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Research Article



# STUDIES WITH RADIOACTIVE ENDOTOXIN. I. THE USE OF $CR^{51}$ TO LABEL ENDOTOXIN OF *ESCHERICHIA COLI*[1]

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The endotoxins in different species of gram negative bacteria exhibit remarkably similar biologic effects which have been observed in both experimental animals and man (1–5). These effects may be seen after the injection of living bacteria, killed bacteria, or endotoxin itself; they consist of fever, shock, leukocytosis preceded by leukopenia, hypoglycemia, diarrhea, and prostration. Although there are sometimes hemorrhages in the intestine and other viscera, the findings postmortem are not enough to explain the cause of death or to indicate where the endotoxin acts.

As a means of locating the site of action of endotoxin, as well as its fate *in vivo*, a series of experiments have been carried out using endotoxin labelled with radioactive chromium. The endotoxin of *Escherichia coli* was selected for this study because it can be conveniently prepared in large quantities without the hazards associated with more highly pathogenic bacteria. Radioactive chromium proved to be satisfactory by virtue of its firm attachment to endotoxin, its relative safety in handling, and its half-life of 26.5 days. This report deals with the technique of labelling of *E. coli* endotoxin and with the properties of labelled toxin; those which follow describe its distribution *in vivo* under various experimental conditions.

## EXPERIMENTAL METHODS

### I. *Preparation of E. coli Endotoxin*

The method of preparation of *E. coli* endotoxin was based on Boivin and Mesrobeanu's (6) principle that endotoxin can be extracted by trichloroacetic acid at cold temperatures. The bacteria were grown in a synthetic medium modified from that described by Gladstone (7) for growth of *S. typhosa*. For the preparation of 25 liters, the following constituents were used: $KH_2PO_4$ 112.5 grams, $(NH_4)_2SO_4$ 12.5 grams, and $NH_4Cl$ 12.5 grams.

These salts were dissolved in 12 liters of distilled water and 700 ml. 1 N NaOH were added, the volume being made up to 15,000 ml. with distilled water. The reaction was adjusted to pH 7.6 by the addition of 1 N NaOH, 9125 ml. distilled water were added, and the medium was put in 4 liter quantities into 6 flasks of 6 liter size and autoclaved at 115° C. for 20 minutes at 15 lbs. pressure. To each flask the following sterile solutions were added: M/6 $MgSO_4 \cdot 7H_2O$ 40 ml., 50 per cent glucose solution 20 ml., and M/$NaHCO_3$ 10 ml. The solutions of $MgSO_4$ and glucose had been sterilized by autoclaving at 115° C. for 15 minutes at 15 lbs. pressure, and the $NaHCO_3$ was sterilized by Seitz filtration.

Care was taken not to add these solutions more than 24 to 48 hr. before the medium was to be used, as storing for longer periods leads to a reduction in the amount of growth (8).

For inoculating this synthetic medium, a strain of *E. coli* was used which had been isolated from a human infection. It was subcultured in 5 per cent rabbit blood broth and on rabbit blood agar to maintain a smooth culture. One or two smooth colonies from the blood agar plate were transferred to 10 ml. of synthetic medium. After 6 to 8 hours' growth at 37° C., the 10 ml. of culture was used to inoculate 400 to 600 ml. of the synthetic medium and this inoculated medium was allowed to incubate overnight at 37° C. Then 50 to 100 ml. portions of the overnight growth were transferred to each of 6 flasks containing 4,000 ml. of synthetic medium.

*E. coli* grew profusely in this medium. After 24 hours' growth at 37° C., the bacterial bodies were chilled in the deep freeze and removed just as ice began to form. The chilling was necessary to arrest growth until they could be separated in a DeLaval cream separator. If they were not chilled, significant loss of toxicity of both the dried bacterial bodies and the endotoxin occurred as measured by the $LD_{50}$ in mice. By slow addition of the bacterial suspensions to the separator, it was possible to remove the bacteria so effectively that hardly any turbidity remained in the supernatant. The harvested bacteria were removed from the bowl of the separator as a thick moist paste and suspended in enough 95 per cent alcohol to give a final concentration of approximately 70 per cent alcohol. This was followed by two more washings with 95 per cent alcohol, two with acetone, and finally by drying at 37° C. for at least two days.

