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Inhibition of a Mammalian Deoxyribonucleic Acid Nucleotidyltransferase by Actinomycin D

BY H. M. KEIR, H. OMURA AND J. B. SHEPHERD
Department of Biochemistry, University of Glasgow

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The isolation of Actinomycin from a culture of *Streptomyces antibioticus* was reported by Waksman & Woodruff (1940). Various forms of Actinomycin have since been described, one of which, Actinomycin D (Vining & Waksman, 1954), is an orange antibiotic consisting of two pentapeptide chains covalently bound to a chromophoric phenoxazine derivative (Brockmann, 1960). It is a powerful inhibitor of the growth of mammalian cells in tissue culture and its action has been reported by Reich, Franklin, Shatkin & Tatum (1962*a*) to be due to selective and complete inhibition of cellular RNA synthesis. Further work (Reich, Goldberg & Rabinowitz, 1962*b*; Goldberg, Rabinowitz & Reich, 1962) revealed that the site of inhibition was the DNA-dependent RNA-nucleotidyltransferase system (nucleoside triphosphate–ribonucleic acid nucleotidyltransferase, EC 2.7.7.6) and that the Actinomycin D exerted its effect specifically by binding to the guanine residues of the DNA-primer.

Kirk (1960) observed that the antibiotic inhibited the activity of DNA nucleotidyltransferase (deoxynucleoside triphosphate–deoxyribonucleic acid deoxynucleotidyltransferase, EC 2.7.7.7) from *Escherichia coli*, and Hurwitz, Furth, Malamy & Alexander (1962), using highly purified enzyme preparations from *E. coli*, showed that the inhibition of both DNA and RNA nucleotidyltransferases obeyed competitive kinetics with respect to the DNA-primer.

However, no information has yet appeared on the action of Actinomycin D in soluble DNA-synthesizing systems derived from mammalian cells. Keir, Binnie & Smellie (1962) and Keir (1962) have described a partially purified preparation of DNA nucleotidyltransferase from Landschutz ascites-tumour cells, and in the present paper we present evidence indicating that the activity of this enzyme fraction is inhibited competitively by Actinomycin D.

EXPERIMENTAL

Abbreviations. The 5′-triphosphates of deoxyadenosine, deoxycytidine and deoxyguanosine, deATP, deCTP and deGTP respectively; thymidine 5′-triphosphate labelled with ^{32}P in the α-phosphate group, [^{32}P]TTP; thymidine 5′-monophosphate labelled with ^{32}P, [^{32}P]TMP; deoxyribonuclease active at about pH 7·5 in the presence of Mg^{2+} ions and producing 5′-phosphoryl-terminal oligonucleotides, deoxyribonuclease I.

Preparation of enzyme fraction. The enzyme fraction used in these experiments was derived from cells of the Landschutz ascites tumour of the mouse, and its preparation and some of its properties with respect to DNA nucleotidyltransferase and deoxyribonuclease I have been fully described by Keir (1962).

Enzyme assays. The assay for DNA-nucleotidyltransferase activity was based on measurement of incorporation of [^{32}P]TMP from [^{32}P]TTP into an acid-insoluble form in the presence of DNA-primer, deATP, deCTP and deGTP (Gray *et al.* 1960; Keir, 1962). Deoxyribonuclease I was assayed under conditions identical with those used for the assay of DNA nucleotidyltransferase but with the omission of the deoxyribonucleoside triphosphates; activity was measured by following the conversion of DNA into acid-soluble ultraviolet-absorbing fragments (Keir, 1962).

Preparation and base analysis of deoxyribonucleic acid. The DNA of Landschutz ascites-tumour cells was isolated and purified as described by Keir *et al.* (1962); small portions of the dry material were hydrolysed and analysed for adenine, guanine, cytosine, and thymine (Keir & Davidson, 1958), by using the molar extinction coefficients compiled by Burton (1959).

