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Induction of Alkaline Phosphatase in a Subcellular Preparation from *Escherichia coli*

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The synthesis of alkaline phosphatase in the cells of *Escherichia coli* has been shown to be under the control of three genes (Garen & Echols, 1962*a*, *b*). Two of these genes (designated $R1$ and $R2$) control the quantitative aspects of enzyme synthesis, and the third (P) controls the structure of the enzyme. With the wild-type bacteria (Hfr strain K10 of *E. coli* K12) the three genes are present ($R1^+R2^+P^+$), and regulate the synthesis of alkaline phosphatase according to the content of orthophosphate in the medium (Torriani, 1960). With a high concentration of orthophosphate in the medium, wild-type bacteria have only a low content in alkaline phosphatase. When, however, the phosphate becomes limiting, a high content of alkaline phosphatase is produced. A mutation of either the $R1$ or the $R2$ gene produces strains of bacteria that have been termed 'constitutive' mutants because the strain is no longer repressible by high concentrations of orthophosphate.

From other genetic studies Echols, Garen, Garen & Torriani (1961) have shown that, for both $R1$ and $R2$ genes, repressibility is dominant over constitutivity in heterozygous diploids that carry a constitutive gene either *cis* or *trans* to the active structural gene for alkaline phosphatase. The two genes then appear to control the formation of a repressor of alkaline phosphatase synthesis.

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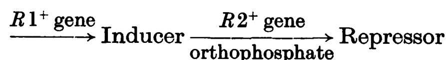
Further, it has been suggested, from a study of the synthetic capacity of $R1^-$ mutants and a partial diploid F′ strain carrying $R1^+P^-$ on the episome and $R1^-R2^+P^+$ on the chromosome (Echols, 1961), that the relation between the genes in control of alkaline phosphatase is of the form:



$$\xrightarrow{R1^+\text{ gene}} \text{Inducer} \xrightarrow[\text{orthophosphate}]{R2^+\text{ gene}} \text{Repressor}$$

It was decided to examine the induction of alkaline phosphatase *in vitro* by using a subcellular membrane fraction prepared from the wild-type strain of *E. coli* after the bacteria had been grown in the presence of orthophosphate. The present paper describes the conditions and factors effecting the induction of alkaline phosphatase in an *E. coli* subcellular preparation (P_1) prepared in a manner similar to that described by Nisman, Fukuhara, Demailly & Genin (1962).

MATERIALS AND METHODS

Materials. Ribonucleoside triphosphates were obtained from Pabst Laboratories (Milwaukee, Wis., U.S.A.), NAD^+, NADH, $NADP^+$, NADPH from Boehringer und Soehne G.m.b.H. (Mannheim, Germany), amino acids (L-form) from California Biochemical Corp. (Los Angeles, U.S.A.), and diethylaminoethylcellulose (DEAE-cellulose) from Eastman Organic Chemicals (U.S.A.). Phosphoenolpyruvate, pyruvate kinase, bovine serum albumin (crystalline), *p*-nitrophenyl phosphate, alkaline phosphatase,

ribonuclease and deoxyribonuclease were all products of Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Radioactive amino acids and $H_3{}^{32}PO_4$ were obtained from the Commissariat à l'Energie Atomique (France).

Penicillin (sodium salt) was supplied by the Laboratoire R. Bellon (Neuilly-sur-Seine, France), and the potassium salt of penicillin G was supplied by Specia (Paris, France).

Difco media were obtained from Difco Laboratories (Detroit, Mich., U.S.A.), peptone and digitonin from Prolabo (Paris), sodium lauryl sulphate from Serlabo (Paris) and actinomycin D from Merck (U.S.A.).

Organisms. The following *Escherichia coli* strains were kindly supplied by Dr A. Garen and Dr H. Echols; the wild-type ($R1^+R2^+P^+$), Hfr strain K10 of *E. coli* K12, and its mutant strains C2 ($R1^-R2^+P^+$) and C4 ($R1^+R2^-P^+$). All strains were maintained by periodic subculture on nutrient agar.

Media. Difco Bacto Antibiotic Medium 3 was prepared and sterilized according to the manufacturer's instructions.

SP2 medium contained 1·2% (w/v) of Difco Bacto Tryptone, 1% (w/v) of peptone, 0·4% of Difco yeast extract, 2% (w/v) of KH_2PO_4, 0·04% of $FeSO_4,7H_2O$ and 0·025% of $MgSO_4,7H_2O$. This mixture was boiled for 1 hr. and adjusted to pH 7·2 with NaOH. After being cooled the mixture was filtered, and the filtrate was reboiled and refiltered when cool. Before the mixture was autoclaved, for 45 min. at 15 lb./in.^{2}, the pH was checked and 1 l. volumes were distributed in 2 l. flasks.

Sucrose medium 1 contained sucrose (0·5M), $MgSO_4$ (15 mM), tris buffer, pH 7·3 (0·02M), and penicillin G (potassium salt) (5000 units/ml.).

Sucrose medium 5 was similar but contained sucrose at a concentration of 1·5M.

Spheroplast formation. Bacteria were inoculated into 500 ml. of Difco Antibiotic Medium 3 and grown with shaking (70 turns/min.) in a water bath at 30° for 14 hr. (late logarithmic phase). The organisms were then mixed with 2·5 l. of SP2 medium and 60 ml. of 40% (w/v) glucose (sterilized separately at pH 4·0). This mixture was incubated with shaking at 30° for 30 min. to restart growth and then 1 l. of 2M-sucrose, 50 ml. of M-$MgSO_4$ and 6×10^6 units of penicillin G (sodium salt) were added. The mixture was equally distributed into three 6 l. flasks and shaken for 2–2·5 hr. until the spheroplasts were formed. Spheroplast formation was verified by contrast-phase microscopy.

Preparation of subcellular fraction P_1. Spheroplasts, membrane fragments and free ribosomes were homogenized by passing ten times through broken-tipped pipettes. Unless otherwise stated, centrifugal forces are the average ($g_{av.}$).

Spheroplasts were centrifuged at 14000*g* for 20 min. in a Servall refrigerated centrifuge (model RC2, rotor GSA). The last traces of phosphate-containing medium were removed by recentrifuging after homogenizing in sucrose medium 1. This was repeated twice. Finally, the spheroplasts were suspended in 120 ml. of sucrose medium 1 and lysed at 4° by adding digitonin (4 g. in 40 ml. of sucrose medium 1). The extinction, *E*, of the suspension was measured before and during lysis by taking 0·2 ml. samples, diluting to 10 ml. with sucrose medium 1 and reading the extinction at 600 mμ in a Beckman model DU spectrophotometer. When the extinction ceased to fall (40–50 min. and 40–50% of the original value), it was assumed that maximum lysis had been attained (Nisman *et al.* 1962).

Before the centrifuging, 0·25 g. of bentonite was added as a nuclease absorbent. The lysate was then centrifuged at 25000*g* for 15 min. (Servall centrifuge, rotor SS-34) and the straw-coloured supernatant, S_1, separated from the pellet of membrane fragments. The pellet was washed thrice by recentrifuging after homogenization in sucrose medium 1. The washings were discarded. Finally the pellet was suspended in sucrose medium 1 or 5, and then centrifuged for 15 min. at 1000*g* (at 4°) in the Martin Christ centrifuge (model UJI) to remove unhomogenized material. The supernatant, P_1, consisting of a suspension of membrane fragments, was used for enzyme induction. The pellet was discarded. The volume, *x* (in ml.), of the medium used for this final suspension was calculated from the extinction, *E*, of the spheroplast suspension before the addition of digitonin. The equation for this ($x = 8E$) has been derived empirically so that the P_1 suspension contains approx. 1·4 mg. of protein/ml., 0·4 mg. of RNA/ml. and 0·2 mg. of DNA/ml. The average values, as calculated from 40 P_1 preparations, are: $1·38 \pm 0·40$ mg. of protein/ml., $0·44 \pm 0·15$ mg. of RNA/ml. and $0·17 \pm 0·05$ mg. of DNA/ml.

The method of preparation of P_1 is illustrated in Scheme 1.

Ribosomes. To collect ribosomes, the S_1 preparation was centrifuged at 105000*g* for 240 min. (at 0°) in a Spinco model L ultracentrifuge (rotor 40). The supernatant was used to prepare 'soluble' RNA. The clear-coloured ribosome-containing pellet was suspended in sucrose medium 1 and centrifuged at 25000*g* for 30 min., and the supernatant was recentrifuged at 105000*g* for 240 min. The pellet of the former centrifuging was discarded, and the pellet of the latter centrifuging, containing free ribosomes, was stored at 0° until required.