The dried bacterial bodies were dispersed in 5 times

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their weight of water and shaken with glass beads in a mechanical paint shaker for 15 minutes. The suspension was refrigerated overnight and an equal volume of ice cold .25 M trichloroacetic acid was added the following morning. The centrifuge bottle containing the cold acid suspension was wrapped in towels for insulation, shaken for 15 minutes in the paint shaker and then placed in cracked ice for three hours with frequent shaking by hand. At the end of the period, virtually all of the bacterial residue was removed from the thick suspension by centrifugating in a Spinco ultracentrifuge for 30 minutes at 20,000 r.p.m.; then the slightly opalescent centrifugate was dialyzed in a collodion sac at room temperature against running tap water for 18 hours and then against frequent changes of distilled water for at least 24 hours. The dialysis tubing was next placed in the incubator at 40° C. and the contents concentrated to ½ or ⅛ of the original volume by evaporation with the assistance of an electric fan blowing against the sac. The concentrated solution was passed through a Seitz filter, and the endotoxin was precipitated from the filtrate by the addition of ethyl alcohol to give a final concentration of 68 per cent alcohol by weight. The alcoholic precipitation was allowed to proceed overnight in the refrigerator. The endotoxin thus precipitated, was washed twice with 95 per cent alcohol and twice with ether. It was dried first at 37° C. and then *in vacuo* at 40° C. to a constant weight. The final product, a white powder, was stored in the desiccator with Drierite. The $LD_{50}$ for each batch of dried bacterial bodies and endotoxin was calculated by the method of Reed and Muench (9). Each determination was based on the inoculation of 6 graded doses of endotoxin in 6 groups of mice containing 6 mice per group. Adult white Swiss mice weighing 20 grams were used. The endotoxin reacted negatively to the biuret test for proteins and to the ninhydrin test for proteins, polypeptides, and amino acids.

## II. *Tagging E. coli Endotoxin with Radioactive Chromium* ($Cr^{51}$)[2]

*E. coli* endotoxin has been tagged firmly with hexavalent chromium $Na_2Cr^{51}O_4$ and with trivalent chromium, $Cr^{51}Cl_3$. The endotoxin has been firmly tagged in each of three ways:

A. Adding $Na_2Cr^{51}O_4$ to growing cultures of *E. coli* and extracting the tagged endotoxin.

B. Adding $Na_2Cr^{51}O_4$ to a suspension of the dried bacterial bodies and extracting the tagged endotoxin.

C. Adding $Na_2Cr^{51}O_4$ or $Cr^{51}Cl_3$ to the endotoxin directly. The procedures for accomplishing these tags are as follows:

### A. *Adding $Na_2Cr^{51}O_4$ to growing cultures*

Both synthetic and standard media have been used.

1) *Standard media.* *E. coli* was grown for 48 hours on tryptose agar at 37° C. in modified Blake bottles and washed off with sterile distilled water. The bacterial sediment was washed twice in sterile distilled water and then refrigerated for two days. The bacteria were then suspended in 1,800 ml. of tryptose phosphate broth to which 7.04 mg. of $Na_2Cr^{51}O_4$ (3.24 mc. $Cr^{51}$) were added. After incubation at 37° C. for 12 hours, the bacteria were again sedimented at 1,500 r.p.m. in the centrifuge and washed twice with sterile distilled water. Successive washing was then performed with 70 per cent alcohol, 95 per cent alcohol (2 washings), acetone and ether. The 7.5 gm. of bacteria obtained in this fashion were dried in a vacuum oven at 40° C.

The total mass of 7.5 gm. of bacteria was suspended in 37.5 ml. of distilled water, shaken in a paint shaker for 30 minutes and cooled to 0° C. in an ice bath. The cold bacterial suspension was mixed with an equal volume (37.5 ml.) of ice cold .25 molar trichloroacetic acid and the endotoxin extracted in the manner described in section I.

The precipitate obtained after treatment with 68 per cent alcohol was washed with 95 per cent ethyl alcohol repeatedly until the count on the washes in the scintillation counter[3] equalled that of the background count. The precipitate was further washed in ether-acetone and dried at 40° C. in the vacuum oven. The total yield of endotoxin was 46 mg.

2) *Synthetic medium.* Eighteen liters of the ammonia medium described above were inoculated with a culture of *E. coli*. After 12 hours' incubation at 37° C., 0.7 mc. of $Na_2Cr^{51}O_4$ (.194 mg.) was added to 9 liters of culture and incubated at 37° C. for an additional 16 hours. After 28 hours, ethyl alcohol was added to a concentration of 20 per cent by weight, all bacteria were separated in the DeLaval cream separator in the manner described, and washed with alcohol, acetone, ether, and dried. The yield of tagged bacteria was 7.2 gm. and of non-tagged bacteria 5.6 gm.

Endotoxin was obtained by extraction with trichloroacetic acid in the cold by the method described above to give 22.9 mg. of tagged endotoxin and 20.3 mg. of non-tagged endotoxin.