RESULTS AND DISCUSSION

The partially purified DNA-nucleotidyltransferase fraction used in these experiments showed a linear reaction rate for at least 3 hr. (cf. Keir, 1962). The effects of increasing concentrations of Actinomycin D on the activity of the nucleotidyltransferase, after 2 hr. incubation periods, are illustrated in Fig. 1. In this system, 50 μg. of DNA-primer was used, i.e. 153 μm-moles of total deoxyribonucleotide, or 32 μm-moles of guanine deoxyribonucleotide as indicated by base analysis (Table 1). Since the lowest amount of Actinomycin D giving 100% inhibition was about 45 μg. (about 36 μm-moles), it would appear that complete inhibition was effected when the molar ratio of Actinomycin D to guanine residues in the primer approached unity. This seems to agree well with the finding that guanine residues are the sites responsible for binding of the antibiotic to DNA (Goldberg *et al.* 1962), and suggests that there are no long stretches of guanine-free regions in the ascites-cell DNA; otherwise, saturating concentrations of Actinomycin D might permit a limited incorporation reaction to take place. The fact that no inhibition was observed in the presence of only 1 μg. of Actinomycin D might be taken to indicate successful competition by DNA-primer at high concentration with the DNA–Actinomycin D inhibitor complex at low concentration. Reich *et al.* (1962*a*) have presented evidence indicating that the DNA–Actinomycin D complex is almost entirely undissociated. Therefore the concept of successful competition at low concentrations of the complex would seem to be more reasonable than the alternative possibility that, at low concentrations of the antibiotic, the amount bound to the DNA is negligible.

The activity of the DNA-nucleotidyltransferase preparation was assayed at various concentrations of DNA-primer, with and without Actinomycin D at a concentration of 8 μg./ml. (Fig. 2). In agreement with studies by Keir *et al.* (1962), the curves relating incorporation to DNA concentration did not fit a rectangular hyperbola. The enzyme preparation contains a small amount of deoxyribonuclease-I activity (sufficient to render acid-soluble 5% of the DNA-primer in 3 hr. at a DNA concentration of 200 μg./ml.; Keir, 1962), and we have observed that this activity is not significantly affected by Actinomycin D (8 μg./ml.). At the lower DNA-primer concentrations given in Fig. 2, significant proportions of primer and product may have been lost during the nucleotidyltransferase assays. Reasonable estimates of these losses based

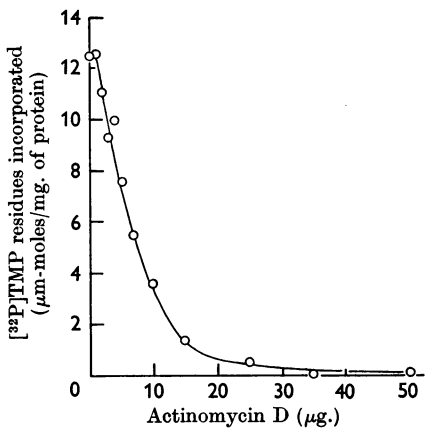

Fig. 1. Inhibition of DNA-nucleotidyltransferase activity by Actinomycin D. Each reaction mixture (0·25 ml.) contained: 10 μmoles of tris–HCl buffer, pH 7·5; 1 μmole of $MgCl_2$; 0·08 μmole of EDTA; 9 μmoles of KCl; 0·5 μmole of potassium phosphate buffer, pH 7·5; 50 μg. of thermally denatured DNA (from Landschutz ascites-tumour cells); 50 μm-moles each of deATP, deCTP, deGTP and [^{32}P]TTP ($1{\cdot}6 \times 10^6$ counts/min./μmole); 80 μg. of protein from a partially purified DNA-nucleotidyltransferase fraction from Landschutz ascites-tumour cells; and the indicated amounts of Actinomycin D. Incubation was at 37° for 2 hr.

Table 1. *Base composition of the deoxyribonucleic acid of Landschutz ascites-tumour cells*

Experimental details are given in the text. The results are presented as molar ratios of bases relative to adenine as 10.

Adenine	10	Ratio: $\frac{\text{adenine}}{\text{thymine}}$	= 1·02	Ratio: $\frac{\text{adenine + thymine}}{\text{guanine + cytosine}}$ = 1·33
Thymine	9·86			
Guanine	7·15	Ratio: $\frac{\text{guanine}}{\text{cytosine}}$	= 0·92	
Cytosine	7·79			