The method of preparation of the ribosomes is illustrated in Scheme 1.

Preparation of membrane ribonucleic acid and membrane total nucleic acids. The following manipulations were all carried out between 0° and 4°. The methods employed were similar to those used by Nisman *et al.* (1962). Membrane nucleic acids (RNA and DNA) were extracted before the extraction of membrane RNA.

The membrane fragments, after the second washing, were suspended in 20 ml. of sucrose medium 1 and stirred with a magnetic stirrer. To separate the DNA and RNA from protein, 10 ml. of 10% (w/v) sodium lauryl sulphate was added, followed 45 sec. later by 30 ml. of water-saturated phenol (neutralized to pH 6·8 with N-NaOH). The mixture was stirred another 10 min. and then 60 ml. of water was added. After 15 min. of further stirring the mixture was centrifuged at 1000*g* for 15 min. The aqueous supernatant was treated with 200 ml. of ethanol (at −20°). This solution was left at −10° for 24 hr., then centrifuged at 1000*g* to collect the precipitated nucleic acids. These were washed five times with 75% (v/v) ethanol (at −10°) and then suspended in 10–20 ml. of a solution containing KCl (2M), tris buffer, pH 7·3 (0·01M), and $MgCl_2$ (1 mM). The suspension was dialysed against 2 l. of the suspending solution for 48 hr. with three changes of liquid. The non-diffusible material, containing membrane RNA and DNA, was stored at 0°.

Membrane RNA was prepared from the total nucleic acids by diluting the total nucleic acids, before dialysis (see above), with 2 vol. of water and treating with deoxyribonuclease (20 μg./ml.) for 60 min. at 4°. The enzyme was destroyed by heating at 93° for 4 min. The RNA was

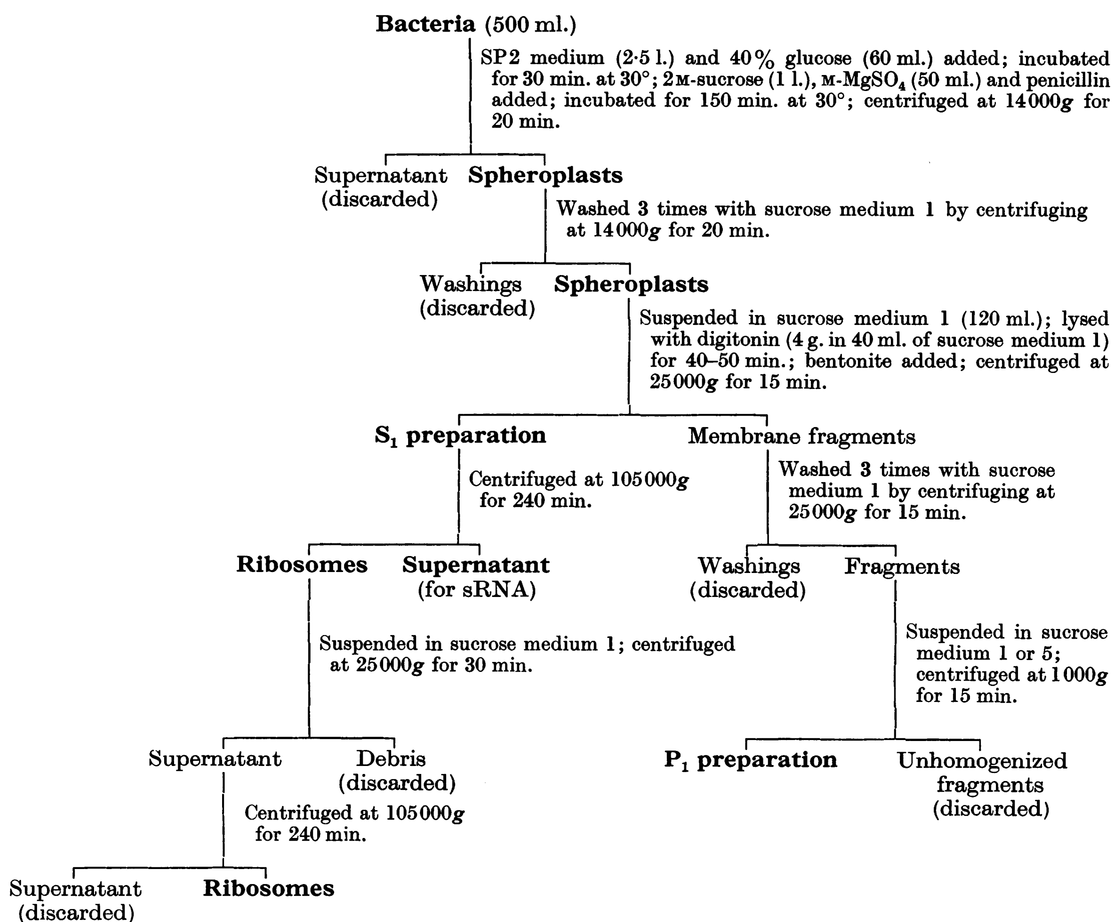
precipitated by adding ethanol cooled to −20° until a final concentration of 70% (v/v) was reached and leaving the mixture at −10° for 24 hr. The RNA was collected by centrifuging, washed with 70% (v/v) ethanol (at −10°) and suspended in the minimum quantity of 0·01 M-tris buffer, pH 7·3, containing $MgCl_2$ (0·1 mM). Insoluble RNA was removed by centrifuging at 20000g for 60 min., and the rest was used immediately or stored at 0°.

The method of preparation of membrane RNA and membrane total nucleic acids is illustrated in Scheme 2.

Soluble ribonucleic acid. Soluble RNA (sRNA) was prepared from the supernatant, S_1, after free ribosomes had been removed. An equal volume of ethanol (at −20°) was added with stirring to the supernatant, and the precipitate was collected by centrifuging and suspended in 20 ml. of 0·01 M-tris buffer, pH 7·3, containing $MgCl_2$ (1 mM). An equal volume of phenol (water-saturated and neutralized to pH 6·8 with N-NaOH) was added, and the mixture was stirred for 10 min. and then centrifuged at 1000g for 15 min. The aqueous supernatant was mixed into 60 ml. of ethanol (at −20°) and left for 24 hr. at −10°. The sRNA was collected by centrifuging, washed five times with 75% (v/v) ethanol, suspended in 5 ml. of 0·01 M-tris buffer, pH 7·3, containing $MgCl_2$ (1 mM) and dialysed against the same solution for 24 hr. at 4°. The non-diffusible material was stored at 0°.

Incubation conditions for the induction of alkaline phosphatase. The incubation mixture used for the induction of alkaline phosphatase contained (per ml.): tris buffer, pH 7·3, 100 μmoles; amino acid mixture, 0·1 ml.; sucrose, 300 μmoles; $CaCl_2$, 6 μmoles; ATP, UTP, GTP, CTP, 0·2 μmole of each; $NADP^+$, 3·0 μm-moles; penicillin, 5000 units; glucose, 3 μmoles; phosphoenolpyruvate, 1 μmole; pyruvate kinase, 0·05 μmg. (i.e. sufficient to hydrolyse 0·3 μmole of phosphoenolpyruvate/hr. at 37°); KCl, 100 μmoles; DNA (56 μg.) and RNA (100 μg.) extracted from the P_1 preparation of the C4 *E. coli* strain (total nucleic acids); P_1 preparation having 10–100 μg. of protein.

The amino acid mixture contained (per ml.): alanine, 7·5 μmoles; arginine, 5·8 μmoles; asparagine, 6·5 μmoles; aspartic acid, 11·3 μmoles; cysteine, 1·95 μmoles; glutamic



Scheme 1. Preparation of P_1 fraction. For an explanation of the Scheme see the text. Homogenizations and centrifuging with sucrose media 1 and 5 were performed at 0–4°.