### B. *Addition of $Na_2Cr^{51}O_4$ to a suspension of the dried bacterial bodies and then extracting the tagged endotoxin*

Modified Blake bottles lined with tryptose agar were inoculated with *E. coli* and incubated at room temperature (27° C.). After 24 hours, the growth was washed off with sterile distilled water. The bacteria were separated from the distilled water in the centrifuge and then washed with 95 per cent ethyl alcohol twice and acetone-ether once. The residue was brought to constant weight by drying at 40° C. in the vacuum oven and storing in a desiccator with calcium chloride. Then 5.3 gm. of dried bacteria were suspended in 100 ml. of distilled water to which were added 4 mc. of $Na_2Cr^{51}O_4$ (1.7 mc.

[2] Radioactive Pharmaceuticals Division, Abbott Laboratories, Oak Ridge, Tennessee.

[3] Radioactivity in all experiments was measured by a gamma sensitive sodium-iodide thallium scintillating well-type crystal.

per mg.). The mixture of bacterial bodies and $Cr^{51}$ was incubated at 37° C. for 48 hours and dialyzed four days against running tap water followed by 24 hours against distilled water to remove free $Cr^{51}$. The volume was concentrated to 50 ml. *in vacuo* and the suspension chilled to 0° C. The chilled suspension of bacteria was mixed with ice cold 0.25 M trichloroacetic acid, and the endotoxin extracted in the manner described. The yield was 39.5 mg.

To test the stability of the bond between $Cr^{51}$ and endotoxin, the entire volume of tagged endotoxin solution was dialyzed against distilled water at 4° C. for 10 days. Under these circumstances less than 1 per cent of the radioactivity on the endotoxin appeared in the dialysate water during a period of 24 hours.

C. *Adding $Na_2Cr^{51}O_4$ and $Cr^{51}Cl_3$ to the endotoxin directly*

1) *Addition of $Na_2Cr^{51}O_4$ and $Cr^{51}Cl_3$ to non-tagged endotoxin.* *E. coli* endotoxin was prepared from bacteria grown in the synthetic ammonia medium as described above and suspended in water in a concentration of 5 mg. per ml. It was filtered through a Seitz filter to sterilize the endotoxin. The $LD_{50}$ for mice before and after filtration was .25 mg.

Two equal samples of endotoxin were incubated at 37° C. for 24 hours with $Cr^{51}$ as follows:

a. 7.0 ml. endotoxin (35 mg.) + 0.4 mc. (0.5 ml.) $Cr^{51}Cl_3$

b. 7.0 ml. endotoxin (35 mg.) + 0.4 mc. (0.5 ml.) $NaCr^{51}O_3$

At the end of 24 hours' incubation, each solution was dialyzed against distilled water at room temperature until there was no longer any significant loss of radioactivity into the dialysate water. The results may be summarized as follows:

| Source of tag | Mg. of endotoxin | Counts per sec. per mg. Scintillation counter | $LD_{50}$ for mice |
|---|---|---|---|
| $Na_2CrO_4$ | 35 | 593.6 | .25 mg. |
| $CrCl_3$ | 35 | 4,771.2 | Not lethal |

The loss of toxicity was attributed to inactivation of the endotoxin upon exposure to the low pH (pH = 2) of the solution of $CrCl_3$. Precautions were taken, therefore, to prevent inactivation of endotoxin by the acidity of $CrCl_3$. Accordingly, the endotoxin was suspended in a phosphate chloride buffer (pH = 7) before addition of $CrCl_3$. The formula of the buffer was: .3530 g · $NaH_2PO_4$ + .6390 g $Na_2HPO_4$ + .172 g NaCl dissolved in 1,000 ml. of water.

Furthermore, to avoid possible loss of endotoxin into the bath during dialysis, in amounts which could not be determined easily, it was decided to rid the endotoxin of free chromium by repeated washes with absolute alcohol in which the endotoxin is insoluble. Two lots of endotoxin were suspended in sterile phosphate chloride buffer and incubated in the presence of $Cr^{51}$ for 24 hours at 37° C. as follows:

Lot A

100 mg. endotoxin plus .56 mg. $Cr^{51}Cl_3$ (.165 mc.)
200 mg. endotoxin plus 1.12 mg. $Na_2Cr^{51}O_4$ (1.057 mc.)
100 mg. endotoxin plus no chromium

Lot B

115 mg. endotoxin plus .77 mg. $Cr^{51}Cl_3$ (.170 mc.)
115 mg. endotoxin plus .505 mg. $Na_2Cr^{51}O_4$ (.355 mc.)
85 mg. endotoxin plus no chromium

After 24 hours' incubation, each sample of endotoxin was reprecipitated by adding ethyl alcohol to a concentration of 68 per cent by weight and separated in the ultracentrifuge at 30,000 r.p.m. for 15 minutes. Each sample was stored in 95 per cent alcohol at 4° C. and then washed repeatedly with absolute alcohol for eight days until no radioactivity appeared in the wash. Then the endotoxin was washed in ether and dried *in vacuo* at 40° C. until it reached constant weight. The radioactivity and $LD_{50}$ of each sample were measured and listed in Table II (Samples 5–8).