on published data for the same enzyme fraction (Keir, 1962) vary from 50 % at the lowest DNA concentration (4 μg./ml.) to 5 % at the highest DNA concentration (200 μg./ml.). Interference of this kind could account for the sigmoid character of the activity–DNA-concentration curves in Fig. 2. With respect to the curve for the Actinomycin D-inhibited system, an additional factor must be considered. At the concentrations of antibiotic and primer used in the experiment described in Fig. 2, it would be expected that every primer molecule would have one or more guanine residues in complexes with Actinomycin D. At low primer concentrations, most or all of the primer guanine residues would be in complexes in this way and priming activity would be very low. On the other hand, at the higher primer concentrations, there would be large stretches of primer not in complexes with the antibiotic, and capable of full priming activity. It seems reasonable, therefore, in constructing the activity–substrate-concentration curves, to make allowance for that proportion of primer which is in complexes and which will not possess primer activity. The Actinomycin D (2 μg. or 1·61 μm-moles) would reasonably be expected to form a complex with 1·61 μm-moles of guanine residues in the primer; this represents about 2·5 μg. of DNA. The DNA concentrations presented in Fig. 2 were adjusted to allow for these small losses of DNA-primer by enzymic degradation and by complex-formation with Actinomycin D, and the data were redrawn as a double-reciprocal plot (Lineweaver & Burk, 1934). Linear plots were obtained for the control and Actinomycin-inhibited assays (Fig. 3); moreover, the two plots intersected on the vertical axis, and it would therefore appear that the inhibitory effect exerted by the antibiotic is competitive.

The extent of inhibition of DNA nucleotidyltransferase by Actinomycin D varied somewhat from preparation to preparation of the enzyme. Though this phenomenon is not yet understood, it

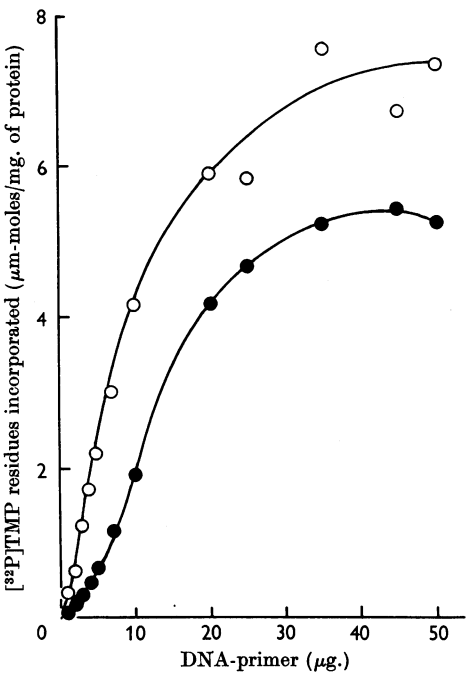

Fig. 2. Inhibition of DNA-nucleotidyltransferase activity by Actinomycin D, at various concentrations of DNA-primer. The assay conditions were as described in Fig. 1 with these exceptions: thermally denatured DNA was included at the concentrations shown, the specific activity of the [^{32}P]TTP was 16×10^6 counts/min./μmole, and each reaction mixture contained 209 μg. of protein from the enzyme fraction. Incubation was at 37° for 2 hr. ○, Control (no addition); ●, 2 μg. of Actinomycin D added.

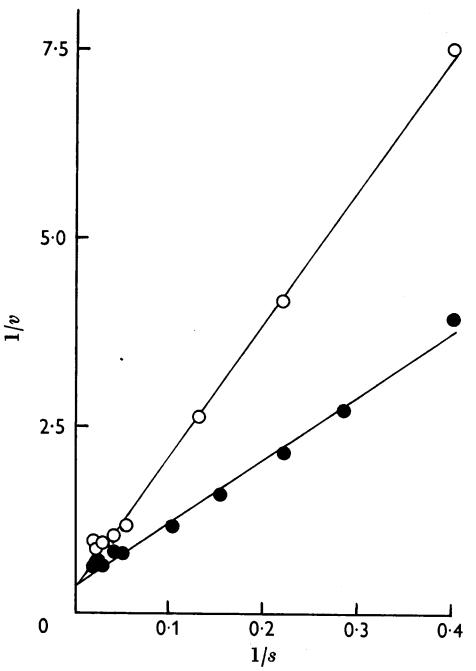

Fig. 3. Competitive inhibition of DNA-nucleotidyltransferase activity by Actinomycin D. The results given in Fig. 2 were adjusted to allow for the small amount of DNA-primer lost by deoxyribonuclease-I action. In addition, allowance was made for the binding of 2·5 μg. of DNA-primer by 2 μg. of Actinomycin D in the inhibited assays. The values were then plotted according to the reciprocal method of Lineweaver & Burk (1934). v, μm-moles of [^{32}P]-TMP incorporated/assay; s, μg. of thermally denatured DNA-primer/assay adjusted as described above. ●, Control (no addition); ○, 2 μg. of Actinomycin D added.

