RIBONUCLEOSIDE TRIPHOSPHATE ACCUMULATION ON AMINO ACID STARVATION OF "STRINGENT"[1] ESCHERICHIA COLI.

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INTRODUCTION

This paper reports the quantitative estimation of the acid-soluble nucleoside triphosphate pool of a stringent strain of Escherichia coli by two different methods. Extracts are made with 5% (w/v) trichloroacetic acid (TCA) in one method and with 0.40 M $HClO_4$ in the other. Both methods indicate similar amounts of nucleotides in normal, growing cells; but from amino acid starved cells, the method using TCA gives lower values than the method using $HClO_4$. We observe a marked increase in the intra-cellular nucleoside triphosphate contents on amino acid starvation when extracts are made with $HClO_4$.

Amino acid starvation of stringent strains causes an abrupt decrease in the rate of accumulation of ribonucleic acid (RNA), (Stent and Brenner, 1961). Under such conditions, continued synthesis of the nucleoside triphosphates, coupled with their decreased utilisation as precursors of RNA, should cause them to accumulate. The higher concentrations of nucleoside triphosphates produced by such an accumulation should then be effective in providing increased feed-back for inhibition of nucleotide biosynthesis.

Edlin and Neuhard (1967) reported that biosynthesis of nucleotides was inhibited on amino acid starvation of a stringent strain of Escherichia

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[1] "Stringent" strains are those in which ribonucleic acid synthesis is greatly reduced on starvation for a required amino acid. (Stent and Brenner, 1961).

coli: but they observed a decrease, not an increase, in the intra-cellular nucleotide pool, as extracted with TCA. These results are thus not consistent with the known mechanisms for regulation of nucleotide biosynthesis by feed-back inhibition, assuming a nucleotide pool in a single compartment. The present studies arose from an attempt to interpret this anomaly. Our results, with a different strain of Escherichia coli and somewhat different growth conditions from those of Edlin and Neuhard (1967), indicate that the influence of amino acid starvation on nucleotide pools and nucleotide biosynthesis is consistent with known feed-back effects.

METHODS

(a) Bacterial Growth. Experiments were carried out with a stringent strain of Escherichia coli (strain ABLA supplied by Mrs. B. Tyler) which had reverted from thr^- leu^- $thiamin^-$ to thr^+ leu^- $thiamin^-$. The bacterial cells were grown in a glucose-salts minimal medium which contained per litre: 0.2 g Na_2SO_4; 5.0 g NaCl; 0.3 mg $FeSO_4$; 0.2 g sodium citrate; 2.2 mg $CaCl_2$; 0.185 g $MgSO_4$; 1.0 g $(NH_4)_2SO_4$; 0.5 mg thiamin; 0.75 g KCl; 1.21 g 2-amino-2-(hydroxy-methyl)-1:3-propanediol, (tris) as free base (obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.); 24.0 mg KH_2PO_4; 4.0 g glucose; 20.0 mg leucine. (pH adjusted to 7.5 with concentrated HCl). Intra-cellular nucleotides were labelled with approximately 10 μCi/ml of $[^{32}P]$-orthophosphate (Australian Atomic Energy Commission, Lucas Heights, N.S.W., Australia) to give a specific activity of approximately 60-70 μCi/μmole. Culture density was followed with a Klett-Summerson photo-electric colorimeter using filter No. 42 transmitting light at 400-465 mμ. Under these conditions, 1.0 Klett unit corresponded to 0.7 μg protein/ml of culture as determined by the method of Lowry, Rosenbrough, Farr and Randall (1951). Crystalline bovine serum albumin was used as a standard. Aeration was aided by shaking cultures at 37°C in a New Brunswick Metabolyte shaker bath with gyratory shaker action at 300 rev./minute. For starvation experi-

ments, the cells were grown in 3 μg/ml of the required amino acid (leucine) which permitted exponential growth for approximately 40 Klett units, followed by an abrupt cessation of growth due to the exhaustion of the added amino acid. This period of growth (3-4 generations) also served to label the cells uniformly with [^{32}P]-orthophosphate. The procedure allowed the sampling of starved cultures without the necessity of harvesting and washing the cells to initiate amino acid starvation. Starvation initiated in this way was effective in reducing, by at least 90%, the incorporation of exogenous 2-[^{14}C]-uracil (supplied by The Radiochemical Centre, Amersham, England) into acid-insoluble material.

(b) Chromatography of Extracts. Fractionation of the cell extracts was carried out in two dimensions on poly(ethyleneimine)-cellulose thin layers spread on vinyl sheets (Randerath and Randerath, 1966). Avicel SF microcrystalline cellulose powder (manufactured by FMC Corporation, American Viscose Division, Marcus Hook, Pennsylvania, U.S.A. and supplied by John Beith and Co., (Vic) Pty. Ltd., Melbourne, Victoria, Australia) was used at a final concentration of 20% (w/v) with poly(ethyleneimine) (adjusted to pH 6.0 with concentrated HCl) at a concentration of 1.33% (w/v). The solvent system used was a modification of that described by Neuhard (1966).