2) *Addition of $Na_2Cr^{51}O_4$ to endotoxin already tagged with chromate ions.* Endotoxin tagged as described in section B above by addition of $Na_2Cr^{51}O_4$ to dead bacterial bodies, was used in this experiment to determine if the tag could be increased by additional exposure. The endotoxin was suspended in water in a concentration of 5 mg. per ml. A total of 10 mg. of tagged endotoxin was incubated for 24 hours at 37° C. with 0.5 mc. of $Na_2Cr^{51}O_4$ and dialyzed against distilled water in parallel with an equal amount of the same batch of tagged endotoxin which had been exposed to no additional $Na_2Cr^{51}O_4$. The dialysis was carried out at 4° C. for 10 days. At that time, no loss of radioactivity was detected in the dialysis bath. The radioactivity of the two samples is given in Table II, number 3A.

## III. *Experiments to Provide Evidence that the Tag is Stable and that Radioactivity is Proportional to Toxicity*

This problem was approached in three ways:

1) Labelled endotoxin was dialyzed during storage for as long as one month and the dialysate examined for free $Cr^{51}$ by measuring the radioactivity. In addition, radioactive endotoxin was washed repeatedly in 68 per cent alcohol and the alcoholic wash tested for radioactivity.

2) Suspensions of radioactive labelled endotoxin were subjected to vigorous physical treatment in an effort to rupture the bond. This procedure, consisting of differential ultracentrifugation and alcoholic precipitation, also served as a test for the homogeneity of the material. If the fractions of endotoxin separated by these procedures were of different composition, and if they possessed different affinities for $Cr^{51}$, it would be expected that the radioactivity per unit of toxicity would vary from fraction to fraction.

3) Serial dilutions of radioactivity were made to learn whether simple dilution of radioactivity was accompanied

by proportional changes in toxicity. This procedure was necessary in order to predict whether the dilution occurring after *in vivo* injection would invalidate radioactivity as a measure of toxin.

The first endotoxin employed was labelled with $Na_2Cr^{51}O_4$. A total of 58 mg., giving 6,064 counts per second in the scintillation counter, was suspended in 23.5 ml. of phosphate buffer. Three fractions of the suspension were separated as follows:

Fraction I was the sediment obtained by centrifugation for one hour at 40,000 r.p.m. in the ultracentrifuge.

Fraction II was obtained by treating the clear supernatant from part I with ethyl alcohol to give a final concentration of 68 per cent by weight, and by ultracentrifugation at 20,000 r.p.m. for 30 minutes. The precipitate was then resuspended in 20 ml. of the phosphate buffer.

Fraction III was the supernatant from part II. It was dialyzed against phosphate buffer for 72 hours after the alcohol had first been removed by evaporation at 37° C. The volume was brought up to 20 ml. with the necessary quantity of phosphate buffer.

Six serial two fold dilutions of each of the three fractions and of the original untreated parent endotoxins were made in phosphate buffer so that the approximate $LD_{50}$ of each fraction could be determined in terms of radioactivity. The radioactivity of each diluted portion was determined and then 0.5 ml. portions of each of the 6 serial dilutions were inoculated intraperitoneally into each of 5 mice. The mortality rate was recorded during 36 hours for the 30 mice receiving each fraction.

The experiment was then repeated using a second endotoxin. This was divided into two portions: one was labelled with $Na_2Cr^{51}O_4$ and the other with $Cr^{51}Cl_3$. A total of 60 mg. of chromate-labelled endotoxin gave 18,540 counts per second in the scintillation counter and a total of 80 mg. of chromic-labelled endotoxin gave 146,560 counts per second. These samples of endotoxin were then treated in the same way as the first endotoxin.

## RESULTS

### I. *Preparation of endotoxin*

Table I summarizes the results of preparing 9 lots of endotoxin in a synthetic medium.

A comparison of the toxicities of each lot of endotoxin with the toxicity of dried bacterial bodies, from which they were extracted, is expressed as the ratio of their $LD_{50}$'s. The ratio of $LD_{50}$ of the whole bacterial body to the $LD_{50}$ of purified endotoxin varies in different batches from 9.05 to 2.50. This would suggest that at least 1/2.5 to 1/9 of the dried bacterial cell is composed of endotoxin. The average dried cell would contain at least 1/4.6 (22 per cent) endotoxin according to this type of calculation. It is emphasized that these are minimum values because of the possibility that endotoxin might lose activity during its extraction.

### II. *Tagging E. coli endotoxin with radioactive chromium ($Cr^{51}$)*

The results are summarized in Table II. The number of mcg. of atomic chromium bound to each mg. of endotoxin was calculated from its radioactivity, expressed per second. This calculation could be made from the specific activity (mc. per mg.) of the $Cr^{51}$ and the determination of the number of counts per mg. of atomic chromium. The chromium atom constitutes exactly ⅛ of each molecule of $CrCl_3$ and $Na_2CrO_4$. Each mc. gave 1,650 counts per second in the scintillation counter. All results are corrected for decay.