may be related in some measure to the age of the enzyme preparation used in individual experiments, for after storage at 0° for a few days the activity begins to fall until, after about 1 month, only a very low level of activity can be detected. The full explanation of this is not known, but the enzyme preparations from ascites-tumour cells appear to contain an inhibitor of deoxyribonuclease I (Keir, 1962), the activity of which deteriorates on storage at 0° (cf. Keir & Smith, 1963); this is manifested by a corresponding increase in activity of deoxyribonuclease I. Different concentrations of this inhibitor and of deoxyribonuclease I in the DNA-nucleotidyltransferase preparation could cause variations in the degree of degradation of primer and product during assay of the nucleotidyltransferase, and could certainly account for the observed differences of activity among the various nucleotidyltransferase preparations before and after storage. These considerations might also explain, at least in part, the differences in the susceptibility of the enzyme preparations to inhibition by Actinomycin D; i.e. nuclease action might release, from the primer, oligonucleotide segments of various lengths (some bearing Actinomycin D units and others not), and thus influence the reaction rate of the nucleotidyltransferase. The effects of inclusion of various oligonucleotide preparations in the standard DNA-nucleotidyltransferase assay system have been described by Keir (1962).

A further factor that might contribute to differences of activity and degrees of inhibition obtained with different enzyme preparations relates to the condition of the DNA-primer. In the experiments described, the concentrations of the DNA solutions during thermal denaturation to produce primer (i.e. single-stranded DNA) varied from 0·5 to 1·3 mg./ml. These differences might be expected to result in the production of primer solutions containing different proportions of single-stranded material (Keir *et al.* 1962). Reich *et al.* (1962*b*) found that both native (double-stranded) and thermally denatured DNA formed complexes with Actinomycin D; therefore it might reasonably be expected that variations would exist with regard to the relative amounts of Actinomycin D in complexes with double-stranded and single-stranded DNA.

Concurrent studies with partially purified preparations of DNA nucleotidyltransferase from calf thymus have revealed the presence of two distinct DNA nucleotidyltransferases, one which gives optimum activity with deATP, deCTP, deGTP and [^{32}P]TTP, and the other with [^{32}P]TTP alone (Krakow, Coutsogeorgopoulos & Canellakis, 1962; Keir & Smith, 1963). The former, 'replicative' DNA nucleotidyltransferase, catalyses extensive incorporation of deoxyribonucleotide into terminal and non-terminal positions of DNA primer, but the latter, 'terminal' DNA nucleotidyltransferase, incorporates [^{32}P]TMP extensively into terminal positions only of DNA-primer (Keir, Shepherd & Hay, 1963). Assays for 'terminal'-nucleotidyltransferase activity in a variety of other mammalian tissues have failed to show degrees of incorporation of the same magnitude as those observed for [^{32}P]TMP incorporation in calf thymus (J. B. Shepherd & H. M. Keir, unpublished work). Landschutz ascites-tumour cells had particularly low incorporation patterns from [^{32}P]TTP alone (deATP, deCTP and deGTP omitted), and one experiment of this kind is included in Fig. 4 (△, ▲) for comparative purposes. The low degree of incorporation did not show any dramatic changes in the presence of Actinomycin D. This is in agreement with observations on both nuclear and cytoplasmic fractions from calf thymus (Keir *et al.* 1963) which showed that the 'replicative' DNA nucleotidyltransferase was much more sensitive than the 'terminal' nucleotidyltransferase to the inhibitory action of Actinomycin D. This, perhaps, is not surprising, since binding of the antibiotic at guanine residues along the DNA-primer molecules would certainly be expected to interfere seriously with incorporation of the replicative variety; terminal incorporation, however, would be expected to be

