of glucose is even more striking, in that a clear leveling off at a low cross-section can be seen.

In Table I we show the values of the sensitive volume and the values (corrected

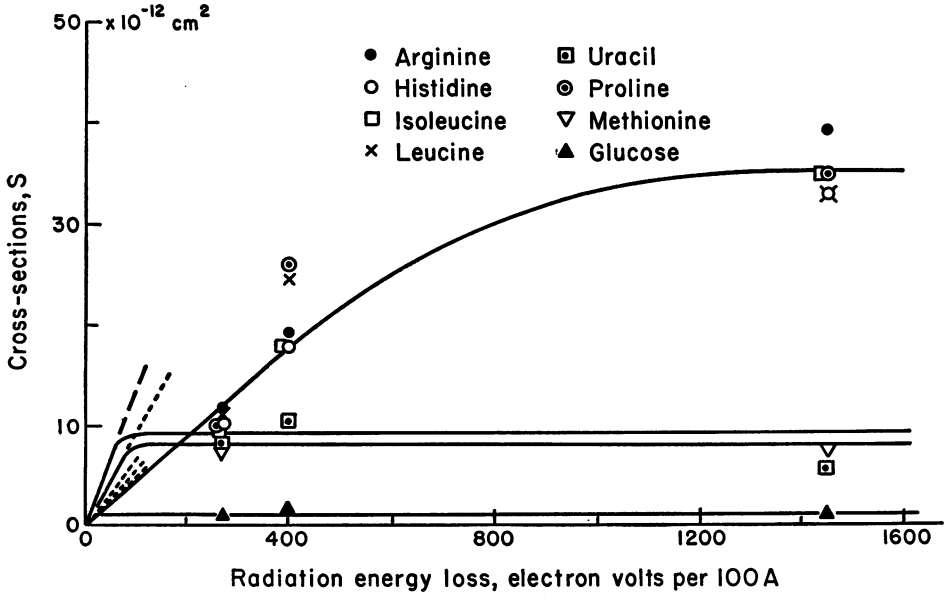

FIGURE 7 A plot of the sensitive cross-section for seven metabolites against the rate of energy loss. The initial slope is found from Co^{60} inactivation. Three groupings appear: arginine, histidine, leucine, isoleucine, and proline are all characterized by high sensitivity at high rates of energy loss; methionine and uracil have rather low sensitivities for such radiation and glucose is consistently low. Probably the first grouping are long thin objects, but the others are more nearly spherical.

for delta rays) of S_o, the maximum cross-section for heavily ionizing particles. In addition, we calculate the appropriate lengths and radii for long cylinders, or spheres according to which is the most suitable approximation. Since the calculation of radii involves $V^{1/3}$ or $A^{1/2}$, a 20 per cent experimental error in the determination of the 37 per cent inhibition dose results in only a 10 per cent error in the linear dimensions of the target.

If we apply the statistical analysis previously used, we can consider the falling aspect of pool size. Roughly speaking, the relation

$$\ln \frac{n}{n_o} = -VI$$

can be held to apply. In most cases the dose necessary to reduce the survival ratio to 0.37 (for which the natural logarithm is -1) is very roughly 500,000 r, giving a value of V of 4×10^{-18}cm³, or a molecular weight of

$$4 \times 10^{-18} \times 1.3 \times 6.03 \times 10^{23} = 3.1 \times 10^{6}$$

assuming material of density 1.3. This is interesting in that it indicates that some rather large, organized molecular structures are responsible for the maintenance of a pool.

DISCUSSION

The effects of radiation which we have roughly measured can be looked at in two ways. The first is essentially empirical and regards radiation as a kind of stress applied to the cell, which can perhaps produce differential effects on the structures that react with various metabolites. Thus one can look simply at any grouping of effects and consider whether they have any significance. Such grouping shows very clearly in the response to heavy particle irradiation. Data in Table I show that the radiation-sensitive elements associated with the uptake of the amino acids arginine, leucine, isoleucine, histidine, and proline fall into one class, characterized by behavior which radiation analysis associates with long, thin, sensitive units, whereas another amino acid, methionine, differs markedly. Methionine incorporation seems to require the intervention somewhere of a much shorter, thicker structure. Uracil seems to behave in the same way. Glucose, on the other hand, appears to be involved with a still smaller, but thick and roughly spherical object.

A second way of looking at the experiments is to consider the results of cell fractionation to see which of the known cellular structures could be involved with the various operations. Such a method is limited by our knowledge of cell components, which is admittedly imperfect, but it is still a useful viewpoint. An *E. coli* cell has a cell wall, a protoplast membrane, two or more "nuclear bodies," a rather organized complement of DNA within the nuclear bodies, and a large number of ribosomal particles with sedimentation constants ranging from 20 to 100 Svedberg units. These ribosomes contain RNA and protein in a tight bonding. There is in addition an amount of soluble RNA of smaller molecular weight, and a large number of enzymes.