It is clear that much more $Cr^{51}$ is bound by endotoxin in the presence of $Cr^{51}Cl_3$ than in the presence of $Na_2Cr^{51}O_4$ under conditions in which the relative concentrations of endotoxin and $Na_2CrO_4$ were the same as those of endotoxin and $CrCl_3$. Under these circumstances, the number of chromate and chromic ions were also approximately the same because the molecular weights of the two compounds were nearly equal. The results also show that much more chromium was bound when $Na_2Cr^{51}O_4$ was incubated with the endotoxin than with the living or dead bacterial cell. In this connection, it was found that the amount of $Cr^{51}$ bound by radioactive endotoxin extracted from dead bacterial cells could be increased by 200 per cent upon subsequent exposure of the extracted endotoxin to $Na_2Cr^{51}O_4$ (Endotoxin 3A, Table II).

The toxicity of the endotoxin as measured by $LD_{50}$ was not changed in any of the experiments by labelling with buffered radioactive chromium. Intravenous injection of 1.0 mg. of the labelled endotoxin was also able to induce the generalized Shwartzman reaction with bilateral cortical necrosis of rabbit kidneys after a preparatory injection of 2.0 ml. of meningococcal filtrate diluted 1:80.[4]

### III. *Evidence that the tag is stable and that radioactivity is proportional to toxicity*

The daily loss of radioactivity in the alcoholic washes or in the dialysis wash was less than 0.1

[4] The meningococcal filtrate was obtained from Dr. Richard T. Smith, Department of Pediatrics, University of Minnesota.

TABLE I

*Summary of results obtained in preparing endotoxin in a synthetic medium* *

| A Lot No. | B $LD_{50}$ (mg.) bacterial bodies | C $LD_{50}$ (mg.) endotoxin | D $\frac{LD_{50}\text{ bacterial cell}}{LD_{50}\text{ endotoxin}}$ | E Wt. bacterial bodies (gm.) | F Estimated total content of endotoxin (gm.) (E/D) | G Wt. endotoxin produced (mg.) | H % yield of total estimated endotoxin (G/F×100) |
|---|---|---|---|---|---|---|---|
| 1 | 1.10 | 0.375 | 1.10/0.375 = 2.93 | 27.7 | 9.45 | 952 | 10.07 |
| 2 | 0.917 | 0.11 | 0.917/0.11 = 8.34 | 7.1 | 0.85 | 166.9 | 19.67 |
| 3 | 0.86 | 0.095 | 0.86/0.095 = 9.05 | 23.7 | 2.62 | 713.6 | 27.27 |
| 4 | 0.312 | 0.058 | 0.312/0.058 = 5.37 | 33.0 | 6.14 | 1,184 | 19.28 |
| 5 | 0.465 | 0.156 | 0.465/0.156 = 2.98 | 25.5 | 8.55 | lost | |
| 6 | 0.34 | 0.085 | 0.34/0.085 = 4.0 | 18.0 | 4.50 | 563 | 12.51 |
| 7 | 0.167 | 0.063 | 0.167/0.063 = 2.65 | 10.0 | 3.77 | 105† | 2.78 |
| 8 | 0.625 | 0.250 | 0.625/0.250 = 2.50 | 16.0 | 6.40 | 510 | 7.90 |
| 9 | 0.312 | 0.094 | 0.312/0.094 = 3.32 | 18.0 | 5.42 | 280 | 5.16 |

* The approximate dilution of endotoxin within the dried bacterial cell can be calculated from the following ratio: $\frac{LD_{50}\text{ of bacterial cell}}{LD_{50}\text{ of endotoxin}}$. Knowing the dilution of endotoxin within the cell, it is possible to calculate the weight of endotoxin in a mass of bacterial bodies from their total weight. It is emphasized that this calculation does not take into account possible loss of toxicity resulting from extraction of endotoxin. For this reason, the estimated content of endotoxin gives only a minimum value. Since the same procedure for extraction was used in all experiments, however, this calculation is valid for comparing the theoretical and total yields in each of the 9 lots. The endotoxin contained .013 mg. nitrogen per gram endotoxin in one of the lots tested. Three other lots contained no nitrogen.

† A portion was lost during processing.

