AN ALTERED ALKALINE PHOSPHATASE FORMED IN THE PRESENCE OF NORLEUCINE

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Received April 13, 1963

Introduction.

Norleucine, when added to exponentially growing cultures of *E. coli* resulted in 'linear' growth, a 50% reduction in the differential rate of incorporation of ^{35}S into protein but no diminution in the differential rate of synthesis of β-galactosidase (Cohen and Munier, 1959). Further, it was shown that norleucine replaced methionine residues in *E. coli* protein (Munier and Cohen, 1959; Cowie *et al*, 1959).

We have confirmed that L-norleucine causes 'linear' growth when added to exponential cultures of *E. coli* C4, whilst the differential rate of synthesis of active alkaline phosphatase (constitutive in strain C4) falls to *c.* 20% of the control value. Evidence is submitted indicating that an alkaline phosphatase possessing altered properties is formed during exposure to norleucine.

Experimental methods and results.

Addition of 3×10^{-3}M L-norleucine to an exponentially growing culture of *E. coli* C4 resulted in 'linear' growth; the differential rate of incorporation of L-(U-^{14}C)-arginine into the trichloroacetic acid (TCA)-insoluble fraction remained the same both in the presence and absence of the analogue. Radioactive norleucine was also incorporated into the TCA-insoluble fraction at a constant differential

rate (Fig. 1a). During the first 45 min. exposure to L-norleucine the differential rate of synthesis of alkaline phosphatase was almost identical with that observed in control cultures, but then fell rather sharply to a value c. 20% of the control rate (Fig. 1b).

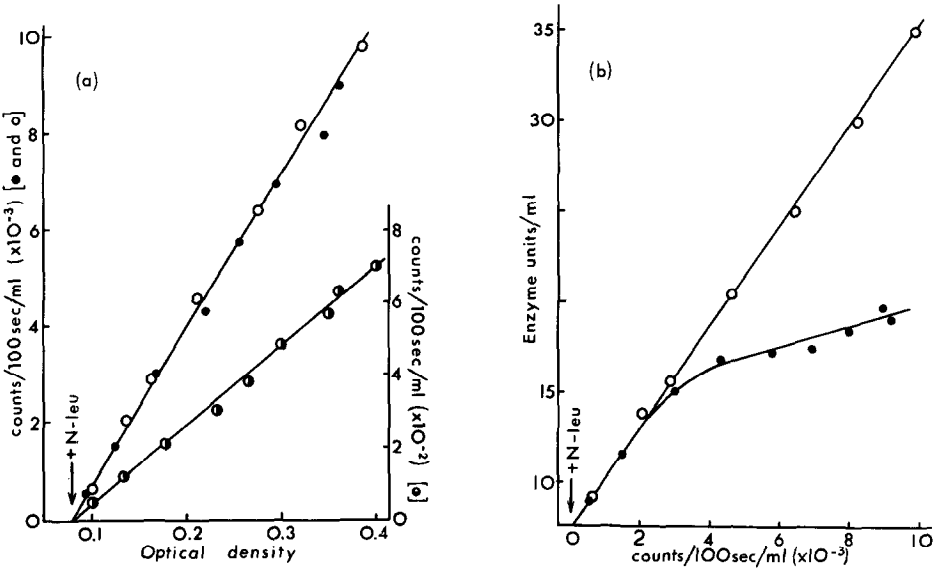

Fig. 1. E. coli was grown in 15 ml. glucose/mineral salts medium (Tristram, 1960) supplemented with L-arginine (3 μmoles) or L-(U-^{14}C)-arginine (2.5 μC; 3 μmoles). L-norleucine (3 x 10^{-3}M) or 3 x 10^{-3}M L-(1-^{14}C)-norleucine (10 μC) was added to exponential cultures, as required.

(a) Incorporation of ^{14}C-norleucine (◑) or ^{14}C-arginine in the presence (●) and absence (○) of ^{12}C-norleucine into the TCA-insoluble fraction (for method, see Neale and Tristram, 1963).

(b) The differential rate of synthesis of alkaline phosphatase in the presence (●) and absence (○) of norleucine. A unit of enzyme activity is defined as the amount of enzyme which, in 1 hr. (37°, pH 7.9) produced 1 μmole p-nitrophenol from p-nitrophenylphosphate (for assay, see Neale and Tristram, 1963). Abcissa: incorporation of ^{14}C-arginine into the TCA-insoluble fraction.