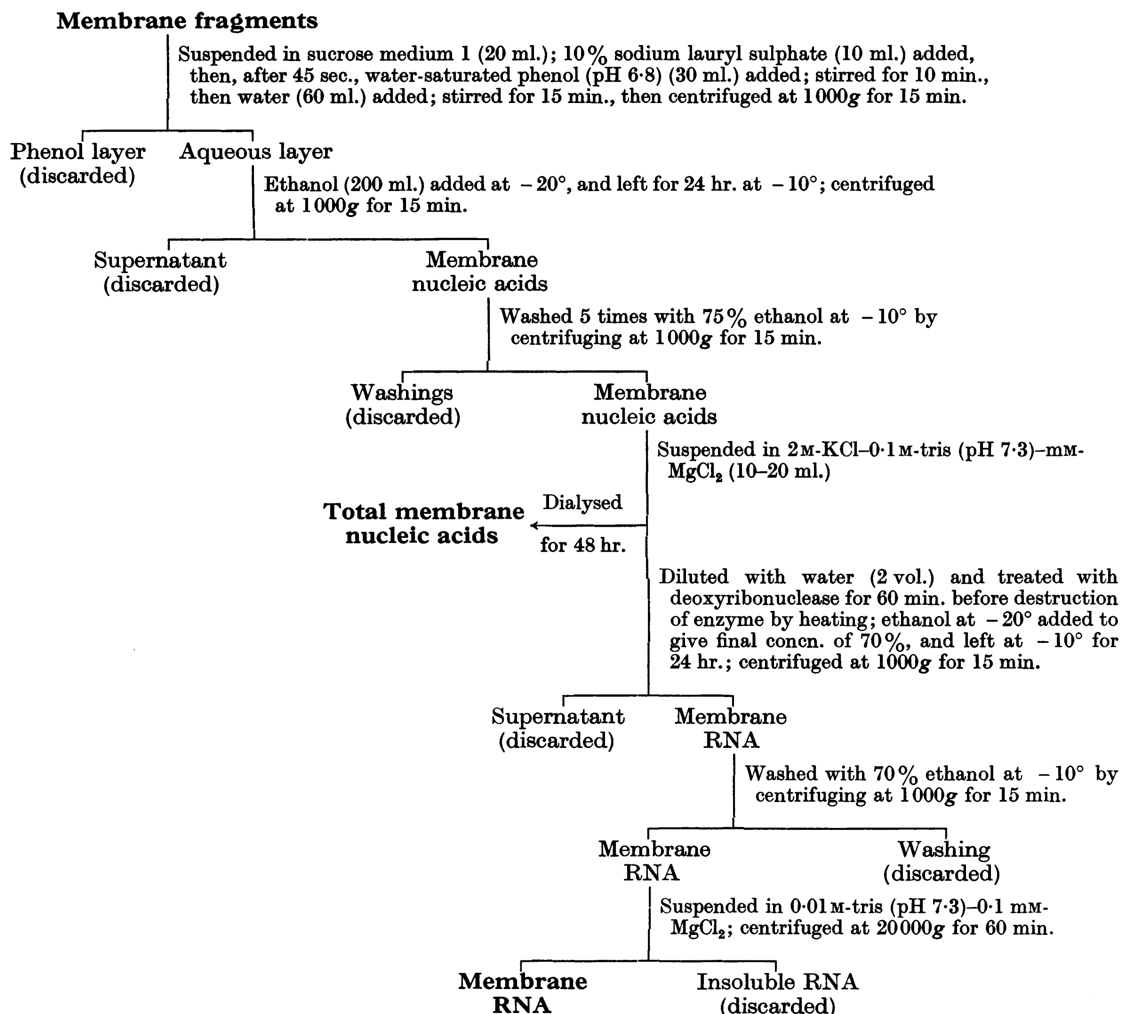
acid, 10·3 μmoles; glutamine, 6·5 μmoles; glycine, 14·5 μmoles; histidine, 2·85 μmoles; leucine, 7·6 μmoles; isoleucine, 7·6 μmoles; lysine, 2·5 μmoles; methionine, 2·5 μmoles; phenylalanine, 4·2 μmoles; proline, 6·0 μmoles; serine, 9·2 μmoles; threonine, 11·6 μmoles; tryptophan, 4·07 μmoles; tyrosine, 2·2 μmoles; valine, 6·5 μmoles; tris buffer, pH 7·3, 100 μmoles.

The incubation mixture was prepared in the order listed above (at 4°), and before incubation the P_1 preparation (usually in sucrose medium 5) was added and thoroughly dispersed by repeated pipettings. Samples (0·5 ml.) were pipetted into 10 ml. tubes (1·5 cm. internal diam.) and the tubes incubated at 37° for various times. Protein synthesis was stopped by transferring the tubes to an ice bath and adding chloramphenicol (500 μg.). The tubes were stored overnight at 0°. Experiments were performed in duplicate. The content of alkaline phosphatase, after the first 60 min. of incubation, always increased linearly for 3 hr. of incubation.

When the incorporation of radioactive amino acids into protein was studied, these amino acids were added in addition to the amino acid mixture. When [α-^{32}P]CTP was incorporated into RNA it also was added in addition to the CTP of the incubation mixture given above.

Protein, ribonucleic acid and deoxyribonucleic acid determinations, and incorporation of radioactive amino acids into proteins. These were all carried out according to the methods of Nisman *et al.* (1962).

Incorporation of cytidine [α-^{32}P]*triphosphate into ribonucleic acid.* The [α-^{32}P]CTP was prepared according to the method of Hurwitz (1959). After incubation of the mixture containing [α-^{32}P]CTP, the reaction was stopped by adding 1 ml. of 10% (w/v) trichloroacetic acid (at 4°). All subsequent manipulations were carried out at 0°. To facilitate



Scheme 2. Preparation of membrane RNA and membrane nucleic acids. For an explanation of the Scheme see the text. Homogenizations and centrifugings with sucrose medium 1 or other media were performed at 0–4°.

precipitation, 1 mg. of albumin was added and the solution centrifuged at 15000*g* for 5 min. in an International centrifuge (model HR-1, rotor 859). The precipitate was washed five times with fresh 10% (w/v) trichloroacetic acid and then dissolved in 1 ml. of N-ammonia. The incorporation of radioactivity into RNA was measured by using a Nuclear-Chicago Corp. model D47 gas-flow counter, after plating and drying the samples to infinite thinness, on aluminium disks. The results, corrected for background radiation and non-specific absorption of CTP, are expressed in terms of μm-moles of [α-^{32}P]CTP incorporated/mg. of protein in the incubation mixture. The non-specific absorption of CTP was calculated from control experiments in which the reaction mixture was not incubated.

Determination of alkaline phosphatase. Alkaline-phosphatase activity was measured in a manner similar to that described by Garen & Levinthal (1960). The incubation mixture (0·5 ml.) was diluted with M-tris buffer, pH 8·0 (0·5 ml.), and allowed to equilibrate at 30°. After 10 min. the enzyme substrate [0·5 ml. of a solution (at 30°) of *p*-nitrophenylphosphate (0·6 mg./ml.) in M-tris buffer, pH 8·0, containing $MgCl_2$ (10 mM)] was added. The incubation at 30° of this mixture was terminated by the addition of 1·5 ml. of M-potassium phosphate buffer, pH 8·0. After the mixture had been cooled to 4°, particulate matter was removed by centrifuging at 15000*g* for 5 min. The liberation of *p*-nitrophenol was measured from the increase in extinction at 410 mμ with a 1 cm. light-path. One unit of enzyme is defined as that activity which liberates, at 30°, 1 μm-mole of *p*-nitrophenol/hr. The results are expressed in terms of units of enzyme/mg. of protein in the incubation mixture. Enzyme activity in commercial alkaline phosphatase and in the *E. coli* preparations used was linear with respect to time and enzyme concentration under the conditions described above.

To determine the enzyme in bacteria or spheroplasts incubated in the presence of phosphate-containing media, care had to be taken to remove phosphate, which might otherwise inhibit enzyme action. To do this a small sample of bacteria was diluted in M-tris buffer, pH 8·0, and sufficient $CaCl_2$ was added to ensure complete precipitation of the phosphate. The amount needed was determined empirically by adding to a series of tubes containing the same amounts of bacteria various quantities of $CaCl_2$, centrifuging and adding more $CaCl_2$ to the supernatant. The minimal amount of $CaCl_2$ which totally precipitated the phosphate was added to the phosphate-containing solution before enzyme assay. The $CaCl_2$, at the concentrations used, did not inhibit the enzyme, nor did the precipitate adsorb the enzyme.

The method of determination of enzyme in the bacteria or spheroplasts was therefore as follows. Samples of bacteria in Difco Antibiotic Medium 3, or spheroplasts in SP2 medium, were diluted with M-tris buffer, pH 8·0, and the organisms lysed with 0·05 ml. of toluene. The suspension was equilibrated for 10 min. at 30° and then the $CaCl_2$ was added. The enzyme was then determined as described above.