TABLE II

*Summary of results obtained in tagging endotoxin* *

| Culture medium | Point at which $Cr^{51}$ was added | Form of $Cr^{51}$ and no. of mc. added | Mg. $Cr^{51}$ added as atomic chromium | Mg. endotoxin recovered | Counts/sec./mg. endotoxin | μc $Cr^{51}$/mg. endotoxin | mcg. Cr/mg. endotoxin | $LD_{50}$ for mice (mg. radioactive endotoxin) | Identification No. |
|---|---|---|---|---|---|---|---|---|---|
| Tryptose agar and broth | Growing culture | 3.24 $Na_2CrO_4$ | 2.34 | 46 | 154.4 | 0.0936 | 0.0665 | 0.25 | 1 |
| Synthetic | Growing culture | 0.7 $Na_2CrO_4$ | 0.194 | 22.9 | 81.6 | 0.049 | 0.0136 | Not tested | 2 |
| Tryptose agar | Dead bacterial bodies | 4.0 $Na_2CrO_4$ | 0.785 | 39.5 | 518 | 0.387 | 0.0763 | 0.25 | 3 |
| Tryptose agar | Radioactive endotoxin prepared in 3 | 0.5 $Na_2CrO_4$ | 0.298 | 12 | 1,552 | 0.610 (.387+ .223) | .2273 (.0763+ .151) | Not tested | 3A |
| Synthetic | Endotoxin | 0.5 $Na_2CrO_4$ | 0.174 | 35 | 1,187.2 | 0.719 | 0.251 | 0.25 | 4 |
| Synthetic | Endotoxin | 0.165 $CrCl_3$ | 0.185 | 58.1 | 4,205 | 2.648 | 2.85 | 0.071 | 5 |
| Synthetic | Endotoxin | 1.057 $Na_2CrO_4$ | 0.369 | 145.4 | 973.5 | 0.59 | 0.206 | 0.129 | 6 |
| Synthetic | Endotoxin | 0.170 $CrCl_3$ | 0.254 | 98 | 2,656 | 1.61 | 2.40 | 0.162 | 7 |
| Synthetic | Endotoxin | 0.355 $Na_2CrO_4$ | 0.166 | 109 | 468.9 | 0.284 | 0.137 | 0.177 | 8 |

* Summary of results obtained in labelling endotoxin by adding $Cr^{51}$ before or after separation of endotoxin from bacteria: Efficiency of tag is best expressed as micrograms of chromium bound per mg. of endotoxin (recorded in third column from right). Note that endotoxin binds 10 times as much trivalent as hexavalent chromium. Micrograms of chromium per mg. of endotoxin are calculated from: 1) radioactivity of endotoxin and 2) specific activity (microcuries/microgram) of chromium compound used for labelling that sample of endotoxin. One microcurie produced 1,650 counts per second in the scintillation counter.