(c) Estimation of Nucleotides. After chromatography, spots corresponding to guanosine triphosphate (GTP), adenosine triphosphate (ATP), cytidine triphosphate (CTP) and uridine triphosphate (UTP) were located by their absorption under a Mineralight ultra-violet lamp (Ultra-Violet Products Inc., San Gabriel, California, U.S.A.). The nucleotides used for markers were from Sigma Chemical Company. Experience showed that these spots always coincided exactly with radioactivity as detected by autoradiography. The spots were cut out, transferred to planchets and counted in a thin-window gas-flow counter. The specific activity of the [^{32}P]-orthophosphate in an experiment was determined by counting 1 μl samples of the medium on pieces of thin layer sheet. The specific activity was then used for the conversion of counts/minute to μmoles of nucleoside triphosphate.

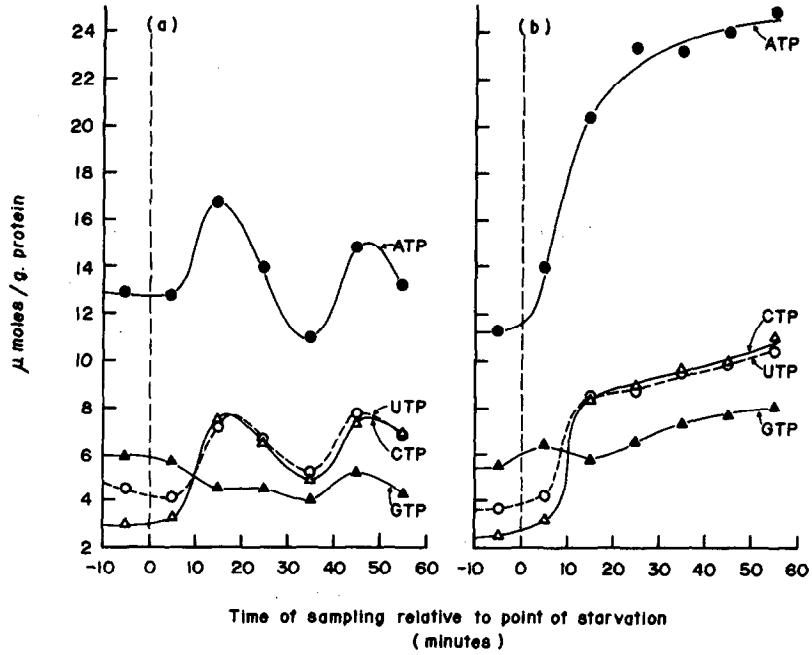

Figure 1. The nucleotide pool of Escherichia coli on amino acid starvation. A culture was grown in 40 ml of minimal medium containing [^{32}P]-orthophosphate and 3 μg of leucine/ml. Approximately 30 minutes before growth was expected to stop through exhaustion of leucine, two samples of 2.0 ml were taken, one for extraction by TCA and the other for extraction by $HClO_4$. The cells were quickly collected on 0.45 μ, 2.1 cm filters (Millipore Corporation, Bedford, Massachusetts, U.S.A.) for transfer to acid. Pairs of samples were taken at 10 minute intervals thereafter. The results for the last seven of nine such pairs of samples are presented. Leucine was exhausted at zero time.

(a). Extraction with 5% TCA. The filter was quickly immersed in 5.0 ml of ice-cold 5% TCA in a round-bottomed 15 ml centrifuge tube, shaken on a vortex mixer for 10 seconds and then left to stand in ice for a further 15 minutes. The tube was then centrifuged in the cold for 10 minutes at 8000 x g. 4.0 ml of the supernatant was then transferred to a 6 x 1 in. test tube and extracted 8 times with equal volumes of ice-cold diethyl ether to remove TCA. A drop of 3 M NH_3 solution was then added and the aqueous phase evaporated to dryness under a stream of nitrogen at 37°C. The dry residue was then redissolved in 0.20 ml of distilled water and 100 μl of a solution of nucleotides added to provide markers. Small samples of extracts were taken before ether extraction and after redissolution of the dry material to allow calculation of the percentage recoveries of radioactivity.

(b). Extractions with 0.40 M $HClO_4$. The filter was quickly immersed in 2.0 ml of ice-cold 0.40 M $HClO_4$ contained in a tapered 15 ml centrifuge tube, shaken on a vortex mixer for 10 seconds and then left to stand in ice for a further 15 minutes. The tube was then centrifuged in the cold for 10 minutes at 2500 x g. A 1.0 ml sample of the supernatant was then neutralized with 0.50 ml of 0.72 M KOH - 0.16 M $KHCO_3$ and left to stand in ice for 15 minutes to allow precipitation of $KClO_4$. From the supernatant, 0.20 ml was taken and mixed with 50 μl of the nucleotide marker solution. Chromatography was carried out as described in "Methods".