Isolation of radioactive alkaline phosphatase. To an incubation mixture (20 ml.) containing more P_1 preparation than usual (400 μg. of protein/ml. and 430 units of alkaline phosphatase/mg. of protein), L-[*Me*-^{14}C]methionine (10 μ-moles; 43 μc) and DL-[1-^{14}C]glutamic acid (40 μmoles; 132 μc) were added. The mixture was incubated in a 250 ml. Erlenmeyer flask with gentle agitation for 4 hr. and then treated in 5 ml. batches for 5 min. in a Raytheon (250 w; 10 kcyc./sec.) ultrasonic disintegrator operating under optimum conditions. The ultrasonically treated material (containing 29600 units of enzyme) was centrifuged at 15000*g* for 20 min.; 28800 units of activity were found in the supernatant. To eliminate radioactive amino acids, 2 ml. of the amino acid mixture, having an additional 40 μmoles of methionine and 160 μmoles of DL-glutamic acid, was added to the supernatant. The total mixture was dialysed against five changes of 0·01 M-tris buffer, pH 7·3, containing $MgCl_2$ (1 mM) during 48 hr. at 4°. The non-diffusible material was concentrated to 2 ml. (28000 enzyme units total) and chromatographed on DEAE-cellulose. The column of DEAE-cellulose (1 cm. × 10 cm.) was washed beforehand with 500 ml. of 0·01 M-tris buffer, pH 7·3. The enzyme was adsorbed on the column and then eluted, by using a Lieberman proportional-feed assembly (Lakshmanan & Lieberman, 1954), by a linear gradient of NaCl ranging from 0 to 0·2 M, buffered at pH 7·3 with 0·01 M-tris. One hundred 2 ml. fractions were collected at 0° at a rate of about 1·0 ml./min. Fractions were analysed for enzyme, radioactivity and protein.

Sucrose-gradient sedimentation of membrane ribonucleic acid and alkaline phosphatase. A 25 ml. gradient of sucrose (range: 20–5%, w/v) was prepared in 30 ml. Spinco centrifuge tubes. The sucrose solutions were prepared in 0·01 M-tris buffer, pH 7·3, containing $MgCl_2$ (0·1 mM). Before the centrifuging, 1 ml. of the solution containing fresh membrane RNA or alkaline phosphatase was placed on top of the gradient. Centrifuging in the Spinco (rotor SW 25·1) was maintained at 25000 rev./min. (90000$g_{max.}$) for 13 hr. at 0°. The gradient was fractionated by piercing the base of the tube and collecting 7-drop fractions (about 0·6 ml.). The RNA was determined in each fraction from the extinction at 260 mμ. The radioactivity was determined after adding to each fraction 2 mg. of albumin, precipitating with 10% (w/v) trichloroacetic acid, centrifuging and washing the precipitate twice with 5% (w/v) trichloroacetic acid; the precipitate was finally dissolved in 1 ml. of N-ammonia, plated and dried to infinite thinness on aluminium disks and counted as described above. The results, corrected for background radiation, are expressed in terms of counts/min. Other determinations were made as indicated above. The RNA sedimentation coefficients are given in Svedberg units (s).

RESULTS

P_1 *preparation.* Tables 1 and 2 demonstrate the loss of alkaline phosphatase during the preparation of the subcellular fraction P_1 from the two strains of *E. coli*, the wild-type and the 'constitutive' mutant C2. The 'constitutive' mutant C4 gave similar results to that of the C2 mutant, although the quantities of enzyme were about twice as high. For the wild-type strain (Table 1), the formation of spheroplasts, under the conditions described in the Methods section, resulted in a 'loss' to the medium of about 35% of the total enzyme; correspondingly for the C2 strain about 55% was 'lost'. With both strains, however, it was found, by differential centrifuging, that some of the enzyme present in

the SP 2 medium could be accounted for by small spheroplasts. The rest of the enzyme present in the SP 2 medium after formation of the spheroplasts was associated with the pellet obtained by centrifuging at 105000*g* of the spheroplast-free SP 2 medium. Very little enzyme was found in the supernatant after this centrifuging (see Tables 1 and 2).

Washing of the spheroplasts of either strain with sucrose medium 1 removed little alkaline phosphatase.

From studies on the action of lysozyme on *E. coli*, Malamy & Horecker (1961) showed that much of the bacterial alkaline phosphatase is located either on the outside of the protoplast membrane or in the cell wall itself. They found that preparation of protoplasts by lysozyme lysis of cell walls liberated most of the bacterial alkaline phosphatase.

The results shown in Tables 1 and 2 indicate that partial liberation of the cell wall during the formation of spheroplasts in the presence of penicillin results in a partial liberation of alkaline phosphatase.

Lysis of the spheroplasts resulted in a further liberation into the S_1 fraction of alkaline phosphatase (48 % or 58 % of the spheroplast content for the wild-type and C 2 strains respectively). The P_1 fractions of both strains contained about 24 % of the total enzyme present in the spheroplasts. The rest of the enzyme, unaccounted for by the S_1 and P_1 fractions, remained in the unhomogenized fraction.

The liberation of protein from the spheroplasts was similar for the two strains. The S_1 fractions contained 47 % or 49 % respectively of the total bacterial protein for the wild-type and C 2 strains, whereas the P_1 fractions contained 24 % or 29 % respectively. The RNA:DNA:protein proportions in the spheroplasts of either strain were approximately 1:0·15:4, whereas for the S_1 fraction the proportions were 1:0·1:3·5, and for the P_1 preparation they were 1:0·25:3; for the intact bacteria of both strains the proportions were 1:0·15:4·6.

Table 1. *Loss of alkaline phosphatase during preparation of the subcellular fraction* P_1 *from* Escherichia coli *K* 10 ($R1^+R2^+P^+$)

The conditions of preparation of the P_1 fraction are described in the Materials and Methods section. Enzyme and protein assays were performed as described in the text.

	Vol. (ml.)	$E_{600\,m\mu}$	Alkaline-phosphatase activity (total units)	(units/mg. of protein)
Bacteria in Difco Antibiotic Medium 3	500	3·410	460000	700
Bacteria in SP 2 medium	3000	0·555	470000	680
Spheroplasts in SP 2 medium	4000	0·480	520000	730
SP 2 medium after removal of spheroplasts	4000	0·100	180000	1800
SP 2 supernatant after centrifuging at 25000*g*	4000	0·020	90000	*
Pellet from 105000*g* centrifuging of SP 2 supernatant	—	—	85000	4000
Spheroplasts before lysis with digitonin	120	12·500	310000	550
S_1 preparation in sucrose medium 1	155	—	149000	560
P_1 preparation in sucrose medium 5	100	—	74000	550

* Insufficient protein was present for analysis.

Table 2. *Loss of alkaline phosphatase during preparation of the subcellular fraction* P_1 *from* Escherichia coli C2 ($R1^-R2^+P^+$)

The methods of preparation of the P_1 subcellular fraction from the C 2 mutant strain of *E. coli* and conditions of assays are the same as in Table 1.

	Vol. (ml.)	$E_{600\,m\mu}$	Alkaline-phosphatase activity (total units)	(units/mg. of protein)
Bacteria in Difco Antibiotic Medium 3	500	4·560	14000000	21000
Bacteria in SP 2 medium	3000	0·755	14500000	20000
Spheroplasts in SP 2 medium	4000	0·660	16000000	21000
SP 2 medium after removal of spheroplasts	4000	0·145	8800000	68000
SP 2 supernatant after centrifuging at 25000*g*	4000	0·033	6900000	*
Pellet from 105000*g* centrifuging of SP 2 supernatant	—	—	6000000	280000
Spheroplasts before lysis with digitonin	120	17·000	6600000	11000
S_1 preparation in sucrose medium 1	155	—	3800000	13000
P_1 preparation in sucrose medium 5	136	—	1560000	9100

* Insufficient protein was present for analysis.

With both strains the ribosome-containing fraction, prepared from the S_1 fraction, contained less than 0·01 % of the alkaline phosphatase of the S_1 fraction. This amount diminished by 40 % after two further washings in sucrose medium 1 but remained constant thereafter. Cowie, Spiegelman, Roberts & Duerksen (1961) have similarly found a small amount of β-galactosidase firmly bound to the ribosomes of *E. coli*. The RNA:DNA:protein proportions in the crude ribosome-containing fraction (prepared as shown in the Materials and Methods section) were 1:0·03:1·5. The proportions in the ribosomes after two further washes in sucrose medium 1 were 1:0·01:0·8. Comparison with the results of Tissières, Watson, Schlessinger & Hollingsworth (1959) indicates that these ribosomes, even after the washes in sucrose medium 1, are contaminated with non-ribosomal protein.

Induction of alkaline phosphatase by the P_1 *fraction.* The induction of alkaline phosphatase by a P_1 fraction prepared from the wild-type ($R1^+R2^+P^+$) strain of *E. coli* is illustrated in Fig. 1. During the first 30–40 min. of incubation there was little increase in the total enzyme; after this lag new enzyme began to appear and within an hour the rate of synthesis reached a maximum. After 4–5 hr. the rate of synthesis decreased, and had completely ceased after 6 hr. of incubation. When actinomycin D was added to the complete system, the formation of enzyme was substantially diminished, but not completely inhibited.