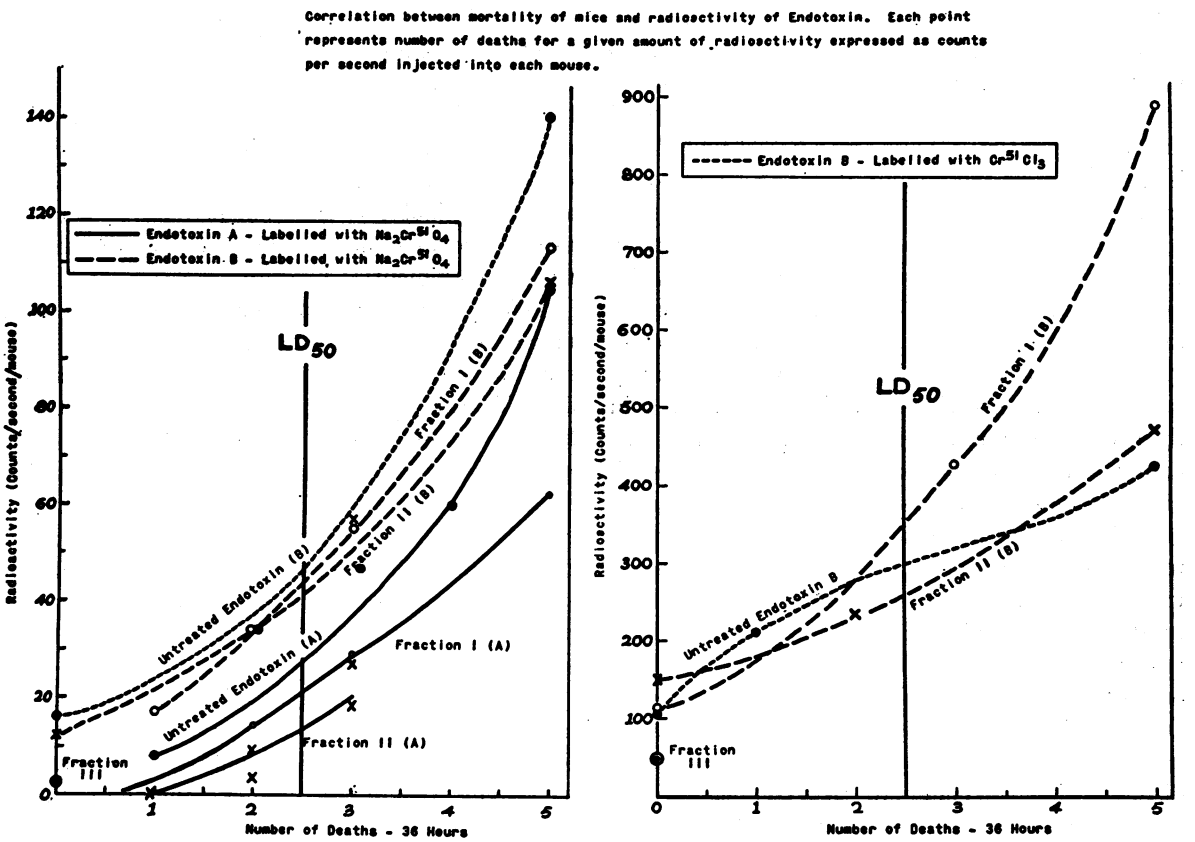


FIG. 1. STABILITY OF CHROMIUM LABELS ON ENDOTOXINS A AND B AFTER ULTRACENTRIFUGATION (FRACTION I) AND ALCOHOLIC REPRECIPITATION (FRACTION II)

Fraction III is the supernate after removal of I and II. Each fraction and the parent endotoxin was divided into 6 serial dilutions and then each dilution inoculated into 5 mice for plotting these mortality curves. All 6 points are not plotted on each curve because only the highest of the dilutions giving 100 per cent mortality is needed. This experiment illustrates not only stability of the tag in the face of vigorous physical manipulation but also a constant ratio between radioactivity and toxicity among the various fractions.

per cent per day for either chromate- or chromic-labelled endotoxin.

In Figure 1, the number of deaths is plotted against the number of counts per second in each sample of endotoxin injected. It is to be noted that for a given endotoxin, the amounts of radioactivity corresponding to the $LD_{50}$ of each the first two fractions were almost identical to that of the original untreated parent endotoxin. Each of the third fractions, consisting of the supernate after sedimentation of endotoxin, contained only slight radioactivity, or none at all, and no toxicity. In other words, radioactivity maintained a constant relationship to toxicity ($LD_{50}$) despite the manipulations to which endotoxin was subjected. It can also be observed that serial dilutions of the endotoxin produced a proportional fall in toxicity and radioactivity. These facts are good evidence that the endotoxin is a homogeneous substance which binds $Cr^{51}$ tightly and evenly.

## DISCUSSION

There can be little doubt, in the case of hexavalent $Cr^{51}$, that endotoxin is more efficiently labelled after it has been separated from the bacterial cell. This conclusion is supported by two observations: 1) Under conditions which employed an excess of chromate ions, much greater radioactivity per milligram of endotoxin resulted when $Cr^{51}$ was added directly to endotoxin than when it was added to growing cultures; 2) radioactive endotoxin, extracted from a suspension of dead bacterial bodies which had been mixed with $Cr^{51}$ gained an amount of bound chromium equal to an

additional 200 per cent upon subsequent exposure of the extracted endotoxin to only 30 per cent as much $Cr^{51}$ as that used originally. This gain in radioactivity indicated that much of the affinity of endotoxin for $Cr^{51}$ remains unsatisfied when $Cr^{51}$ is added before the endotoxin has been removed from the bacterial cell. The high affinity of $Cr^{51}$ for extracellular endotoxin clearly demonstrates a stable union between the two which does not depend on intimate bacterial incorporation of chromium into the endotoxin molecule during growth. Instead, it is likely that chromium was not actively utilized by the growing bacterial cell and that in the presence of multiplying bacteria chromium was bound entirely by endotoxin which already existed. If chromium is not assimilated during bacterial growth, than greater efficiency would be expected in labelling endotoxin after it has been extracted from the cell and separated from other bacterial products. The calculations in section I indicate that in the average bacterial cell of *E. coli,* at least one-fifth of the contents is composed of endotoxin. In a growing culture, which is a mixture of living and dead bacteria, as well as their products of disintegration, the endotoxin thus competes with a large quantity of other bacterial products which undoubtedly also possess an affinity for chromium. These bacterial products may also combine chemically with the endotoxin itself and thus interfere with its union with $Cr^{51}$. The number of chromate ions was calculated to be present in great excess of the estimated available endotoxin in all experiments. The presence, however, of other bacterial products capable of binding chromium could appreciably reduce the ratio of free chromate ion to endotoxin molecules.