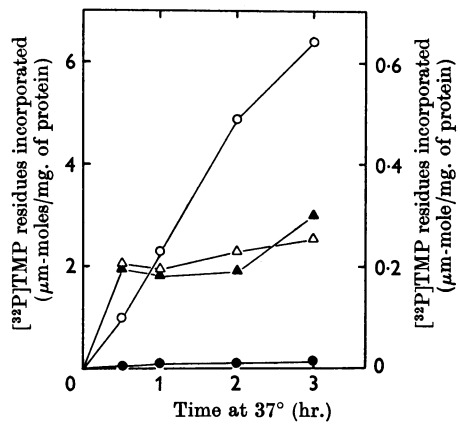

Fig. 4. Action of Actinomycin D on the activities of two DNA-nucleotidyltransferase systems. The assay conditions differed from those described in Fig. 1 in that all reaction mixtures contained 25 μg. of DNA-primer and 150 μg. of enzyme fraction protein. ○, Standard assay; ●, standard assay + 5 μg. of Actinomycin D; the incorporation scale for these two plots is given on the left of the Figure. △, deATP, deCTP and deGTP omitted from the standard assay; ▲, deATP, deCTP and deGTP omitted from and 5 μg. of Actinomycin D added to the standard assay; the incorporation scale for the latter two plots is given on the right of the Figure (i.e. this scale is tenfold smaller than the scale for the standard assay). The specific activity of the [^{32}P]TTP was 11×10^6 counts/min./μmole.

less susceptible, since direct interference would arise only in primer molecules bearing deoxyguanylyl residues at or near the 3′-hydroxy-terminal position. Nevertheless, the limited terminal-addition reaction catalysed by the ascites-tumour-cell enzyme fraction must be susceptible in some measure, for at high Actinomycin D concentrations the incorporation of [^{32}P]TMP can be almost eliminated (Fig. 1). From these considerations, it seems justifiable to conclude that the shape of the curve relating inhibition to Actinomycin D concentration (Fig. 1) can be attributed to complete elimination of replicative incorporation at the lower Actinomycin D concentrations, together with partial (and, at the highest Actinomycin concentrations, complete) inhibition of the more-resistant terminal incorporation.

The competitive inhibition exercised by Actinomycin D in the DNA-nucleotidyltransferase system from *E. coli* (Hurwitz *et al.* 1962) displays several features that are relevant to the mammalian enzyme system described in the present paper. The activity of the bacterial enzyme was remarkably high (11·4 μmoles of deoxycytidine 5′-monophosphate incorporated/mg. of protein/20 min.), and the preparation was inhibited by 50% at a molar ratio of Actinomycin D to DNA-primer guanine residues of 0·50. The 50% inhibition of the fresh mammalian enzyme fraction used (Fig. 1) was given at a molar ratio of Actinomycin D to DNA-primer guanine residues of 0·15. [The guanine content of the DNA of Landschutz ascites-tumour cells and of *E. coli* DNA were taken from Table 1 and Chargaff (1955) respectively.] As the concentration of Actinomycin D was increased, the bacterial enzyme, like the mammalian preparation, showed an initial marked fall in activity at the lower concentrations. As the concentration of antibiotic was further increased, a degree of activity of the enzyme was reached which remained relatively resistant even to high molar ratios of Actinomycin D to DNA-primer guanine residues (0·6–1·6), a feature that is in agreement with our postulate presented above that terminal addition is the predominant reaction at high ratios of Actinomycin D to DNA-primer. The bacterial enzyme can catalyse terminal-addition reactions when presented with only one deoxyribonucleoside triphosphate (Adler, Lehman, Bessman, Simms & Kornberg, 1958). The bacterial and the mammalian enzyme preparations contain deoxyribonuclease activity (Aposhian, Richardson & Schildkraut, 1962; Keir, 1962), but degradative activity is likely to be so insignificant in the powerful bacterial synthetic system that adjustments of the kind summarized in Fig. 3 are not needed to obtain accurate velocity–substrate-concentration curves for the bacterial nucleotidyltransferase (cf. Hurwitz *et al.* 1962). Elliott (1963) has described the inhibition of a DNA-nucleotidyltransferase preparation from *E. coli* by Actinomycin D. The activity of the fraction was very low (3·5 μm-moles of deoxycytidine monophosphate incorporated/mg. of protein/30 min.) relative to the highly purified preparation used by Hurwitz *et al.* (1962), and was highly sensitive to Actinomycin D: 50% inhibition was given at a molar ratio of Actinomycin D to DNA-primer guanine residues of 0·13.

SUMMARY

1. The inhibitory effect of Actinomycin D was studied in a partially purified preparation of deoxyribonucleic acid nucleotidyltransferase from Landschutz ascites-tumour cells. The inhibition exercised by the antibiotic appeared to obey competitive kinetics with respect to the DNA-primer.

2. Relation of inhibition to the molar ratio of Actinomycin D to deoxyribonucleic acid-primer guanine residues revealed that 100% inhibition was achieved at a ratio of about unity.