The exact quantity of enzyme that can be induced in 4 hr. of incubation varies with the P_1 preparation. Usually P_1 preparations from the wild-type strain gave a tenfold increase in enzyme after 4 hr. of incubation (Table 3). Better results have been obtained with P_1 preparations containing a high proportion of RNA and DNA to protein (see the Materials and Methods section). Better induction is also obtained from preparations in which the force of homogenization by pipetting has been minimal. In one experiment in which the homogenizations were effected with a 100 ml. syringe, the RNA:DNA:protein proportions were 1:0·1:3, and the induction of alkaline phosphatase under standard conditions resulted in a 2·5-fold increase in total enzyme after 4 hr. of induction. From this it was apparent that the structure of the P_1 fraction, and its content of DNA, may well be important factors in the process of induction.

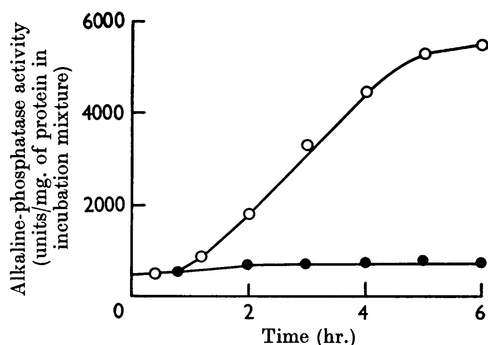


Fig. 1. Induction of alkaline phosphatase by a P_1 preparation from *E. coli* ($R1^+R2^+P^+$). Experimental conditions are given in the text. Actinomycin D (20 μg./ml.) was added to the incubation mixture (at 0°) 45 min. before incubation. The incubation mixture contained (per ml.): 45 μg. of protein, 15 μg. of RNA and 9 μg. of DNA of P_1 preparation (prepared in sucrose medium 5). ○, System not treated with actinomycin; ●, system treated with actinomycin.

Enzymic destruction of DNA (Table 3) also inhibited the subsequent induction of alkaline phosphatase, and quantitatively had a similar effect to that of actinomycin D. It has been shown by Reich, Goldberg & Rabinowitz (1962) and

Table 3. *Induction of alkaline phosphatase by the subcellular fraction* P_1 *prepared from the wild-type* ($R1^+R2^+P^+$) Escherichia coli *strain*

Experimental conditions, determination of alkaline phosphatase and content of complete system are given in the text. The composition of the P_1 fraction, prepared in sucrose medium 5, per ml. of incubation mixture was: 31 μg. of protein, 7 μg. of RNA and 3 μg. of DNA. When additions were made to the complete system, they were added 45 min. before incubation was commenced in order to equilibrate (at 4°).

	Alkaline-phosphatase activity (units/mg. of protein)
Before incubation	
Complete system	490
After incubation (for 4 hr. at 37°)	
Complete system	4810
Complete system + chloramphenicol (1 mg./ml.)	500
Complete system + deoxyribonuclease (20 μg./ml.)	740
Complete system + ribonuclease (30 μg./ml.)	550
Complete system + actinomycin D (20 μg./ml.)	750
System − glucose	1800
System − amino acids	670
System − $MgCl_2$	1500
System − $CaCl_2$	530
System − phosphoenolpyruvate and pyruvate kinase	4200
System − ATP, UTP, GTP and CTP	2200
System − UTP	2400
System − total nucleic acids (C4 constitutive mutant)	3100
System − $NADP^+$	3600
Complete system + ribosome-containing fraction (65 μg. of RNA)	5100
Complete system + soluble RNA (77 μg. of RNA)	5100

Furth, Kahan & Hurwitz (1962) that the antibiotic actinomycin D inhibits RNA polymerase, an enzyme that effects the transcription to RNA (termed 'messenger' or 'informational' RNA) of genetic information contained in DNA The results in Table 3 are interpreted as indicating that alkaline-phosphatase synthesis is substantially decreased if the formation of informational RNA is blocked, either by the destruction of the DNA or through the action of actinomycin D. Ribonuclease also inhibits the formation of alkaline phosphatase (Table 3). The addition of calcium chloride to the incubation mixture gives rise to an increased induction of alkaline phosphatase (Table 3). The amount added is sufficient to precipitate orthophosphate in excess of 1 μmole/ml.

The effect of the omission of calcium chloride from the incubation mixture varied with P_1 preparation, and possibly with its content of orthophosphate. In some cases the omission of calcium chloride led to only a 50 % decrease in new enzyme synthesized. When calcium chloride was added after incubation, it did not affect the assay of enzyme activity. Apparently therefore the inclusion of calcium chloride was effective in the processes of induction.

The role of $NADP^+$ in induction (Table 3) was relatively unspecific: NAD^+, NADH or NADPH (in the place of $NADP^+$) also stimulated. The amount of alkaline phosphatase formed with NAD^+ or NADH (3 μm-moles/ml.) was 85 % of that synthesized in the presence of the same concentration of $NADP^+$. NADPH (3 μm-moles/ml.) completely replaced $NADP^+$ in stimulating induction. Mixtures of the coenzymes were no more effective than $NADP^+$ alone.

Higher concentrations of $NADP^+$ or NADPH inhibited the induction of alkaline phosphatase. In comparison with the standard system, the addition of 30 μm-moles of $NADP^+$ or NADPH/ml. of the incubation mixture resulted in a 10 % decrease in the total alkaline phosphatase synthesized.

The omission of the amino acids from the incubation mixture (Table 3) usually led to a 60–100 % decrease in enzyme synthesis, depending on the P_1 preparation. If the amino acids were omitted from the incubation mixture during the first 45 min. of induction, the capacity for the induction of alkaline phosphatase could not be increased by the subsequent addition of amino acids.

The omission of one or all four of the nucleoside triphosphates from the incubation mixture resulted in the same decrease in enzyme synthesis (Table 3). Once again the actual amount of enzyme synthesized in the absence of the triphosphates varied with the individual P_1 preparation, the range of variation usually being within 30–60 % of the total net alkaline phosphatase synthesized by a system containing the triphosphates. A similar range of variation was found with the omission of total nucleic acids from the *E. coli* C4 mutant strain (Table 3).

The omission of phosphoenolpyruvate and pyruvate kinase from the incubation mixture led to a 10 % decrease in total enzyme, and the omission of magnesium chloride led to a decrease of about 80 % in the induction of alkaline phosphatase (Table 3).

The addition of soluble RNA (sRNA) or the ribosomes (see the Materials and Methods section) to the incubation mixture (Table 3) resulted in less than a 10 % increase in alkaline phosphatase.

Post-incubation effect. Nisman, Demailly, Yapo & Pelmont (1963*b*) have described a post-incubation phenomenon in the induction of β-galactosidase by the P_1 fraction. The addition of chloramphenicol to an incubation mixture resulted in an immediate cessation of the incorporation of L-[Me-^{14}C]methionine into protein, but did not inhibit immediately the synthesis of β-galactosidase when the incubation was continued for another 4 hr.

It was decided to examine the induction of alkaline phosphatase to see if there was a similar post-incubation effect. Incubation of a mixture containing the P_1 preparation from the wild-type bacteria (P_1 content/ml. of incubation mixture: 33 mg. of protein, 11 mg. of RNA and 3 mg. of DNA, and other constituents listed in the Materials and Methods section) resulted in an increase (per mg. of protein) of 4100 units of alkaline phosphatase after 4 hr. of incubation. The addition of chloramphenicol (1 mg./ml.) after 4 hr. to a similar mixture followed by incubation for a further 8 hr. resulted in a total increase in alkaline phosphatase of 4600 units/mg. of protein. In comparison with this 12 % increase in alkaline phosphatase (post-incubation effect) the incorporation of [Me-^{14}C]-methionine during the same 8 hr. was negligible.

When the incubation mixture was kept at 0° for 8 hr. after the addition of chloramphenicol, there was no increase in enzyme or methionine incorporation. It seems then that there is a slight post-incubation effect on the induction of alkaline phosphatase, but in comparison with that on the induction of β-galactosidase it is small. The post-incubation effect on the induction of β-galactosidase has been verified with the P_1 preparation from the wild-type ($R1^+ R2^+ P^+$) bacteria.

Isolation of radioactive alkaline phosphatase. There was the possibility that the observed induction of alkaline phosphatase was not a synthesis but rather an activation of a latent form of the enzyme. To examine this the enzyme was extracted from an incubation mixture containing radioactive amino acids to see whether the enzyme had incorporated the radioactivity.