Both chromate and chromic ions attach themselves to the endotoxin molecule without altering toxicity. Boivin and Mesrobeanu (6) have demonstrated that endotoxin obtained by their method is free of protein and is dissociated into a toxic phospholipid fraction, and a non-toxic polysaccharide fraction. The endotoxin prepared in the present experiments from *E. coli* was also found to be free of protein, polypeptides, and amino acids by the extremely sensitive ninhydrin test. It is possible, therefore, that if chromic ion became attached to the polysaccharide fraction, the chromium would be connected to the toxic moiety through a non-toxic link. Fortunately, from the standpoint of these experiments, the attachment of chromium to the toxic moiety, even if it were an indirect one, is stable and constant as illustrated in the experiments in section III. A remarkably close quantitative correlation exists between toxicity and radioactivity so that it would appear justifiable to use radioactivity as a measure of the amount of tagged endotoxin during tracer experiments.

Before conducting tracer experiments with tagged endotoxin, however, certain precautions must be observed. The importance of avoiding detoxification by the low pH of unbuffered $CrCl_3$ has been emphasized. Use of a phosphate chloride buffer is an excellent safeguard against this difficulty. Care must also be taken against the accumulation of free chromium ion in solutions of endotoxin. All solutions of tagged endotoxin stored for long periods should be checked for unbound chromium immediately before use by dialysis or by washing the reprecipitated toxin.

It is probably important, also, to take precautions against the presence of organic materials in the bacterial media which could contaminate the endotoxin. The liquid synthetic medium is therefore preferred because it avoids the incorporation into the endotoxin of various impurities including the galactose present in agar (8).

## SUMMARY AND CONCLUSIONS

*E. coli* endotoxin has been prepared in a synthetic medium consisting of inorganic salts and dextrose. Dried bacterial cells obtained after growth in this medium were calculated to contain an approximate average of at least 22 per cent endotoxin.

*E. coli* endotoxin has been firmly labelled with hexavalent $Cr^{51}$ by the addition of $Na_2Cr^{51}O_4$ to suspensions of each of the following:

1) actively growing cultures of *E. coli*

2) dead bacterial cells harvested from the culture medium

3) endotoxin after separation from the bacterial cell.

The most efficient means of labelling with $Cr^{51}$ is by addition to endotoxin after separation from the bacterial cell. This provides a high count per mg. endotoxin with a minimum waste of free

chromium. The tag is firm and the $LD_{50}$ of radioactive toxin is the same as corresponding samples of non-tagged endotoxin.

*E. coli* endotoxin has also been labelled with trivalent $Cr^{51}$ by the use of $Cr^{51}Cl_3$. $Cr^{51}Cl_3$ produced a far heavier tag than $Na_2Cr^{51}O_4$ and did not reduce toxicity of endotoxin when precautions were taken to prevent inactivation by low pH.

A close quantitative relationship has been shown to exist between toxicity and radioactivity so that it would appear justifiable to use radioactivity as a measure of the amount of chromate tagged endotoxin during tracer experiments.

## REFERENCES


1. Petrie, G. F., The endotoxin of the meningococcus. J. Hyg., 1937, **37**, 42.
2. Spink, W. W., and Anderson, D., Experimental studies on the significance of endotoxin in the pathogenesis of brucellosis. J. Clin. Invest., 1953, **33**, 540.
3. Brues, A. M., and Shear, M. J., Chemical treatment of tumors. X. Reactions of four patients with advanced malignant tumors to injection of a polysaccharide from *Serratia marcescens* culture filtrate. J. Nat. Cancer Inst., 1944, **5**, 195.
4. Braude, A. I., Siemienski, J., Williams, D., and Sanford, J. P., Overwhelming bacteremic shock produced by gram-negative bacilli; a report of four cases with one recovery. Univ. Mich. Med. Bull., 1953, **19**, 23.
5. Favorite, G. O., and Morgan, H. R., Effects produced by the intravenous injection in man of a toxic antigenic material derived from *Eberthella typhosa:* Clinical, hematological, chemical, and serological studies. J. Clin. Invest., 1942, **21**, 589.
6. Boivin, A., and Mesrobeanu, L., Recherches sur les antigènes somatiques et sur les endotoxines des bactéries: I. Considérations générales et exposé des techniques utilisées. Rev. d'immunol., 1935, **1**, 553. II. L'antigène somatique complet (antigen O) de certaines bacteries est le constituant principal de leur endotoxine. Rev. d'immunol., 1936, **2**, 113. III. Antigène somatique O "complet" et variations bacteriennes. Rev. d'immunol., 1937, **3**, 319.
7. Gladstone, G. P., The antigenic composition and virulence of *Bact. typhosum* grown on a chemically-defined medium. Brit. J. Exper. Path., 1937, **18**, 67.
8. Freeman, G. G., Challinor, S. W., and Wilson, J., The use of a synthetic medium in the isolation of the somatic antigens of *Bact. typhi-murium* and *Bact. typhosum*. Biochem. J., 1940, **34**, 307.
9. Reed, L. J., and Muench, H., A simple method of estimating fifty per cent endpoints. Am. J. Hyg., 1938, **27**, 493.