3. The nucleotidyltransferase was assayed also in the presence of only one triphosphate, thymidine 5′-triphosphate. The very limited incorporation observed in these circumstances probably took place at terminal positions in the primer, and was relatively resistant to the inhibitory action of Actinomycin D.

4. It was concluded that low concentrations of Actinomycin D eliminated nucleotidyltransferase activity of the replicative type, but that much higher concentrations were required to eliminate the remaining and relatively resistant incorporation which was probably of the terminal type.

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The Oxidation of L-Malate by *Pseudomonas* sp.

BY M. J. O. FRANCIS, D. E. HUGHES, H. L. KORNBERG AND P. J. R. PHIZACKERLEY
Departments of Biochemistry and of Clinical Biochemistry, University of Oxford, and Department of Biochemistry, University of Leicester

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Although cell-free extracts of many pseudomonads, such as *Pseudomonas* KB1 (Kogut & Podoski, 1953; Kornberg & Madsen, 1958) and *Pseudomonas* B_2aba (Kornberg & Gotto, 1961), have been shown to be rich in the soluble L-malate dehydrogenase linked to NAD (EC 1.1.1.37), extracts of *Pseudomonas ovalis* Chester do not catalyse the reduction of oxaloacetate by the reduced coenzyme, $NADH_2$. It has, however, been shown that the tricarboxylic acid cycle is the major route for the oxidation of acetate in this organism, and that, during growth on acetate or on precursors of acetate as sole carbon source, the glyoxylate cycle functions as a means of supplying intermediates to that cycle (for review, see Kornberg & Elsden, 1961). Since the oxidation of L-malate to oxaloacetate is obligatory in both the tricarboxylic acid and glyoxylate cycles, an enzymic system capable of catalysing this conversion must be present. It is the main purpose of the present paper to describe some of the properties of the enzyme system which catalyses the oxidation of L-malate in *Ps. ovalis* Chester.

A preliminary account of this work has been presented to The Biochemical Society (Kornberg & Phizackerley, 1961).

MATERIALS AND METHODS

Maintenance and growth of organisms. Cultures of *Ps. ovalis* Chester and of *Ps.* B_2aba were maintained on agar slopes consisting of: potassium phosphate buffer, pH 7·2 (50 mM), ammonium chloride (50 mM), sodium succinate (50 mM), essential salts (4 mg. of $CaCl_2,6H_2O$, 8 mg. of $MgSO_4,7H_2O$, 0·4 mg. of $MnSO_4,4H_2O$ and 0·4 mg. of $FeSO_4,7H_2O$/100 ml. of medium), solidified with 2% (w/v) of agar (Hopkin and Williams Ltd., Chadwell Heath, Essex). Stock cultures of the organism were subcultured every 2 weeks, grown at 30° and stored at 2°.

For growth in liquid medium, a loopful of organisms from a freshly grown slope was suspended in a Carrel culture flask (J. A. Jobling and Co. Ltd., Sunderland) containing 400 ml. of the above-mentioned medium but with the agar omitted. In experiments indicated in the text, other substrates at 50 mM concentration replaced sodium succinate as carbon source. The flasks were shaken at 30° for 16–24 hr. on a reciprocal shaker. Growth of the cells was determined by measurements, in an EEL nephelometer (Evans Electroselenium Ltd., Harlow, Essex), of the light-scattering of samples of the bacterial suspensions, and comparison of the readings with a previously constructed calibration curve. Although the medium was adequate to sustain growth of over 1 mg. dry wt./ml., the bacteria were removed while still in the phase of logarithmic growth, at densities of 0·2–0·4 mg. dry wt./ml. The contents of the Carrel flasks were centrifuged at 10° for 15 min. at 1500 *g*. The cells were washed with 10 mM-potassium phosphate, pH 7·2, centrifuged, and resuspended in this buffer.

Preparation of particles by ultrasonic disintegration. In a typical preparation, 60 ml. of washed cells (approx. 50 mg. dry wt./ml.), suspended in 10 mM-potassium phosphate, pH 7·2, were disrupted by ultrasonic oscillation, in batches of 20 ml., by means of a 600 W Mullard magnetostrictor oscillator operating at 3·5 A and 25 kcyc./sec. for 3 min. Intact cells were removed by centrifuging at 12 000 *g* at 2° for 15 min. The supernatant fluid (referred to below as 'whole ultrasonic extract') was then centrifuged at