The possible formation of altered alkaline phosphatase during exposure of cells to L-norleucine was examined by adding a 'pulse' of L-(U-^{14}C)-lysine (2 μmoles; 10 μC) to a culture (total volume 200 ml.) previously exposed to 3 x 10^{-3}M L-norleucine for 2.5 hr. A similar

amount of ^{14}C-lysine was added to an exponential control culture adjusted to the same optical density and volume. The incorporation of the 'pulse' into TCA-insoluble material was followed (Neale and Tristram, 1963), and corresponded to about 6 min. and 15 min. supply of lysine in the control and norleucine cultures respectively. To another culture (volume 200 ml.) previously exposed to 3×10^{-3}M ^{12}C-norleucine for 2.5 hr. was added L-(1-^{14}C)-norleucine (50 μC), together with sufficient medium to maintain the total norleucine concentration at 3×10^{-3}M.

Alkaline phosphatase was extracted and further purified after conversion of cells to stable protoplasts by the method of Littauer and Eisenberg (1959). After centrifugation at 5° the protoplast pellet was resuspended in M sucrose (in 0.1 M tris, pH 7.4), again centrifuged and the combined centrifugates (in which the enzyme was recovered quantitatively) clarified by a further centrifugation at 5° (45 min; 13,000 x g). Following dialysis against 0.1 M tris, pH 7.4 (24 hr., 4°) and against several changes of 0.01 M tris, pH 7.4 (48 hr., 4°) the alkaline phosphatase was purified by gradient elution from columns of DEAE-cellulose, using a linear gradient of NaCl (0-0.16 M) in 0.01 M tris, pH 7.4 (Garen and Levinthal, 1960).

Such a fractionation of cells exposed to a ^{14}C-lysine 'pulse' in the presence of norleucine yielded a peak of alkaline phosphatase activity (corresponding to enzyme formed before norleucine addition) preceded by a peak of radioactivity which eluted three fractions prior to the enzyme peak (Fig. 2), whereas this radioactive peak was absent in an identical fractionation of material from control cells (Fig. 3a). Fractions 18-22 of the elution illustrated in Fig. 2 were combined, dialyzed against 0.01 M tris, pH 7.4 to remove NaCl, mixed with purified, unlabelled alkaline phosphatase from control cells and rechromatographed as before. The radioactive material again eluted 2-3 fractions ahead of the peak of enzyme activity. Elution of material from control cells

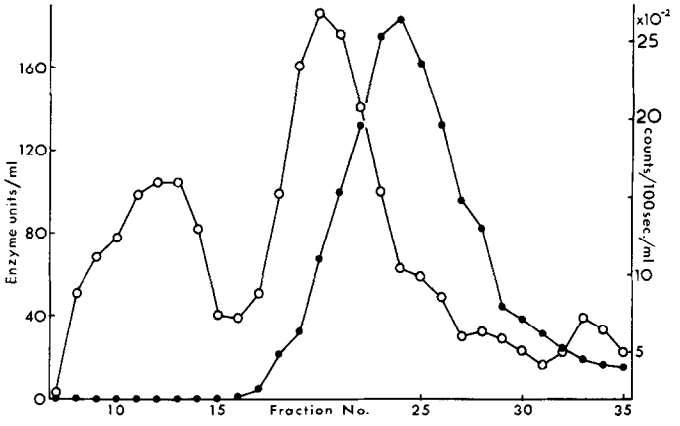

Fig. 2. Fractionation* of crude alkaline phosphatase preparation (see Methods) from cells exposed to 3 x 10^{-3}M L-norleucine and L-(U-^{14}C)-lysine 'pulse' (2 μmoles; 10 μC).

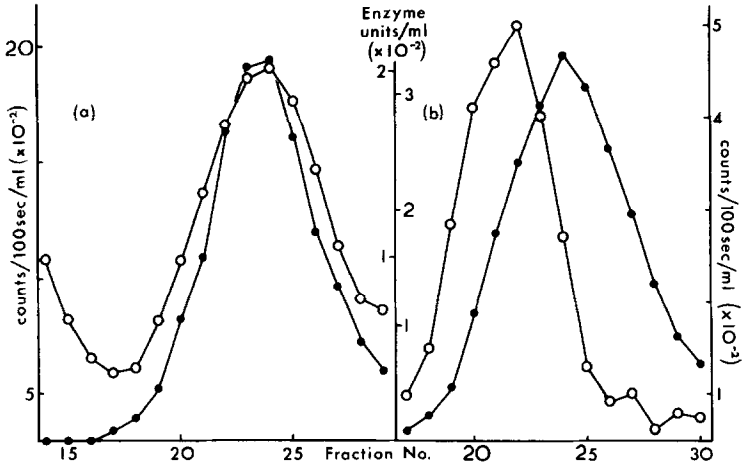

Fig. 3. Fractionation* of alkaline phosphatase preparation from (a) control cells labelled with L-(U-^{14}C)-lysine 'pulse' (2 μmoles; 10 μC) and (b) cells exposed to 3 x 10^{-3}M ^{12}C-norleucine followed, 2.5 hr. later, by L-(1-^{14}C)-norleucine (50 μC; culture volume 200 ml.).