The radiation data enable us to eliminate from this list the whole nucleus, as being too large a radiation target, and the enzymes or soluble RNA as being too small. Interest therefore centers on DNA, large specific RNA, and the ribosomes. The effect of radiation on the uptake of methionine and uracil fits rather remarkably well with the idea that ribosomal particles of sedimentation constant about 80 Svedberg units are involved. On the other hand, the sensitivity found for the five other amino acids studied is much more in agreement with that to be expected from a nucleic acid chain of molecular weight about 4×10^6. Whether this be DNA or RNA, we have no basis for telling. It is possible that a ribonucleoprotein particle, in action, is unrolled in some way, and could therefore result in the long, thin appearance of a radiation target.

Present concepts of protein synthesis postulate the existence of a protein-forming "template." In such a frame of reference, it would be expected that the incorporation of amino acids would be inhibited by destruction of a radiation target identical for all. Our results indicate that at least for five of the six amino acids studied, this indeed is the case. The results for methionine (Kempner and Pollard, 1958) therefore are quite paradoxical. It is extremely difficult to see how these re-

sults could be consistent with any simple model of protein synthesis. Further studies on the radiation inhibition of the incorporation of methionine and other amino acids might be very informative about the mechanism of such synthesis.

The ability of cells to utilize glucose after exposure to each type of radiation used was found to be a simple exponential function of dose. No evidence for a "multiple hit" requirement (Pollard *et al.*, 1955) or for targets of two or more different sizes was found. This implies that all exogenous glucose passes through a common structure which is the most radiation-sensitive element in its biochemical pathways. The target analysis of this unit is given in Table I. If it is assumed that the target is a single macromolecular structure, then a calculation of the expected sedimentation constant leads to a value of 20 to 30S.

We can mention briefly the radiation sensitivity found for the "pool." The fact that radiation does have an effect on the pool seems to dispose of the idea that no more is involved than an inert sieve-like membrane. It is of interest that the doses at which an effect on the pool becomes great are also those at which radioactive label begins to leak out of the cell. Whether the action of radiation is on large molecular units within the cell, or on large units comprising the protoplast membrane itself, we cannot say.

We wish to thank Dr. R. B. Roberts and Dr. E. T. Bolton of the Carnegie Institution of Washington for helpful advice with the technical problem of cell filtration and uptake studies, and Mr. J. Lowry and Miss N. Barrett for help in running the cyclotron.

This work is part of a dissertation presented to the faculty of Yale University in candidacy for the degree of Doctor of Philosophy.

Aided by grants from the John A. Hartford Foundation and the United States Atomic Energy Commission.

Received for publication, October 5, 1960.

REFERENCES

HUTCHINSON, F., 1957, The distance that a radical formed by ionizing radiation can diffuse in a yeast cell, *Radiation Research,* **7,** 473.

HUTCHINSON, F. H., MOROWITZ, H. J., and KEMPNER, E. S., 1957, Evidence concerning the size of amino acid incorporation structures in *E. coli, Science,* **126,** 310.

KEMPNER, E. S., and BISBEE, F. A., 1958, Simple, inexpensive, automatic sample changer and recorder, *Nucleonics,* **16,** 87.

KEMPNER, E., and POLLARD, E. C., 1958, *in* Microsomal particles and protein synthesis, (R. B. Roberts, editor), Washington, D. C., Washington Academy of Sciences.

MCQUILLEN, K., ROBERTS, R. B., and BRITTEN, R. J., 1959, Synthesis of nascent protein by ribosomes in *Escherichia coli, Proc. Nat. Acad. Sc.,* **45,** 1437.

ORE, A., 1957, Interpretation of radiation results based on target theory, *Radiation Research,* **6,** 27.

POLLARD, E. C., 1959, Radiation inactivation of enzymes, nucleic acids, and phage particles, *Rev. Mod. Physics,* **31,** 273.

POLLARD, E. C., 1960, Theory of the action of ionizing radiation on bacteria, *Am. Naturalist,* **94,** 71.

POLLARD, E. C., and BARRETT, N., 1959, Study of β-galactosidase using ionizing radiation, *Radiation Research* **11**, 781.

POLLARD, E. C., GUILD, W. R., HUTCHINSON, F., and SETLOW, R. B., 1955, The direct action of ionizing radiation on enzymes and antigens, *Progr. Biophysics and Biophysic. Chem.*, **5**, 72.

ROBERTS, R. B., COWIE, D. B., ABELSON, P. H., BOLTON, E. T., and BRITTEN, R. J., 1957, Studies of biosynthesis in *Escherichia coli, Carnegie Institution of Washington, Pub. No. 607.*