An incubation mixture containing L-[Me-^{14}C]-methionine and DL-[1-^{14}C]glutamic acid was incubated with gentle agitation at 37° for 4 hr. The total alkaline phosphatase was extracted and fractionated on a DEAE-cellulose column by eluting with a linear gradient of sodium chloride (see the Materials and Methods section for details). The enzyme and radioactivity content of the first 60 fractions are shown in Fig. 2. The alkaline phosphatase was eluted from the column during the early stages of chromatography.

After a similar induction, extraction and chromatography, the alkaline-phosphatase fractions were collected and rechromatographed under similar conditions, and the main alkaline-phosphatase fractions were combined. The enzyme was concentrated by flash-evaporation and centrifuged on a sucrose gradient (see the Materials and Methods section). The result is shown in Fig. 3. The alkaline-phosphatase fractions on the gradient contained radioactivity, although some radioactivity was also found in fractions that did not contain the enzyme. It seems therefore that there is synthesis of the enzyme during induction.

Role of ribonucleic acid in induction. The role of RNA in the process of induction was studied first by examining the effect of actinomycin D on RNA and protein synthesis and then the effect of specific RNA fractions on induction.

(*a*) Action of actinomycin D. Fig. 4 illustrates the inhibition, by actinomycin, of the incorporation of [α-^{32}P]CTP into RNA during the first 60 min. of incubation. Comparison of the incorporations of [α-^{32}P]CTP into RNA after 30 min. of incubation showed that actinomycin, added at zero time, resulted in an 80% inhibition of RNA synthesis. The addition of the antibiotic after zero time also inhibited, by an amount depending on the time of addition (Fig. 4). In the absence of actinomycin, the highest rate of incorporation of [α-^{32}P]CTP was

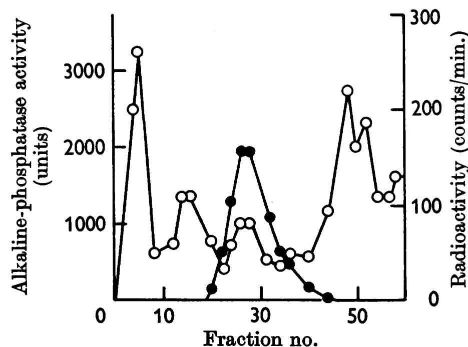


Fig. 2. Fractionation on DEAE-cellulose of P_1 protein after the induction of alkaline phosphatase. The composition of the incubation mixture, conditions of incubation, extraction of protein and fractionation on a DEAE-cellulose column, and determinations of enzyme (●) and radioactivity (○), are given in the Materials and Methods section. The P_1 fraction was prepared from the wild-type ($R1^+R2^+$-P^+) strain.

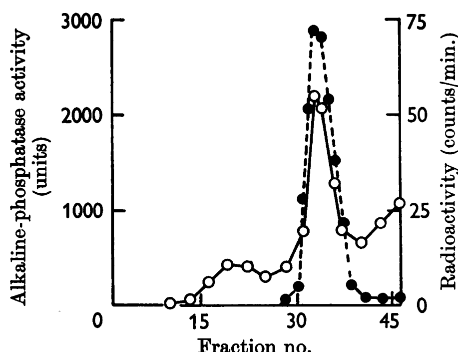


Fig. 3. Sucrose-gradient sedimentation of newly synthesized alkaline phosphatase. The extraction of alkaline phosphatase after induction and preliminary purification by chromatography on DEAE-cellulose before sedimentation on sucrose gradient were made under the same conditions as in Fig. 2. After chromatography, the main alkaline-phosphatase fractions were combined, concentrated by flash-evaporation and centrifuged on a sucrose gradient (see the Materials and Methods section). Fractions of the gradient were assayed for alkaline phosphatase (●) and radioactivity (○).

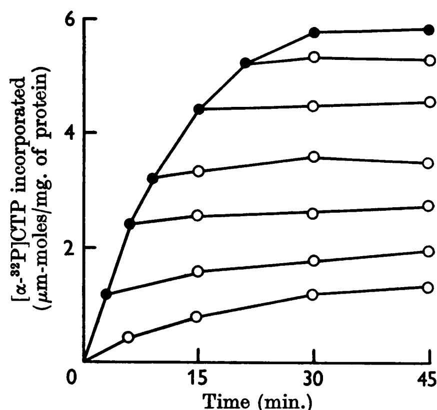


Fig. 4. Action of actinomycin D on RNA synthesis. The composition of the incubation mixture [containing 15μm-moles of [α-^{32}P]CTP (specific activity 4×10^7 counts/min. per μmole)/ml.] and determination of radioactivity are given in the Materials and Methods section. Samples (0·5 ml.) of incubation mixture were incubated at 30° for various times, the incubation being stopped by the addition of 1 ml. of 10% (w/v) trichloroacetic acid (at 0°). Actinomycin (10μg./0·5 ml.) was added as indicated. ○, Systems with actinomycin; ●, systems without actinomycin.

Table 4. *Effect of actinomycin D on the synthesis of alkaline phosphatase and total proteins by the* P_1 *fraction prepared from the wild-type* ($R1^+ R2^+ P^+$) Escherichia coli *strain*

From the P_1 preparation the incubation mixture contained 91 mg. of protein, 37 mg. of RNA and 11 mg. of DNA, and 425 units of alkaline phosphatase/mg of protein. The incubation mixture also contained 0·8 μmole (2·8 μc) of L-[Me-^{14}C]methionine/ml. of incubation mixture. The conditions of incubation, determination of alkaline phosphatase and the incorporation of radioactivity into protein are given in the text.

	Protein					Alkaline phosphatase				
	Incorporation of L-[Me-^{14}C]-methionine (μm-moles/mg. of total protein)				Net synthesis at 240 min. (%)	Activity (units/mg. of total protein)				Net synthesis at 240 min. (%)
Incubation time (min.) ...	60	120	180	240		60	120	180	240	
Incubation time before addition of actinomycin D (min.)										
0	0·31	0·51	0·73	0·96	16	495	630	740	750	7
3	0·39	0·93	1·16	1·56	26	510	820	1100	1500	24
6	0·48	0·89	1·38	1·81	31	530	950	1400	1800	31
9	0·53	0·93	1·50	1·99	34	550	1000	1500	2000	35
12	0·72	1·13	1·64	2·01	34	570	1100	1600	2300	42
15	0·73	1·25	1·86	2·46	42	590	1100	1600	2400	44
21	0·83	1·47	2·20	2·86	48	630	1300	2000	2800	53
30	1·05	1·83	2·54	3·30	56	680	1400	2200	3200	62
45	1·22	2·10	3·00	3·95	67	720	1700	2500	3500	69
60	1·63	2·52	3·31	4·20	71	750	1700	2700	3800	75
120	1·42	3·05	4·15	5·24	89	690	2000	3400	4500	91
180	1·53	3·36	4·72	5·73	97	740	2200	3700	4700	96
Not added	1·53	3·20	4·61	5·91	100	730	2100	3600	4900	100

during the first 6 min. of incubation. After 30–45 min. there was very little further incorporation of [α-^{32}P]CTP.

The incorporation of [α-^{32}P]CTP in the presence of the antibiotic increased slowly throughout the 60 min. of incubation (Fig. 4). This indicated that there was an incorporation of [^{32}P]phosphate which was not inhibited by actinomycin. Whether this represents an incorporation into RNA (or other acid-insoluble product) whose formation is not stopped by actinomycin, or represents metabolism of the [α-^{32}P]CTP and incorporation of the [^{32}P]-phosphate into non-specific acid-insoluble products, cannot be determined from these results.

Protein synthesis, as determined from the incorporation of L-[Me-^{14}C]methionine was linear with respect to time before and after the addition of actinomycin, but the rate of enzyme synthesis decreased after the addition (Table 4). Comparison of the incorporation of L-[Me-^{14}C]methionine into protein showed that the addition of actinomycin at zero time resulted in an 84% inhibition of protein synthesis (Table 4). The induction of alkaline phosphatase, with or without the addition of actinomycin at zero time (Table 4), was similar to that shown in Fig. 1. Delay in the addition of the antibiotic resulted in increased induced synthesis of alkaline phosphatase. However, when it was added after the first 60 min. of incubation the rate of induction decreased.

The effect of actinomycin on the synthesis of alkaline phosphatase, after the first 3–6 min. of incubation, closely paralleled its effect on protein synthesis (Table 4).

It is evident therefore that, under the conditions of incubation used, the first 6 min. of incubation are the most important for RNA synthesis and that RNA synthesis is inhibited by the addition of actinomycin. Similar results have been reported by Pelmont, Yapo, Demailly & Nisman (1963).