RESULTS AND DISCUSSION

The results presented in Figure 1 are the direct comparison of the nucleoside triphosphate pool as estimated by the TCA procedure (a) and the $HClO_4$ procedure (b). Up to 5 minutes before the onset of leucine starvation the two methods give similar results. From starved cells, however, the estimates of amounts of nucleotides by the TCA method are lower and more variable. The results presented are representative of those obtained from a number of other experiments using both methods of extraction with samples from either the same or different cultures. Extraction with $HClO_4$ gave consistent results which indicated that the contents of ATP, CTP and UTP, but not GTP, rose sharply on amino acid starvation. With TCA extractions, fluctuating results were usual within an experiment. Results also varied from experiment to experiment, with the effect of amino acid starvation ranging from slight increases in the nucleoside triphosphate pool to large decreases, similar to those observed by Edlin and Neuhard (1967). We have found the TCA procedure for estimation of nucleotides to be unsatisfactory because of the variability of the results and also the fact that there is an appreciable loss of radioactivity, ranging from 10%-30% of the total counts, when the TCA extracts are treated with ether to remove TCA and then evaporated to dryness.

The results obtained by extraction with $HClO_4$ demonstrate that amino acid starvation of this stringent strain of Escherichia coli approximately doubles the intra-cellular contents of ATP, CTP and UTP. If such an increase produces a proportional increase in their concentrations at the sites of nucleotide synthesis within the cell, then it might well provide a sufficient increase in feed-back inhibition to account for observed decreases in rates of nucleotide biosynthesis (Edlin and Neuhard, 1967) during amino acid starvation. Presumably, the pools of these three nucleotides rise as a result of a continuation of their biosynthesis at a rate in excess of their decreased rate of utilisation for RNA synthesis.

The observations reported here on the effect of amino acid starvation

are in close agreement with those of Goldstein, Brown and Goldstein (1960). Edlin and Broda (1968) have recently reported experiments on the effect of growth at high temperature on the nucleoside triphosphate contents of two strains of Eschericia coli possessing a temperature sensitive valyl-tRNA synthetase. Allowing for the effect of higher temperature alone on the nucleotide contents, their results, obtained by the TCA method, suggest that inhibition of the valyl-tRNA synthetase gave increases in the content of all the ribonucleoside triphosphates, except GTP, in one strain, but not in the other.

All the results have the similarity that GTP is the nucleoside triphosphate which is relatively lowest in content after deprivation of the cells of an amino acid or an amino acyl-tRNA. One basis for this effect may derive from the interrelationships of the pathways for purine nucleotide biosynthesis and from their relationship to that for histidine biosynthesis (Magasanik, 1962). Guanosine diphosphate (GDP) inhibits the GTP-dependent conversion of inosine monophosphate (IMP) to adenylosuccinate in the synthesis of adenosine monophosphate (Wyngaarden and Greenland, 1963) and we observe that the GDP/GTP ratio drops markedly immediately on amino acid starvation. This change could alter the utilisation of IMP towards production of adenine nucleotides as compared with guanine nucleotides. A resulting imbalance in concentrations of the nucleotides would be difficult to overcome while the utilisation of ATP, for incorporation into RNA or for biosynthesis of histidine (no longer in demand for protein synthesis), remained greatly decreased by the lack of an amino acid. Another possibility arises from the report of Raina and Cohen (1966) of an accumulation of polyamines on amino acid starvation. Our observations suggest that some factor related to amino acid starvation decreases the estimate of nucleotide contents by the TCA method. Conceivably, increased contents of polyamines may bind nucleotides to made them less readily extractable. Such binding appears likely to be greater for purine derivatives, particularly those of guanine, since the binding of guanine derivatives to polylysine and polyarginine (Wagner and Arav, 1968) and

to poly(ethyleneimine)-cellulose thin layers (Randerath and Randerath, 1964) has been shown to be stronger, as compared with other nucleotides, under a variety of conditions. Thus, decreased estimates of nucleotide contents in amino acid starved cells could possible be an artifact produced by increased polyamine content, particularly with regard to guanine nucleotides and the use of the TCA method.

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REFERENCES

Edlin, G., and Broda, P. (1968). Bacteriol. Rev. (in press).
Edlin, G., and Neuhard, J. (1967). J. Mol. Biol., 24, 225.
Goldstein, D.B., Brown, B.J. and Goldstein, A. (1960). Biochim. Biophys. Acta, 43, 55.
Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951). J. Biol. Chem., 193, 265.
Magasanik, B. (1962). In I.C. Gunsalus and R.Y. Stainer (Eds.), "The Bacteria" Vol. 3. Academic Press, New York and London, p. 295.
Neuhard, J. (1966). Biochim. Biophys. Acta, 129, 104.
Raina, A. and Cohen, S.S. (1966). Proc. Nat. Acad. Sci., Wash., 55, 1587.
Randerath, K. and Randerath, E. (1964). J. Chromatog., 16, 111.
Randerath, K. and Randerath, E. (1966). J. Chromatog., 22, 110.
Stent, G.S. and Brenner, S. (1961). Proc. Nat. Acad. Sci., Wash., 47, 2005.
Wagner, K.G. and Arav, R. (1968). Biochemistry, 7, 1771.
Wyngaarden, J.B. and Greenland, R.A. (1963). J. Biol. Chem., 238, 1054