*Two ml. fractions were collected from an 18 x 1 cm. DEAE-cellulose column during elution with a linear gradient of NaCl in 0.01 M tris, pH 7.4 (0-0.16 M NaCl in 120 ml.).

Radioactivity (scintillation counter)

Alkaline phosphatase activity

exposed to the ^{14}C-lysine 'pulse' yielded coincident peaks of enzyme activity and radioactivity (Fig. 3a). Identical fractionation of the cells exposed to L-(1-^{14}C)-norleucine resulted in an elution pattern similar to that obtained after exposure to unlabelled norleucine plus a ^{14}C-lysine 'pulse' (compare Fig. 2 and 3b).

The material present in the radioactive peak eluted from the column represented in Fig. 2 was tested for ability to react with anti-alkaline phosphatase serum. One ml. portions of fractions 18 and 19 (Fig. 2) were pooled and increasing amounts placed in a series of tubes, together with a solution of highly purified normal (unlabelled) alkaline phosphatase (containing c.100 enzyme units/ml.). After allowing sufficient time for precipitation the enzyme activity and radio-activity was assayed before and after removal of the precipitate (Table I). At enzyme levels below the equivalence point (about 18 enzyme units with 0.05 ml. antiserum) about 75% of the total radio-activity was precipitated by anti-alkaline phosphatase serum and it is probable that the non-precipitated radioactivity represented contaminating protein not related to alkaline phosphatase. Above the equivalence point some of the enzyme activity remained in solution, together with a higher proportion of the radioactivity. Addition of more antiserum to tubes 5-7 resulted in precipitation of all residual enzyme activity and much of the residual radioactivity. Similar precipitin tests on material from the radioactive peak obtained after exposure of cells to ^{14}C-norleucine (Fig. 3b) also led to the conclusion that this peak is due to a protein immunologically related to alkaline phosphatase.

Discussion.

It is apparent that the addition of norleucine to cultures of *E. coli* C4 caused a decrease in the differential rate of active alkaline phosphatase synthesis although there was no change in the differential rate of protein formation, as measured by ^{14}C-arginine incorporation. After the differential rate of synthesis of alkaline

TABLE I

Precipitation of 'norleucine' enzyme by anti-alkaline phosphatase serum

Tube	Enzyme soln. (ml)		Enzyme units			Radioactivity		
	Normal	'Norleu'	Total	Filtrate	Pptd. %	Total	Filtrate	Pptd. %
1	0.038	0.075	3.6	0	100	744	182	76
2	0.075	0.150	8.2	0	100	1620	360	78
3	0.100	0.200	12.0	0	100	1874	499	74
4	0.125	0.250	15.0	0	100	2195	768	65
5	0.150	0.300	19.8	1.5	92	2768	1167	58
6	0.175	0.350	24.7	6.7	73	3287	1833	44
7	0.200	0.400	27.8	11.3	59	3806	2259	41

All tubes contained anti-alkaline phosphatase serum (0.05 ml.) and enzyme soln. (as indicated) in a final volume of 1.5 ml. 0.01 M tris, pH 7.4 containing 0.15 M NaCl. Tubes were maintained at 37° (1 hr.) and then at 4° (48 hr.) before removal of precipitates from a 1 ml. aliquot by filtration through Oxoid membrane filters (2.5 cm. diam.). Enzyme activity and radioactivity were assayed on suitable samples of filtered and unfiltered material from each tube. (Anti-alkaline phosphatase serum is a non-neutralizing antiserum, the activity of the enzyme being unaffected by combination with antibody.) Radioactivity was determined on infinitely thin samples plated on ground glass planchettes (2.5 cm. diam.) and counted on a scintillation counter (Isotope Developments Ltd.), the activities being expressed as counts/1000 sec. Enzyme preparations: Normal - purified (unlabelled) alkaline phosphatase from control cells; 'Norleu' - pooled fractions 18 and 19 from column in Fig. 2.

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phosphatase had fallen to a low level as a result of exposure to ^{12}C-norleucine the addition of either ^{14}C-lysine or ^{14}C-norleucine resulted in the appearance of a new radioactive peak which eluted prior to normal alkaline phosphatase and was not present in control cell extracts (Fig. 2, 3). Immunological tests showed that the new peak reacted with normal anti-alkaline phosphatase serum. It is considered that exposure to norleucine caused the synthesis of alkaline phosphatase molecules containing norleucine and possessing altered enzymic activity and physico-chemical properties. The properties of this altered enzyme are under detailed investigation.

Acknowledgements.

The authors wish to thank Dr. A. Garen of the Biology Department, Massachussetts Institute of Technology, Cambridge, Mass. for a culture of *E. coli* C4. They also thank the Royal Society for a grant made to Professor D. Lewis, F.R.S. for the purchase of apparatus. One of the authors (S.N.) acknowledges the award of a Keddy Fletcher-Warr Studentship of the University of London.

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