(*b*) *Nature of the ribonucleic acid synthesized by the* P_1 *fraction.* The nature of the RNA synthesized during the incubation procedure was examined after extracting it and subjecting it to sucrose-gradient sedimentation. The conditions of extraction and centrifuging of RNA are important to the resultant sedimentation characteristics of the RNA (Gros *et al.* 1961). From experiments on the effects of the pulse-labelling of *E. coli* cells, they found that newly formed RNA, extracted in the presence of sodium lauryl sulphate and centrifuged on a gradient containing 0·1 mM-magnesium chloride, sedimented with 8s characteristics. It was free from other RNA (unlabelled) which was extracted at the same time (23s, 16s and 70s material).

The incorporation of [α-^{32}P]CTP into RNA was examined to see if the synthesis of RNA *in vitro* corresponded to the synthesis *in vivo*.

An incubation mixture containing [α-^{32}P]CTP and a P_1 preparation from the wild-type bacteria

was incubated at 30° for 30 min. Before the extraction of the nucleic acids and preparation of the membrane RNA from them (see the Materials and Methods section), 'carrier' membrane nucleic acids were added. The 'carrier' nucleic acids had been freshly extracted from the membrane P_1 preparation of the wild-type bacteria. A control experiment was carried out, by the same procedure, with a reaction mixture that had not been incubated (Fig. 5*b*).

The two membrane-RNA preparations were fractionated by sucrose-gradient sedimentation and the fractions assayed for radioactivity and RNA (from the extinction at 260 mμ) (Figs. 5*a* and 5*b*).

Comparison with the work of Spiegelman (1961) and Gros *et al.* (1961) indicated that the three RNA peaks that separated during centrifuging corresponded to 23s, 16s and 8–4s RNA (see Figs. 5*a* and 5*b*).

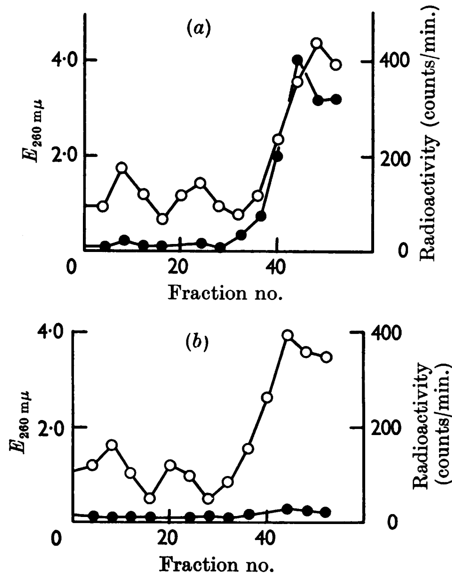


Fig. 5. Sucrose-gradient sedimentation of membrane RNA: (*a*) incubated; (*b*) not incubated. Membrane RNA was extracted before (*a*) and after (*b*) 30 min. of incubation at 30° of a reaction mixture (20 ml.) containing 15μm-moles of [α-^{32}P]CTP (specific activity $4·5 \times 10^7$ counts/min. per μmole)/ml., and a P_1 preparation prepared from the wild-type bacteria (88μg. of protein, 28μg. of RNA and 15μg. of DNA/ml.). Before extraction 'carrier' nucleic acids were added (RNA, 8·55 mg.; DNA, 5·1 mg.), and the total nucleic acids extracted and membrane RNA samples prepared (see the Materials and Methods section and the text). The membrane RNA was fractionated by sedimentation of a sucrose gradient: 7-drop fractions (about 0·6 ml.) were collected and assayed for radioactivity (●) and RNA (○) (see the Materials and Methods section). From left to right the three RNA peaks corresponded to 23s, 16s and 8–4s RNA (see the text).

There was almost no incorporation of radioactivity in the control experiment where the P_1 preparation had not been incubated (Fig. 5*b*). For the experiment in which the P_1 preparation was incubated for 30 min., almost all the [α-^{32}P]CTP incorporated was found in the RNA having the lowest sedimentation coefficient (8–4s) (Fig. 5*a*). Only a small fraction of the radioactivity was incorporated into the heavier RNA fractions (23s and 16s).

These results indicate that, even with a relatively long period of RNA synthesis by the P_1 fraction, almost all of the newly formed RNA which is extracted (under the conditions given in the Materials and Methods section) is of a low-molecular-weight type. Apparently little of the 23s and 16s RNA is synthesized.

Fractionation of the membrane RNA from the mutant C4 *E. coli* strain resulted in a similar RNA centrifuging pattern. The three major RNA fractions were collected and their effects on the induction of alkaline phosphatase determined by using a P_1 preparation from the wild-type bacteria (Table 5). The 23s RNA fraction did not stimulate the induction of alkaline phosphatase; the 16s RNA fraction stimulated only slightly; the 8–4s RNA fraction gave a 70% increase in new enzyme when compared with a control without added RNA. However, the stimulation in terms of increase of alkaline phosphatase was not as great as that resulting from the addition to the incubation mixture of either the unfractionated total mutant-C4-membrane RNA or the mutant-C4 total nucleic acids. Neither was the stimulatory effect, in terms of increase of alkaline phosphatase/100 μg. of RNA added, as great for the 8–4s RNA as for the total mutant-C4 membrane-RNA fraction. These facts indicate that there was a loss of activity during the fractionation procedure, although in terms of weight there was little RNA loss.

The total nucleic acids from the wild-type *E. coli* strain (at a concentration of 100 μg./ml. of incubation mixture) did not stimulate the induction of alkaline phosphatase. The total wild-type strain membrane RNA (1 mg./ml.) or 8–4s membrane RNA (see Table 5) both slightly stimulated the induction of alkaline phosphatase.

Finally, the effect of membrane RNA on induction by an incubation mixture that had been treated with actinomycin was determined. The actinomycin was equilibrated with the incubation mixture for 20 min. at 0° before the addition of the RNA and incubation at 37°. The addition of the mutant-C4 total nucleic acids, even in the presence of actinomycin, slightly increased the synthesis of alkaline phosphatase (Table 5). When a much larger quantity of mutant-C4 total membrane RNA was added, there was a larger stimulation of induction.

Table 5. *Effect of ribonucleic acid on the incubation of alkaline phosphatase*

The composition of the incubation mixture, preparation of total nucleic acids and membrane RNA, conditions of incubation, and determinations of RNA, DNA and alkaline phosphatase are given in the text. The incubation mixture, except where shown, did not contain the usual total nucleic acids. The incubation mixture contained (per ml.): 41 μg. of protein, 12 μg. of RNA and 5 μg. of DNA originating from the P_1 preparation of the wild-type ($R1^+R2^+P^+$) *E. coli* strain. Actinomycin (20 μg./ml.) was added 45 min. before incubation. The mutant-C4 or wild-type total membrane RNA was fractionated on a sucrose gradient, the fractions containing the 23s, 16s or 8–4s RNA (see the text) were combined and the RNA was concentrated by precipitation with ethanol and centrifuging at 25000g.

Incubation mixture	Nucleic acids (μg./ml. of incubation mixture)		Alkaline-phosphatase activity	
	RNA	DNA	(units/mg. of protein)	(net increase in units/100 μg. of RNA added)
1. Without actinomycin				
Before incubation				
System − C4 total nucleic acids	12	5	520	—
After incubation				
System − C4 total nucleic acids	12	5	2500	—
System + C4 total nucleic acids	105	61	4600	2300*
System + C4 membrane RNA (total)	1020	5	7100	430
System + C4 membrane RNA (23s)	250	5	2400	—
System + C4 membrane RNA (16s)	130	5	2600	80
System + C4 membrane RNA (8–4s)	650	5	4200	270
System + wild-type membrane RNA (total)	880	5	3100	70
System + wild-type membrane RNA (8–4s)	520	5	2600	20
2. With actinomycin				
System − C4 total nucleic acids	12	5	660	—
System + C4 total nucleic acids	105	61	760	110
System + C4 membrane RNA (total)	1020	5	1910	120
System + C4 membrane RNA (8–4s)	650	5	1300	100
System + wild-type membrane RNA (total)	880	5	740	10

* The effect of added DNA (mutant-C4 total nucleic acids) has not been taken into account in calculating the net increase of alkaline phosphatase/100 μg. of RNA added.

A similar stimulation, though not as large, was also obtained by addition of the mutant-C4 8–4s membrane RNA. The wild-type-strain 8–4s membrane-RNA fraction only gave a slight stimulation of induction (Table 5).

These preliminary experiments suggest, in agreement with the results found with whole cells, that transcription of genetic information present in the DNA is mediated in induction by an RNA that has a low molecular weight—under the conditions of extraction and centrifuging mentioned in the Materials and Methods section. How this RNA is active in mediating induction cannot be ascertained from these experiments.

DISCUSSION

The present paper describes the synthetic properties of an *E. coli* subcellular fraction (P_1) in relation to the induction of alkaline phosphatase. Two main questions arise from this work. First, what is the nature of the P_1 fraction and how is it related to its ability to synthesize alkaline phosphatase? Secondly, what differences exist between the induction of alkaline phosphatase and that of β-galactosidase in subcellular preparations from *E. coli*?

Nature of the P_1 fraction. In the preparation of the P_1 fraction some of the bacterial alkaline phosphatase is associated with particulate material obtained during the preparation of the spheroplasts. The composition of this material has not been investigated. The enzyme has also been found in the S_1 fraction (obtained as a supernatant on centrifuging the total lysate of the digitonin-treated spheroplasts). The rest of the enzyme was found to be associated with the membranes themselves. In comparison with the whole bacteria or spheroplasts the P_1 preparation contains less alkaline phosphatase/mg. of protein.

Also in comparison with the whole bacteria, spheroplasts or ribosomes the P_1 preparation has high ratios of DNA to RNA and to protein. It also contains much of the bacterial RNA, although most of the bacterial RNA is freed from it after removal of the S_1 fraction which contains free ribosomes and soluble RNA (Wachsmann, Fukuhara & Nisman, 1960).

For the P_1 preparation to support the induction of an enzyme, two primary criteria have to be satisfied. The osmotic stability of the P_1 fraction has to be ensured and an inducer has to be present. For the induction of β-galactosidase, the P_1 preparation is stabilized by the presence of potassium

chloride (Nisman, Kayser, Demailly & Genin, 1961) and the inducer is isopropyl β-D-thiogalactoside. [Nisman *et al.* (1963*b*) demonstrated that, though isopropyl β-D-thiogalactoside and thiomethyl β-D-galactoside were effective in inducing β-galactosidase in a subcellular *E. coli* fraction similar to that described above, melibiose was not. All three are effective in inducing bacteria (see Monod & Cohn, 1952). Spheroplasts are also induced for β-galactosidase by melibiose (J. Pelmont, personal communication). These facts indicate among other things that, even if spheroplasts or bacteria are present in trace amounts in the P_1 fraction, they do not contribute to the process of induction. The presence of penicillin, the stimulatory effect of nucleic acids on the induction of alkaline phosphatase and the inhibition by actinomycin agree with this conclusion.]

With the induction of alkaline phosphatase the preparation is stabilized osmotically with sucrose and the system becomes induced under the conditions of incubation described in the Materials and Methods section. The form and action of the inducer have yet to be examined.

When these two criteria have been satisfied, the P_1 preparation is able to synthesize protein. The amount, however, is negligible without the addition of magnesium chloride and amino acids. The presence of Mg^{2+} ions is known to affect the structure of RNA (Simpson, 1962) as well as to aid the action of certain enzymes operative in RNA synthesis (Grunberg-Manago, 1962). Amino acids are needed not only for protein synthesis but also for RNA stability (Nisman, Pelmont, Demailly & Yapo, 1963*a*).

The energy requirements of the P_1 preparation are under investigation. The contribution of glucose in this respect as well as that of phosphoenolpyruvate may be important. The presence of $NADP^+$ and its stimulatory effect on the induction of alkaline phosphatase may similarly be related to the energy requirements. It is also possible that $NADP^+$ has a more direct role in protein synthesis. Rothman & Byrne (1963) have shown that the alkaline-phosphatase molecule consists of two polypeptide chains joined by cystine linkages. There is the possibility, which needs investigation, that $NADP^+$ aids in the formation of these cystine linkages.

Assay of the amino acid-activating enzymes in the P_1 preparation, prepared as described in the Materials and Methods section, has indicated that they are all present in trace amounts (J. Pelmont, personal communication).

The form of the RNA in the P_1 fraction has not been fully investigated. However, the P_1 preparation contains sufficient protein-synthesizing sites to make the addition of ribosomes or soluble RNA unnecessary. Nisman *et al.* (1962) have shown that an *E. coli* fraction containing ribosomes is able to synthesize proteins. Whether ribosomes are the only sites of protein synthesis in the cell or P_1 fraction remains to be demonstrated.

The P_1 preparation is able to support induced enzyme synthesis and there is evidence for a preceding synthesis of 'informational' RNA. It has also been found that inhibition of RNA synthesis by actinomycin D results in an inhibition of the induction of alkaline phosphatase, but that induction can occur even in the presence of actinomycin if a specific RNA is added.

Differences between the induction of alkaline phosphatase and that of β-galactosidase. Nisman *et al.* (1963*a*) have shown that the induction of β-galactosidase in a preparation similar to that described above is also dependent on a preceding RNA synthesis. Apart from the nature of the inducers, the main difference between the induction of β-galactosidase and that of alkaline phosphatase is the post-incubation effect. For the induction of β-galactosidase post-incubation, after the addition of chloramphenicol, results in a two- or three-fold increase in total enzyme. For the induction of alkaline phosphatase there is at most an increase, after post-incubation, of 10–15% of the total quantity of enzyme. This may be related to the fact that alkaline phosphatase has a molecular weight of about 80000 (Garen & Levinthal, 1960) whereas β-galactosidase is reported to have a molecular weight of about 520000 (Sund & Weber, 1963). Nisman *et al.* (1963*b*) have suggested that the post-incubation effect in the induction of β-galactosidase is related to a polymerization of enzyme sub-units.

SUMMARY

1. The ability of a subcellular fraction, prepared from the wild-type strain of *Escherichia coli* ($R1^+R2^+P^+$), to synthesize alkaline phosphatase was examined. A subcellular fraction (P_1) was prepared from penicillin-produced spheroplasts of *E. coli* after lysis with digitonin. The fraction contains a high ratio of DNA to protein and consists mainly of membrane fragments.

2. The P_1 fraction contains about 10–15% of the original bacterial alkaline phosphatase; some of the enzyme is liberated from the bacteria during the preparation of the spheroplasts and some after lysis of the spheroplasts by digitonin.

3. Conditions of incubation are described in which the P_1 fraction synthesizes alkaline phosphatase.

4. Actinomycin D inhibits the synthesis of RNA and the induction of alkaline phosphatase in the P_1 fraction. The inhibition can be partially overcome by the addition of a specific RNA extracted from a constitutive mutant of the wild-type strain.

5. Partial purification of the enzyme synthesized *in vitro* indicates that there is enzyme synthesis during induction.

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Biochem. J. (1964) **90**, 391

Metabolism of Polycyclic Compounds

23. THE METABOLISM OF PYRENE IN RATS AND RABBITS*

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Elson, Goulden & Warren (1945) showed that when pyrene is given to rats there is an increased urinary excretion of sulphuric acid esters, glucosiduronic acids and neutral sulphur. Harper (1957) found that the hydrocarbon is hydroxylated in rats and mice to 1-hydroxypyrene and 1,6- and 1,8-dihydroxypyrene. A compound that yielded pyrene with mineral acid was also detected. In the present work it has been shown that pyrene is also converted into *trans*-4,5-dihydro-4,5-dihydroxypyrene and into *N*-acetyl-*S*-(4,5-dihydro-4-hydroxy-5-pyrenyl)-L-cysteine, and that the latter compound is the main source of the pyrene liberated when the urines of treated animals are acidified. The oxidation products of pyrene with perbenzoic acid gave, on reaction with *N*-acetylcysteine, a small amount of the synthetic mercapturic acid.

* Part 22: Boyland & Sims (1962*c*).

EXPERIMENTAL

Absorption spectra. These were measured in ethanol solution on a Perkin-Elmer model 137 ultraviolet spectrophotometer.

Melting points. These are uncorrected.

Chromatography. Except where stated paper chromatography was carried out on Whatman no. 1 paper by downward development for 18 hr. in butan-1-ol–propan-1-ol–aq. 2N-NH_3 (2:1:1, by vol.) The dried chromatograms were examined under ultraviolet light and dipped in either the platinic iodide reagent of Toennies & Kolb (1951) or in a 0·2% (w/v) solution of ninhydrin in acetone and heated to 70% for 10 min. Other chromatograms were sprayed with a solution of diazotized *p*-nitroaniline (0·2% in 4N-HCl), heated to 80° for 5 min. and sprayed with aq. Na_2CO_3.

Thin-layer chromatograms were prepared by coating glass plates with a film of silica gel G (E. Merck A.-G., Darmstadt, West Germany) of 0·25 mm. thickness. The chromatograms were developed for 10 cm. with: *A*, hexane–